Tomato Atypical Receptor Kinase 1 Is Involved in the Regulation of Preinvasion Defense1[OPEN]

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Tomato Atypical Receptor Kinase 1 (TARK1) is a pseudokinase required for postinvasion immunity. TARK1 was originally identified as a target of the Xanthomonas euvesicatoria effector protein Xanthomonas outer protein N (XopN), a suppressor of early defense signaling. How TARK1 participates in immune signal transduction is not well understood. To gain insight into TARK1’s role in tomato (Solanum lycopersicum) immunity, we used a proteomics approach to isolate and identify TARK1-associated immune complexes formed during infection. We found that TARK1 interacts with proteins predicted to be associated with stomatal movement. TARK1 CRISPR mutants and overexpression (OE) lines did not display differences in light-induced stomatal opening or abscisic acid-induced stomatal closure; however, they did show altered stomatal movement responses to bacteria and biotic elicitors. Notably, we found that TARK1 CRISPR plants were resistant to Pseudomonas syringae pathovar tomato strain DC3000-induced stomatal reopening, and TARK1 OE plants were insensitive to P. syringae pathovar tomato strain DC3118 (coronatine deficient)-induced stomatal closure. We also found that TARK1 OE in leaves resulted in increased susceptibility to bacterial invasion. Collectively, our results indicate that TARK1 functions in stomatal movement only in response to biotic elicitors and support a model in which TARK1 regulates stomatal opening postelicitation.

Pathogens have developed a number of different strategies to invade and multiply within host plant tissues, and plants have evolved countermeasures to combat encroaching pathogens. One of the first stages dictating antagonistic interactions between host and pathogen begins with the perception of microbial patterns called microbe-associated molecular patterns (MAMPs), which are detected by cell-surface-localized pattern recognition receptors (PRRs; Jones and Dangl, 2006). This response initiates signaling cascades that generate reactive oxygen species (ROS), activate mitogen-activated protein kinases, and transcribe defense-related genes aimed to limit pathogen growth and host invasion.

For leaf-associated bacteria, early recognition events often occur at the surface of the leaf and ports of entry into leaf tissue. Bacteria gain access to the nutrient-rich extracellular spaces within leaf tissue (the apoplast) by invading wounds and natural openings. One of the primary structures that gate access to the apoplast and restrict pathogen invasion are guard cell pores called stomata. Given that a primary role of stomata is to exchange CO2 and water with the environment (Kim et al., 2010), leaf tissues are vulnerable to microbial invasion during periods of transpiration and photosynthesis. Detection of bacterial MAMPs by PRRs within leaves ultimately leads to stomatal closure, a defense response referred to as stomatal immunity (Melotto et al., 2006; Zeng and He, 2010).

Most of the molecular mechanisms describing stomatal immunity against phytopathogenic bacteria come from studies using Arabidopsis (Arabidopsis thaliana) and Pseudomonas syringae as a model host-pathogen system. For example, PRR protein complexes in Arabidopsis have been shown to be of particular importance in stomatal immunity especially in the context of ROS production and signaling. The NADPH oxidase RESPIRATORY BURST OXI-DASE HOMOLOG D (RBOHD) plays a critical role in ROS production in leaves (Nühse et al., 2007; Zhang...
et al., 2007; Mersmann et al., 2010). RBOHD was also shown to be required for MAMP-induced stomatal closure (Mersmann et al., 2010; Macho et al., 2012). RBOHD is part of an important PRR complex involving the flagellin receptor FLAGELLIN SENSING2 (FLS2) and the receptor-like cytoplasmic kinase BOTRYTIS-INDUCED KINASE1 (BIK1; Li et al., 2014). MAMP perception through FLS2, as well as the EF-TU RECEPTOR (EFR), leads to RBOHD phosphorylation by BIK1 and this regulation of RBOHD activity induces stomatal closure and contributes to resistance to surface-inoculated bacteria (Kadota et al., 2014; Li et al., 2014). These studies highlight core Arabidopsis protein complexes involved in regulation of stomatal movement. By contrast, there are limited data describing protein complexes involved in this process outside of the Brassicaceae.

In addition to MAMP perception, the plant hormones abscisic acid (ABA), salicylic acid (SA), and jasmonic acid Ile (JA-Ile) play integral roles in regulating stomatal movement. ABA is known to induce stomatal closure and is required for P. syringae pathovar tomato strain DC3000 (Pst) and MAMP-induced stomatal closure in multiple plant species (Melotto et al., 2006; Du et al., 2014). In Arabidopsis, the ABA-deficient mutant aba3-1 does not close stomata in response to Pst, or to the bacterial elicitors flagellin (Flg22) and lipopolysaccharide (Melotto et al., 2006). A similar response has been observed with the tomato ABA biosynthesis mutant notabilis (not), where Pst-induced stomatal closure was not observed (Du et al., 2014). Treatment with the defense hormone SA has also been shown to induce stomatal closure (Khokon et al., 2011; Zeng et al., 2011). Arabidopsis mutants defective in SA biosynthesis (ics1 and eds5/sid1/sord3) and SA signaling (npr1) are compromised in bacterial- and MAMP-induced stomatal closure (Melotto et al., 2006; Zeng and He, 2010; Zeng et al., 2011). By contrast, JA-Ile, the most active form of JA produced in plants in response to wounding, promotes stomatal opening. Direct application of JA-Ile to dark-treated Ipomea tricolor stomata induced stomatal opening (Okada et al., 2009). Arabidopsis coronary insensitve1 (coi1) mutants, which lack the JA-Ile receptor, have constitutively smaller stomata apertures (Sheard et al., 2010; Panchal et al., 2016a). These pharmacological and genetic studies highlight the importance of ABA and SA as positive regulators of stomatal immunity, and of JA-Ile as a negative regulator.

Pathogen manipulation of stomata has been most extensively shown with Pst through the secretion of small molecules and delivery of effector proteins into host plant cells (Melotto et al., 2017). The Pst small molecule coronation (COR) has been well characterized for its role in inducing stomatal reopening. COR is a hormone mimic that closely resembles JA-Ile (Krumm et al., 1995; Staswick and Tiryaki, 2004; Melotto et al., 2006; Okada et al., 2009). Both JA-Ile and COR have been shown to bind to the Arabidopsis JA coreceptor COI1 (Katsir et al., 2008). When COR binds to COI1, downstream signaling leads to the induction of NAC transcription factors that repress SA biosynthesis genes and induce SA metabolism genes, thereby suppressing SA accumulation and promoting stomatal opening (Zheng et al., 2012; Du et al., 2014; Gimenez-Ibanez et al., 2017). Thus, COR functions as a bacterial virulence factor by interfering with stomatal immunity and SA-dependent defense responses.

In this study, we investigated the role of the pseudokinase Tomato Atypical Receptor Kinase1 (TARK1) in the regulation of stomatal movements and preinvasion immune responses in tomato plants in response to pathogen challenge. TARK1 is a Leu-rich repeat receptor-like kinase (LRR-RLK) belonging to the LRR XIIb RLK subfamily (Sakamoto et al., 2012). TARK1 was originally identified in a screen to identify targets of XopN, a Xanthomonas euvesicatoria (Xe) virulence factor secreted by the type III secretion system. TARK1 was shown to be required for tomato immunity and leaf symptom development in response to Xe infection. While the function of TARK1 has remained elusive, it is known to be localized to the plasma membrane and to possess a pseudokinase domain (Kim et al., 2009).

Pseudokinases like TARK1 are prevalent in many plant species, representing 13% of all Arabidopsis kinases and 20% of Arabidopsis RLKs (Castells and Casacuberta, 2007). There is growing evidence that pseudokinases in plants and animals play important roles in signaling cascades and can serve as scaffolding proteins that either directly or indirectly regulate protein-protein interactions (Langeberg and Scott, 2015). In plants, multiple pseudokinases have been associated with diverse biological processes ranging from the regulation of immune complex formation and signaling, to modulation of ABA-based signaling during seedling development (Lewis et al., 2013; Blaum et al., 2014; Halter et al., 2014a, 2014b; Kumar et al., 2017). Recent work has also shown that the LRR-RLK pseudokinase GUARD CELL HYDROGEN PEROXIDE-RESISTANT1 (GHR1) plays an important role in stomatal closure (Hua et al., 2012; Sierla et al., 2018).

Here we provide evidence linking TARK1 to stomatal immunity in tomato. Using a proteomics approach, we discovered that TARK1 interacts with tomato proteins predicted to be associated with stomatal movement and disease resistance signaling. Analysis of transgenic tomato mutant lines with altered TARK1 levels revealed that TARK1 is required for stomatal responses triggered by biotic elicitors, supporting a model in which TARK1 regulates stomata opening postelicitation. Our findings implicate TARK1 in both pre- and postinvasion immunity in tomato and describe components of putative TARK1-associated complexes operating during pathogen-triggered immunity.

RESULTS

TARK1 Interacts with Proteins Associated with Stomatal Movement

Previously, it was shown that TARK1 is involved in tomato disease resistance in response to Xe pathogenesis.
Reduction in TARK1 mRNA expression using a hairpin RNA construct led to enhanced susceptibility to *Xe* infection in postinvasion immunity assays (Kim et al., 2009). TARK1-silenced plants also presented fewer disease symptoms in response to *Xe* infection, indicating that TARK1 plays a role not only in immune signaling in response to *Xe* but also in disease symptom development. Considering that TARK1 plays a role in tomato immunity but lacks any detectable kinase activity in vitro (Kim et al., 2009), we hypothesized that TARK1 interacts with plasma membrane proteins, potentially PRRs, or other membrane-associated proteins to regulate and/or amplify immune signal transduction post-bacterial invasion.

To test this hypothesis, we used an immunoprecipitation (IP)-proteomics approach to purify and identify TARK1-associated protein complexes. In service to this, we generated TARK1 transgenic overexpression (OE) lines by transforming VF36 tomato (*Solanum lycopersicum*) plants with a P35S::TARK1-GFP construct containing the 35S Cauliflower Mosaic Virus promoter constitutively expressing TARK1 with a C-terminal GFP epitope tag (TARK1-GFP; Fig. 1A). The TARK1 OE line used in this study constitutively expresses TARK1-GFP in uninfected leaves (Fig. 1B).

To identify TARK1 immune complexes formed during pathogen-triggered immunity (PTI), TARK1 OE leaves were inoculated with a high dose of the *Xe* type-3 secretion system (T3SS) mutant *Xe* ΔhrcV. This strain lacks a key structural component necessary for the T3SS to deliver effector proteins from the pathogen to the host (Rossier et al., 1999). *Xe* ΔhrcV infection represents activation of PTI because tomato plants susceptible to wild-type *Xe* are now able to detect *Xe* pathogen-associated...
molecular patterns in the absence of Xe effector proteins and mount a stronger basal tomato defense response (Taylor et al., 2012). Notably, we detected more TARK1-GFP protein in TARK1 OE leaves infected with Xe ΔhrcV (Fig. 1B) using polyclonal peptide antibodies that recognize the putative juxtamembrane domain (amino acids 295–312), the region N-terminal to the pseudokinase domain (Fig. 1A). We also detected accumulation of the endogenous TARK1 protein in wild-type and TARK1 OE leaves infected with Xe ΔhrcV, but not in uninfected leaf tissue (Fig. 1B). These data reveal that TARK1 protein levels are regulated in tomato leaves in response to biotic stress.

Twenty-four hours following Xe ΔhrcV inoculation, TARK1-GFP was purified from infected TARK1 OE tomato leaves using GFP magnetic agarose beads. Wild-type leaves, or leaves overexpressing GFP alone, were used as controls. After isolation of TARK1-associated complexes, protein samples were analyzed using liquid chromatography tandem mass spectrometry analysis (LC-MS/MS). Of the peptides detected, we selected for candidate proteins that were enriched in the TARK1-GFP IP over the GFP control IP. We then selected for proteins whose mRNA abundance had increased during PTI (>1.8-fold increase). We determined this by utilizing a tomato RNA-sequencing data set that compared a high-dose Xe ΔhrcV infection to mock treatment (Stork, 2014). We also excluded proteins with known or proposed functions (Gene Ontology designations; Ashburner et al., 2000; The Gene Ontology Consortium, 2019) linked to chloroplast function and photosynthesis. Using these criteria, we identified 17 candidate proteins that associated with TARK1 during PTI (Supplemental Table S1).

Gene ontology enrichment analysis of the candidate tomato proteins did not reveal a significant enrichment in any biological process. When we ran the same analysis using the closest Arabidopsis homologs of the candidate proteins, there was a substantial enrichment in proton transmembrane transport. To expand our search, we bioinformatically mined available online datasets and literature to uncover potential biological functions associated with each tomato candidate and its closest Arabidopsis homolog. From these analyses, we found that the 17 candidates fell into three major categories—defense, membrane and vesicle transport, and mitochondrial function (Supplemental Table S1).

Of the defense related candidates, three proteins (plasma membrane H+-ATPase HA4 [Solyc07g017780], lipoxygenase LOX8 [Solyc08g029000], and LRR-RLK [Solyc04g074000; herein referred to as RLK15]) share homology with Arabidopsis proteins that regulate stomatal functions (Fig. 1C). Previous phylogenetic analysis utilizing tomato and Arabidopsis H+-ATPase sequences found that tomato HA4 clusters closest with Arabidopsis AHA1 and AHA2 (Liu et al., 2016), which are known to play important roles in guard cell movement during pathogen attack. Mutant Arabidopsis leaves with constitutively active AHA1 possess open stomata that do not close in response to ABA or PTI induced stomatal closure (Merlot et al., 2007; Liu et al., 2009). LOX8’s closest homolog in Arabidopsis is LIPOXYGENASE1 (LOX1) based on BLASTp (Altschul et al., 1997) and phylogenetic analysis, which places tomato LOX8 and Arabidopsis LOX1 in the 9-LOX subfamily (Upadhyay and Mattoo, 2018). Studies of Arabidopsis LOX1 indicate that it is required for resistance to Pst spray inoculation and MAMP-induced stomatal closure (Montillet et al., 2013). Tomato RLK15’s closest homolog in Arabidopsis is MDS1-INTERACTING RECEPTOR LIKE KINASE2 (MIK2). RLK15 and MIK2 are found within LRR-RLK subfamily XIIb as determined by sequence alignment of all tomato and Arabidopsis LRR-RLK kinase domain sequences (Sakamoto et al., 2012). In Arabidopsis, MIK2 has been shown to interact with the heteromeric guanine nucleotide-binding (G) protein β-subunit AGB1, which is required for RAPID ALKALINIZATION FACTOR1 (RALF1)-induced stomatal closure (Yu et al., 2018). In addition to interaction with MIK2, AGB1 was also shown to interact with RECEPTOR-LIKE KINASE1 (RLK1), the closest Arabidopsis homolog of TARK1.

To confirm interaction between TARK1 and HA4, RLK15, or LOX8, we fused the HA epitope to TARK1 (TARK1-3xHA) and GFP to the candidate interactors (GFP-HA4, RLK15-GFP, and LOX8-GFP) and then overexpressed pairs of proteins in Nicotiana benthamiana using Agrobacterium tumefaciens-mediated transient expression. Co-IP assays were then performed using GFP magnetic agarose beads. A LRR-RLK homologous to TARK1 (referred to as TARK1-Like or TARK1L; Solyc11g011020) was tagged and used as a control to assess specificity of the interactions. TARK1L is predicted to encode a RLK that shares 67% similarity to TARK1 at the amino acid level. It has five LRRs and the same sequence variation as TARK1 for the conserved catalytic residue in subdomain VIb in the kinase domain, indicating that it may be a pseudokinase (Kim et al., 2009). TARK1-3xHA was enriched in the co-IPs with GFP-HA4 or RLK15-GFP compared to the co-IPs with TARK1L-3xHA (Fig. 1D). However, LOX8 interaction with TARK1 was not detected using this assay (Fig. 1D).

Collectively, our biochemical studies suggest that TARK1 associates with multiple proteins during PTI (Supplemental Table S1). Confirmation of TARK1 interaction with HA4 and RLK15 provides additional evidence for complex formation inside plant cells and reveals a potential functional link between TARK1 and stomatal movement.

TARK1 Regulates Preinvasion Defense

To investigate TARK1’s potential role in stomatal movement, we generated transgenic tark1 mutant tomato lines in the VF36 background using CRISPR/Cas9-mediated genome editing. Mutants were produced using a single guide RNA targeting nucleotides...
94 to 113 of TARK1. The mutant allele used in this study has a 1-bp insertion after nucleotide 109 in the coding region of TARK1 that results in a predicted premature stop after amino acid 42 (Fig. 1A). This tark1 mutant is herein referred to as TARK1 CR. We were unable to detect the accumulation of the full-length TARK1 protein in TARK1 CR leaves inoculated with Xe ΔhrcV using polyclonal antibodies that recognize a TARK1 peptide located in the juxtamembrane domain (Fig. 1B). These data suggest that TARK1 CR is a loss-of-function mutant line.

Following the generation of the TARK1 CR lines, we determined whether TARK1 was involved in light-induced stomatal opening. We adapted an established leaf imaging assay that is used for Arabidopsis (Chitrakar and Melotto, 2010) to monitor stomatal apertures in intact tomato leaf tissue. Leaf pieces of the wild type, TARK1 CR, and TARK1 OE lines were floated on control buffer and then stomatal apertures were measured every hour for 6 h after the transition from dark to light. At 1 h post light exposure, all genotypes had closed stomata (Supplemental Fig. S1A), indicating that neither TARK1 CR nor TARK1 OE had constitutively open stomata. Maximal stomatal apertures were similar between genotypes and this was reached at 3 to 4 h post light exposure (Supplemental Fig. S1A). These data suggest that there is no difference in maximal stomatal aperture or light-induced stomatal opening among the genotypes.

We then treated leaves with ABA to determine whether there was a difference in stomatal closure responses. ABA is a key signaling hormone associated with both abiotic and biotic stress responses, and it induces stomatal closure (Zhang, 2014). We first determined the dose response of tomato stomata to ABA treatment to find the optimal concentration for our stomatal assays to capture hypo- or hyperclosure responses (Supplemental Fig. S2A). Leaf pieces were then floated on control buffer or buffer containing 10 µM ABA and stomatal apertures were measured 4 h post-treatment (Supplemental Fig. S1B). No significant differences in the extent of stomatal closure were detected, indicating that TARK1 is not involved in ABA-induced stomatal closure.

**TARK1 CR Mutants Are More Sensitive to SA and Flg22**

Considering that TARK1 was previously shown to be a positive regulator of tomato immunity in association

![Figure 2](image_url)
with bacterial infection (Kim et al., 2009), we examined stomatal movements in response to the defense hormone SA and two MAMPs, the bacterial flagellin peptide Flg22 and the fungal polysaccharide chitin. We chose chitin to determine whether TARK1 could be involved in general defense rather than bacteria-specific responses. Dose response assays were performed with wild-type stomata to optimize effective concentrations of elicitors for these assays (Supplemental Fig. S2, B–D). Following this, leaf pieces of wild-type, TARK1 CR, and TARK1 OE lines were floated on control buffer, 100 μM SA, and 10 μM Flg22 or 10 μg mL⁻¹ chitin, and stomatal apertures were measured 4 h posttreatment. TARK1 CR stomata were significantly more closed in response to both SA and Flg22 when compared to wild-type and TARK1 OE stomata (Fig. 2, A and B). Chitin induced stomatal closure in all three genotypes, and apertures were not significantly different (Fig. 2C). These data indicate that the TARK1 CR mutant is more sensitive to SA and Flg22, and that chitin perception is unaffected. Moreover, they suggest that TARK1 is involved in stomatal movement that occurs in response to elicitors detected during bacterial infection.

**Pst-Induced Stomatal Reopening Is Affected in Both TARK1 CR and TARK1 OE Plants**

To determine whether TARK1 is involved in bacteria-induced stomatal closure, leaf pieces of wild-type, TARK1 CR, and TARK1 OE lines were floated on stomatal buffer (control) or a 1 × 10⁸ colony-forming units (CFU) mL⁻¹ suspension of *Pst* wild type or *Pst* DC3118 (COR−) in water. This *Pst* COR− strain is commonly used to assay for defects in stomatal immunity, because *Pst* COR− is unable to produce coronatine, which is required for the *Pst* wild type to reopen stomata after their closure in response to *Pst* invasion (Melotto et al., 2006). In our assay system, we found that wild-type stomata typically close by 1 h post *Pst* treatment and reopen 4 h post *Pst* treatment in a COR-dependent manner (Fig. 3, A and B), consistent with previous reports (Melotto et al., 2006; Zhang et al., 2008; Montillet et al., 2013; Panchal et al., 2016b). TARK1 CR stomata closed in response to both the *Pst* wild type and *Pst* COR− at 1 h posttreatment; however, stomatal apertures were significantly smaller at 4 h posttreatment compared to the 1-h timepoint (Fig. 3B). Response to bacteria-delivered COR was impaired for TARK1 CR stomata. At both timepoints, TARK1 OE stomata treated with *Pst* wild type or *Pst* COR− were more open than wild-type stomata (Fig. 3, A and B), indicating that TARK1 OE stomata are not responsive to either treatment (Fig. 3A). To support these findings, we repeated the pathogen assays with two additional tomato lines—a TARK1 CR mutant line that is predicted to produce a premature stop after 88 amino acids, referred to as TARK1 CR line 2, and a TARK1 OE line that constitutively expresses TARK1-HA (Supplemental Fig. S3A; Taylor et al., 2012). TARK1 CR line 2 plants produced no detectable TARK1 protein, while TARK1-HA plants produced a greater amount of TARK1-HA than endogenous TARK1 protein (Supplemental Fig. S3, B and C). The stomatal responses for TARK1 CR line 2 and TARK1-HA OE leaves (Supplemental Fig. S3, D–G) were similar to those obtained for TARK1 CR line 1 and TARK1-GFP OE leaves, respectively (Fig. 3). Collectively, our stomatal assays using the wild type and four mutant tomato lines indicate that TARK1 plays a role in stomatal opening or regulating stomatal closure in response to *Pst* treatment.

**Stomata in Tomato Lines Close in Response to COR and JA-Ile**

To further investigate the COR-insensitive phenotype in the TARK1 CR line, we treated leaf pieces with a commercially available source of COR from Sigma. Apertures of stomata for all three genotypes treated with 0.1 ng μL⁻¹ of COR were significantly smaller than those treated with control buffer (Fig. 3C), suggesting that COR treatment alone induces stomatal closing in tomato. This finding was unexpected, as COR treatment (0.1–2.5 ng μL⁻¹) did not cause stomatal closing in Arabidopsis (Melotto et al., 2006). We tested different concentrations of COR (0.01–1 ng μL⁻¹) and detected COR-dependent stomatal closing 2 to 4 h posttreatment (Supplemental Fig. S4A). We also tested COR from a different source, Carol Bender Consulting, and that COR preparation also induced stomatal closure (Supplemental Fig. S4B). These data suggest that the tomato response to exogenous application of COR is different from those reported in Arabidopsis.

In addition, neither source of COR reopened stomata in response to ABA-induced closure (Supplemental Fig. S4B). Considering that COR is a JA-Ile mimic, we also tested whether JA-Ile caused stomatal closure. Wild-type stomatal apertures treated with 1, 10, or 100 μM JA-Ile were significantly smaller than the control aperture at 4 h posttreatment (Supplemental Fig. S4C). These assays show that wild-type tomato stomata are sensitive to COR or JA-Ile elicitation and result in stomatal closure under the conditions tested.

Despite these findings, we attempted to test whether COR treatment could induce stomatal reopening in wild-type stomata treated with *Pst* COR− in our assay. Previous studies in Arabidopsis have shown that exogenous application of COR is sufficient to complement the *Pst* COR− mutation (Panchal et al., 2016b; Ishiga et al., 2018). We confirmed this using COR from Sigma. Stomatal apertures in Arabidopsis leaves treated with *Pst* COR− were significantly smaller compared to those in leaves treated with *Pst* COR− plus exogenous COR (Supplemental Fig. S4D), consistent with previous results (Panchal et al., 2016b; Ishiga et al., 2018). By contrast, we found that exogenous application of COR to tomato leaves was unable to complement the *Pst* COR− mutation in our stomatal assays (Fig. 3D). In addition, we found that COR application suppressed stomatal

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reopening mediated by the Pst wild type (Fig. 3D). Our data thus indicate that exogenous application of COR produces a different and distinct phenotype in tomato compared to those established for Arabidopsis (Melotto et al., 2006; Desclos-Theveniau et al., 2012; Panchal et al., 2016b; Ishiga et al., 2018). It should be noted, however, that Pst-delivered COR induces stomatal reopening in tomato (Fig. 3B), which is consistent with previous studies.

Collectively, these data reveal that COR and (±)JA-Ile both trigger stomata closing in VF36 tomato plants. The fact that stomata from wild-type, TARK1 CR, and TARK1 OE plants all close in response to exogenous COR treatment suggests that all genotypes are sensitive to COR. However, because we were unable to observe COR-induced stomatal opening in our assays, we cannot rule out the possibility that TARK1 CR plants are resistant to COR-induced stomatal opening during Pst invasion.

Role of TARK1 in Pre- and Postinvasion Defenses against Pseudomonas

We next determined whether the differences in stomatal movement responses impacted bacterial invasion by assessing pre- and postinvasion infection outcomes. To study bacterial invasion of leaf tissue, we spray-inoculated individual leaves of wild-type, TARK1 CR, and TARK1 OE plants with a $1 \times 10^8$ CFU mL$^{-1}$ suspension of Pst wild type or Pst COR−. Bacterial growth and disease symptoms were monitored for multiple leaves from a single leaf of the same age on different plants to control for variation in disease progression dependent on leaf development.

![Figure 3](image-url)

**Figure 3.** Stomatal movement in leaves of wild-type (WT), TARK1 CRISPR (CR), and TARK1 OE plants in response to Pst and COR treatment. A and B, Stomatal apertures of leaf pieces from wild-type, TARK1 CR, and TARK1 OE plants were floated on water alone (control) or with Pst DC3000 (Pst WT) or the COR-deficient strain Pst DC3118 (Pst COR−) and measured at 1 (A) and 4 h (B) posttreatment. C, Stomatal apertures of leaf pieces floated on MES buffer (pH 6.15; control) or 0.1 ng mL$^{-1}$ COR in MES buffer at 4 h posttreatment. D, Stomatal apertures measured in control (MES buffer; black), COR (Sigma; blue), Pst wild type (in water; red), Pst wild type + COR (0.5 ng mL$^{-1}$; orange), Pst COR− (in water; gray), and Pst COR− + COR (purple) 4 h posttreatment. Approximately 60 to 100 apertures were measured per individual plant from the indicated genotypes per independent experiment. Box and whisker plots represent data aggregated from three independent experiments (n = 247–300 apertures; see Supplemental Table S3 for exact numbers). Whiskers represent the range, boxes represent the interquartile range split by the median, circles represent individual data points, and letters over bars represent statistical significance determined by one-way ANOVA (P < 0.05).
TARK1 OE leaflets infected with Pst wild type had a significantly higher number of bacteria and greater disease symptom development compared to similarly infected wild-type leaflets at 4 dpi postinoculation (Fig. 4, A and B). TARK1 CR leaflets infected with Pst wild type had titers of bacteria and extent of symptom development similar to those observed in infected wild-type leaflets at 2 and 4 dpi post spray (Fig. 4, A and B; Supplemental Fig. S5). TARK1 OE leaflets also harbored significantly more Pst COR—bacteria at 4 dpi when compared to wild-type leaflets. These data indicate that OE of TARK1 enhances susceptibility to Pst epiphytic growth and/or invasion.

We also utilized the TARK1 CR and TARK1 OE lines to study postinvasion defense in tomato leaves following Pst challenge. Individual leaflets of wild-type, TARK1 CR, and TARK1 OE leaves of the same age were hand-inoculated with a 1 × 10^4 CFU mL^-1 suspension of Pst wild type and Pst COR— and bacterial titers then were measured at 4 dpi. For hand-inoculated leaves, both TARK1 OE and TARK1 CR leaflets harbored similar titers of Pst wild type and Pst COR— at 4 dpi when compared to the infected wild type (Supplemental Fig. S6A). These data indicate that TARK1 does not affect resistance to Pst infection within the apoplast.

Role of TARK1 in Pre- and Postinvasion Defense against Xanthomonas

Considering that TARK1 is involved in resistance to Xe infection (Kim et al., 2009), we determined whether TARK1 plays a role in preinvasion resistance to Xe infection. Wild-type, TARK1 CR, and TARK1 OE plants were infected with Xe using spray- and hand-inoculation procedures similar to those described above for Pst infections. For spray-inoculated leaves, only TARK1 OE leaflets sprayed with Xe wild type had a significantly higher number of bacteria at 12 dpi compared to similarly infected wild-type leaflets (Fig. 4C). In terms of disease symptoms, wild-type leaves sprayed with Xe wild type developed more evenly distributed bacterial spot symptoms (i.e. necrotic lesions) compared to TARK1 CR leaves (Fig. 4D) despite both genotypes having similar bacteria titers (Fig. 4C). By contrast, the TARK1 OE leaves containing the highest titer of Xe wild type (Fig. 4C) developed bacterial spot lesions in dense clusters that were not uniformly distributed (Fig. 4D). These data indicate that reducing or overexpressing TARK1 protein levels in tomato leaves impacts the development and severity of bacterial spot disease symptoms.

For hand-inoculated leaves, individual leaflets of wild-type, TARK1 CR, and TARK1 OE leaves of the same age were hand-inoculated with a 1 × 10^5 CFU mL^-1 suspension of Xe wild type or Xe ΔxopN and bacterial titers were measured at 12 dpi. The titer of Xe wild type was significantly higher than the titer Xe ΔxopN in the wild type (Supplemental Fig. S6B), consistent with our prior work demonstrating that XopN is a virulence factor required for maximal Xe growth in VF36 tomato leaves (Roden et al., 2004; Kim et al., 2009; Taylor et al., 2012). Notably, the titer of Xe ΔxopN was
significantly higher in TARK1 CR leaflets, but not in TARK1 OE leaflets, compared to similarly infected wild-type leaflets (Supplemental Fig. S6B). These data show that TARK1 is required for restriction of *Xe* ΔxopN growth in the extracellular spaces of leaf tissue (i.e. postinvasion or apoplastic immunity) and that OE of TARK1 does not enhance this resistance under the conditions tested. These pre- and postinvasion studies reveal that OE of TARK1 leads to enhanced *Xe* invasion and that loss of TARK1 function impairs apoplastic immunity in the absence of XopN.

**DISCUSSION**

The goal of this study was to further investigate the role of the pseudokinase TARK1 in tomato immunity. Previous work uncovered a role for TARK1 in the positive regulation of postinvasion immunity (also referred to as apoplastic immunity) in tomato leaves in response to bacterial infection (Kim et al., 2009). We hypothesized that TARK1 might interact with PRRs or other membrane-associated proteins in leaf cells to regulate and/or amplify defense signal transduction during PTI. By purifying TARK1 complexes formed during PTI (Fig. 1C), we were able to identify several TARK1-interacting proteins and link TARK1 function to preinvasion immunity. Two of the proteins confirmed to interact with TARK1, plasma membrane H^+-ATPase HA4 and LRR-RLK RLK15, are predicted to be involved in stomatal function (Fig. 1D). By using loss-of-function and TARK1 OE lines, we now provide evidence that, in addition to its function in apoplastic immunity, TARK1 plays a role in the regulation of stomatal movement during bacterial infection.

While TARK1 is not required for light-induced stomatal opening or ABA-induced stomatal closure in tomato leaves (Supplemental Fig. S1), it is required for stomatal movement in response to bacterial invasion. TARK1 CR stomata are hypersensitive to Flg22- and SA-induced stomatal closure (Fig. 2, A and B). Moreover, TARK1 CR stomata close in response to *Pst* but do not reopen in response to *Pst*-delivered COR (Fig. 2B). This suggests that TARK1 CR stomata are either insensitive or resistant to COR. TARK1 CR leaves were not altered in their susceptibility to *Pst* at 2 or 4 d post-spray inoculation (Fig. 4A; Supplemental Fig. S5), indicating that the reduction in stomatal apertures measured during elicitation did not change infection outcomes. This suggests that reduced stomatal apertures triggered by *Pst* are not narrow enough to restrict bacterial entry over the course of the infection. By contrast, OE of TARK1 enhances *Pst* invasion, in a COR-independent manner, resulting in higher titers within the leaf tissue and, as a consequence, more disease symptoms (Fig. 4B). These latter results indicate that lack of closure of stomata in response to *Pst* or *Pst* COR– treatment (Fig. 3B) impacts the host’s ability to resist bacterial invasion. Our data suggest that TARK1 functions either as a positive regulator of stomatal opening or a negative regulator of stomatal closure.

We were unable to test the possibility that TARK1 may be involved in COR- or JA-Ile-induced stomatal reopening. We found that tomato stomata close in response to both COR and JA-Ile, which is contradictory to what others have shown in Arabidopsis and tomato (Melotto et al., 2006; Okada et al., 2009; Ortigosa et al., 2019). We confirmed that our COR stock elicited the expected stomatal responses in Arabidopsis (Supplemental Fig. S4D), validating that our elicitation conditions and imaging methods captured known stomatal aperture changes reported in the literature. It is important to note that there is controversy in the field as to whether the JA pathway promotes stomatal opening or closure (Sühi et al., 2004; Munemasa et al., 2007; Speth et al., 2009; Montillet et al., 2013). Although there is one study showing that JA-Ile treatment causes stomatal opening (Okada et al., 2009), we were unable to reproduce this finding using similar concentrations of JA-Ile (Supplemental Fig. S4). A major difference in our stomatal assay is that we image tissue directly and do not perform epidermal leaf peels. It is possible that differences in stomatal phenotypes may be dependent on the growth conditions, species, and/or cultivars tested.

While regulation of stomatal closing in response to biotic and abiotic stresses is well understood (Murata et al., 2015), less is known about the regulation of stomatal opening. Most of our knowledge comes from the study of blue light-induced stomatal opening. Blue light perception by phototropin receptors leads to the initiation of signaling cascades that eventually lead to activation of the plasma membrane H^+-ATPase AHA1 via phosphorylation of key residues and interaction with a 14-3-3 protein (Shimazaki et al., 2007; Hayashi et al., 2010; Yamauchi et al., 2016). Activation of AHA1 causes membrane hyperpolarization (Shimazaki et al., 2007; Marten et al., 2010) and activation of K^+ channels, which alters ion levels within the cell (Lebaudy et al., 2008; Kim et al., 2010). This causes a decrease in water potential and water uptake, triggering stomatal opening (Inoue et al., 2010; Marten et al., 2010). Considering that TARK1 interacts with the putative H^+-ATPase HA4, we hypothesize that TARK1 may be regulating HA4 activity or interaction with other HA4-associated proteins at the plasma membrane to regulate stomatal apertures in response to bacterial invasion.

For biotic interactions, the l-type lectin receptor kinase V.5 (LecRK-V.5) was shown to be a negative regulator of stomatal closure (Desclos-Theveniau et al., 2012), revealing that plants possess mechanisms to reverse bacteria-induced stomatal closure to maintain homeostasis. Genetic evidence indicates that LecRK-V.5 regulates stomatal immunity upstream of ROS production. LecRK-V.5 mutants have constitutively smaller stomatal apertures in the absence of elicitation. Also, LecRK-V.5 OE stomata close in response to *Pst* COR– and then reopen (Desclos-Theveniau et al., 2012). While both LecRK-V.5 and TARK1 mutants are affected in stomatal opening in response to bacteria; the phenotypes are distinct. This indicates that the role of
TARK1 in regulation of stomatal movement is likely via a different mechanism.

How TARK1 functions as a pseudokinase to participate in defense signaling is not yet clear. A few plant pseudokinases have been shown to serve as decoy substrates for pathogen enzymes playing key roles in pathogen recognition and activation of effector triggered immunity (Lewis et al., 2013; Wang et al., 2015). Other pseudokinases serve as scaffolding proteins to mediate protein-protein interactions. Interestingly, LRR-RLK pseudokinase GHR1 is required for stomatal closure through the activation of SLAC1 (Hua et al., 2012), which is thought to occur via GHR1-mediated protein interactions (Sierla et al., 2018). It is possible that TARK1 may be serving as a scaffold to recruit other proteins at the plasma membrane that have a direct influence over the regulation of stomatal movement. For instance, TARK1 may function as a scaffold that facilitates the interaction of the H+–ATPase HA4 with another protein that either activates or inhibits HA4’s activity. One candidate may be TTF1, a 14-3-3 protein previously shown to be a target of the Xe effector protein XopN. Based on in vitro biochemical data, Taylor et al. (2012) proposed that XopN promotes interaction between TARK1 and TTF1 to interfere with one or both of their functions. Alternatively, but not mutually exclusively, TARK1 could serve as a substrate for kinases like the LRR-RLK RLK15. Differential phosphorylation of TARK1 could facilitate the assembly or disassembly of TARK1 protein complexes involved in pre- and postinvasion immunity. Future work will be aimed at elucidating the role(s) of the TARK1-associated complexes identified in this work to understand the underlying mechanisms by which this pseudokinase coordinates immune signaling in tomato.

Taken together, our findings establish a role of TARK1 in both pre- and postinvasion immunity. For preinvasion immunity, OE of TARK1 in tomato leaves affects stomatal immunity and leads to enhanced bacterial invasion. This phenotype does not require bacteria-delivered COR. In the absence of TARK1, stomata are more sensitive to biotic elicitors and play a role in preinvasion defense after bacterial invasion. This work provides avenues to elucidate the role of this pseudokinase in the regulation of stomatal opening during PTI.

**MATERIALS AND METHODS**

**Plant Constructs, Transformation, and Mutagenesis**

To generate TARK1-GFP and GFP OE transgenic lines, pGWB5(TARK1-GFP, Kim et al., 2009) and pGWB5(GFP, Nakagawa et al., 2007) were mobilized into Agrobacterium tumefaciens strain LBA4404 and strains were used to transform the VF36 tomato (Solanum lycopersicum) line using standard methods (McCormick, 1991). TARK1-HA OE lines were previously published (Taylor et al., 2012).

To generate the TARK1 CRISPR mutant, CRISPR/Cas9 sites were selected by CRISPR-P 2.0 (http://chi.hszu.edu.cn/CRISPR2/; Liu et al., 2017). To generate sgRNA constructs for TARK1, sgRNAs were PCR amplified from pDONR207(AHL5/single-guide RNA [sgRNA]) with attL1 and attL2; from Jeffery L. Dangl) using primer sets sgRNAfor/sgRNArev. PCR products were self-ligated and recombined into pMIDC832x35sCas9-HA-NLS with Gateway cassette; Jeon et al., 2020) using LR clonase II (ThermoFisher). Final plasmids were transformed into Agrobacterium LBA4404 and strains were used for tomato transformations (McCormick, 1991). Homozygous mutant T1 plants were selected using PAGE-based genotyping (Zhu et al., 2014). To confirm mutations, CRISPR/Cas9 target sites were PCR amplified from genomic DNA using the primer set TARK1forrev and products were sequenced by the Sanger method (Genewiz).

For transient protein OE, TARK1 interacting proteins were amplified by PCR using their respective primer sets (Supplemental Table S2) and cloned into pENTR/D-TOPO, then recombined into the pEAG-QWB85 or pEAG-QWB86 destination vector via recombination reaction, creating pEAG-QWB86(HA4), pEAG-QWB85(RLK15), and pEAG-QWB85(LOX8). To make GFP fusion constructs in the pEAG vector (Sainsbury et al., 2009), gateway cassette-GFP or GFP-gateway cassette regions were PCR amplified from pGWB5 or pGWB6 (Nakagawa et al., 2007) using PCR primer sets GWB5for/GWB5rev or GWB6for/GWB6rev, respectively. PCR products were digested with EcoRV and NruI + EcoRV, respectively, and cloned into NruI + Stul sites in pEAG-HT to generate pEAG-QWB5 or pEAG-QWB6.

**TARK1 Peptide Antibody Production**

A synthetic peptide (ATEHNHDEVDIFSDKKVRV) corresponding to residues 295 to 312 in the juxtamembrane domain of TARK1 was synthesized and conjugated to keyhole limpet hemocyanin carrier protein and used to generate polyclonal antisera from rabbits (Covance).

**Bacterial Strains and Culture Conditions**

Strains used in this study were as follows: Pseudomonas syringae pathovar tomato strain DC3000 (Pst wild type), strain Pst DC3118 (the coronatine-deficient mutant, COR–), Xanthomonas euvesicatoria 85-10 (Xe wild type), Xe ΔhrcV (type-3 secretion-deficient mutant), Xe ΔhrcF (type-3 secretion-deficient mutant), and Xe ΔxopN. Pst and Xe strains were grown on nutrient yeast glycerol agar (Turner et al., 1984) at 28°C. Pst DC3000, Xe wild type, Xe ΔhrcV, and Xe ΔhrcF antibiotic selection was 100 μg mL−1 rifampicin (Rif) while Pst DC3118 was 100 μg mL−1 rif + 50 μg mL−1 kanamycin and Xe ΔxopN was 100 μg mL−1 Rif + 50 μg mL−1 spectinomycin.

**Bacterial Infection Assays**

For spray inoculation assays, a 1 × 108 CFU mL−1 suspension of Pst wild type or COR– (in 1 mM MgCl2) or Xe (in 10 mM MgCl2) with 0.02% (v/v) silwet L-77 (Helena Chemical Company) was sprayed on leaves until they were dripping and plants were then placed in chambers at high humidity (>95%) for 48 h. Plants were kept in a glass house under 16 h of light/day at 28°C. Leaflets from the same leaf were used for each experiment. For each strain analyzed, four leaf discs (0.5 cm2) per treatment per time point were collected from one leaflet, pooled, ground in 1 or 10 mM MgCl2, and then spotted on nutrient yeast glycerol agar plates in triplicate to determine the bacterial titer in each sprayed leaflet. Three biological replicates (i.e. two leaflets per plant and three plants per genotype) were used per experiment. For hand-inoculation assays, leaves were hand-inoculated with either Pst strains (1 × 108 CFU mL−1 in 1 mM MgCl2) or Xe strains (1 × 108 CFU mL−1 in 10 mM MgCl2) using a needle-less syringe. Infected plants were kept in glasshouse under 16 h of light/day at 28°C. Bacterial growth was measured as described above.
Transient Protein Expression in Nicotiana benthamiana

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10 mM KCl) for 3 h under light to allow stomata to fully open, as described (Melotto et al., 2006; Chitrakar and Melotto, 2010). Leaf pieces were then floated on 10 μM ABA (Sigma), 100 μM SA (Sigma), 10 μM Flg22 peptide (Protein and Nucleic Acid Facility, Stanford University), COR (purified from P. syringae pathovar glycinea; Sigma and Carol Bender Consulting) or (±)JA-ile (Cyamine Chemical) in stomatal buffer or 1 x 10^4 CFU mL^-1 of Pst DC3000 or Pst DC3118 (Zhao et al., 2003) in water (Melotto et al., 2006). ±JA-ile is a mixture of two of possible stereoisomers of JA-ile: (1R,2R) and (1S,2S). All treatments involving ABA contained 0.05% (v/v) ethanol and COR contained 0.0025% (v/v) MeOH. At the indicated time points, leaf pieces were removed to a microscope slide containing sterilized water and observed under a fluorescent microscope (Leica DM5000 B). Widths of stomatal apertures were measured using ImageJ software (Tsai et al., 2011). Completely closed stomata were reported as a value of 1 μm.

Statistical Analysis

Each experiment was conducted at least two times unless stated otherwise. Statistical significances were based on one-way ANOVA followed by Tukey’s honestly significant difference (HSD) mean-separation test using SPSS Statistics for Macintosh version 25.0 (IBM).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: NM_001247651.2 (TARK1), NM_001324146.1 (HA4), NM_001372087.1 (NRC4a), XM_003121863.5 (RLK15), XM_001279325.2 (GAPDH), XM_004234937.4 (Heat shock protein), XM_004244842.4 (LOX8), NM_001247220.2 (Calnexin-like protein), XM_004230682.4 (Coatomer subunit protein1), XM_004230813.4 (Coatomer subunit protein2), NM_001279338.2 (Mitochondrial phosphate carrier protein), XM_004236916.3 (ATP synthase subunit β), NM_001306138.1 (Mitochondrial ADP/ATP carrier protein), XM_004235953.4 (α/β hydrolase fold protein), NM_001321342.1 (Cys synthase protein), XM_004256042.4 (Ubiquinol-cytochrome c reductase iron-sulfur subunit); and XM_004246336.4 (Glutathione S-transferase).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Light-induced stomatal opening and ABA-induced stomatal closure in wild-type, TARK1 CR, and TARK1 OE leaves.

Supplemental Figure S2. Characterization of ABA-, SA-, Flg22-, and chitin-induced stomatal closure in wild-type tomato leaves.

Supplemental Figure S3. Stomatal movement in the TARK1 CR mutant line 2 and TARK1 OE (TARK1-HA) leaves behave similarly to TARK1 CR mutant line 1 and TARK1 OE (Fig. 3) leaves.

Supplemental Figure S4. Characterization of the effects of COR treatment on stomatal apertures in tomato and Arabidopsis leaves.

Supplemental Figure S5. Phenotypes of wild-type, TARK1 CR, and TARK1 OE plants 2 d after bacterial spray inoculation.

Supplemental Figure S6. Apoplastic immunity in wild-type, TARK1 CR, and TARK1 OE plants.

Supplemental Table S1. List of candidate TARK1 interacting proteins identified by MS.

Supplemental Table S2. Primer sequences used for experimental procedures.

Supplemental Table S3. Number of stomata apertures measured for each figure.

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Communoprecipitation Assays

IP of TARK1-GFP or GFP tagged TARK1 interacting proteins was performed as described, with modifications (Kadota et al., 2016). Tomato TARK1-GFP and GFP OE leaves were hand-inoculated with a suspension (1 x 10^6 CFU mL^-1) of Xe d Alevim in 10 mL MgCl2. Tissue was collected 24 h later, frozen, and ground in liquid N2. Ground tissue was added to extraction buffer at a 3:1 ratio. Final concentrations of buffer components were 150 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1.5 mM Na2VO4, 10 mM dithiothreitol, 1 mM Na2MoO4, 1.5 mM NaF, 1 mM EDTA, 1 mM protease inhibitor cocktail (Sigma), 0.5% (v/v) IGEPAL (detergent), and 1 mM phenylmethylsulfonyl fluoride. Tissue was solubilized in buffer at 4°C with rotation for 1 h. Samples were spun down at 15,000g at 4°C for 20 min. The supernatant was filtered and incubated with 50 μL of GFP TRAP magnetic agarose beads (Chromotek) at 1.5 h at 4°C with rotation. Beads were removed and washed with extraction buffer lacking detergent. Proteins were eluted with 0.2 mL Gly (pH 2.5). Experiments were performed in triplicate. Wild-type plants were used as a control in TARK1-GFP IPs 1 and 2, and GFP OE plants were used as controls for TARK1-GFP IP 3.

For validation of TARK1 interaction with candidate proteins, TARK1-3xHA or TARK1-Like-3xHA (control) were transiently expressed with GFP-HA, RLK15-GFP, or LOX8-GFP in N. benthamiana leaves and then purified as described above.

Mass Spectrometry

Mass spectrometry (MS) was performed at the Stanford University MS Facility (http://mass-spec.stanford.edu). Protein was precipitated using four volumes of cold acetone at −80°C overnight. The supernatant was removed, and the resulting pellet was reconstituted and reduced with 10 μL dithiothreitol at 55°C for 30 min. Proteins were alkylated with 30 μL acrylamide for 30 min at room temperature. Digestion was performed using Trypsin/LysC (Promega) overnight at 37°C, and the resulting peptides were desalted, dried, and then reconstituted.

Peptides were analyzed by liquid chromatography (LC) using a NanoAcuity UPLC (Waters) followed by MS using an Orbitrap Elite MS (Thermo Scientific) equipped with a captive spray emitter source (Michrom Bioresources). For a typical LC/MS experiment, a flow rate of 450 nL min^-1 was used, where mobile phase A was 0.2% (v/v) formic acid in water and mobile phase B was 0.2% (v/v) formic acid in acetonitrile. Analytical columns were prepared in-house with an internal diameter of 100 μm packed with Dr. Maisch 1.8-μm C18 stationary phase to a length of ∼20 cm. Peptides were directly injected into the analytical column using a gradient (2% to 45% B, followed by a high-B wash) of 80 min. MS was operated in a data-dependent fashion using collision-induced dissociation fragmentation for MS/MS spectra generation collected in the ion trap.

For data analysis, the RAW data files were checked using Preview (Protein Metrics) to verify the success of injection and sample quality. They were then processed using Byonic v2.6.49 (Protein Metrics) to identify peptides and infer proteins using the S. lycopersicum database from Uniprot, including isoforms. Proteolysis with Trypsin/LysC was assumed to be fully specific with up to two missed cleavage sites. Precursor mass accuracies were held within 1 ppm with fragment ions held within 0.4 D. Proteins were held to a false discovery rate of 1%, using standard approaches described previously (Elias and Gygi, 2007).

Stomatal Assays

Leaf pieces were cut from leaflets of fully expanded leaves of 4- to 5-week-old tomato plants and floated on stomatal buffer (25 mM MES-KOH [pH 6.15] and 10 μM Cl-) for 3 h under light to allow stomata to fully open, as described (Mudgett et al., 2000). Tomato tomatoes were hand-inoculated with a suspension (0.8 x 10^6 CFU mL^-1) of two strains in induction media. Plants were incubated at room temperature under continuous low light for 2 d.

Statistical Analysis

Each experiment was conducted at least two times unless stated otherwise. Statistical significances were based on one-way ANOVA followed by Tukey’s honestly significant difference (HSD) mean-separation test using SPSS Statistics for Macintosh version 25.0 (IBM).

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