Mai1 Protein Acts Between Host Recognition of Pathogen Effectors and Mitogen-Activated Protein Kinase Signaling

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The molecular mechanisms acting between host recognition of pathogen effectors by nucleotide-binding leucine-rich repeat receptor (NLR) proteins and mitogen-activated protein kinase (MAPK) signaling cascades are unknown. MAPKKK α (M3K α) activates MAPK signaling leading to programmed cell death (PCD) associated with NLR-triggered immunity. We identified a tomato M3K α -interacting protein, SIMai1, that has 80% amino acid identity with *Arabidopsis* brassinosteroid kinase 1 (AtBsk1). SIMai1 has a protein kinase domain and a C-terminal tetratricopeptide repeat domain that interacts with the kinase domain of M3K α . Virus-induced gene silencing of *Mai1* homologs in *Nicotiana benthamiana* increased susceptibility to *Pseudomonas syringae* and compromised PCD induced by four NLR proteins. PCD was restored by expression of a synthetic *SIMai1* gene that resists silencing. Expression of AtBsk1 did not restore PCD in *Mai1*-silenced plants, suggesting SIMai1 is functionally divergent from AtBsk1. PCD caused by overexpression of M3K α or MKK2 was unaffected by *Mai1* silencing, suggesting *Mai1* acts upstream of these proteins. Coexpression of *Mai1* with M3K α in leaves enhanced MAPK phosphorylation and accelerated PCD. These findings suggest

Mai1 is a molecular link acting between host recognition of pathogens and MAPK signaling.

Keywords: bacterial speck disease, NLR-triggered immunity, plant immunity, *Pseudomonas syringae*, signal transduction, tomato

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Plants use intracellular immune receptors to detect and respond to specific virulence (effector) proteins that pathogens translocate into the host cell as part of their infection process (Maekawa et al. 2011; Qi and Innes 2013). These receptors are typically members of the family of nucleotide-binding leucine-rich repeat receptors (NLRs) and may have either a coiled-coil (CC) or a Toll-interleukin-1 receptor (TIR) domain (Jones et al. 2016; Takken and Govers 2012). In some cases, additional NLRs promote the function of sensor NLRs, although their mechanisms are unknown (Dong et al. 2016; Wu et al. 2015, 2017). Nucleotide binding is thought to maintain NLRs in an equilibrium state with ATP hydrolysis shifting the NLR to an ADP-bound inactive state and detection of a pathogen effector shifting the NLR to the ATP-bound activated state (Zhang et al. 2017). Oligomerization of NLRs mediated by the CC or TIR domain is often essential for NLR activation (Gutierrez et al. 2010; Wang et al. 2019a and b). The molecular mechanisms that promote signaling upon NLR activation are not well-understood (Zhang et al. 2017). TIR-NLRs often rely on the EDS1 lipase-like protein, whereas CC-NLRs often rely on the NDR1 integrin-like protein, suggesting these two classes of NLRs might involve different early signaling partners (Aarts et al. 1998). Recently a CC-NLR, NRG1, has emerged as a candidate for an early TIR-NLR-specific signaling component (Brendolise et al. 2018; Castel et al. 2019; Qi et al. 2018; Wu et al. 2019).

The interaction of tomato and *Nicotiana benthamiana* with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* is a model system for investigating the molecular basis of NLR activation and associated signaling (Ntoukakis et al. 2014; Pedley and Martin 2003). The CC-NLR Prf acts in concert with host protein kinases Pto and Fen to activate NLR-triggered immunity (NTI) (Gutierrez et al. 2010; Mucyn et al. 2009). Pto and Fen bind the *P. syringae* pv. *tomato* effectors AvrPto or AvrPtoB, possibly by acting as ‘decoys’ of the virulence targets of these effectors (Abramovitch and Martin 2005; Martin 2012; van der Hoorn and Kamoun 2008). Pto and Fen interact constitutively with an N-terminal domain of Prf, and the interaction

contributes to the stabilization of both the NLR and Pto/Fen (Mucyn et al. 2006, 2009; Xiao et al. 2007). Pto and Fen can autophosphorylate and this activity appears to be required for changing the kinases into an active form but is not necessary for downstream signaling (Jia et al. 1997; Mathieu et al. 2014). Like other NLRs, Prf likely cycles between inactive (ADP-bound) and active (ATP-bound) forms (Du et al. 2012). As with other NLR-mediated pathosystems, little is known about early signaling steps following Prf activation. Recently however, two NLRs, NRC2 and NRC3, and a cytoplasmic kinase, Epk1, of the GmpK6/AtMRK1 family have been shown to be required for Pto/Prf-associated PCD and resistance to *P. syringae* pv. *tomato*, although where exactly in this NTI pathway these proteins operate is unknown (Pombo et al. 2014; Wu et al. 2016, 2017).

Host responses activated by Pto/Prf are typical of NTI and include phosphorylation of mitogen-associated protein kinases (MAPKs) (Meng and Zhang 2013; Pedley and Martin 2005), transcriptional reprogramming (Jia and Martin 1999; Pombo et al. 2014), generation of reactive oxygen species (Chandra et al. 1996), and localized programmed cell death (PCD) (Coll et al. 2011; Du et al. 2012), which is thought to inhibit spread of the pathogen in host tissues. The role and mechanisms associated with MAPK signaling have been well-characterized in the Pto/Prf pathway. Virus-induced gene silencing (VIGS) of two tomato MAPKK genes (*SIMKK1* and *SIMKK2*) and two MAPK genes (*SIMPK2* or *SIMPK3*) compromised Pto/Prf-mediated resistance and initially revealed a role for MAPK cascades in this pathway (Ekengren et al. 2003; Tena et al. 2001). NTI-associated MAPK signaling is also important in *Arabidopsis* and rice (Agrawal et al. 2003; Colcombet and Hirt 2008; Meng and Zhang 2013; Rasmussen et al. 2012; Tsuda et al. 2009). A subsequent VIGS screen in *N. benthamiana*, which tested the effect of silencing more than 2,400 randomly chosen cDNAs on NTI, identified one MAPKKK, M3K α , as playing an important role in Pto/Prf-mediated immunity (del Pozo et al. 2004). Silencing of *M3K α* abolished PCD associated with Pto/Prf activation as well as cell death associated with *P. syringae* pv. *tomato*-related disease symptoms (del Pozo et al. 2004). In Pto/Prf-expressing leaves, transient expression of AvrPto or M3K α revealed that both increased the activity of SIMPK2 and SIMPK3 (del Pozo et al. 2004; Pedley and Martin 2004). M3K α is a member of group A2 in the MEKK (MAPK/ERK kinase) family, and this group has since been found to contain other members that function in NTI (Hashimoto et al. 2012). Here, we describe the identification and characterization in *N. benthamiana* of a tomato M3K α -interacting protein, Mail, a receptor-like cytoplasmic kinase (RLCK), and present data suggesting it acts as a molecular link between early pathogen recognition events and MAPK signaling.

RESULTS

SIMai1 interacts via its TPR domain with the kinase domain (KD) of SIM3K α .

The tomato M3K α (SIM3K α) protein was used as a bait in a yeast two-hybrid screen of a cDNA prey library generated from Rio Grande-PtoR tomato leaves inoculated with *P. syringae* pv. *tomato* (Fig. 1A) (Oh and Martin 2011; Zhou et al. 1995). From this screen, 18 clones were identified that contained sequences derived from the same gene, which was called *Mai1* (*M3K α -interacting 1*; Solyc04g082260). The tomato Mail (SIMai1) protein is a predicted RLCK with 497 amino acids. The most similar protein in *Arabidopsis* is BRASSINOSTEROID-SIGNALING KINASE 1 (AtBSK1) (Supplementary Fig. S1), which was originally identified as a substrate of the BRI

brassinosteroid receptor and later implicated in immunity (Shi et al. 2013a and b; Tang et al. 2008; Yan et al. 2018). In rice, there are two proteins related to SIMai1, OsBSK1-1 and OsBSK1-2, with OsBSK1-2, also having a reported role in immunity (Wang et al. 2017). Subsequent assays showed that SIMai1 interacts with the SIM3K α KD and not with its N- or C-terminal domains (Fig. 1B). Immunoblotting showed that all proteins were expressed (Supplementary Fig. S2).

SIMai1 has residues in its N terminus that are predicted to be myristoylated or palmitoylated (i.e., MGCC), a central KD, and a region of tetratricopeptide repeats (TPR) in the C terminus (Fig. 1C). AtBSK1 was reported to be an active kinase in vitro, and an amino acid substitution in its ATP binding site (K104E) compromised its role in resistance to a fungal pathogen (Shi et al. 2013b). Like all BSK proteins, SIMai1 lacks specific

Fig. 1. Tomato Mail (SIMai1) interacts via its tetratricopeptide repeat (TPR) domain with the SIM3K α kinase domain (KD). **A**, Schematic of tomato M3K α (SIM3K α) showing the N- and C-terminal domains (NTD and CTD) and the KD along with the amino acid coordinates. **B**, SIMai1 was tested in a yeast two-hybrid (Y2H) assay for interaction with SIM3K α full-length and its NTD, KD, and CTD subdomains. **C**, Schematic of SIMai1 showing the KD, TPR domain, and myristoylation/palmitoylation motif (MGCC) along with amino acid coordinates. **D**, SIMai1, its KD and TPR subdomains, and the SIMai1(R430Q) variant were tested for interaction with SIM3K α -KD in a yeast two-hybrid assay. For all Y2H assays, SIMai1 was expressed as the prey protein fused to the activation domain in pJG4-5 and SIM3K α was expressed as the bait protein fused to the LexA DNA-binding domain in pEG202. Dark patches indicate a positive interaction.

Fig. 2. SIM3K α interacts with SIM1a1 but not with other tomato brassinosteroid-signaling kinases (BSKs). **A**, Phylogenetic analysis of tomato (*Solanum lycopersicum* [Solyc]) and *Arabidopsis thaliana* (At) BSK family members based on gene coding sequences. Numbers next to the branches indicate the percentage of trees in which the associated taxa are clustered together, and the tree is drawn to scale with the branch lengths measured in the number of substitutions per site. **B**, Interaction of tomato BSK proteins with SIM3K α in a yeast two-hybrid assay, using full-length tomato BSKs with the SIM3K α -kinase domain (KD). Yeast were grown in medium lacking leucine and tryptophan (–L–W) or lacking leucine, tryptophan, histidine, and adenine (–L–W–H–A). Empty vectors served as negative controls. Growth on –L–W–H–A medium indicates a positive interaction. **C**, Interaction of tomato BSKs and SIM3K α in a split luciferase complementation assay in *Nicotiana benthamiana* leaves measured by quantitative luminescence. Protein expression was driven by a 35S promoter. Results shown are means $\pm$ standard deviation of three technical replicates. The asterisk indicates a significant difference using a Student's *t* test ($P < 0.01$). Similar results were observed in three independent experiments. A N-terminal fragment of luciferase (NLuc) was fused to the tomato BSK proteins and C-terminal fragment of luciferase (CLuc) was fused to SIM3K α -KD. Relative light units are shown.

amino acid sequences that are essential for catalysis (GxGxxG, HRD, and DFG motifs), which suggests it may be a pseudokinase (Supplementary Fig. S3) (Bayer et al. 2009; Grüter et al. 2013; Kwon et al. 2019; Sreeramulu et al. 2013). Consistent with this observation and with a recent report about AtBSK1 (Neu et al. 2019), we were unable to detect SIMail kinase activity using multiple in-vitro assay conditions, including conditions previously published for AtBSK1 kinase activity (Zhao et al. 2019). TPR motifs consist of a degenerate 34–amino acid consensus sequence that forms two antiparallel alpha helices (Blatch and Lässle 1999) and domains often mediate protein-protein interactions (Blatch and Lässle 1999; Kwon et al. 2009; Nyarko et al. 2007). The SIMail TPR domain alone was sufficient for interacting with the SIM3Kα KD (Fig. 1D). The abolished immunity-related functions in *Arabidopsis bsk1-1* mutants were associated with an R443Q substitution in the TPR domain (Shi et al. 2013b), but the comparable substitution (R430Q) in SIMail did not affect its interaction with SIM3Kα. Immunoblotting showed that all proteins were expressed.

SIM3K α interacts only with SIMai1 among the seven BSK proteins in tomato.

The tomato genome has seven *BSK* gene family members, compared with the 12 *BSK* genes present in the *Arabidopsis* genome (Fig. 2A) (Sreeramulu et al. 2013). The transcript abundance of three of the tomato *BSK* genes, including *SIMail*, increases upon activation of the Pto/Prf pathway in tomato (Supplementary Table S1). Although the seven tomato *BSK* proteins have highly similar TPR sequences (Supplementary Fig. S4), only *SIMail* interacted with *SIM3Kα* in a yeast two-hybrid assay (Fig. 2B). The same specificity of the *SIM3Kα* and *SIMail* interaction was observed in a split luciferase complementation assay, in which *SIM3Kα* and the tomato *BSK* proteins were expressed in *N. benthamiana* leaves using the 35S CaMV (cauliflower mosaic virus) promoter (Fig. 2C). Immunoblotting showed that all proteins were expressed.

SlMai1 interacts with a subset of tomato M3Ks in yeast.

In an initial effort to explore the role of SIMa1, an additional yeast two-hybrid screen of the tomato cDNA library was conducted using SIMa1 as the bait. This screen identified a 14-3-3 protein (TFT3; Solyc04g074510) and, interestingly, another immunity-associated M3K (M3K γ 2; Solyc02g065110) (Hashimoto et al. 2012). We therefore tested the possible interaction of SIMa1 with additional SIM3Ks. Pairwise yeast two-hybrid assays were performed, using the KDs of six additional SIM3Ks in the MEKK, ZIK, and RAF families as the prey proteins (Supplementary Fig. S5) (Ichimura et al. 2002; Wu et al. 2014). Three of these SIM3Ks interacted strongly with SIMa1. All five SIMa1-interacting SIM3Ks are in the MEKK family. The transcript abundance of two of these interacting SIM3Ks (SIM3K α and Solyc04g079400) is increased during flgII-28-induced pattern recognition receptor-triggered immunity (PTI) as well as during the Pto/Prf-mediated immune response in tomato. A distinguishing amino acid motif is not present in the KDs of the SIM3Ks that interact with SIMa1.

SlMai1 is localized to the cell periphery.

Myristoylation and palmitoylation sites, as are predicted in the SIMa1 N-terminus, can promote localization of proteins to the plasma membrane (PM). With confocal microscopy, a Mail-yellow fluorescent protein (YFP) fusion protein was observed to be localized to the plant cell periphery, similar to another RLCK (Solyc10g085990) that has predicted myristoylation and palmitoylation sites (Supplementary Fig. S6).

Substitutions in the putative SIMail myristoylation site (G2A) or palmitoylation sites (C3S/C4S) altered its localization, as indicated by YFP signal appearing in the nucleus and the cytoplasm. Immunoblotting showed each protein was expressed similarly in *N. benthamiana*.

Silencing of *Mai1* homologs in *N. benthamiana*, using two *SIMail* fragments, compromises PCD and NTI associated with Pto/Prf and PCD induced by other CC-NLR proteins.

The transcript abundance of many genes with established roles in the Pto/Prf pathway increases upon activation of the Pto/Prf pathway (Supplementary Fig. S7). The transcript abundance of *SIMail* also increases during this NTI response, and this observation, along with the SIMail interaction with SIM3K α , suggested that SIMail might contribute to plant immunity. To test this hypothesis, two nonoverlapping fragments (*SIMail-1* and *SIMail-2*) from the *SIMail* gene, each of which is predicted to silence all four of the *SIMail* homologs present in *N. benthamiana*, were used for tobacco rattle virus (TRV)-based VIGS (Fig. 3A; Supplementary Fig. S8). Silencing was confirmed using *SIMail*-specific primers in semiquantitative reverse transcription (RT)-PCR. *SIM3K α* and *SIMKK2*, whose silencing is known to compromise NTI-associated PCD (del Pozo et al. 2004), and the empty TRV vector were included as controls. Silencing of the *Mai1* homologs in *N. benthamiana* (*NbMai1*), using either *SIMail-1* or *SIMail-2*, or of tomato *M3K α* (*SIM3K α*) and *N. tabacum* *MKK2* (*NtMKK2*), compromised the PCD normally observed when AvrPto + Pto or AvrPtoB₁₋₃₈₇ + Pto were coexpressed by agroinfiltration (Fig. 3A and C).

N. benthamiana recognizes the effector HopQ1, which is present in *Pseudomonas syringae* pv. *tomato* DC3000, and deletion of this effector allows DC3000 to cause disease on this species (Wei et al. 2007). Additionally, a *N. benthamiana* line (R411b) is available that stably expresses both Pto and Prf, thus conferring a strong NTI response to DC3000 through recognition of AvrPto or AvrPtoB (Balmuth and Rathjen 2007). To test the role of Mai1 in Pto/Prf resistance to *P. syringae* pv. *tomato*, the *SIMail-1* and *SIMail-2* VIGS constructs were used to silence the *NbMai1* homologs in *N. benthamiana* R411b, the plants were inoculated with DC3000 Δ hopQ1 or DC3000 Δ hopQ1 Δ avrPto Δ avrPtoB, and the bacterial populations were measured. The DC3000 Δ hopQ1 strain (which expresses AvrPto and AvrPtoB) showed significantly increased growth (16-fold more) in *NbMai1*-silenced leaves as compared with unsilenced R411b leaves (Fig. 3B). In contrast, the DC3000 Δ hopQ1 Δ avrPto Δ avrPtoB strain grew to the same population level in *NbMai1*-silenced and unsilenced leaves. These results support a role for Mai1 in disease resistance to *P. syringae* pv. *tomato* mediated by Pto/Prf. A comparable difference in avirulent *P. syringae* pv. *tomato* growth has been reported for *N. benthamiana* silenced for *Prf* (approximately 30-fold) (Lu et al. 2003; Pombo et al. 2014) or *NRC2/3* (approximately 10-fold) (Wu et al. 2016) compared with unsilenced plants.

We have reported previously that silencing of *M3K α* or *TFT7* in *N. benthamiana* compromises PCD that is induced by several NLR protein/effector pairs (del Pozo et al. 2004; Oh et al. 2010). The *SIMail-1* and *SIMail-2* VIGS constructs were therefore used to silence the *NbMai1* homologs in *N. benthamiana*, and the silenced leaves were agroinfiltrated with three CC-NLR gene/effector pairs that activate NTI-associated PCD: *Arabidopsis* *RPP13* and the oomycete effector *ATR13*, potato *Gpa2* and the nematode effector *RBP-1*, and potato *Rx2* and the potato virus X coat protein (Oh and Martin 2011). Each of these pairs caused PCD in the empty TRV vector control plants, and this response was reduced by silencing of

NbMai1, *M3K α* , or *MKK2* (Fig. 3C). These experiments indicate that Mai1 acts in a pathway shared by several CC-NLR proteins that target diverse pathogens.

Silencing of *NbMai1* homologs does not affect PCD that occurs in *N. benthamiana* leaves upon expression of *M3K α* or *MKK2*.

A MAPK cascade involving *M3K α* and *MKK2* acts downstream of Pto/Prf and plays an important role in activating NTI (del Pozo et al. 2004; Pedley and Martin 2004). Expression of *M3K α* in *N. benthamiana* causes PCD, and this PCD can be compromised by silencing *MKK2* (del Pozo et al. 2004). Additionally, expression of the constitutively active form of *MKK2* (*MKK2^{DD}*) also causes PCD in *N. benthamiana* (Oh et al. 2010). To test the position of Mai1 function in the Pto/Prf pathway, *SIMail-1*, *SIMail-2*, *SIM3K α* , and *NtMKK2* VIGS constructs were used for silencing in *N. benthamiana*, and constructs that encode *SIM3K α* or *NtMKK2^{DD}* were agroinfiltrated into silenced leaves. No decrease in PCD was observed in leaves silenced with *SIMail-1* and *SIMail-2* and agroinfiltrated with *SIM3K α* or *NtMKK2^{DD}*, as compared with the control leaves (Fig. 4). As expected, leaves silenced with the *SIM3K α* or *NtMKK2* constructs reduced PCD caused by agroinfiltration of *SIM3K α* and silencing with the *NtMKK2* construct compromised PCD caused by *NtMKK2^{DD}* (Fig. 4). These experiments, along with the observations above, indicate that Mai1 acts with *M3K α* and upstream of *MKK2* to regulate NTI-associated PCD.

A synthetic *SIMail* gene complements PCD impairment in *N. benthamiana* silenced with *SIMail-1* and *SIMail-2*.

To verify that the phenotypes we observed were not due to silencing of an ‘off-target’ gene, we developed a synthetic version of *SIMail* (*synSIMail*) with a divergent DNA sequence that would make it resistant to silencing, yet encode an identical amino acid sequence (Supplementary Fig. S9). *N. benthamiana* leaves silenced with the *SIMail-1* and *SIMail-2* VIGS constructs were agroinfiltrated with *SIMail* or *synSIMail* constructs expressing cMyc-tagged SIMail proteins, and the proteins were detected by immunoblotting. As expected, SIMail protein did not accumulate in *NbMai1*-silenced leaves expressing the unaltered *SIMail* gene but did in leaves expressing *synSIMail* (Fig. 5A). These observations indicate that *synSIMail* resists the silencing of the *NbMai1* genes caused by the *SIMail-1* and *SIMail-2* VIGS constructs. SIMail proteins accumulated in unsilenced control plants expressing either *SIMail* or *synSIMail* (Fig. 5A).

Changes in nucleotides were introduced into *SIMail* and *synSIMail* (Supplementary Table S2) to alter amino acid residues for the myristoylation motif (G2A) and the putative ATP binding site (K91M), to test whether these sites are important for SIMail function (a Mai1[C3S/C3S]-myc protein was also tested, but, for unknown reasons, it did not express well). As expected, the variant proteins accumulated in unsilenced control leaves, but only the proteins encoded by *synSIMail* sequences accumulated in silenced leaves (Fig. 5A).

Next, each of these constructs was agroinfiltrated into leaves of wild-type *N. benthamiana* silenced with the *SIMail-1* and *SIMail-2* VIGS constructs or unsilenced leaves along with AvrPtoB₁₋₃₈₇ to induce Pto/Prf-dependent PCD. As expected, in unsilenced control leaves expression of AvrPtoB₁₋₃₈₇ with each of the *SIMail* and *synSIMail* constructs caused PCD similar to levels observed when AvrPtoB₁₋₃₈₇ was expressed with a *YFP* control (Fig. 5B and C). However, in leaves silenced with *SIMail-1* and *SIMail-2*, PCD occurred in areas agroinfiltrated with *synSIMail* and *synSIMail*(K91M) but not in areas agroinfiltrated with *SIMail* or a *YFP* control (Fig. 5B and

C). Areas agroinfiltrated with *synSIMail(G2A)* developed PCD, but it was reduced compared with *synSIMail* or *synSIMail(K91M)* (Fig. 5B and C). In combination with our other localization data, these observations suggest that the glycine-2 myristoylation motif of SIMail contributes to SIMail function in NTI-associated PCD. Finally, consistent with our inability to detect SIMail kinase activity, the ability of the SIMail(K91M) protein to fully complement *Mai1* silencing indicates that kinase activity of Mai1 does not play a role in NTI-associated PCD.

AtBSK1 does not restore PCD in *N. benthamiana* plants silenced with *SIMail-1* and *SIMail-2*.

In light of the relatively high amino-acid sequence identity between SIMail and AtBSK1, we tested whether AtBSK1 could restore immunity-associated PCD in *N. benthamiana* plants silenced with the *SIMail-1* and *SIMail-2* VIGS constructs. An alignment of the AtBSK1 DNA sequence with the *SIMail-1* and *SIMail-2* VIGS constructs revealed little overall similarity suggesting AtBSK1 might evade silencing by these constructs (Supplementary Fig. S10). In fact, an immunoblot

Fig. 3. Mai1 contributes to programmed cell death (PCD) and disease resistance induced by Pto/Prf and PCD induced by several coiled coil-nucleotide-binding leucine-rich repeat receptor (NLR) proteins. **A**, The tobacco rattle virus (TRV) virus-induced gene silencing (VIGS) system was used in *Nicotiana benthamiana* to silence *NbMai1* homologs (using two nonoverlapping constructs, *SIMail-1* and *SIMail-2*), or genes known to reduce PCD (*SIM3Kα* and *SIMKK2*), or a TRV-only control. Using the 35S CaMV (cauliflower mosaic virus) promoter, PCD elicitors were expressed by agroinfiltration into leaves of silenced plants. Numbers below photos indicate the number of spots observed with cell death compared with the total number of spots infiltrated from three replicates ($n = 22$ to 28). Photographs representative of the most common response in each treatment are shown. **B**, *N. benthamiana* plants that stably express *Pto* and *Prf* (R411b plants) were silenced with the *SIMail-1* and *SIMail-2* VIGS constructs or the TRV control and were infiltrated with *Pseudomonas syringae* pv. tomato DC3000 Δ hopQ1 (Δ hopQ1-1) or DC3000 Δ avrPto Δ avrPtoB Δ hopQ1 (Δ avrPto Δ avrPtoB Δ hopQ1-1). Bacterial populations were determined on days 0 and 2. Significance was determined using analysis of variance with a Tukey's post hoc multiple comparisons test, and letters indicate significant differences between treatments ($P < 0.001$). Results shown are the individual values from each plant ($n = 3$) and standard deviation, and means are shown with a horizontal line. Data shown are representative of three biological replicates ($n = 9$). **C**, VIGS and agroinfiltration of the indicated NLR and Avr cell death elicitors was performed, as in A, with all constructs expressed from the 35S CaMV promoter. Indicated is the number of spots observed with cell death compared with the total number of spots infiltrated from three replicates ($n = 22$ to 28).

comparing SIMai1, synSIMai1, and AtBsk1 expression in *Mai1*-silenced and control plants showed that AtBSK1 protein was expressed in both the control and *Mai1*-silenced plants (Supplementary Fig. S11). We then tested whether AtBSK1 could complement *Mai1*-silenced plants, using the same conditions as described in Figure 5. Unlike synSIMai1, AtBSK1 was unable to restore PCD, suggesting that AtBSK1 and SIMai1 are functionally distinct or, possibly, that AtBSK1 acts with other proteins that are not present or are too divergent in *N. benthamiana*.

Overexpression of SIMai1 with SIM3K α in *N. benthamiana* accelerates development of PCD and increases activation of MAPKs, and the SIMai1 myristoylation motif contributes to these responses.

We reported previously that coexpression in *N. benthamiana* leaves of M3K α with its interacting partner TFT7, a 14-3-3 protein, led to increased accumulation of M3K α and also accelerated the time at which PCD occurred (Oh et al. 2010). We performed a similar experiment using agroinfiltration of *N. benthamiana* leaves to coexpress SIM3K α or SIM3K α -KD with SIMai1 (using the *synSIMai1* construct) or YFP as a control. When scored at 48 and 72 h after SIM3K α or SIM3K α -KD protein induction with estradiol, PCD occurred faster in leaves coexpressing either of the SIM3K α proteins with SIMai1 as compared with the SIM3K α with YFP control infiltrations (Fig. 6A). As expected, no PCD occurred in leaf areas agroinfiltrated with a kinase-inactive SIM3K α -KD(K231M) variant (Fig. 6A). Five days after agroinfiltration, PCD occurred in all agroinfiltrated areas in which it was expected (Supplementary Fig. S12).

The enhanced PCD in this experiment raised the possibility that SIMai1 interaction with SIM3K α increases accumulation of one or both of these proteins or enhances downstream MAPK activity. To test these hypotheses, the SIM3K α and SIMai1 constructs were agroinfiltrated into *N. benthamiana* leaves, and SIM3K α expression was induced with estradiol 2 days after infiltration. At 12, 24, and 30 h after induction with estradiol, total proteins were extracted and analyzed by immunoblotting. No difference in the abundance of SIM3K α -KD or SIMai1 protein was observed when they were coexpressed as compared with coexpression with YFP. Employing similar conditions but sampling leaves at 2 h after estradiol induction of the SIM3K α constructs, we used an antibody that specifically detects phosphorylated (activated) MAPKs and observed that NbMAPK phosphorylation was substantially increased when SIMai1 was coexpressed with SIM3K α -KD; a slight increase in

NbMAPK phosphorylation was observed upon coexpression of SIMai1 with full-length SIM3K α (Fig. 6B). Consistent with the complementation experiments in Figure 5, coexpression of SIMai1(G2A) with SIM3K α did not cause an increase in MAPK phosphorylation, whereas coexpression of SIMai1(K91M) with SIM3K α was just as effective as SIMai1 in enhancing NbMAPK phosphorylation (Fig. 6C). We attempted to test the requirement of the SIMai1 TPR domain for PCD enhancement and activation of M3K α , but a SIMai1 protein lacking the TPR domain was poorly expressed in *N. benthamiana* leaves (Supplementary Fig. S13).

DISCUSSION

We discovered that the tomato *Mai1* protein interacts in vivo in both a yeast two-hybrid system and a plant split luciferase complementation assay with tomato M3K α , a previously described positive regulator of NTI (del Pozo et al. 2004). The SIMai1 TPR domain and the SIM3K α KD are sufficient for mediating their interaction. Although all seven tomato BSK proteins have a TPR domain, SIM3K α interacts only with SIMai1. In contrast, SIMai1 interacts with at least five members of the MEKK family. The predicted SIMai1 fatty acylation sites play a role in its localization to the cell periphery. Experiments in *N. benthamiana* indicated that, like SIM3K α and TFT7, *Mai1* is a positive regulator of PCD induced by several CC-NLR proteins. Complementation assays using a synthetic *SIMai1* gene that resists silencing induced by the *SIMai1-1* and *SIMai1-2* VIGS constructs confirmed that SIMai1 contributes to immunity-associated PCD and indicated that fatty acylation of SIMai1 but not kinase activity is important for its function. Importantly, we found that *Mai1* contributes to *P. syringae* pv. *tomato* resistance conferred by Pto/Prf. Coexpression of SIMai1 with SIM3K α -KD does not affect the accumulation of either protein, although it does enhance phosphorylation of downstream MAPKs. The observed effect was stronger with SIM3K α -KD than full-length M3K α , which may be due to the higher expression levels of SIM3K α -KD compared with SIM3K α , as shown previously (del Pozo et al. 2004), and the short expression time frame (2 h). Together, these observations reveal *Mai1* as a new component of the plant immune system, acting between CC-NLR proteins and a MAPK signaling pathway that contributes to PCD and resistance to *P. syringae* pv. *tomato*.

The first indication that BSK1-like proteins might play a role in NTI came from the discovery that AtBSK1 occurs in a complex containing RPS2, although this observation has not

Fig. 4. *Mai1* acts upstream of M3K α and MKK2. Representative photographs of the cell death observed 5 days postagroinfiltration of the cell death elicitors SIM3K α or constitutive-active NtMKK2^{DD} into *N. benthamiana* leaves that were silenced using the indicated VIGS constructs. Expression of cell-death elicitors was driven by a 35S cauliflower mosaic virus promoter. Indicated are the number of spots observed with cell death compared with the total number of spots infiltrated from three replicates ($n = 22$ to 38). Photographs representative of the most common response in each treatment are shown.

been investigated further (Qi et al. 2011). Subsequent studies in *Arabidopsis* showed that both *bsk1-1* and *mapkkk5* mutants were more susceptible to DC3000 strains expressing AvrRpt2 or AvrRphB, which are recognized by the CC-NLRs RPS2 and RPS5, respectively (Shi et al. 2013b; Yan et al. 2018). However, the *bsk1-1* and *mapkkk5* mutants were similarly more susceptible to virulent DC3000, so the specific connection to NTI was unclear. In *N. benthamiana*, silencing of *NbBSK1*, *NbMKK2*, and *NbSIPK* blocked brassinosteroid-induced tobacco mosaic virus resistance (Deng et al. 2016). We tested whether AtBSK1 could restore PCD in *N. benthamiana* plants silenced with the *SIMai1-1* and *SIMai1-2* VIGS constructs and found that AtBSK1, though expressed well in these plants, could not restore PCD like synSIMai1, suggesting that SIMai1 and AtBSK1 have at least some distinct functions in *Arabidopsis* and tomato. However, it is also possible that the failure of AtBSK1 to complement *Mai1*-silencing is due to protein incompatibility between *Arabidopsis* and tomato. Our observations that Mai1 plays a role in PCD associated with several CC-NLR proteins and contributes to *P. syringae* pv. *tomato* resistance conferred by the Pto/Prf pathway support a role in NTI signaling, and the interaction of Mai1 with M3K α sheds lights on the mechanism for its function in enhancing MAPK activation leading to immunity-associated PCD.

Our finding that the SIMai1 TPR domain is necessary and sufficient for interaction with SIM3K α is consistent with the known role of TPRs in mediating protein-protein interactions for diverse biological processes including immune responses (SGT1 [Azevedo et al. 2006]) and hormone signaling (AtTRP1 [Lin et al. 2009]) in plants. The *Arabidopsis* *bsk1-1* mutant allele encodes a protein with an R443Q substitution in its TPR domain, yet this alteration did not affect the interaction of AtBSK1 with FLS2 (Shi et al. 2013b). We made the

comparable substitution (R430Q) in SIMai1 and observed no effect on the interaction of SIMai1 with SIM3K α in a yeast two-hybrid assay. Further studies are therefore needed to determine whether this residue plays a role in SIMai1 function. The SIMai1 TPR domain consists of approximately 100 amino acids and contains three TPR motifs. The amino acid sequences of the TPR domains for the seven tomato BSK proteins are remarkably similar, although there are 13 residues unique to SIMai1. Specificity for the interaction of the SIMai1 TPR domain with the KD of SIM3K α should facilitate the future identification of the specific TPR amino acids important for binding SIM3K α and may provide insight into how this interaction enhances MAPK activation.

The KDs of five of the nine tested SIM3Ks interacted with SIMai1. While this work focused on the role of SIMai1 in NTI, these multiple interactions might explain how Mai1 (and BSK1 proteins in other species) may contribute to both PTI and NTI responses, and future work will address whether SIMai1 plays a role in PTI. Some of the SIM3Ks that interacted with SIMai1 have been associated with diverse plant immune responses. For example, M3K α , in addition to contributing to CC-NLR pathways, also plays a role in resistance to *Plantago asiatica* mosaic virus in *N. benthamiana* (Hashimoto et al. 2012; Komatsu et al. 2010). In addition, the RLCK AtPBL27 interacts with the KD of AtM3K α and the C-terminal domain of AtMAPKKK5 (M3K γ), with the latter interaction providing a molecular link between chitin perception via the CERK1 receptor and downstream MAPK signaling (Yamada et al. 2016). M3K γ and M3K α also act in a M3K β > M3K α > M3K γ PCD-inducing signaling pathway in *N. benthamiana* (Hashimoto et al. 2012). Since TPR domains can promote self-association (Nyarko et al. 2007), it would be interesting to test if Mai1 self-association facilitates the interaction of M3K α and M3K γ

Fig. 5. Synthetic *SIMai1* complements cell death impairment in *Nicotiana benthamiana* plants silenced with the *SIMai1-1* and *SIMai1-2* virus-induced gene silencing (VIGS) constructs. **A**, Immunoblot analysis showing that proteins accumulate from the expression of constructs encoding synthetic *SIMai1* (*synSIMai1*) but not *SIMai1* in *N. benthamiana* plants silenced with the VIGS constructs *SIMai1-1* or *SIMai1-2*. SIMai1-cMyc fusion proteins were detected using an anti-cMyc antibody. Equal loading was confirmed by Ponceau S stain. **B**, Cell death induced by AvrPtoB₁₋₃₈₇ was recovered by SIMai1 proteins expressed from *synSIMai1* or *synSIMai1(K91M)* in wild-type *N. benthamiana* plants silenced with the VIGS constructs *SIMai1-1* or *SIMai1-2*. All constructs were agroinfiltrated into *N. benthamiana* leaves. Expression was driven with a 35S promoter. Photographs are representative of three independent experiments with similar results. Indicated are the number of spots observed with cell death compared with the total number of spots infiltrated from three replicates ($n = 19$ or 20). Photographs representative of the most common response in each treatment are shown.

in this pathway. In *Arabidopsis*, the M3K YODA regulates several immune responses, and a constitutive-active version of this protein confers broad-spectrum resistance to multiple pathogens, including *P. syringae* pv. *tomato* (Sopeña-Torres et al. 2018). We did not test the interaction of SIMai1 with the YODA

homolog from tomato (Soly08g081210), although SIMai1 did interact with a related protein, YODA2 (Soly03g025360). Interestingly, it was recently reported that the AtBSK1 TPR domain interacts with the KD of YODA and plays a role in embryonic patterning in *Arabidopsis* (Neu et al. 2019). SIMai1

Fig. 6. Overexpression of SIMai1 with *SIM3Kα* in *Nicotiana benthamiana* accelerates development of programmed cell death and increases activation of mitogen-activated protein kinases (MAPKs), and the SIMai1 myristoylation motif contributes to these responses. **A**, Host responses to agroinfiltration of *SIM3Kα* (full length), *SIM3Kα-KD*, or *SIM3Kα-KD-K231M* with *synSIMai1* or YFP (yellow fluorescent protein gene). Expression was driven using a 35S cauliflower mosaic virus promoter for the *SIMai1* and YFP constructs and by an estradiol-inducible system for the *SIM3Kα* constructs. Each image is from an individual infiltration and plant, shown at 48 and 72 h post-estradiol induction (hpi) of the *SIM3Kα* variants. Indicated are the number of spots that showed cell death compared with the total number of infiltrated spots from three replicates ($n = 28$). Photographs representative of the most common response in each treatment are shown. **B**, Immunoblot from coexpression of *SIM3Kα* and *synSIMai1* in leaves of *N. benthamiana*. Phosphorylated MAPKs in *N. benthamiana* (Nb p-MAPKs) were detected using an anti-pERK antibody. An anti-cMyc antibody was used to detect the synthetic SIMai1-cMyc constructs, and anti-hemagglutinin (HA) was used to detect the *SIM3Kα*-HA constructs. **C**, Immunoblot as in B but from coexpression of *synSIMai1* variants (G2A and K91M) with *SIM3Kα*.

did not interact with SIM3K ϵ which, like SIM3K α and SIMai1, acts downstream of multiple NLRs including Pto/Prf (Melech-Bonfil and Sessa 2010). M3K ϵ has been suggested to act in a parallel pathway and not redundantly to M3K α , and our data suggest it probably uses a different mechanism to activate MAPKs (Melech-Bonfil and Sessa 2010). A distinguishing amino-acid motif is not present in the KDs of the five SIMai1-interacting SIM3Ks, as compared with the four that did not interact with SIMai1, but the fact that the five interact with SIMai1 suggests they likely have a common structural feature. In the future, the interaction of SIMai1 with only a subset of the MEKK family of SIM3Ks should facilitate the identification of one or more features in the KD that are involved in the interaction with the SIMai1 TPR domain and may help reveal how this interaction enhances MAPK activation.

The interactions of SIMai1 with multiple tomato M3Ks, including some that do not have known roles in immunity, raise the possibility that SIMai1 (and BSK1-related proteins in other species, as supported by the interaction of BSK1 with YODA in *Arabidopsis* [Neu et al. 2019]) might have additional functions in other plant processes. In *Arabidopsis*, AtBSK1 is a substrate of the BRI1 receptor kinase that initiates the brassinosteroid signaling pathway; this pathway has been completely defined and does not involve a M3K (Clouse 2011). *Arabidopsis* plants with loss-of-function mutations in *BSK1* and rice plants with reduced expression of BSK1-2 do not have pronounced growth defects, although leaves of the *Arabidopsis bsk1-1* mutant are slightly narrower than in wild-type plants and an AtBSK1 genomic clone can complement this phenotype (Shi et al. 2013b; Sreeramulu et al. 2013; Yan et al. 2018). We have observed that *N. benthamiana* plants silenced with the *SIMai1-1* and *SIMai1-2* VIGS constructs are slightly smaller than unsilenced control plants and have slightly larger leaves. As part of this work, we generated a CRISPR-induced mutation in *SIMai1* in tomato and found that plants carrying homozygous *mail* mutations grew more slowly than wild-type plants and showed severe morphological defects with brittle, thin leaves that had distorted shapes (Supplementary Fig. S14). Experiments to test the immunity responses of the *mail* tomato plants were inconclusive because even mock inoculation caused the leaves to fall off. Interestingly, *mail* mutant tomato plants that happened to be in a greenhouse sprayed with the insecticide Overture developed severe necrosis and subsequent stunting, as compared with wild-type or *Mail/mail* heterozygous plants. We were only able to recover one *mail* mutant line, and additional characterization of multiple independent mutants will be needed to verify that the *mail* mutation is responsible for these phenotypes; however, our preliminary observations suggest that *SIMai1* has a role in development or stress responses, or both, in tomato, which is reminiscent of the dual roles of several other proteins in both immunity and development (Huot et al. 2014; Lin et al. 2013).

There are contrasting results in the literature about kinase activity of BSK proteins. We were unable to detect SIMai1 autophosphorylation or its phosphorylation of the generic kinase substrate myelin basic protein, using multiple in-vitro kinase assay conditions. BSK proteins lack key amino acids that are required for kinase activity and, thus, have been predicted to be pseudokinases with putative roles as scaffold proteins (Bayer et al. 2009; Grüter et al. 2013; Kwon et al. 2019; Sreeramulu et al. 2013). Although AtBSK1 also lacks these motifs, three papers report that AtBSK1 autophosphorylates and can transphosphorylate AtMAPKKK5 and that the ATP binding site was required for its role in resistance to a fungal pathogen and PTI (Shi et al. 2013b; Yan et al. 2018; Zhao et al. 2019). We attempted to reproduce the kinase activity reported for AtBSK1 but were unable to see activity under our kinase assay conditions or those used for AtBSK1 (Zhao et al.

2019). Similarly, a recent report describing the interaction of AtBSK1 with YODA was unable to detect kinase activity for AtBSK1 (Neu et al. 2019). The lack of SIMai1 kinase activity is consistent with other reports of the inability to detect kinase activity for six of the *Arabidopsis* BSKs (Bayer et al. 2009; Sreeramulu et al. 2013) and with our complementation experiments that showed that disruption of the canonical ATP binding site of SIMai1 did not impact its ability to restore PCD. Further experiments are needed to investigate the contrasting results of SIMai1 and AtBSK1 kinase activity.

Both our microscopy and complementation experiments suggest that SIMai1 function requires its localization to the cell periphery, at least for its involvement in the Pto/Prf pathway. Fatty acylation and targeting to the PM plays an important role in the function of many immunity-associated proteins (Boyle and Martin 2015; Boyle et al. 2016). AtBSK1 is localized to the PM, and substitutions in its putative myristoylation site (G2A) disrupt this localization and its association with FLS2 (Shi et al. 2013a and b). In the Pto/Prf pathway, both AvrPto and Pto have an N-terminal myristoylation site that is required for their function, and AvrPto is known to localize to the PM (Martin 2012); interestingly, AvrPto has been reported to interact with AtBSK1 (Xiang et al. 2011). It is possible that localization of SIMai1 to the cell periphery promotes its association with certain NLR protein complexes. We observed no interaction of SIMai1 with Pto in a yeast two-hybrid assay, although we were unable to test its possible interaction with Prf in these experiments, since Prf does not express well in yeast. If Mail does occur in NLR protein complexes, then it is likely SIMai1 is not localized exclusively to the PM, as AvrPtoB is not PM-localized and CC-NLRs are known to exist in multiple sub-cellular compartments (Qi and Innes 2013).

The SIMai1 interaction with SIM3K α and its ability to enhance MAPK signaling provide some initial insight into the mechanism of SIMai1 in the Pto/Prf pathway and open several avenues to further investigate early signaling events acting between NLRs and MAPK signaling. Our current data show that SIMai1 acts directly with SIM3K α but additional experiments are needed to further understand how SIMai1 functions to connect CC-NLR recognition of pathogen effectors to the MAPK signaling cascade (a proposed model is presented in Supplementary Figure S15). However, we demonstrate that Mail is a key player in CC-NLR mediated immunity. AtBSK1 has been found in a complex with RPS2 (Qi et al. 2011) and, as mentioned above, it is possible that SIMai1 resides in the Pto/Prf complex and perhaps in other CC-NLR complexes. Such an association could stabilize the complexes, facilitate NLR oligomerization, or promote the interaction of NLRs with other proteins, for example Prf with Pto and Fen. SGT1 interacts with Prf (Kud et al. 2013), and the association of the TPR domain in SGT1 and the SIMai1 TPR domain could play a role in SIMai1 function. The Epk1 kinase is a component of the Pto/Prf pathway that also acts upstream or with M3K α (Pombo et al. 2014). In addition, the NLR proteins NRC2 and NRC3 act in the Pto/Prf signaling pathway, although it is unknown whether they act upstream of MAPK signaling (Wu and Kamoun 2019; Wu et al. 2016, 2017). Investigation of the relationship of Mail to Epk1 and NRC2 and NRC3 is an important future goal for understanding the molecular links between host recognition of effectors and MAPK signaling.

MATERIALS AND METHODS

Yeast two-hybrid assays.

Protein interaction and β -galactosidase assays were performed as described previously (Oh et al. 2010). For the yeast two-hybrid screen using full-length Mail as the bait protein in

pEG202, a prey library generated from Rio Grande-PtoR tomato leaves inoculated with *P. syringae* pv. *tomato* DC3000, as previously described (Zhou et al. 1995), was used to screen for SIMail1 interacting proteins. Tomato BSK genes and M3K α -KD were analyzed in the GAL4 system using bait and prey vectors pGBKT7 and pGADT7, respectively. Further details may be found in the Supplementary Methods.

Phylogenetic analyses.

Sequence alignments were performed using MUSCLE (Edgar 2004) and maximum likelihood trees were generated with MEGA7 (Kumar et al. 2016).

Split luciferase complementation assay.

Transient protein expression and luciferase assays were performed as described (Teper et al. 2018). *Agrobacterium tumefaciens* strains carrying NLuc and CLuc constructs were infiltrated into leaves of *N. benthamiana*. Leaves coexpressing different constructs were examined for luciferase (LUC) activity 40 h after infiltration (before cell death occurred in the case of SIMail1 and M3K α). Quantitative LUC activity was determined by a Veritas Microplate Luminometer (Promega, Madison WI, U.S.A.) and reported as relative light units.

Virus-induced gene silencing (VIGS).

The TRV vector derivatives were transformed into *A. tumefaciens* GV2260 and prepared for infection as previously described (Chakravarthy et al. 2010). Cell death, transient expression, and bacterial growth assays were performed five-to-six weeks after agroinfiltration with TRV. To confirm silencing of the *NbMail* orthologs using the *SIMail-1* and *SIMail-2* VIGS constructs, semiquantitative RT-PCR was performed as in (del Pozo et al. 2004) using *NbEF1 α* -specific primers as a loading control.

Cell death assays.

The various cell death elicitors were agroinfiltrated in *N. benthamiana* TRV-VIGS plants as previously described (Oh et al. 2010). Expression of the cell death elicitors was induced with estradiol at 48 or 62 h post infiltration (Figs. 3, 4, 5, and 6). All cell death data were visually collected and scored blindly. Further details may be found in Supplementary Text.

In vitro protein kinase assays.

MBP- or GST- tagged protein plasmids were transformed into BL21 cells for in vitro protein expression. Details on the expression and purification of the proteins can be found in the Supplementary Methods. Kinase assays were performed for 30 min at room temperature in 20 μ L of kinase reaction buffer (50mM HEPES, pH 7.5, 10mM MgCl₂ and/or 10mM MnCl₂ and/or 10mM CaCl₂, and 3 μ g myelin basic protein) containing 2 μ Ci [γ -³²P], or were performed exactly as described in (Zhao et al. 2019), using 5 μ g of each of the proteins. Reactions were stopped by adding SDS-PAGE sample buffer. Samples electrophoresed on a 10% SDS-PAGE gel. Gels were dried using a gel dryer and radiolabeled proteins were visualized on autoradiography film or a phosphoimaging system.

Generation of synthetic Mail1 and sequence variants.

The synthetic *SIMail* sequence was initially designed using the Integrated DNA Technologies (IDT) Codon Optimization Tool, and individual nucleotides were then changed such that no fragment longer than 11 nucleotides was identical to the *SIMail* sequence. Synthetic *SIMail* variants were generated by PCR amplification using oligonucleotides encoding the

amino acid changes. Further cloning details can be found in the Supplementary Methods.

Immunoblots of transiently expressed proteins in *N. benthamiana*.

Total protein was extracted from *A. tumefaciens*-infiltrated leaves as previously described (Hind et al. 2016), and 10 μ g was loaded on SDS-PAGE before transferring to PVDF membrane (Merck Millipore). SIMail1-myc or synSIMail1-myc proteins were detected using anti-Myc antibodies (GenScript) and chemiluminescent ECL Plus substrate (Thermo Fisher Scientific). Phosphorylated (activated) MAPK proteins were detected using antiphospho-p44/42 MAPK T202/Y204 (pERK) antibodies (Cell Signaling). SIM3K α proteins were detected using anti-hemagglutinin antibodies (Krackeler). Membranes were stained with Ponceau S (Sigma Aldrich) to verify equal loading.

Bacterial population assays.

Six-week-old *SIMail-2*-silenced or control-silenced plants were infiltrated with *P. syringae* pv. *tomato* strains DC3000 Δ hopQ1, DC3000 Δ avrPto/ Δ avrPtoB/ Δ hopQ1, DC3000 Δ avrPto/ Δ avrPtoB, or DC3000 Δ avrPto/ Δ avrPtoB/ Δ hopQ1, using a 1:10,000 dilution at an optical density at 600 nm of 0.5 (approximately 1×10^4 CFU/ml). Three leaf discs (each 0.33 cm²) from each replicate plant were taken 2 h after infiltration (day 0) and 2 or 3 days later and were homogenized in 10 mM MgCl₂ to determine the bacterial populations via serial dilution plating. A two-tailed Student's *t* test was used to calculate the *P* values.

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AUTHOR RECOMMENDED RESOURCES

Boyce Thompson Institute *Nicotiana benthamiana* genomics resources: <https://btiscience.org/our-research/research-facilities/research-resources/nicotiana-benthamiana>
IDT Codon Optimization Tool: <https://www.idtdna.com/pages/tools>
Plant Genome Editing Database (PGED): <http://plantcrispr.org/cgi-bin/crispr/index.cgi>
Sol Genomics Network (SGN) database: <http://solgenomics.net>
Tomato Functional Genomics Digital expression (RNA-seq) experiment list database: <http://ted.bti.cornell.edu/cgi-bin/TFGD/digital/home.cgi>
SGN VIGS tool: <http://vigs.solgenomics.net>

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