Xanthomonas T3S Effector XopN Suppresses PAMP-Triggered Immunity and Interacts with a Tomato Atypical Receptor-Like Kinase and TFT1

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XopN is a virulence factor from Xanthomonas campestris pathovar vesicatoria (Xcv) that is translocated into tomato (Solanum lycopersicum) leaf cells by the pathogen’s type III secretion system. Xcv △xopN mutants are impaired in growth and have reduced ability to elicit disease symptoms in susceptible tomato leaves. We show that XopN action in planta reduced pathogen-associated molecular pattern (PAMP)-induced gene expression and callose deposition in host tissue, indicating that XopN suppresses PAMP-triggered immune responses during Xcv infection. XopN is predicted to have irregular, α-helical repeats, suggesting multiple protein–protein interactions in planta. Consistent with this prediction, XopN interacted with the cytosolic domain of a Tomato Atypical Receptor-Like Kinase1 (TARK1) and four Tomato Fourteen-Three-Three isoforms (TFT1, TFT3, TFT5, and TFT6) in yeast. XopN/TARK1 and XopN/TFT1 interactions were confirmed in planta by bimolecular fluorescence complementation and pull-down analysis. Xcv △xopN virulence defects were partially suppressed in transgenic tomato leaves with reduced TARK1 mRNA levels, indicating that TARK1 plays an important role in the outcome of Xcv–tomato interactions. These data provide the basis for a model in which XopN binds to TARK1 to interfere with TARK1-dependent signaling events triggered in response to Xcv infection.

INTRODUCTION

Plant immunity to bacterial pathogens requires a complex detection and signaling network. Insight to key components of this network has come from the study of early signaling events that occur during Pseudomonas syringae–Arabidopsis thaliana interactions (Jones and Dangl, 2006). Early host immune responses involve the perception of the invading microbe and the activation of specific defense signal transduction pathways that lead to local and systemic resistance in plants (Durrant and Dong, 2004). Pattern recognition receptors (PRRs) at the host cell surface recognize conserved molecular structures on the extracellular microbe (Zipfel, 2008). These structures are referred to as pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (Zipfel, 2008). Examples of bacterial PAMPs include flagellin (Felix et al., 1999) and elongation factor EF-Tu (Kunze et al., 2004), which activate the leucine-rich repeat transmembrane receptor kinases FLS2 (for FLAGELLIN SENSITIVE2) and EFR (for EF-Tu RECEPTOR) (Chinchilla et al., 2006; Zipfel et al., 2006), respectively. PRR activation stimulates signaling that leads to the generation of reactive oxygen species, the induction of mitogen-activated protein kinase (MAPK) cascades, the modulation of host gene transcription, and the deposition of callose at the plant cell wall (Brown et al., 1995; Asai et al., 2002; Torres et al., 2002; Hauck et al., 2003; Tao et al., 2003). These host responses are associated with plant immunity and are referred to as PAMP-triggered immunity (PTI) (Jones and Dangl, 2006).

Phytopathogenic bacteria (e.g., Pseudomonas, Xanthomonas, Ralstonia, and Erwinia) evolved the type III secretion (T3S) system to dampen basal defense responses (Lindgren et al., 1986; Bonas et al., 1991; Van Gijsegem et al., 1995; Ham et al., 1998). The T3S system is required for host infection by these pathogens and is widely conserved among bacterial pathogens (Alfano and Collmer, 2004; Coburn et al., 2007). The T3S system allows the secretion and translocation of effector proteins across the bacterial and host cell membranes (Troisfontaines and Cornelis, 2005). The function of most T3S effectors is not known; however, they are critical factors required for bacterial growth and colonization in distinct eukaryotic hosts.

Recent biochemical studies revealed that several P. syringae T3S effectors contribute to pathogen virulence by suppressing...
PTI (Göhre and Robatzek, 2008). AvrPto blocks PTI by binding to and inhibiting FLS2 and EFR kinase activity (Xiang et al., 2008). AvrPtoB, an E3 ubiquitin ligase (Abramovitch et al., 2006), promotes the degradation of FLS2 (Göhre et al., 2008), whereas both AvrPtoB and AvrPto target Arabidopsis (At) BAK1 (for BRI1-ASSOCIATED RECEPTOR KINASE1), a positive regulator of FLS2 signaling (Shan et al., 2008). HopM1 promotes proteasome-mediated degradation of At MIN7, an ADP ribosylation factor guanine nucleotide exchange factor, resulting in the inhibition of vesicle trafficking that is necessary for the host to mount the polarized cell wall–based defense (Nomura et al., 2006). HopU1 is a mono-ADP-ribosyltransferase that modifies the Arabidopsis RNA binding protein GRP7, interfering with RNA metabolism and the activation of PTI (Fu et al., 2007). Finally, HopAI1 is a unique phosphothreonine lyase that suppresses Arabidopsis MAPK signaling controlling PTI by dephosphorylating the MAPKs MKP3 and MKP6 (Zhang et al., 2007). These studies demonstrate that successful phytopathogenic bacteria use a suite of T3S effectors to overcome PTI.

Little is known about how Xanthomonas campestris pathovar vesicatoria (Xcv) employs its T3S effectors to suppress PTI. Xcv is the causal agent of bacterial spot on tomato (Solanum lycopersicum) and pepper (Capsicum annuum) plants (Jones et al., 1998). Xcv strain 85-10 is predicted to have at least 25 effector proteins based on genetic studies (Bonas et al., 1989; Swords et al., 1996), an Xcv T3S effector screen (Roden et al., 2004), and bioinformatic analysis of the sequenced Xcv genome (Thieme et al., 2005). The detailed study of AvrBs3 from Xcv strain 82-8 and XopD from Xcv strain 85-10, however, has revealed that modulation of host nuclear events is critical for Xcv pathogenesis. In susceptible pepper, AvrBs3 activates host transcription and promotes hypertrophy of leaf mesophyll cells (Kay et al., 2007), whereas in resistant pepper, AvrBs3 modulation of host transcription results in the activation of the Bs3 resistance protein, a putative flavin monooxygenase (Römer et al., 2007). XopD encodes a SUMO protease that represses host transcription at the late stages of infection in tomato (Kim et al., 2008). Thus, AvrBs3 and XopD both appear to alter the downstream nuclear events associated with PTI. How Xcv modulates the early signaling events associated with PTI is not yet clear.

In previous work, we showed that XopN is a novel effector protein that is widely conserved among Xanthomonas species (Roden et al., 2004). XopN proteins from Xcv and Xanthomonas campestris pv. campestris are required for maximal pathogen growth and symptom production in tomato and radish (Raphanus sativus), respectively, demonstrating the importance of this effector class in bacterial pathogenesis (Roden et al., 2004; Jiang et al., 2008). Although the function of XopN is not known, its structure is predicted to contain antiparallel, α-helical tandem repeats (Roden et al., 2004). Proteins containing this type of fold participate in multiple protein–protein interactions by serving as protein scaffolds or adapters (Burack and Shaw, 2000; Bhattacharyya et al., 2006). This led us to hypothesize that XopN’s virulence role might be to physically associate and interfere with one or more signaling components that comprise the host’s defense machinery. The goal of this study was thus twofold. First, we wanted to determine if XopN functions to suppress plant immunity during susceptible Xcv–tomato interactions. Second, we wanted to isolate XopN-interacting proteins from tomato to identify host virulence targets and potential signaling pathways modulated by Xcv.

Here, we provide evidence that XopN is a suppressor of PTI in tomato (i.e., PAMP-triggered gene expression and callose deposition). Moreover, we show that XopN physically interacted with two types of proteins with predicted functions in signal transduction: Tomato Atypical Receptor-like Kinase1 (TARK1) and four Tomato Fourteen-Three-Three isoforms (TFT1, TFT3, TFT5, and TFT6). Xcv ΔxopN virulence defects were partially suppressed in transgenic tomato leaves with reduced TARK1 mRNA levels, indicating that TARK1 plays an important role in the outcome of Xcv–tomato interactions. These data provide the basis for a model in which XopN binds to TARK1 to interfere with TARK1-dependent signaling events triggered in response to Xcv infection.

RESULTS

XopN Null Mutants Exhibit Reduced Xcv Growth and Symptom Production

Previously, we showed that a functional XopN locus is required for maximal Xcv multiplication in susceptible tomato VF36 leaves (Roden et al., 2004). The mutant Xcv strain analyzed in that study contained the tetracycline gene in the middle of the xopN open reading frame (ORF). The resulting Xcv mutant strain contained the 5′ region of the xopN ORF and therefore was not a null mutant (Roden et al., 2004). In this study, we constructed a xopN null mutant (designated as ΔxopN) to confirm that deletion of the entire xopN ORF is alone sufficient to reduce Xcv virulence. The entire xopN ORF in Xcv strain 85-10 was replaced with the spectinomycin (Sp) resistance gene by homologous recombination to produce ΔxopN. Strain virulence was assessed by completely hand-infiltrating susceptible VF36 tomato leaves with a 105 colony forming units (cfu)/mL suspension of wild-type Xcv or Xcv ΔxopN (each containing the pVS61 vector subsequently used to introduce the wild-type gene for complementation). Individual leaves of the same age on the same branch were infiltrated with different Xcv strains and then compared with each other for each experimental test. At least three healthy plants were used for each experiment, and the analysis was repeated at least three times. Thus, the growth curve data represents the average Xcv titer detected in multiple susceptible tomato plants over the time course.

As expected, the Xcv ΔxopN null mutant exhibited reduced bacterial titers (Figure 1A) and disease symptoms (Figure 1B) in susceptible tomato leaves relative to the wild-type Xcv strain. Reduced disease symptoms are defined as fewer bacterial lesions (i.e., spots associated with bacterial spot disease in tomato) and less tissue chlorosis and necrosis (Stall, 1995). In susceptible Xcv–tomato interactions, bacterial growth increases significantly between 0 and 4 d after inoculation (DAI) (Gassmann et al., 2000; Roden et al., 2004; Kim et al., 2008). Virulent strains continue to grow within tomato leaves until 8 to 12 DAI when multiplication is reduced and the tissue becomes saturated with
bacteria (Gassmann et al., 2000; Roden et al., 2004; Kim et al., 2008). Xcv strains impaired in virulence generally exhibit wild-type growth at 3 to 4 DAI but then exhibit changes in bacterial multiplication and leaf symptoms after 4 DAI (Gassmann et al., 2000; Roden et al., 2004; Metz et al., 2005; Kim et al., 2008). We found that Xcv ΔxopN growth was significantly reduced at 6 DAI relative to wild-type Xcv growth (Figure 1A). The low titer of Xcv ΔxopN in the leaf tissue (Figure 1A) directly correlated with reduced disease symptoms (Figure 1B). Xcv ΔxopN strains carrying the broad host vector pVSP61 containing the xopN promoter and ORF (Xcv ΔxopN [xopN-HA]) exhibited wild-type growth at 10 DAI (Figure 1A) and symptom production in tomato leaves at 12 DAI (Figure 1B), indicating that the ΔxopN mutant phenotype was fully complemented. Thus, XopN is a virulence factor required for full Xcv pathogenesis in tomato.

XopN Suppresses mRNA Levels of Tomato Pathogenesis-Related Genes

Reduced Xcv ΔxopN virulence in susceptible tomato leaves suggested that XopN might be a negative regulator of basal immunity. To address this, we monitored mRNA levels for Pathogenesis-Related (PR) genes during Xcv infection. Our objective was to determine if XopN specifically or generally affects the transcription and/or abundance of PR mRNAs. We assayed six different tomato genes: PR-1b1, PR-Q'b, Tsi-1, GST, PR-13, and PR-P69. PR-1b1 (also referred to as SENU4) encodes the basic PR-1 isoform and its mRNA levels increase in response to salicylic acid (SA) and aging (John et al., 1997; Block et al., 2005). PR-1b1 mRNA levels were shown to increase in susceptible tomato leaves by 6 to 8 d after Xcv inoculation (Ciardi et al., 2000). PR-Q'b encodes a class III ß-1,3-glucanase (Domingo et al., 1994). Tsi-1 encodes the tomato homolog of the potato (Solanum tuberosum) STH-2 gene (Sree Vidy et al., 1999). GST encodes glutathione S-transferase (Harvey et al., 2008). PR-13 encodes a thionin that mediates resistance to P. syringae pv tomato (Pst) strain DC3000 in Nicotiana attenuata (Rayaparam et al., 2008). PR-P69 encodes a subtilisin-like endoprotease that is induced by viruses (Vera and Conejero, 1988).

The mRNA abundance of the aforementioned genes was monitored in tomato leaves infected with Xcv and Xcv ΔxopN for 4 and 6 d. XopN-dependent changes in mRNA abundance were detected between 4 and 6 DAI (Figure 2), the time period when Xcv and Xcv ΔxopN titers started to differ within the leaf tissue (Figure 1A). For example, PR-1b1 mRNA levels were higher in leaves infected with Xcv ΔxopN compared with leaves infected with Xcv or 10 mM MgCl2 (Figure 2). Similar trends were observed for PR-Q'b, Tsi-1, and GST mRNA levels. By contrast, the mRNA levels for PR-13 and PR-69 did not change significantly in the presence or absence of XopN (Figure 2). These data indicate that XopN suppresses the mRNA levels of some, but not all, PR genes during Xcv infection in tomato leaves.

XopN Suppresses PAMP-Triggered Gene Expression in Tomato

We next tested whether or not XopN could suppress PTI in tomato. Genes induced in response to bacterial PAMPs defining PTI have not been well established in tomato. However, candidate PAMP-induced genes have been identified based on a survey of gene expression patterns analyzed during Pst DC3000–tomato interactions (Thara et al., 1999), Xcv–tomato interactions (Mayrose et al., 2006), and flagellin-treated Arabidopsis plants (Navarro et al., 2004). Four candidate genes were analyzed in this study to determine if they are induced in response to Xcv PAMPs and represent PTI marker genes in tomato. These included the following: (1) PTI5, which encodes a transcription factor in the ethylene response factor family (Zhou et al., 1997); (2) GRAS2, which encodes a transcription factor linked to abiotic and biotic stress (Mayrose et al., 2006); (3) WRKY28, which encodes a tomato homolog of the Arabidopsis WRKY28 transcription factor (Navarro et al., 2004); and (4) LRR22, which encodes a tomato homolog of the Arabidopsis LRR22 receptor-like kinase (Navarro et al., 2004).
To monitor gene induction, individual VF36 tomato leaves on the same branch were inoculated with 10 mM MgCl\textsubscript{2} containing a 2 \times 10^{8} \text{cfu/mL} suspension of Xcv, Xcv Δ\textit{xopN}, or Xcv Δ\textit{hrpF}, a mutant that lacks the putative T3S translocon HrpF required for effector translocation into plant cells (Büttner et al., 2002). Total RNA was isolated from the inoculated tissue at 6 h after inoculation (HAI), and marker gene mRNA abundance was determined by quantitative real-time RT-PCR. Relative gene expression values for each treatment were normalized to the expression value in each sample, and relative expression values were determined against the average value of the sample infiltrated with 10 mM MgCl\textsubscript{2} at 4 DAI. Averages of two independent experiments are shown. Error bars indicate SD.

PTI5, WRKY28, LRR22, and GRAS2 mRNA abundance was also monitored in Xcv-infected tissue at 4 and 6 DAI using the same RNA sample generated for Figure 2. Interestingly, the mRNA levels of PTI5, WRKY28, and LRR22 were higher in tomato leaves inoculated with Xcv Δ\textit{xopN} compared with wild-type Xcv (see Supplemental Figure 1 online). These data show that \textit{xopN}-dependent suppression of PTI5, WRKY28, and LRR22 mRNA abundance correlates with an increase in Xcv titer in tomato leaves.

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XopN Suppresses Callose Deposition

We next tested whether or not XopN could suppress callose deposition, a cell wall–based defense triggered in response to PAMPs in infected host leaves (DebRoy et al., 2004). We used two well-characterized Pst DC3000 strains (wild-type and ΔCEL) (DebRoy et al., 2004) to study changes in callose deposition in response to XopN. Wild-type Pst DC3000 strongly suppresses callose deposition by employing two T3S proteins encoded by the conserved effector locus (CEL), AvrE, and HopM1. Pst DC3000 ΔCEL mutants fail to suppress callose deposition and are less virulent (DebRoy et al., 2004). To express XopN in Pst DC3000 and ΔCEL, we ligated the *Pseudomonas* T3S signal sequence (contained within amino acids 1 to 100) from the avrRpt2 gene to the 5′ end of the xopN gene to create the fusion protein AvrRpt2-100-XopN. Susceptible VF36 tomato and Arabidopsis Columbia-0 (Col-0) leaves were hand-infiltrated with a 108 cfu/mL suspension of Pst DC3000 AvrRpt2-100-CEL, AvrRpt2-100, or ΔCEL AvrRpt2-100-XopN and then stained for callose after 12 h.

As expected, tomato leaves infected with ΔCEL expressing AvrRpt2-100 contained more callose deposits (88 ± 21 per mm2) than leaves infected with Pst DC3000 expressing AvrRpt2-100 (25 ± 4 per mm2) (Figure 4A). Tomato leaves infected with ΔCEL expressing AvrRpt2-100-XopN contained fewer callose deposits (33 ± 11 per mm2) compared with leaves infected with ΔCEL expressing AvrRpt2-100 but had more callose deposits relative to leaves infected with Pst DC3000 expressing AvrRpt2-100 (Figure 4A). We next monitored XopN-dependent effects on callose deposition in Arabidopsis. Callose deposition is more robust in Arabidopsis leaves compared with that observed in tomato leaves. Consistent with our findings in tomato, Arabidopsis leaves infected with ΔCEL expressing AvrRpt2-100-XopN contained fewer callose deposits (922 ± 258 per mm2) compared with leaves infected with ΔCEL expressing AvrRpt2-100 (1523 ± 206 per mm2) (Figure 4B). Taken together, these data show that XopN action in planta is sufficient to suppress callose deposition in both tomato and Arabidopsis leaves.

To determine if XopN-dependent suppression of callose deposition impacts pathogen replication, we monitored the growth of ΔCEL strains with and without XopN in Arabidopsis Col-0 leaves. The bacterial titer of ΔCEL expressing AvrRpt2-100-XopN was significantly higher than ΔCEL expressing AvrRpt2-100 (Figure 4C). XopN-dependent reduction in callose deposition (Figure 4B) correlated with increased bacterial growth (Figure 4C). These data suggest that XopN suppresses cell wall–based defenses in Arabidopsis.

XopN Interacts with TFT Isoforms and a Tomato Atypical Receptor-Like Kinase

XopN is predicted to have at least seven irregular, tandem repeats (Figure 5A), likely positioned as antiparallel α-helices that stack on each other to form a solenoid (Roden et al., 2004). Based on its putative structural fold, we hypothesized that XopN may function as a protein scaffold or a protein adapter inside plant cells to promote and/or interfere with the organization of signaling complexes during Xcv infection.

To test this hypothesis, we performed a yeast two-hybrid screen to identify host binding partners of XopN. LexA-XopN served as the bait and a tomato cDNA library as the prey. We screened 108 primary yeast transfectants and identified 14 cDNAs representing eight different proteins (Table 1). Three tomato interactors (SGN-U320014, SGN-U316698, and SGN-U313819) shared homology with a putative metal binding protein, a seed maturation-like protein, and a chloroplast inner envelope membrane protein, respectively (Table 1). These interactors were not further characterized in this study. The other cDNA clones encode proteins known or predicted to be involved with signaling. These cDNAs were isolated multiple times, defining two major classes of XopN-interacting proteins. Class I cDNAs encode four different TFT (or 14-3-3) proteins (Table 1). Two of the TFT isoforms (TFT1 and TFT6) are known to be pathogen inducible in tomato (Roberts and Bowles, 1999). Class II cDNAs (SGN-U328133) encode the cytosolic domain (CD) of a putative leucine-rich repeat receptor-like kinase (LRR-RLK), designated as TARK1. Two different cDNAs encoding the CD of TARK1 were isolated: TARK1-CD (amino acids 269 to 605) and TARK1-329-605 (amino acids 329 to 605) (Table 1, Figure 5B).

XopN binding to TARK1-CD was the strongest interaction detected in the yeast screen demonstrated by intense blue staining on GAL-UHT-L-X-gal selection media and fast growth on GAL-UHTL (Figure 5B). XopN interaction with TFTs appeared to be weaker than the XopN/TARK1-CD interaction given that growth was observed on GAL-UHTL plates but only light blue staining was observed on GAL-UHT-L-X-gal plates. XopN/TFT1 interaction was consistently stronger than the interactions observed with the other TFT isoforms. Based on this and the fact that TFT1’s expression is induced during pathogen attack (Roberts and Bowles, 1999), TFT1 was selected as a candidate XopN interactor for further analysis in planta.

Since there are >600 LRR-RLKs in the Arabidopsis genome (Shiu and Bleeker, 2001) and likely more in the tomato genome, we next sought to address the binding specificity of XopN to the CD of TARK1. We tested potential XopN interactions with the kinase domain (KD) of Sl BRI1 and the complete CD of Sl FLS2, two *S. lycopersicum* (Sl) LRR-RLKs associated with brassinosteroid and flagellin signaling, respectively (Montoya et al., 2002; Robatzek et al., 2007). We also tested XopN binding to the CD of a TARK1-like protein, TARK1-L, which was identified in the Sol Genomics Network cDNA database (www.sgn.cornell.edu). TARK1-like shares 65% similarity and 50% identity with TARK1 at the amino acid level. Protein interactions were not detected in yeast between XopN/SI BR11-KD, XopN/SI FLS2-CD, or XopN/TARK1-like under the conditions tested (Figure 5C), although each protein domain was stably expressed in yeast (see Supplemental Figure 2 online). This demonstrates that XopN physical interaction with the CD of TARK1 in yeast is highly specific. Therefore TARK1, in addition to TFT1, was selected for additional in planta studies.

Localization of XopN, TARK1, and TFT1 in Planta

We next determined if the localization patterns of XopN, TARK1, and TFT1 overlap in planta, which would indicate that the proteins could interact within the plant cell. Fluorescent protein–tagged fusions of XopN, TARK1, and TFT1 (yellow
fluorescent protein [YFP]-XopN, TARK1-green fluorescent protein [GFP], and YFP-TFT1) were overexpressed in *Nicotiana benthamiana* using the *Agrobacterium tumefaciens*–mediated transient expression system. Protein localization was then determined using confocal microscopy. YFP-XopN was diffuse throughout the plant cytoplasm but not observed in the plant nucleus (Figure 6A). YFP-XopN also appeared to be associated with the plant plasma membrane (PM). This pattern was distinct from that of the YFP control (Figure 6A). YFP was localized in both the cytoplasm and nucleus. TARK1-GFP was localized to the PM, whereas YFP-TFT1 localized to both the plant cytoplasm and nucleus (Figure 6A). Plasmolysis was performed to better visualize the localization of TARK1 and XopN. After plasmolysis, TARK1-GFP was closely associated with the PM, consistent with the prediction that TARK1 is a transmembrane LRR-RLK (see Supplemental Figure 3 online). YFP-XopN localization was less distinct, showing association with the PM and cytoplasm (see Supplemental Figure 3 online). Fractionation of similarly infected tissue by centrifugation showed that TARK1-HA was localized to a detergent-soluble pellet, whereas XopN-6xHis was enriched in the pellet but also present in the water-soluble supernatant (see Supplemental Figure 5 online). These results demonstrate that the localization pattern of XopN overlaps with its putative binding partners, TARK1 and TFT1, and suggests that these proteins could physically interact with each other within the plant cell.

**Visualization of Protein Interactions in Planta by Bimolecular Fluorescence Complementation**

We next used the bimolecular fluorescence complementation (BiFC) assay to monitor XopN/TARK1 and XopN/TFT1 interactions in planta (Walter et al., 2004). For the BiFC assay, the
nonfluorescent N-terminal domain of YFP (nYFP) and the nonfluorescent C-terminal domain of CFP (cCFP) were fused to the N- and C-terminal ends of the test proteins (i.e., XopN, full-length TARK1, and TFT1). Interactions between the respective fusion proteins were then tested in N. benthamiana using the Agrobacterium-mediated transient expression assay. Representative BiFC confocal microscopy images showing XopN/TARK1 and XopN/TFT1 interaction are presented in Figure 6B. Coexpression of XopN-nYFP and TARK1-cCFP resulted in bright fluorescence near the PM (Figure 6B). Fluorescence was only observed when the cCFP domain was fused to the C terminus of the TARK1, the domain predicted to reside in the plant cytoplasm. Coexpression of XopN-nYFP and TFT1-cCFP resulted in bright fluorescence in the cytoplasm and close to the PM (Figure 6B), similar to the localization pattern of YFP-XopN alone (Figure 6A). The fluorescence pattern for XopN-nYFP + TFT1-cCFP was more diffuse than that observed for XopN-nYFP + TARK1-cCFP localization (Figure 6B). As a control, we tested physical interaction between XopN and Arabidopsis phosphoinositide-specific phospholipase C (PLC2) (Figure 6B), a protein that is localized to the plant PM (see Supplemental Figure 3 online). Coexpression of XopN-nYFP and PLC2-cCFP did not result in fluorescence above background levels (Figure 6B), indicating that these two proteins do not interact in this assay. All of the fusion proteins analyzed were stably expressed in N. benthamiana (see Supplemental Figure 4B online). Taken together, these BiFC studies show that XopN interacts with both TARK1 and TFT1 inside plant cells and that localization is occurring near the PM.

**Affinity Purification of XopN with TARK1 and TFT1 in Plant Extracts**

Considering that multiple factors (e.g., protein expression levels, rates of protein folding, and/or protein stability) contribute to the detection of protein interactions by BiFC (Lalonde et al., 2008), we next confirmed that XopN interacts with TARK1 and TFT1 in N. benthamiana extracts using a Ni-NTA affinity pull-down assay. N. benthamiana leaves were hand-infiltrated with a 6 × 10^8 cfu/mL suspension of A. tumefaciens coexpressing XopN-6xHis and TARK1-HA (Figure 7A) or XopN-6xHis and TFT1-HA (Figure 7B). A solubilized total protein extract was isolated from leaves 48 HAI, and then XopN-6xHis was pulled-down using Ni-NTA agarose beads. Protein gel blot analysis shows that TARK1-HA copurified with XopN-6xHis (Figure 7A). Similarly, TFT1-HA copurified with XopN-6xHis (Figure 7B). Weak binding of TARK1-HA and TFT1-HA to the Ni-NTA agarose beads was observed; however, both proteins were highly enriched following the XopN-6xHis pull down. These studies demonstrate that XopN interacts with both TARK1 and TFT1 inside plant cells and that localization is occurring near the PM.

**TARK1 is an Inactive Kinase**

The TARK1 protein consists of 605 amino acids and is predicted to encode a protein with a cleavable N-terminal signal peptide, a single transmembrane domain, and a cytoplasmic Ser/Thr KD (Figure 8A). The extracellular domain contains five LRRs, placing it in the LRR III RLK subfamily classified in the Arabidopsis...
genome (Shiu and Bleecker, 2001). Whereas nothing is known about this protein in tomato, the closest TARK1 homologs in Arabidopsis, AtRKL1 and AtRLK902, have been previously characterized in terms of their kinase activity and tissue-specific expression patterns (Tarutani et al., 2004b). Both proteins were reported to possess weak autophosphorylation activity in vitro, suggesting they might function as kinases in vivo (Tarutani et al., 2004a). AtRKL1 is expressed at low levels in leaves and high levels in guard cells and hydathodes (Tarutani et al., 2004b), two portals used by bacteria, including *Xanthomonas*, to enter leaf tissue.

Comparison of TARK1’s KD to conserved subdomains typically found in eukaryotic kinases (Hanks and Hunter, 1995) revealed that two crucial Asp residues in subdomains VIB and VII are replaced with Asn and Glu (see Supplemental Figure 6A online). In active kinases, the Asp in subdomain VIB is predicted to be the catalytic base, whereas the Asp in subdomain VII comprises the Asp-Phe-Gly triplet involved in γ-phosphate transfer. The substitutions in TARK1’s KD are conservative; however, similar substitutions were found in the maize atypical receptor kinase, an LRR-RLK lacking detectable kinase activity (Llompart et al., 2003). By contrast, LRR-RLKs with reported kinase activity (e.g., Sl BRI1, Sl FLS2, At RKL1, and At RLK902) contain one or both conserved Asp residues (see Supplemental Figure 6A online). Thus, we designated this protein as TARK1 to reflect its unusual amino acid sequence in subdomains VIB and VII.

<table>
<thead>
<tr>
<th>Tomato Gene IDa</th>
<th>Protein Product or Putative Homology</th>
<th>Predicted Coding Sequenceb</th>
<th>cDNA Length Isolated</th>
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<tr>
<td>TFT1</td>
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<tr>
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<td>14-3-3, isoform 3</td>
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aSGN nomenclature refers to Unigene designation for available tomato ESTs provided by Sol Genomics Networks at http://www.sgn.cornell.edu.
bPredicted coding sequence listed in base pairs.
To determine if TARK1 encodes an active kinase, we performed an in vitro autophosphorylation assay using the wild-type CD and a mutated CD of TARK1 purified from *Escherichia coli* [i.e., GST-TARK1-CD and GST-TARK1(K365E)-CD; see Supplemental Figure 6B online]. Lys-365 is the invariant residue in subdomain II that is known to be required for ATP binding and maximal kinase activity in diverse protein kinases (Hanks and Hunter, 1995). Lys-365 in TARK1-CD was mutated to Glu. Purified GST and GST-SlBRI1-KD were used as negative and positive controls, respectively (see Supplemental Figure 6B online). We also tested the activity of the CD of At RLK902 (GST-AtRLK902-CD) because it was reported to have weak kinase activity despite substitutions of critical amino acids in subdomain VIB and VII (Tarutani et al., 2004a). Recombinant GST-TARK1-CD and GST-AtRLK902-CD did not exhibit in vitro autophosphorylation activity under the conditions tested despite stable expression of the recombinant protein domains (see Supplemental Figures 6B and 6C online). By contrast, GST-SlBRI1-KD exhibited robust autophosphorylation activity (see Supplemental Figure 6C online). We note that weak kinase activity was detected for all of the test proteins in an autoradiograph exposed overnight (see Supplemental Figure 6C online). However, further inspection indicated that the weak kinase activity did not comigrate with GST-TARK1-CD or GST-TARK1(K365A)-CD (see Supplemental Figure 7 online). Therefore, we conclude that the weak kinase activity that was detected is likely an *E. coli* kinase that copurified with GST-TARK1-CD, GST-TARK1(K365A), and possibly GST-AtRLK902. These data indicate that TARK1-CD does not have autophosphorylation activity in vitro and supports the prediction that TARK1 is an atypical protein kinase or dead kinase.

**XopN Binds to an LXXLL Motif in TARK1**

We next determined the regions in TARK1 that are required for the XopN/TARK1 interaction. We performed deletion analysis using the minimal cytoplasmic region of TARK1 shown to interact with XopN (Tarutani et al., 2004a). Recombinant GST-TARK1-CD was mutated to Glu. Purified GST and GST-SIBRI1-KD were used as negative and positive controls, respectively (see Supplemental Figure 6B online). We tested the activity of the CD of At RLK902 (GST-AtRLK902-CD) because it was reported to have weak kinase activity despite substitutions of critical amino acids in subdomain VIB and VII (Tarutani et al., 2004a). Recombinant GST-TARK1-CD and GST-AtRLK902-CD did not exhibit in vitro autophosphorylation activity under the conditions tested despite stable expression of the recombinant protein domains (see Supplemental Figures 6B and 6C online). By contrast, GST-SIBRI1-KD exhibited robust autophosphorylation activity (see Supplemental Figure 6C online). We note that weak kinase activity was detected for all of the test proteins in an autoradiograph exposed overnight (see Supplemental Figure 6C online). However, further inspection indicated that the weak kinase activity did not comigrate with GST-TARK1-CD or GST-TARK1(K365A)-CD (see Supplemental Figure 7 online). Therefore, we conclude that the weak kinase activity that was detected is likely an *E. coli* kinase that copurified with GST-TARK1-CD, GST-TARK1(K365A), and possibly GST-AtRLK902. These data indicate that TARK1-CD does not have autophosphorylation activity in vitro and supports the prediction that TARK1 is an atypical protein kinase or dead kinase.

**XopN Binds to an LXXLL Motif in TARK1**

We next determined the regions in TARK1 that are required for the XopN/TARK1 interaction. We performed deletion analysis using the minimal cytoplasmic region of TARK1 shown to interact with XopN. **(A)** Schematic of TARK1 protein. SP, signal peptide; TMD, transmembrane domain; LEDLL, LXXLL motif in proximal membrane region. **(B)** Analysis of XopN interactions with select C-terminal domains of TARK1. EGY48 pSH18-34, pEG202-nls(lexA:xopN) was transformed with pJG4-5 alone (vector), pJG4-5 containing TARK1 cytosolic domain (TARK1-CD), the LXXLL motif + kinase domain (TARK1329-605), or the kinase domain (TARK1337-605). Strains were plated on inducing (GAL/-UHT/X-Gal or GAL/-UHTL) and noninducing (GLC/-UHT) medium and then incubated at 30°C for 3 to 4 d. **(C)** XopN interaction with TARK1 LXXLL site-directed mutants. The LEDLL motif was mutated by replacing the conserved Leu residues with Ala. EGY48 pSH18-34, pEG202-nls(xopN) was transformed with pJG4-5 containing no insert (vector), TARK1329-605 (LEDLL), or two Ala substitution mutants (LEDAAA) and (AEDLL). Strains were analyzed as in (A).
with XopN in yeast (Figure 5B). We discovered that XopN interacted with TARK1-CD and TARK1 in yeast (Figure 8B). The removal of eight amino acids (i.e., VFGL dood) abolished XopN binding to TARK1. Intriguingly, this peptide contains an LXXLL motif (where L is leucine and X is any amino acid), which is a signature motif that has been shown to be important in receptor–ligand interactions (Plevin et al., 2005). The LXXLL motif is adjacent to the predicted ATP binding site located between amino acids 343 and 365 (Saraste et al., 1990).

To determine if the LXXLL motif is required for XopN/TARK1 interaction, two mutant proteins were constructed in which the Leu residues in the N terminus (N) of XopN (Figure 8C). These data show that the LXXLL motif in the proximal membrane region of TARK1 is required for TARK1/XopN physical interaction in yeast. Inspection of the protein sequences for TARK1-L, SI BR1, and SI FLS2 identified similar LXXLL motifs adjacent to their respective ATP binding sites. However, XopN does not interact with TARK1-L, SI BR1, or SI FLS2 (Figure 5C), indicating that the LXXLL motif in TARK1 is necessary but likely not sufficient for interaction with XopN. Interestingly, all of the putative TARK1 homologs in Arabidopsis contain the signature LXXLL motif (see Supplemental Figure 9 online), suggesting that this sequence may be functionally conserved for LRR-RLKs in the LRR III subfamily.

TARK1 Binds to the N-Terminal Domain of XopN

Deletion analysis of XopN was performed to determine the domains that interact with TARK1. A series of N- and C-terminal deletions of XopN were constructed and then tested in a directed yeast two-hybrid assay with TARK1 (Figure 9A). All of the mutant proteins were equally expressed in yeast (see Supplemental Figure 10 online). TARK1-CD only interacted with polypeptides containing the N terminus (N) of XopN (Figure 9B). TARK1-CD did not interact with the M3 mutant lacking XopN amino acids 1 to 177 (Figure 9B), indicating that some of those residues are necessary for TARK1 binding.

An LXXLL Motif in the N Terminus of XopN Is Required for TARK1 Binding

Next, we inspected XopN’s sequence to determine if it also contains a signature LXXLL motif that could facilitate interaction with TARK1. Intriguingly, an LXXLL motif (LGALL) resides between amino acids 61 and 65 in XopN, the region required for binding to TARK1 (Figure 9A). Site-directed mutagenesis was performed to substitute the two C-terminal Leu residues in the LGALL motif to Ala residues, creating the sequence LGAAA (Figure 9A). In yeast, the LGAAA mutant was expressed (see Supplemental Figure 10 online), but physical interaction with TARK1-CD was not detected (Figure 9B), indicating that the LXXLL motif is critical for XopN/TARK1 binding in this assay.

The importance of the LXXLL motif in XopN for binding to TARK1 was further supported by pull-down analyses in N. benthamiana. Leaves were hand-infiltrated with a 6 × 10^8 cfu/mL suspension of A. tumefaciens coexpressing the XopN mutant protein LGAAA-6xHis and TARK1-HA or XopN-6xHis and TARK1-HA. A solubilized total protein extract was isolated from leaves 48 HAI, and then the His-tagged proteins were affinity purified using Ni-NTA agarose beads. Protein gel blot analysis showed that the LGAAA-6xHis protein is stably expressed in N. benthamiana leaves (Figure 9C). The LGAAA-6xHis mutant, however, did not pull down TARK1-HA, whereas TARK1-HA copurified with the wild-type XopN-6xHis protein (Figure 9C).

XopN LXXLL Motif Is Required for Full Xcv Virulence

To determine if the LXXLL motif in XopN is required for XopN-dependent virulence in tomato, an epitope-tagged version of the XopN mutant protein (LGAAA-HA) was introduced into the Xcv ΔxopN null strain and then tested for complementation of the ΔxopN growth defect. Susceptible VF36 tomato leaves were hand-infiltrated with a 10^5 cfu/mL suspension of Xcv ΔxopN expressing LGAAA-HA, vector, or XopN-HA. The expression of LGAAA-HA in Xcv ΔxopN was similar to wild-type XopN-HA (see Supplemental Figure 11 online). Compared with wild-type XopN, LGAAA-HA expression did not fully rescue the growth defect of Xcv ΔxopN at 10 DAI (Figure 9D). Growth of Xcv ΔxopN (LGAAA-HA) was ~50% that of Xcv ΔxopN (xopN-HA). Therefore, the LXXLL motif in XopN is required to promote full XopN-dependent virulence in tomato leaves.

We also assessed the impact of the LGAAA mutation on XopN-dependent suppression of callose deposition in Arabidopsis. Pst DC3000 ΔCEL expressing AvrRpt2_1-100-XopN(LGAAA) suppressed callose deposition in tomato and Arabidopsis leaves to the same extent as Pst ΔCEL expressing AvrRpt2_1-100-XopN (Figures 4A and 4B). Furthermore, the respective strains grew to similar levels within the infected Arabidopsis leaves (Figure 4C). Therefore, the LXXLL motif at the N terminus of XopN is not required for XopN-dependent suppression of cell wall–based defenses in planta.

Silencing TARK1 mRNA Expression Suppresses Xcv ΔxopN Phenotypes

Given that XopN is a virulence factor that suppresses PTI in tomato, we hypothesize that TARK1 encodes a defense receptor that is a target of XopN virulence. If TARK1 is a positive regulator of defense signaling, then reducing TARK1 levels in planta should suppress the reduced virulence phenotype of the Xcv ΔxopN strain in tomato leaves (Figure 1). To test this model, we engineered transgenic tomato lines that are silenced for TARK1 mRNA expression. TARK1 was silenced using a hairpin RNA construct containing the 3’ untranslated region (UTR) of the TARK1 gene, designated hp-TARK1. The 3’ UTR region was used to prevent the silencing of related LRR-RLK family members in tomato. Five independent T2 transgenic lines were
characterized to measure TARK1 mRNA levels and quantify bacterial growth and symptom production. Figure 10 shows the phenotypes for two T2 tomato lines (i.e., lines 1 and 2) with reduced TARK1 mRNA levels determined by quantitative real-time RT-PCR (Figure 10A). The phenotypes of the TARK1 silenced lines were compared with an unsilenced transgenic plant (Figure 10B). The hp-TARK1 construct appeared to be specific for the TARK1 gene, as it reduced TARK1 mRNA levels in lines 1 and 2 without affecting the mRNA levels of a homologous tomato gene that we designated TARK1-like or TARK1-L (Figure 10A).

Bacterial growth curves were performed using wild-type Xcv and the Xcv ΔxopN mutant and the hp-TARK1 transgenic lines to determine whether the reduction in TARK1 expression can suppress the growth defect of Xcv ΔxopN. We found that in the control line containing TARK1 mRNA, the titer of wild-type Xcv was ∼11-fold higher than that of Xcv ΔxopN at 12 DAI (Figure 10B, left panel), consistent with our earlier observations (Figure 1A). By contrast, the titer of wild-type Xcv on both TARK1-silenced lines was only ∼2.5-fold higher than that of the Xcv ΔxopN mutant at 12 DAI (Figure 10B, left panels). This suggested that the growth defect of Xcv ΔxopN mutant was partially suppressed when TARK1 mRNA expression was reduced. In addition, we found that the difference in symptom development (i.e., the timing and extent of leaf chlorosis and necrosis) between wild-type Xcv and Xcv ΔxopN was less pronounced on the TARK1-silenced lines than on the control line (Figure 10B, right panels). At 12 DAI, TARK1-expressing control leaves infected with wild-type Xcv had already begun to collapse due to tissue necrosis, whereas leaves infected with the Xcv ΔxopN mutant had become chlorotic but showed no signs of necrosis. By contrast, TARK1-silenced leaves for lines 1 and 2 infected with either wild-type Xcv or Xcv ΔxopN showed less symptom development at 12 DAI despite high bacterial titer. Wild-type Xcv
infected leaves of lines 1 and 2 were beginning to exhibit chlorosis, but necrosis was not observed (Figure 10B). These data suggest that TARK1 contributes to disease symptom production in tomato since necrosis was reduced in TARK1-silenced leaves infected with wild-type Xcv (Figure 10B).

We next determined if the growth of Xcv and XcvΔxopN in the TARK1-silenced lines was significantly different than that detected in the unsilenced control lines. Bacterial growth curves (Figure 10C) were performed with three plants expressing TARK1 and three plants silenced for TARK1 expression (Figure 10D), and the average number of bacteria in the leaves was determined at 8 and 12 DAI. The bacterial titers of Xcv and XcvΔxopN in leaves expressing TARK1 mRNA were significantly different at 8 DAI. By contrast, no significant difference in growth was observed for Xcv and XcvΔxopN in TARK1-silenced leaves (Figure 10C). At 12 DAI, Xcv levels were significantly higher than XcvΔxopN in both lines; however, the fold-change was different. Xcv was ~13-fold higher than XcvΔxopN in the control line, whereas Xcv was only fourfold higher than XcvΔxopN in the TARK1-silenced line (Figure 10C). Collectively, these results suggest that the silencing of TARK1 mRNA expression in tomato leaves can partially suppress the virulence defects exhibited by the XcvΔxopN null mutant, supporting a role for TARK1 in immune signaling and symptom development.

DISCUSSION

In this work, we show that the Xcv T3S effector XopN is a suppressor of PTI in Arabidopsis and tomato. Few studies have carefully assessed the time at which PTI is activated during Xcv–tomato interactions and when such defenses inhibit pathogen growth. Xcv T3S-deficient strains can grow in tomato leaves for 2 to 4 DAI, albeit at levels much lower than wild-type Xcv, before growth is inhibited by basal defenses (Kocal et al., 2008). Xcv's T3S system and its effectors are required to fully suppress the level of PTI mRNAs induced in tomato leaves in response to Xcv PAMPs (Figure 3). Taken together, these data suggest that PTI is activated early in response to Xcv infection, but suppression of bacterial growth in infected tissue is not detectable by growth curve analysis until 2 to 4 DAI.
Consistent with this timing, we found that Xcv and Xcv ΔxopN multiplication were quite similar in tomato leaves up to 4 DAI despite the fact that XopN suppresses PTI (Figure 1). This indicates that deletion of one effector protein does not significantly alter bacterial growth before day 4 (Figure 1). A significant reduction in Xcv ΔxopN growth was only apparent between 4 and 6 DAI, highlighting the earliest time point at which the loss of XopN virulence activity can be detected in Xcv growth curves. Reduced Xcv ΔxopN growth between 4 and 6 DAI correlated with high PTI mRNA levels in the Xcv ΔxopN infected leaves at 6 DAI (see Supplemental Figure 1 online). These data suggest that XopN-dependent suppression of PTI contributes to bacterial growth and symptom production, although its contribution is less than that of the full Xcv effector arsenal.

In tomato, PTI appears to be the major factor that limits Xcv growth during the mid stage of infection (6 to 8 DAI) because bacterial titer can be significantly higher in tomato lines with reduced SA levels (Kim et al., 2008). That is, nutrients do not appear to be limiting in the leaf tissue at this stage of the interaction. Efforts are currently being made to establish robust markers for physiological and transcriptional changes that represent early, mid, and late stages of the interaction. Such markers will be useful to define the precise contribution of individual effectors in Xcv pathogenesis.

The isolation of XopN-interacting proteins, TARK1 and TFT1, suggests that XopN might be interfering with signaling components at or near the plant PM. We speculate that XopN might be tethered at the PM by associating with TARK1 via its N-terminal domain. If this were the case, then XopN could potentially interact with other host proteins via its C-terminal domain functioning like a protein scaffold or adapter. Scaffolds are α-helical repeat-containing proteins that assemble components of a pathway and quantitatively control signal inputs and outputs (Burack and Shaw, 2000; Bhattacharyya et al., 2006). Little is known about scaffolds in plants; however, several unknown proteins with predicted α-helical repeats are present in plant genomes, hinting that protein scaffolds may serve similar regulatory roles in plants as they do in yeast and mammals. Future work will address whether or not XopN mimics a plant scaffold to facilitate interactions between TARK1 and possibly TFT1 and if such interactions promote or inhibit plant signal transduction.

TARK1 deletion analysis identified an important LXXLL signature motif that is required for binding to XopN (Figure 8C). The LXXLL motif is conserved in all of the TARK1 homologs present in the Arabidopsis LRRIII subfamily of LRR-RLKs (see Supplemental Figure 9 online). LXXLL motifs are found in a number of coactivators that control nuclear receptor activation and are known to form α-helical loops that facilitate binding to the surface of receptors with bound ligands (Savkur and Burris, 2004; Plevin et al., 2005). Interestingly, an LXXLL motif was also identified in the N terminus of XopN. The XopN LXXLL motif was critical for binding to TARK1 (Figure 9C) and important for XopN-dependent virulence (Figure 9D). The LXXLL motif in XopN, however, was not required for XopN-dependent suppression of callose deposition in tomato or Arabidopsis (Figures 4A and 4B). This suggests that TARK1-dependent signaling events might be distinct from XopN-dependent modulation of PTI responses. Alternatively, XopN could be binding simultaneously to multiple host proteins (e.g., TFT1) for which LXLL mutations in the N terminus of XopN only disrupts physical interaction with TARK1. Future work will address these possibilities.

What is the function of TARK1? Also, how does XopN binding to TARK1 alter its function? We are just beginning to address these questions, though hints to TARK1 function have emerged from our TARK1 silencing studies in tomato. Reduced TARK1 mRNA expression in transgenic tomato leaves partially suppressed the virulence defect attributed to the Xcv ΔxopN mutant strain (Figure 10). This suggests that TARK1 might function as a positive regulator of defense signaling in tomato. Interestingly, we consistently observed reduced symptom development in tomato lines with reduced TARK1 mRNA levels infected with Xcv compared with tomato lines expressing TARK1 mRNA infected with a similar level Xcv (Figure 10B). These findings suggest that TARK1 signaling events are not only required to inhibit Xcv growth but also influence the timing and severity of disease symptom production in highly infected tomato leaves.

TARK1 protein features and localization to the PM are consistent with those shared by other cell surface receptors. The closest TARK1 homologs in Arabidopsis are in the LRRIII subfamily of LRR-RLKs (Shiu and Bleecker, 2001). TARK1 is a non-RD kinase (Dardick and Ronald, 2006). It lacks the conserved Arg and Asp residues found in RD kinases (Dardick and Ronald, 2006). Non-RD kinases have been shown to be PRRs or proteins that signal through PRRs. This suggests that TARK1 might encode a PRR. The short extracellular domain of TARK1 containing five LRRs, however, indicates that TARK1 might interact with primary receptors to amplify and/or regulate signal transduction, perhaps by playing a role analogous to that of BAK1 in PTI signaling (Chinchilla et al., 2007; Heese et al., 2007; Kemmerling et al., 2007). Considering that TARK1 does not exhibit kinase activity in vitro (see Supplemental Figure 6 online), we suspect that this atypical receptor might be participating in phosphorylation-independent interactions with other signaling components. The prevalence of atypical receptor-like kinases in plants suggests that phosphorylation-independent mechanisms might be important for plant signaling (Castells and Casacuberta, 2007).

It is compelling that XopN also interacts with several tomato 14-3-3 isoforms in yeast. Two studies revealed that 14-3-3s are recruited by pathogens to evade the immune system and promote pathogenesis. The Pseudomonas aeruginosa T3S effector ExoS exhibits increased ADP-ribosylating activity on small GTPases by binding to cellular 14-3-3s to interfere with cytoskeleton changes during infection (Henriksson et al., 2002). In a different mode of action, the fungal toxin fuscoicin stabilizes the binding of 14-3-3s to a plant PM H(+)-ATPase resulting in an increase in proton pump activity (Wurtele et al., 2003). Our modeling studies of XopN have not revealed any indications that XopN is an enzyme. Thus, we favor the model that XopN binding to 14-3-3s alters the stability or affinity of XopN interactions with other host proteins or perhaps the affinity of 14-3-3s for their substrates. A prime candidate would be TARK1.

In conclusion, we provide evidence that XopN is an important virulence factor that suppresses PTI during Xcv–tomato interactions. This is the first report of a Xanthomonas T3S effector capable of suppressing PTI. We also provide data that XopN
interacts with TARK1, a LRR-RLK that is partially required for resistance against Xcv infection in a XopN-dependent manner. Collectively, these data suggest a model in which XopN binds to TARK1 to interfere with TARK1-dependent signaling responses triggered in tomato leaves in response to Xcv infection.

METHODS

Bacterial Strains, Growth, and Matings

Strains used in this study were as follows: Escherichia coli DH5α and TOP10; Agrobacterium tumefaciens C58C1 pCH32; and Xanthomonas campestris pv vescatoria (Xcv) strain 85-10. E. coli and A. tumefaciens were grown on nutrient yeast glycerol agar (NYGA) (Sambrook) at 37°C and 28°C, respectively. Xcv strains were grown on yeast nutrient agar (YNA) containing 10 μg/mL rifampicin (Rif), 10 μg/mL tetracycline (Tc), and/or 50 μg/mL kanamycin (Km). A. tumefaciens antibiotic selection was 50 μg/mL Tc, 35 μg/mL Km, and/or 50 μg/mL spectinomycin (Sp). E. coli antibiotic selection was 50 μg/mL carbenicillin and/or 50 μg/mL Km. Vectors were mobilized from E. coli into Xcv and A. tumefaciens by triparental mating using standard methods.

PCR and DNA Constructions

PCR was used to engineer restriction sites for construct gene fusions. PCR-generated DNA fragments were cloned into pCR-BluntII-TOPO vector creating pCRII-DOWN. Primer sets used for PCR are listed in Supplemental Table 1 online. Conditions used for PCR and cloning details will be available on request. The sequence of all DNA constructs was confirmed that homologous recombination occurred at the xopN locus.

Construction of the Xcv ΔxopN Null Mutant

The Sp resistance gene was used to replace the xopN gene in Xcv strain 85-10 by homologous recombination, creating Xcv ΔxopN. A 1.5-kb promoter fragment upstream of the xopN ORF (UP) was PCR amplified as an EcoRI-BamHI fragment and then cloned into pENTR/D-TOPO (pENTD) creating pENTD(UP). A 1.6-kb fragment downstream of the xopN ORF (DOWN) was PCR amplified as a BamHI-BglII fragment and cloned into pCR-BluntII-TOPO vector creating pCR-DOWN. Primer sets BS11/BS12 and BS13/BS14 were used to amplify the upstream and downstream regions, respectively. To create pENTD (ΔxopN), the BamHI/BglII fragment of pCRIIDOWN was inserted into the BamHI site of pENTD(UP). Then, a 1.5-kb Sp cassette was cloned into the BamHI site between the upstream and downstream sequences in pENTD (ΔxopN), creating pENTD (ΔxopN:Sp). The 4.6-kb ΔxopN:Sp fragment in pENTD (ΔxopN:Sp) was recombined into the suicide vector pLVC18-RIc (gift from Brian Staskawicz’s lab) by a Gateway LR reaction (Invitrogen). pLVC18 (ΔxopN:Sp) was moved into Xcv 85-10 by triparental mating. Strains were selected for growth on NYGA containing 100 μg/mL Rif, 50 μg/mL Sp, and for loss of growth on NYGA containing 10 μg/mL Tc. PCR and sequence analysis confirmed that homologous recombination occurred at the xopN locus.

Constructs for Xcv ΔxopN Complementation Analysis

The xopN promoter (690 bp 5’ of the ATG) and the xopN ORF (1 to 2202 bp) were PCR amplified as one fragment from Xcv 85-10 genomic DNA using primer set JR170/JR227. This DNA fragment (~690 to ~2202) was cloned into the pCR-BluntII-TOPO vector, creating pCRII(xopN-xopN-HA). The EcoRI fragment from pCRII(xopN-xopN-HA) was subcloned into pVS61 vector creating pVS61(xopN-xopN-HA). This plasmid was then moved into Xcv 85-10 ΔxopN by triparental mating.

Bacterial Growth Curves

To monitor Xcv growth in planta, Solanum lycopersicum cultivar VF36 leaves were hand-inoculated by complete infiltration of the leaf tissue with a 1 × 10^8 cfu/mL suspension of bacteria in 10 mM MgCl2 using a needleless syringe. Leaves of the same age on the same branch were used for each experimental test. Plants were kept under 16-h light/day at 28°C. For each Xcv strain analyzed, four leaf discs (0.5 cm^2) per treatment per time point were collected from one leaf, pooled, ground in 10 mM MgCl2, and then spotted on NYGA plates in triplicate to determine the bacterial load in each inoculated leaf. Three biological replicates (i.e., three plants) were used, and the experiment was repeated at least three times. The average bacterial titer ± SD is reported.

Analysis of Xcv PAMP-Triggered Gene Expression in Tomato

Four genes (PTI1, WRKY28, LRR22, and GRAS2) found to be induced in S. lycopersicum (Rio Grande prf3 tomato plants) by Pseudomonas syringae pv tomato DC3000 ΔhrcC (Mudgett and Staskawicz, 1999) PAMPs at 6 hAI were analyzed for Xcv-induced responses in VF36 tomato plants. Primer sequences used for quantitative real-time RT-PCR are listed in Supplemental Table 1 online. Individual VF36 tomato leaves on the same branch were hand-inoculated with a 2 × 10^8 cfu/mL suspension of bacteria in 10 mM MgCl2 or 10 mM MgCl2 alone. Four strains were analyzed: (1) Xcv pVS61 = Xcv wt; (2) Xcv ΔxopN pVS61 = Xcv ΔxopN; (3) Xcv ΔxopN pVS61(xopN) = Xcv ΔxopN (xopN); and (4) Xcv ΔhrpF pVS61 = Xcv ΔhrpF. Xcv strain 85-10 ΔhrpF was obtained from Brian Staskawicz. Total RNA was isolated from individual leaves at 6 hAI. Analysis was repeated in four independent experiments.

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from leaves using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Five micrograms of RNA were used for cDNA synthesis. Quantitative real-time RT-PCR was performed using the cDNA and gene-specific primers (see Supplemental Table 1 online). Each cDNA was amplified by quantitative PCR using HotStart-IT SYBR Green qPCR Master Mix (USB) and the MJ Opticon 2 (Bio-Rad). Tomato ACTIN expression was used to normalize the expression value in each sample, and relative expression values were determined against buffer or wild-type Xcv using the comparative Ct method (2^-ΔΔCt).

Construction of AvrRpt2_1-100-XopN Fusion and Calllose Staining Assay

The BamHI-EcoRI fragment from pCRII(xopN) was cloned into the BamHI-EcoRI site of pVS61( avrRpt2_1-100) (gift from Brian Staskawicz’s lab) to create pVS61( avrRpt2_1-100-xopN). Plasmids were conjugated into Pst DC3000 and ΔCEL (gift from Sheng Yang He’s lab) by triparental mating. Calllose staining was performed as described (DebRoy et al., 2004) with the following modifications. VF36 tomato leaves and Arabidopsis Col-0 leaves were completely infiltrated with a 2 × 10^8 cfu/mL suspension of bacteria in 10 mM MgCl2 using a 1-mL syringe. Strains analyzed were Pst DC3000 pVS61( avrRpt2_1-100), ΔCEL pVS61( avrRpt2_1-100), ΔCEL pVS61( avrRpt2_1-100-xopN), and ΔCEL pVS61( avrRpt2_1-100-xopN (LGAAAA)). Tissue samples were collected after 12 h infection in the light and then stained for calllose. Images were taken to 1.5 cm from the site of inoculation using a fluorescence microscope. Calllose deposits were counted using ImageJ Software (Abramoff et al., 2004).

Yeast Constructs and Two-Hybrid Screen

PCR and primer set JR78/JR79 were used to engineer an EcoRI and XbaI restriction site at the 5’ and 3’ end of xopN, respectively. The PCR
product was cloned into pZeroBlunt (Invitrogen), and the DNA sequence was verified. The xopN gene was subcloned into the EcoRI and SalI sites of the LexA bait vector pEG202nls (courtesy of Barbara Baker) creating the pEG202nls(xopN). This plasmid was transformed into Saccharomyces cerevisiae strain EGY48 ura3 trp1 his3 lexA operator-LEU2 containing pSH18-34 or EGY48 pJ101 to assess LexA-NLS-XopN–mediated protein expression, transcriptional activity, repressor activity, and Lex requirement as described (Ausubel et al., 1996).

For library screening, EGY48 pSH18-34, pEG202nls(xopN) was transformed with the pJG4-5 prey vector containing a Tobacco Mosaic Virus-infected tomato cDNA library amplified with a poly T primer (courtesy of Barbara Baker). Cells were plated on complete medium (CM) minus -URA, -HIS, -TRP and grown at 30°C to select for primary transformants. Colonies were scraped from plates, washed, and suspended in 15% glycerol, and then stored in 1-mL aliquots at -80°C.

Primary transformants were thawed, added to 9 mL galactose/raffinose (Gal/Raf) CM -URA, -HIS, -TRP (GAL/-UHT), and grown at 30°C for 4 h while shaking. Cultures were plated on Gal/Raf CM -URA -HIS -TRP -LEU (GAL/-UHTL) to select for tomato cDNA clones interacting with XopN. Colonies were picked and stumped onto dilated Gal/Raf CM -URA -HIS -TRP X-gal plates (GAL/-UHT-X-gal) to determine the strength of interactions. Yeast strains were also selected on media containing glucose for negative controls: GLC/-UHT-X-Gal and GLC/-UHTL. Colony PCR using dark and light blue colonies, indicative of strong and weak interactions, respectively, was performed to amplify cDNAs using pJG4-5 primer set J163/J164 flanking the cDNA insertion site. Amplified inserts were digested with TaqI or Hhal and unique clones were sequenced. cDNA sequences were analyzed by BLAST (Altschul et al., 1997) and Sol Genomics Network (www.sgn.cornell.edu). Individual plasmids were isolated and retransformed into Saccharomyces cerevisiae EGY48 pSH18-34, pEG202nls(xopN) to confirm interactions. We also tested XopN interaction with the CDs of tomato BR11 (SI BR11), FLS2 (SI FLS2, courtesy of Silke Robatzeck), and a tomato TARK1-like protein (TARK1-L) using pJG4-5(SIBR11-CD), pJG4-5(SISFLS2-CD), and pJG4-5(TARK1L-CD), respectively.

To create pJG4-5(SIBR11-CD), pJG4-5(SISFLS2-CD), and pJG4-5(TARK1L-CD), the coding regions for the CDs were amplified by PCR using primer sets (XL44/XL24, XL88/XL89, and JG393/JG394, respectively), cloned into pENTR/DTOPO, and then subcloned into pJG4-5. Plasmids were then introduced into yeast strain EGY48 pSH18-34 pEG202nls(lexA-xopN), and interactions were tested as described above.

Yeast Protein Extraction

Yeast cells were resuspended in lysis buffer (1.85 M NaOH and 7% 2-mercaptoethanol) and then proteins were precipitated in 10% trichloroacetic acid. Protein pellets were washed in 1 M Tris and then resuspended in 8 M urea sample buffer.

Protein Gel Blot Analysis

Proteins were separated by SDS-PAGE and analyzed by immunoblot analysis as described (Mudgett et al., 2000). Proteins were visualized by chemiluminescence using anti-HA (Covance), anti-GFP (BD Biosciences), anti-6xHis (Qiagen), anti-XopN (see below), and anti-LexA (Affinity Bioreagents) antibodies, peroxidase-conjugated secondary antibodies (Bio-Rad), and ECL reagent (GE Biosciences).

XopN Antibody Production

To create 6xHis-XopN, the xopN ORF in pENTR(xopN) was recombined into pDEST17 by a Gateway LR reaction. 6xHis-XopN protein was overexpressed in E. coli BL21 IRNA cells and purified using Ni-NTA agarose (Qiagen) under denaturing conditions. Polyclonal antisera were raised in rabbits using the purified 6xHis-XopN fusion protein (Covance).

YFP Fusions and BiFC Constructs for Expression in Nicotiana benthamiana

To create YFP-XopN, the xopN ORF was cut with EcoRI and XbaI and cloned into the binary vector pEZKR-LCY (courtesy of David Ehrhardt) to generate pEZKR-LCY(xopN). To create YFP-TFT1, the TFT1 ORF was cloned into pEZKR-LCY cut with KpnI and BamHI to generate pEZKR-LCY(TFT1). To create TARK1-GFP, the entire ORF (amino acids 1 to 662) was amplified with primer set JR231/JR221, cloned into pENTR/DTOPO, and recombined into the Gateway binary destination vector pGWBS (courtesy of T. Nakagawa), generating pGWBS(TARK1). For pLC2-GFP, Arabidopsis PLC2 (AT3g08510) was PCR amplified with primer set SLF/SRL, cloned into pDONR221, and then recombined into pX-YFP_GW to create pPLC2__YFP.

For the BiFC constructs, TFT1, XopN, and TARK1 were PCR amplified (primer sets GB3/GB4, GB1/GB2, and GB11/GB12, respectively), cloned into the Gateway entry vector pENTR/DTOPO, and then recombined into the Gateway binary destination vectors pXNGW, pXNGW, pXCGW, and pXCWG (courtesy of Wolf Frommer) using LR clonase (Invitrogen). Each protein was independently tagged with cCFP and nYFP at either the N or C terminus. The cCFP and nYFP domains also contain the 6xHis epitope tag. The binary vector backbone is derived from pZP312, which contains a single 35S cauliflower mosaic virus promoter and terminator derived from pRT100. For PLC2-cCFP, Arabidopsis PLC2 in pDONR221 was recombined into pXCWG.

A. tumefaciens–Mediated Transient Protein Expression in N. benthamiana

All binary plasmids were transformed into A. tumefaciens strain C58C1 pCH32 (Mudgett et al., 2000) for transient protein expression in N. benthamiana. Strains were grown overnight at 28°C on Luria agar medium containing the appropriate antibiotics. Bacteria were collected, resuspended in media (10 mM MES, pH 5.6, 10 mM MgCl₂, and 150 μM acetosyringone; Acros Organics), and then incubated 2 h at room temperature before inoculation. Leaves were hand-inoculated with suspension of either one (0.6 × 10⁸ cells/mL) or two (1.2 × 10⁹ cells/mL) strains in induction media. Plants were incubated at room temperature under continuous low light for 2 to 4 d.

Microscopy

A. tumefaciens–infected N. benthamiana leaves were analyzed at 48 HAI. Leaf discs were placed on a slide and visualized using a ×60 objective lens on an inverted microscope (TE2000; Nikon). Fluorescence microscopy was performed with a trichroic filter set (Chroma) using excitation at 493 ± 17 nm with image collection through band emission filter 530 ± 40 nm. Images were collected with a cooled CCD camera (Princeton Instruments) at 12-bit precision (Diagnostic Instruments).

 Constructs and Assay for Protein Pull-Down Analysis in Plant Extracts

Expression vectors for Ni-NTA affinity pull-down assays were constructed by cloning XopN-His6 into the HindIII and XbaI sites of pEZKR-LCY, creating pEZKR(XopN-His6). For single expression constructs, TARK1-HA and TFT1-HA were cloned into the XbaI and SacI sites of the pATC940 vector. For double expression constructs, TARK1-HA and TFT1-HA were cloned into the Gateway destination vector pGWBS and recombined into the Gateway binary destination vector pGWB5 containing the 35S cauliflower mosaic virus promoter and terminator.
35S-TARK1-HA-NOS and 3SS-TFT1-HA fragments were then subcloned into the SpeI site of pEZRK(XopN-His6), creating pEZRK(XopN-His6 + TARK1-HA) and pEZRK(XopN-His6 + TFT1-HA), respectively. All constructs were transformed into A. tumefaciens strain C58C1 pCH32 for transient expression in N. benthamiana.

Proteins were coexpressed in N. benthamiana leaves via the A. tumefaciens–mediated transient expression assay described above. After 48 h, leaves were frozen in liquid N2 and then pulverized in extraction buffer (50 mM NaH2PO4, pH 8, 20 mM imidazole, and 0.15% Triton X-100). Samples were vortexed and centrifuged for 10 min at 18,000g at 4°C. The soluble extracts were incubated with 25 μL of a 50% slurry of Ni-nitrilotriacetic acid Superflow agarose (Qiagen). Agarose was recovered by centrifugation and washed three times with extraction buffer, and proteins were eluted with 25 μL of sample buffer (Mudgett et al., 2000) and then analyzed by gel blot analysis.

TARK1 Site-Directed Mutagenesis

The coding sequence for the CD of TARK1 was amplified with primer set JR231/JR220 from tomato VF36 cDNA and cloned into pEDT to create pEDT(XopN-HA). pEDT(XopN-HA) was recombined into the pEG202nls destination vector (gift from Brian Staskawicz’s lab) to create pEG202nls(TARK1-HA) and pEG202nls(Zahul for technical assistance, and laboratory members for critical reading of the natural text.)

Construction of XopN Deletion Mutants for Yeast Two-Hybrid Analysis

Six xopN deletion mutants (i.e., N, C, M4, M5, and M6) were amplified by PCR (using primer sets BS1/BS4, BS3/BS2, BS5/BS2, BS6/BS2, BS1/BS7, and BS1/BS8, respectively), cloned into pENTR/D-TOPO, and then recombined into the pEG202nls destination vector (gift from Brian Staskawicz’s lab) via a Gateway LR reaction to create pEG202nls(TARK1-CD AEDLL).

Construction of LGAAA Mutant for Expression in Xcv ΔxopN

Overlapping PCR was used to create a DNA fragment containing the xopN promoter and the xopN ORF containing the LGAATA mutation. The 5′ region of xopN(-690 bp to +148bp) was PCR amplified from pVSP61(PxopN-xopN-HA) using primer set JR170/JR15. The ORF of xopN (LGAATA)-HA was PCR amplified from pEDT(xopN-LGAATA)-HA using primer set BS1/BS227. The 5′ fragment and mutated ORF were used as templates in overlapping PCR and the respective product was cloned into pCRII, creating pCRII(PxopN-xopN(LGAATA)-HA). The EcoRI fragment was then subcloned into pVSP61 to create pVSP61(p-xopN(LGAATA)-HA). The vector was then introduced into Xcv 85-10 ΔxopN by triparental mating.

TARK1 Silencing Constructs and Transgenic Tomato Lines

The 3′ region (429 bp) of TARK1 containing the 3′ UTR region was PCR amplified using primer set JR235/JR236, and the product was cloned into pCRII creating pCRII(3′ UTR). The BamHI-XbaI and KpnI-XhoI fragments were then subcloned into pKANNIBAL to create pKANNIBAL(hp-TARK1). The NotI fragment was then subcloned into pRT278 (Gleave, 1992), creating pRT278(hp-TARK1). The construct was mobilized into the A. tumefaciens strain LBA4404 and used for generating the stable TARK1-silenced VF36 tomato by using standard tomato transformation method (McCormick, 1991). The transformants were analyzed by RT-PCR to measure the TARK1 mRNA levels using the primer set JR195/JR221.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative, GenBank/EMBL, or Sol Genomics Network cDNA (www.sgn.cornell.edu) databases under the following accession numbers: AM039952 (xopN), M68248 (PR-1b/SGN4U), X74906 (PR-Q’b), Y15846 (Sti-1), DQ986037 (GST), U20591 (PR-13), X95270 (PR-P69), AT1G48480 (At RKL1), AT3G17840 (At RKL902), P30206 (TFTI), CAB65693 (TFT3), P30210 (TFT5), P30211 (TFT6), FJ176293 (TARK1), SGN-U320509 (TARK1-L), AAN8509 (SI BR1/SR160), SGN-U327754 (SI FLS2), SU317953 (LR22), DQ399825 (GRAS2), SGN-U320188 (WRKY28), and U89256 (PT1S).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. XopN Suppresses the mRNA Levels of PTI Marker Genes during Xcv Infection.

Supplemental Figure 2. Protein Gel Blot Analysis of Proteins Isolated from the Strains Described in Figure 5.

Supplemental Figure 3. Microscopy of Plasmolyzed Plant Cells to Monitor Protein Association with the Plasma Membrane.

Supplemental Figure 4. Protein Gel Blot Analysis of N. benthamiana Leaves Inoculated with A. tumefaciens Strains Used for Analyses in Figure 6.

Supplemental Figure 5. Subcellular Fractionation of XopN-6xHis and TARK1-HA in N. benthamiana Leaves.

Supplemental Figure 6. TARK1 Does Not Exhibit Kinase Activity in Vitro.

Supplemental Figure 7. TARK1 Cytosolic Domain Copurified with an E. coli Protein Kinase.

Supplemental Figure 8. Protein Gel Blot Analysis of Wild-Type TARK1-CD and Two Different LXXL Mutant Proteins Expressed in Yeast.

Supplemental Figure 9. LXXLL Is a Conserved Motif Preceding the Predicted P-Loop in TARK1 and Several Putative Homologs in the LRRIII Subfamily of LRR-RLKs from Arabidopsis.

Supplemental Figure 10. Protein Gel Blot Analysis of XopN Mutant Proteins Isolated from Yeast Strains Described in Figure 9B.

Supplemental Figure 11. Protein Gel Blot Analysis of Wild-Type XopN-HA and LGAATA-HA Expressed in Xcv ΔxopN Bacterial Extracts.

Supplemental Table 1. PCR Primers Used in This Study.

Supplemental Methods.

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**Xanthomonas T3S Effector XopN Suppresses PAMP-Triggered Immunity and Interacts with a Tomato Atypical Receptor-Like Kinase and TFT1**

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