Functional Analysis of Plant Defense Suppression and Activation by the *Xanthomonas* Core Type III Effector XopX

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Many phytopathogenic type III secretion effector proteins (T3Es) have been shown to target and suppress plant immune signaling but perturbation of the plant immune system by T3Es can also elicit a plant response. XopX is a “core” *Xanthomonas* T3E that contributes to growth and symptom development during *Xanthomonas euvesicatoria* infection of tomato but its functional role is undefined. We tested the effect of XopX on several aspects of plant immune signaling. XopX promoted ethylene production and plant cell death (PCD) during *X. euvesicatoria* infection of susceptible tomato and in transient expression assays in *Nicotiana benthamiana*, which is consistent with its requirement for the development of *X. euvesicatoria*-induced disease symptoms. Additionally, although XopX suppressed flagellin-induced reactive oxygen species, it promoted the accumulation of pattern-triggered immunity (PTI) gene transcripts. Surprisingly, XopX coexpression with other PCD elicitors resulted in delayed PCD, suggesting antagonism between XopX-dependent PCD and other PCD pathways. However, we found no evidence that XopX contributed to the suppression of effector-triggered immunity during *X. euvesicatoria*–tomato interactions, suggesting that XopX’s primary virulence role is to modulate PTI. These results highlight the dual role of a core *Xanthomonas* T3E in simultaneously suppressing and activating plant defense responses.

Phytopathogenic bacteria of the genus *Xanthomonas* cause disease in diverse crop systems worldwide. *Xanthomonas euvesicatoria* (formerly *X. campestris* pathovar *vesicatoria*) (Jones et al. 2004) causes bacterial spot disease in tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) (Jones et al. 1998). During infection of susceptible plants, *X. euvesicatoria* infiltrates the apoplastic space of foliar tissue through natural openings or wounds and multiplies rapidly there. *X. euvesicatoria* utilizes type III secretion (T3S) to translocate type III effector proteins (T3Es) directly into the hosts’ cells (Buttner 2012), and functional T3S is required for maximal *X. euvesicatoria* growth in its hosts (Bonas et al. 1991). Genomic analyses have identified nine “core” T3Es that are found in almost all sequenced *Xanthomonas* strains (Hajri et al. 2009; Jalan et al. 2011; Moreira et al. 2010; Potnis et al. 2011). The

conservation of these core T3Es suggest that they serve a critical role in *Xanthomonas* pathology, and insight gained by the study of these core T3Es may aid in the development of disease mitigation strategies (Dangl et al. 2013; Potnis et al. 2011).

XopX is one such core T3E. It was originally identified in a screen of *X. euvesicatoria* genes that conferred the ability to cause plant cell death (PCD) in *Nicotiana benthamiana* to the non-PCD-eliciting bacterium *X. campestris* pv. *campestris* 8004 (Metz et al. 2005). XopX is delivered into the plant cell by T3S and required for full virulence of *X. euvesicatoria* GM98-38 on tomato and pepper (Metz et al. 2005). Despite the ability of XopX to elicit PCD in *N. benthamiana* when delivered by bacteria, transgenic *N. benthamiana* expressing XopX are viable, more susceptible to *Xanthomonas* and *Pseudomonas* spp., and develop more severe symptoms during infection (Metz et al. 2005). This suggests that XopX elicitation of PCD is linked to its virulence activity rather than evidence of avirulence activity. Although XopX’s function in the plant cell during infection is unknown, its ortholog from *X. oryzae* pv. *oryzae* was recently shown to suppress *X. oryzae* pv. *oryzae* LipA-elicited callose deposition in rice (Sinha et al. 2013). This latter evidence suggests that XopX may contribute to bacterial virulence by suppressing plant immune signaling. Given that XopX is cytotoxic when expressed in yeast (Salomon et al. 2011), it is likely that XopX targets a broadly conserved eukaryotic cell process that is required for viability.

We are interested in identifying a specific role for XopX in *X. euvesicatoria* pathogenesis. Like many phytopathogenic bacteria, *X. euvesicatoria* maintains a hemibiotrophic lifestyle that requires the pathogen to suppress or evade plant defense responses but avoid killing its host at early stages of infection (Doidge 1921). During infection, the detection of conserved microbe-associated molecular patterns (MAMPs) such as bacterial flagellin by plant cell surface receptors elicits a limited plant defense response called pattern-triggered immunity (PTI) (Jones and Dangl 2006). It is well known that phytopathogenic bacteria employ T3Es to suppress PTI (Boller and He 2009). In response, plants exploit the pathogen’s requirement of a living host by activating PCD during effector-triggered immunity (ETI), an elevated defense response elicited when plant disease resistance proteins detect the presence or activity of specific T3Es (Spoel and Dong 2012). However, T3Es can also suppress ETI (Jones and Dangl 2006). Thus, the interaction between T3Es and the plant immune system is complex and multilayered, and the specific combination of T3Es deployed by the pathogen is a critical determinant of the outcome of a plant-pathogen interaction.

In addition to its role during ETI in resistant plants, PCD leads to host tissue necrosis, which is a symptom and eventual outcome of disease caused by hemibiotrophic pathogens in susceptible plants. The regulation of this PCD (by host or patho-

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gen) is not well understood (Badel et al. 2006; Cohn and Martin 2005; del Pozo et al. 2004). In tomato, the phytohormones salicylic acid (SA) and ethylene (ET) are critical, positive regulators of PCD and symptom development that occurs during infection by *X. euvesicatoria* and *Pseudomonas syringae* pv. *tomato* DC3000 (Cohn and Martin 2005; Lund et al. 1998; O'Donnell et al. 2001, 2003). Our previous work revealed that *X. euvesicatoria* employs the T3E XopD as a “tolerance factor” to suppress SA- and ET-dependent defense and symptom development in tomato (Kim et al. 2008, 2013). The T3E XopJ was also shown to suppress SA accumulation, resulting in the delay of tissue senescence during *X. euvesicatoria* infection of pepper (Ustun et al. 2013). By contrast, the T3Es AvrPto and AvrPtoB are responsible for activating ET production during *P. syringae* pv. *tomato* infection of tomato, which affects symptom development (Cohn and Martin 2005). Currently, it is not known whether individual *X. euvesicatoria* T3Es are also responsible for promoting ET or SA production.

In this study, we focused on assessing the role of XopX in regulating PCD, manipulating phytohormone signaling, and suppressing immunity during PTI and ETI. These three critical functions help to distinguish the role that individual T3Es play within the context of a given T3E repertoire (Cunnac et al. 2009). We provide evidence that XopX contributes to *X. euvesicatoria* virulence by suppressing specific aspects of plant immunity (i.e., reactive oxygen species [ROS] accumulation)

but simultaneously activates plant defense responses and PCD. A similar pattern of dual behavior (i.e., suppression of plant immunity coupled to activation of plant defense and PCD) was previously identified for the *P. syringae* pv. *tomato* core T3E AvrE1 and led to the model that plants can respond to T3E virulence function with a “default to death and defense” strategy (Badel et al. 2006; Lindeberg et al. 2012). Our results for XopX provide further support for this model and highlight important considerations for evaluating how individual T3Es contribute to the outcome of plant–microbe interactions.

RESULTS

Confirmation of XopX contribution to *X. euvesicatoria* virulence using *X. euvesicatoria* 85-10.

The contribution of XopX to *X. euvesicatoria* growth in tomato and pepper was previously demonstrated using *X. euvesicatoria* GM98-38 (Metz et al. 2005). In order to take advantage of the genome sequence and annotation of *X. euvesicatoria* 85-10 during our study of XopX (Thieme et al. 2005), we used an *X. euvesicatoria* 85-10 strain with a genomic deletion in the *xopX* coding sequence ($\Delta xopX$) (Sonnewald et al. 2012). Consistent with prior work, $\Delta xopX$ grew less and caused reduced leaf chlorosis and necrotic spots during infection of ‘VF36’ tomato, which is susceptible to *X. euvesicatoria* 85-10 (Supplementary Fig. S1A and B). We genetically com-

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics ^a	Source or reference
Bacteria		
<i>Xanthomonas euvesicatoria</i>		
Wild-type	Strain 85-10; Rif ^r	Minsavage et al. 1990;
$\Delta xopX$	Strain 85-10; deletion in <i>xopX</i> ; Rif ^r	Thieme et al. 2005
$\Delta hrcV$	Strain 85-10; deletion in <i>hrcV</i> ; Rif ^r	Sonnewald et al. 2012
$\Delta avrRxxv$	Strain 85-10; deletion in <i>avrRxxv</i> ; Rif ^r	Rossier et al. 1999
$\Delta xopX \Delta avrRxxv$	Derived from $\Delta xopX$; deletion in <i>avrRxxv</i> ; Rif ^r	This work
<i>Pseudomonas syringae</i> pv. <i>tomato</i>		
$\Delta hrcU$	Strain DC3000; deletion in <i>hrcU</i> ; Rif ^r	This work
<i>Agrobacterium tumefaciens</i>		
C58C1, pCH32	C58 with Ti plasmid cured; pCH32 is derivative of pCC113 with <i>virE</i> ; Rif ^r Tet ^r	Mudgett and Staskawicz 1999
EHA105	C58 with pEHA105 (pTiBo542 Δ T-DNA); Rif ^r	Tai et al. 1999
GV2260	C58 with pGV2260 (pTiB6S3 Δ T-DNA); Rif ^r	Hood et al. 1993
<i>Escherichia coli</i>		
DH5 α	F–80 Δ lacZ Δ M15 Δ (lacZYA-argF)U169 <i>endA1 deoR recA1 hsdR17</i> (rK–mK+) <i>phoA supE44 λ-thi-1 gyrA96 relA1</i>	McBride and Summerfelt 1990
HB101, pRK600	F– Δ (<i>gpt-proA</i>)62 <i>leuB6glnV44 ara-14 galK2 lacY1 (mcrC-mrr) rpsL20 Str^r xyl-5 mtl-1 recA13 thi-1</i> ; pRK600 is Cmp ^R derivative of RK2013	Life Technologies, Carlsbad, CA
<i>Acinetobacter</i>		
ADPWH _{lux}	Insertion of <i>luxCDABE</i> between <i>salA</i> and <i>salR</i>	Kessler et al. 1992
Plasmids		
pCR8/GW/TOPO	Spc ^r	Huang et al. 2005
pJET1.2/blunt	Amp ^r	Life Technologies, Carlsbad, CA
pDEST17	Amp ^r	Thermo Scientific, Waltham, MA
pDEST17(<i>xopX</i>)	pDEST17 with <i>xopX</i> fusion to N-terminal 6xHis tag; Amp ^r	Life Technologies, Carlsbad, CA
pLVC18-RfC	Gateway destination suicide vector; Tet ^r	Roden et al. 2004
pLVC18-RfC($\Delta avrRxxv$)	pLVC18 derivative with fusion of genomic regions upstream and downstream of <i>avrRxxv</i> ; Tet ^r	This work
pDSK519	Kan ^r	Keen et al. 1988
pDSK519(<i>xopX</i> ₂₆₂₃)	pDSK519 with <i>xopX</i> including upstream and downstream regions; Kan ^r	This work
pGWB6	Kan ^r	Nakagawa et al. 2007
pGWB6(<i>xopX</i>)	pGWB6 with <i>xopX</i> and 35S promoter; Kan ^r	This work
pBTEx	Kan ^r	Frederick et al. 1998
pBTEx(<i>Pto</i> ; <i>avrPto</i>)	pBTEx with <i>Pto</i> and <i>avrPto</i> , both with 35S promoter; Kan ^r	Frederick et al. 1998
pBTEx(<i>Bax</i>)	pBTEx with <i>Bax</i> and 35S promoter; Kan ^r	G. Sessa
pER8	Spc ^r	Zuo et al. 2000
pER8(SIMKKK Δ a)	pER8 with Spc ^r	G. Sessa
pER8(SIMKK2 ^{DD})	Spc ^r	G. Sessa
pER8(SIMPK1)	Spc ^r	G. Sessa

^a Rif^r, Tet^r, Str^r, Cmp^r, Spc^r, Amp^r, and Kan^r indicate resistant to rifampicin, tetracycline, streptomycin carbenicillin, spectinomycin, ampicillin, and kanamycin, respectively.

plemented $\Delta xopX$ by transforming it with a broad-host-range plasmid bearing the *xopX* locus from *X. euvesicatoria* 85-10 (pDSK519[*xopX*₂₆₂₃]) (Table 1). pDSK519(*xopX*₂₆₂₃) restored $\Delta xopX$ to wild-type (WT) levels of *X. euvesicatoria* growth and disease symptoms in infected tomato 'VF36' leaves. To confirm the contribution of XopX protein expression to *X. euvesicatoria* virulence, we generated XopX antisera for use in

monitoring XopX protein levels in *X. euvesicatoria* cultures. Plasmid-borne *xopX* restored XopX protein expression to $\Delta xopX$, although at higher levels than that detected for the WT. These results support the requirement of XopX for full *X. euvesicatoria* virulence (Metz et al. 2005) and allowed us to use *X. euvesicatoria* 85-10 (referred to from now on as WT) and $\Delta xopX$ for further study of XopX function.

Fig. 1. XopX contribution to plant cell death (PCD) and phytohormone accumulation during *Xanthomonas euvesicatoria* infection of susceptible tomato. **A**, Tomato VF36 leaves were inoculated with the wild type (WT) and $\Delta xopX$ at 2×10^8 CFU ml^{-1} in specific regions of the same leaflet or **B** to **D**, whole leaflets were inoculated with WT, $\Delta xopX$, or $\Delta hrcV$ at 2×10^8 CFU ml^{-1} or 1 mM $MgCl_2$ (mock). **A**, Leaflets were harvested 1, 2, or 3 days postinoculation (dpi) and photographed before (left insets) and after (right insets) staining with trypan blue to highlight regions of PCD. **B**, Four leaf disks from each treatment were harvested at 1, 2, or 3 dpi, washed, and floated on 5 ml of distilled water for 4 h with gentle shaking prior to measuring the conductivity of bathing water. Values are mean conductivity of bathing water ($\mu S\ mm^{-2}\ leaf\ mL^{-1}$ bathing water) $\pm$ standard deviation (SD) ($n = 3$ plants). **C**, Inoculated whole leaflets were harvested 48 h postinoculation (hpi) and placed into separate sealed vials with minimal damage. Ethylene (ET) gas in headspace around the leaflet was quantified by gas chromatography over the course of 1 h. Values are mean ET concentration ($nL\ ET\ g^{-1}\ leaf\ h^{-1}$) $\pm$ SD ($n = 4$ plants). **D**, Leaf disks from inoculated leaflets were harvested 48 hpi and total (left) and free (right) salicylic acid (SA) were quantified using the *Acinetobacter* sp. ADPWH_{lux} biosensor. Values are mean total or free SA ($\mu g\ SA\ g^{-1}$ leaf) $\pm$ SD ($n = 4$ plants). **E**, Tomato VF36 leaves were inoculated with *X. euvesicatoria* WT, $\Delta xopX$, or $\Delta hrcV$ at 2×10^8 CFU ml^{-1} . Leaf bacteria were enumerated for each treatment 0, 6, 24, and 48 hpi. Values are mean leaf bacteria (CFU cm^{-2} leaf) $\pm$ SD ($n = 4$). Different letters above bars indicate statistically significant differences as determined by two-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD), $P < 0.05$ (B and E) or one-way ANOVA and Tukey's HSD, $P < 0.05$ (C and D); n.s. = no significant differences among treatments.

XopX is required for *X. euvesicatoria*-induced ET and PCD in susceptible tomato.

Previous studies have shown that some *Pseudomonas* T3Es required for full pathogenicity also promote PCD (i.e., the functionally redundant T3E pairs AvrE1/HopM1 and AvrPto/AvrPtoB), possibly as a consequence of their virulence function (Badel et al. 2006; Cohn and Martin 2005). Given that XopX is required for symptom development and tissue necrosis during *X. euvesicatoria* infection of tomato, we tested whether or not XopX is required for *X. euvesicatoria*-induced PCD in tomato by performing trypan blue staining of tomato leaves inoculated with *X. euvesicatoria*. High-titer (2×10^8 CFU ml⁻¹) inoculation of *X. euvesicatoria* into susceptible tomato leaves results in PCD and tissue collapse 3 to 5 days postinoculation (dpi) (Fig. 1A). The extent of cell death can be visualized by the relative amount of trypan blue stain taken up by dead cells. As expected, tomato leaves inoculated with WT were heavily stained with trypan blue by 3 dpi (Fig. 1A). However, tomato leaves inoculated with $\Delta xopX$ had lower levels of staining (Fig. 1A), suggesting that XopX is required for *X. euvesicatoria*-induced PCD in tomato.

To quantify XopX's contribution to *X. euvesicatoria*-induced PCD in tomato, we measured electrolyte leakage from leaves inoculated with 1 mM MgCl₂ (mock) or WT, $\Delta xopX$, or $\Delta hrcV$ (an *X. euvesicatoria* 85-10 mutant unable to secrete T3Es) at 2×10^8 CFU ml⁻¹ (Rossier et al. 1999). At 3 dpi, electrolyte leakage from leaves inoculated with WT was significantly higher than the mock treatment (Fig. 1B). By contrast, electrolyte leakage from $\Delta hrcV$ -inoculated leaves was similar to the mock

treatment (Fig. 1B), indicating that the secretion of T3Es is required for *X. euvesicatoria*-induced PCD. Electrolyte leakage from $\Delta xopX$ -inoculated leaves was similar to electrolyte leakage from $\Delta hrcV$ -inoculated leaves and the mock treatment (Fig. 1B), suggesting that T3S of XopX causes PCD or that XopX is required for PCD caused by another T3E.

During both *X. euvesicatoria* and *P. syringae* pv. *tomato* infection of susceptible tomato, PCD and the resulting symptom development is positively regulated, in part, by host factors, such as the phytohormones ET and SA (del Pozo et al. 2004; Lund et al. 1998; O'Donnell et al. 2001, 2003). In fact, specific *X. euvesicatoria* T3Es suppress ET and SA signaling to mitigate symptom development in susceptible plants and promote *X. euvesicatoria* growth (Kim et al. 2008, 2013; Ustun et al. 2013). Although ET and SA signaling are activated during ETI in resistant plants, less is known about how these phytohormones are activated by T3Es in susceptible plants (Bari and Jones 2009). Previously, the elicitation of PCD by AvrPto and AvrPtoB was linked to their promotion of ET biosynthesis (Cohn and Martin 2005). Because XopX is required for *X. euvesicatoria*-induced PCD (Fig. 1A and B), we next tested the role of XopX in promoting ET and SA production in susceptible tomato leaves. Consistent with previous reports (O'Donnell et al. 2001, 2003; Kim et al. 2013), inoculation of WT at 2×10^8 CFU ml⁻¹ significantly increased ET accumulation in tomato leaves compared with mock inoculation by 2 dpi (Fig. 1C). A low level of ET was detected in $\Delta hrcV$ - and mock-inoculated leaves (Fig. 1C), demonstrating that *X. euvesicatoria*-induced ET requires the secretion of T3Es. The ET level in

Fig. 2. Plant cell death (PCD) and phytohormone accumulation in *Nicotiana benthamiana* leaves after transient expression of green fluorescent protein (GFP):XopX. *Agrobacterium tumefaciens* strains bearing binary vectors for GFP or GFP:XopX expression were inoculated into *N. benthamiana* leaves at 4×10^8 CFU ml⁻¹. **A**, Leaves were harvested at 3 days postinoculation (dpi) and photographed before (top) and after (bottom) staining with trypan blue to indicate regions of PCD. **B**, Four leaf disks from each treatment were harvested 20 h postinoculation (hpi), washed, and floated on 5 ml of distilled water. Conductivity of bathing water was measured at 24 hpi and every subsequent 24 h for 5 days. Values are mean conductivity of bathing water ($\mu S\ mm^{-2}\ leaf\ ml^{-1}$ bathing water) $\pm$ standard deviation (SD) ($n = 3$ plants). **C**, Inoculated regions were harvested at 24 and 48 hpi and placed into separate sealed vials with minimal damage. Ethylene (ET) gas in headspace around leaf was quantified by gas chromatography over the course of 1 h. Values are mean ET concentration ($nL\ ET\ g^{-1}\ leaf\ h^{-1}$) $\pm$ SD ($n = 3$ plants). **D**, Leaf disks were harvested 24 and 48 hpi and total (left) and free (right) salicylic acid (SA) was quantified using the *Acinetobacter* sp. ADPWH_{lux} biosensor. Values are mean total or free SA ($\mu g\ SA\ g^{-1}\ leaf$) $\pm$ SD ($n = 3$ plants). **B** to **D**, Asterisks above bars indicate statistically significant differences as determined by two-way analysis of variance and Tukey's honestly significant difference, $P < 0.05$.

$\Delta xopX$ -inoculated leaves was 26% of that in WT-inoculated leaves on average, indicating that XopX alone accounts for the majority of the ET accumulation induced by WT (Fig. 1C). However, the ET level in $\Delta hrcV$ -inoculated leaves was still significantly lower than that in $\Delta xopX$ -inoculated leaves (only 6% of WT), suggesting that other *X. euvesicatoria* T3Es contribute to ET accumulation, albeit to a lesser extent.

Total and free SA levels in WT-inoculated leaves were significantly higher than that in $\Delta hrcV$ - and mock-inoculated leaves (Fig. 1D), suggesting that SA accumulation during *X. euvesicatoria* infection is also primarily T3S dependent. This accumulation was not dependent on XopX because SA levels in $\Delta xopX$ -inoculated leaves were similar to those in WT-inoculated leaves (Fig. 1D).

Therefore, the results of our PCD and phytohormone analyses in *X. euvesicatoria*-inoculated tomato leaves indicate that the primary tomato phenotypes associated with the presence of XopX within the plant cell are the production of ET (Fig. 1D and E) and the activation of PCD (Fig. 1A and B). We also quantified *X. euvesicatoria* growth in tomato leaves to account for the contribution of bacterial load to PCD and ET production during these experiments. Prior to the onset of PCD and tissue collapse at 3 dpi, WT and $\Delta xopX$ populations increased approximately 10-fold and were present at similar titers at 0, 6, and 24 h postinoculation (hpi), although WT was significantly higher than $\Delta xopX$ at 48 hpi (Fig. 1E). By contrast, $\Delta hrcV$ grew less and was significantly lower than both WT and $\Delta xopX$ at 48 hpi (Fig. 1E). Because $\Delta xopX$ and $\Delta hrcV$ elicited similar PCD (Fig. 1B) and ET (Fig. 1C) phenotypes but had different growth patterns (Fig. 1E), bacterial titer alone does not explain the lack of PCD and ET elicited by the $\Delta xopX$ strain. Therefore, these data suggest that the difference in PCD and ET production between WT- and $\Delta xopX$ -inoculated leaves was primarily due to the presence of XopX. To confirm this hypothesis, we next expressed XopX in planta to determine whether XopX alone is sufficient for elicitation of PCD and ET.

Transient XopX expression promotes ET and PCD in *N. benthamiana*.

The bacterial genetic approach for studying XopX described above may be complicated by the presence of other *X. euvesicatoria* T3Es as well as the growth defect of the $\Delta xopX$ strain. Therefore, to study XopX function in isolation, we transiently transformed *N. benthamiana* leaf cells to express XopX with an N-terminal green fluorescent protein (GFP) tag (GFP:XopX) and then monitored PCD and ET production. We chose *N. benthamiana* as the host because *Agrobacterium*-mediated transient transformation of *N. benthamiana* is more efficient than tomato. Consistent with the requirement of XopX for *X. euvesicatoria*-induced PCD in tomato (Fig. 1), transient expression of XopX in *N. benthamiana* was previously reported to cause PCD either on its own or in conjunction with an unidentified, T3S-dependent factor (Metz et al. 2005; Salomon et al. 2011). In our experiments, transient expression of GFP:XopX alone caused weak PCD in *N. benthamiana* leaves (Fig. 2A). Macroscopic PCD was usually not visible before 3 to 4 dpi, although trypan blue staining and electrolyte leakage measurements of regions expressing GFP:XopX (Fig. 2A and B) provided evidence of detectable PCD beginning 48 hpi.

Next, we used this transient expression system to test whether the presence of XopX in the plant cell is sufficient to promote ET production. Transient expression of GFP:XopX but not GFP induced ET accumulation in *N. benthamiana* leaves (Fig. 2C) prior to the onset of XopX-induced PCD. Therefore, XopX is both required (*X. euvesicatoria*-inoculated tomato, Fig. 1D) and sufficient (transient expression in *N. benthamiana*) to elicit ET production prior to PCD. By contrast,

total SA accumulation was similar in regions expressing GFP and GFP:XopX. Free SA accumulation was slightly less in regions expressing GFP:XopX than in those expressing GFP (Fig. 2D). This slight suppression of free SA accumulation might be a consequence of XopX-triggered ET production, because data from *Arabidopsis thaliana* suggests that ET signaling can antagonize SA signaling (Kunkel and Brooks 2002).

XopX promotes PTI gene transcript accumulation but suppresses PTI-associated ROS.

The finding that XopX promotes ET production and PCD is consistent with the requirement of XopX in promoting *X. euvesicatoria* symptom development but it does not explain how XopX contributes to *X. euvesicatoria* growth. Although AvrPto and AvrPtoB were shown to elicit ET and PCD during *P. syringae* pv. *tomato* infection of tomato (Cohn and Martin 2005), subsequent research has suggested that their primary role is suppression of plant immune signaling by plant cell receptor-kinases (Gimenez-Ibanez et al. 2009; Gohre et al. 2008; Xiang et al. 2008). Indeed, a primary function of phytopathogenic T3Es in promoting bacterial growth is the suppression of plant immune signaling at different nodes (Mukhtar et al. 2011). Because XopX is also required for full *X. euvesicatoria* growth in susceptible plants, we next sought to explicitly test whether XopX suppresses PTI signaling during *X. euvesicatoria* infection of susceptible tomato by monitoring the transcript levels of known PTI genes (i.e., *SLPti5*, *SLWRKY28*, *SILRR22*, and *SIGRAS2*) in response to *X. euvesicatoria* with or without XopX. Previously, transcripts of these genes were found to increase in response to flagellin and infection by T3S mutants of *P. syringae* pv. *tomato* and *X. euvesicatoria*, and were suppressed by the *X. euvesicatoria* T3E, XopN (Kim et al. 2009; Nguyen et al. 2010). We used quantitative polymerase chain reaction (qPCR) to quantify the transcript levels of these genes in tomato 'VF36' leaves inoculated with 1 mM MgCl₂ or a high titer (2×10^8 CFU ml⁻¹) of WT, $\Delta xopX$, or $\Delta hrcV$ at 6 and 12 hpi. As expected, $\Delta hrcV$ treatment induced transcript accumulation for all four PTI genes relative to the mock treatment, and this induction was suppressed by WT treatment (Fig. 3A). *SLPti5* and *SILRR22* were significantly lower in WT-inoculated leaves than in $\Delta hrcV$ -inoculated leaves, while *SLWRKY28* and *SIGRAS2* were slightly—though not significantly—lower in WT than $\Delta hrcV$ (Fig. 3A). Surprisingly, PTI gene transcripts tended to be lower in $\Delta xopX$ -inoculated leaves than in WT-inoculated leaves (Fig. 3A). A significant difference was observed for *SILRR22* (Fig. 3A). Thus, XopX appears to weakly promote, rather than suppress, a plant defense response in tomato, at least at the level of transcript accumulation.

We next tested whether XopX is sufficient to suppress PTI elicited by *P. syringae* pv. *tomato* using transient expression of XopX in *P. syringae* pv. *tomato*-infected *N. benthamiana* leaves. We monitored XopX-dependent suppression of PTI prior to the onset of XopX-induced PCD in this system. Treatment of *N. benthamiana* leaves with T3S-deficient *P. syringae* pv. *tomato* ($\Delta hrcU$) triggers transcript accumulation of the PTI genes *NbAcre31* and *NbPti5* (Nguyen et al. 2010). We treated *N. benthamiana* leaves transiently expressing GFP or GFP:XopX with a T3S-deficient *P. syringae* pv. *tomato* strain ($\Delta hrcU$) at 2×10^8 CFU ml⁻¹ and quantified PTI gene transcript accumulation. *P. syringae* pv. *tomato* $\Delta hrcU$ treatment triggered accumulation of *NbAcre31* and *NbPti5* transcripts compared with a mock treatment in leaf tissue expressing GFP although, for *NbPti5*, this increase was not statistically significant (Fig. 3B). Gene transcript levels were the same (*NbAcre31*) or higher (*NbPti5*) in the *P. syringae* pv. *tomato* $\Delta hrcU$ -inoculated leaf tissue expressing GFP:XopX compared with that

expressing GFP (Fig. 3B). However, GFP:XopX expression alone triggered accumulation of these transcripts, and *P. syringae* pv. *tomato* Δ *hrcU* treatment did not have a further effect on their accumulation (Fig. 3B). XopX induction of PTI gene expression in *N. benthamiana* is consistent with our results using *X. euvesicatoria* in tomato and further suggests that XopX itself promotes defense in planta.

Finally, we sought to test the effect of XopX on extracellular ROS production, a nontranscriptional PTI response. Application of flg22, a 22-amino-acid peptide derived from flagellin, to *N. benthamiana* leaf tissue elicits the rapid accumulation of extracellular ROS which, as in *Arabidopsis thaliana*, requires a plasma membrane-localized NADPH oxidase, NbRbohD (Segonzac et al. 2011). We treated leaf disks transiently expressing GFP or GFP:XopX with 200 nM flg22 and quantified the resulting ROS burst. By contrast to PTI transcript accumulation, GFP:XopX but not GFP alone was sufficient to prevent flg22-induced ROS burst (Fig. 3C). This confirms that XopX

presence in the plant cell suppresses at least one PTI output. However, this suppression activity is contradictory to the activation of plant defense gene transcription by XopX.

XopX interferes with PCD induced by Pto-AvrPto, mitogen-activated protein kinase (MAPK) signaling, or Bax.

Although there is an overlap in the signaling components and outputs involved in PTI and ETI (Thomma et al. 2011; Tsuda and Katagiri 2010), the ability to suppress both PTI and ETI appears to be restricted to a subset of phytopathogenic T3Es (Feng et al. 2012; Guo et al. 2009). Such dual activity suggests that these T3Es either target a regulatory node common to both pathways or have multiple plant targets or suppression activities. Therefore, we assessed the versatility of XopX in suppressing plant immune responses by testing its ability to suppress ETI. In *N. benthamiana*, transient coexpression of the *P. syringae* pv. *tomato* T3E AvrPto and its cognate resistance

Fig. 3. Effect of XopX on pattern-triggered immunity (PTI) responses in tomato and *Nicotiana benthamiana*. **A**, Tomato VF36 leaves were inoculated with *Xanthomonas euvesicatoria* wild type (WT), Δ *xopX*, or Δ *hrcV* at 2×10^8 CFU ml⁻¹ or 1 mM MgCl₂ (mock). Samples were harvested 6 and 12 h postinoculation (hpi) for RNA extraction and cDNA synthesis. Transcript levels for known tomato PTI gene markers (i.e., *SIPti5*, *SIWRKY28*, *SILRR22*, and *SIGRAS2*) were quantified by quantitative polymerase chain reaction (qPCR). Relative fold change in transcript abundance for each gene was calibrated to the average Δ cycle threshold (Δ C_T) values from the control treatment (mock) with *SICTIN* as the internal control (Livak and Schmittgen 2001). Values are mean relative fold induction ($2^{-\Delta\Delta C_T}$) $\pm$ standard deviation (SD) ($n = 4$ plants). **B**, *Agrobacterium tumefaciens* strains bearing binary vectors for green fluorescent protein (GFP) or GFP:XopX expression were inoculated into *N. benthamiana* leaves at 4×10^8 CFU ml⁻¹. At 24 hpi, intact leaves were inoculated with 1 mM MgCl₂ (mock) or 2×10^8 CFU ml⁻¹ of *Pseudomonas syringae* pv. *tomato* Δ *hrcU*. Leaf tissue was harvested 6 h after Δ *hrcU* treatment for RNA extraction and cDNA synthesis. Transcript levels for known PTI gene markers (i.e., *NbAcre31* and *NbPti5*) were quantified by qPCR using the comparative C_T method. Relative fold change in transcript abundance for each gene was calibrated to the average Δ C_T values from the control treatment (empty vector + mock), with *NbPP2A* as the internal control. Values are mean relative fold induction ($2^{-\Delta\Delta C_T}$) $\pm$ SD ($n = 4$ plants). **C**, GFP or GFP:XopX were expressed in *N. benthamiana* leaves as in B. At 24 hpi, three leaf disks were harvested and floated on distilled water in individual wells of a 96-well plate overnight under continuous light. Water was removed and replaced with horseradish peroxidase solution with 200 nM flg22 (flg22, top) or without flg22 (mock, bottom). H₂O₂ production was quantified for 90 min after flg22 treatment using a plate reader luminometer. Each plate read lasted approximately 50 s. Values for each read are mean relative light units (RLU) produced $\pm$ SD ($n = 4$ plants). Different letters above bars indicate statistically significant differences as determined by two-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD), $P < 0.05$ (A) or one-way ANOVA and Tukey's HSD, $P < 0.05$ (B); n.s. = no significant differences among treatments.

(R) protein, Pto, initiates ETI signaling that culminates in PCD (Oh and Martin 2011a). We coexpressed Pto-AvrPto in *N. benthamiana* with either GFP or GFP:XopX and monitored PCD. Surprisingly, despite the fact that XopX induces weak PCD (Fig. 2A and B), GFP:XopX delayed PCD activated by Pto-AvrPto (Fig. 4, left).

We next sought to exploit knowledge of the Pto-AvrPto ETI signaling pathway to determine the signaling node at which XopX may be acting (Oh and Martin 2011a). In *N. benthamiana* NbMAPKKK α , its downstream substrate NbMKK2, and MAPK NbSIPK and NbWIPK are required for PCD activated by Pto recognition of AvrPto (Oh and Martin 2011a). Additionally, transient expression of SIMAPKKK α or the constitutively active version of SIMKK2 (SIMKK2^{DD}) both activate PCD (del Pozo et al. 2004; Oh and Martin 2011b), as does transient expression of a tomato MAPK, SIMPK1 (Fig. 4),

which acts downstream in this pathway (Pedley and Martin 2004). To determine the effect of XopX expression on MAPK signaling in *N. benthamiana*, we coexpressed estradiol-inducible constructs of the tomato orthologs of each of the MAPK components with either GFP or GFP:XopX. In all cases, GFP:XopX delayed PCD caused by induction of the MAPK components (Fig. 4, middle).

Previously, the ability of *Pseudomonas* T3Es (including AvrPtoB) to suppress ETI was linked to general PCD suppression activity, including PCD elicited by transgenic expression of the mammalian apoptotic protein, Bax (Abramovitch et al. 2003; Jamir et al. 2004). To test the ability of XopX to suppress or antagonize PCD generally, we coexpressed the mammalian apoptotic protein Bax with either GFP or GFP:XopX. Again, GFP:XopX coexpression with Bax delayed Bax-induced PCD (Fig. 4, right).

Fig. 4. Transient coexpression of green fluorescent protein (GFP):XopX and plant cell death (PCD) elicitors in *Nicotiana benthamiana*. *Agrobacterium tumefaciens* strains bearing binary vectors for GFP or GFP:XopX expression were co-inoculated into *N. benthamiana* leaves with strains bearing binary vectors for expression of PCD elicitors: Pto-AvrPto, mitogen-activated protein kinase (MAPK) signaling components, or Bax. **A**, Representative images of co-inoculations 3 days postinoculation (dpi) (Pto-AvrPto and Bax) or 5 dpi (MAPK components; 4 days after estradiol treatment). Circle indicates region of inoculation and color of circle indicates amount of tissue collapse (magenta = full, blue = partial, yellow = none or limited). **B**, Summary of tissue collapse scores for treatments shown in **A**. Bars indicate percentage of replicates ($n = 18$) in each category of tissue collapse using the same color scale as in **A**. Asterisks over bars indicate statistically significant difference in distribution of replicates among tissue collapse categories between GFP and GFP:XopX co-inoculations with each PCD elicitor as determined by Wilcoxon signed-rank test, $P < 0.05$. Final *A. tumefaciens* concentrations in Pto-AvrPto co-inoculations were 2×10^8 CFU ml^{-1} for all strains; in MAPK, component co-inoculations were 2×10^8 CFU ml^{-1} (GFP, GFP:XopX, SIMPK1, and empty) or 1×10^8 CFU ml^{-1} (SIMAPKKK α and SIMKK2^{DD}); and, in Bax, co-inoculations were 4×10^8 CFU ml^{-1} for all strains. MAPK component constructs were driven by estradiol-inducible promoters and estradiol was applied by spraying 24 h postinoculation. All other constructs were driven by the 35S promoter.

Activation of the NtMEK2 (the tobacco ortholog of SIMKK2) elicits ROS accumulation upstream of PCD, an effect also linked to Bax-elicited PCD (Kawai-Yamada et al. 2004; Liu et al. 2007). Therefore, we next monitored PCD-associated ROS accumulation by cytological staining of H₂O₂ with 3,3'-diaminobenzidine (DAB). Indeed, GFP:XopX coexpression with NtMEK2^{DD} in *N. benthamiana* resulted in reduced DAB staining compared with coexpression with GFP (Fig. 5A), suggesting that XopX acts upstream of ROS accumulation to suppress PCD.

We also analyzed whether XopX could suppress PCD by affecting the stability or abundance of PCD elicitors. Immunoblot analysis of hemagglutinin (HA)-tagged MAPK compo-

nents resulted in detectable protein levels in both GFP- and GFP:XopX-expressing regions (Fig. 5B). Interestingly, all MAPK component protein levels were consistently lower in leaf tissue expressing GFP:XopX than in leaf tissue expressing GFP, correlating with reduced PCD in those regions (Fig. 5B). Notably, reduction of MAPK component protein levels occurred prior to tissue collapse and had a negative correlation with the level of PCD (i.e., XopX reduced protein accumulation and PCD), suggesting that this effect was not due to PCD per se. Furthermore, XopX did not affect total protein levels, as determined by Ponceau staining of immunoblots and similar levels of nonspecific bands in immunoblots for both GFP and GFP:XopX samples (Fig. 5B). These data suggest that XopX

Fig. 5. Reactive oxygen species (ROS) accumulation and protein levels during green fluorescent protein (GFP):XopX coexpression with mitogen-activated protein kinase (MAPK) signaling components in *Nicotiana benthamiana*. **A**, *Agrobacterium tumefaciens* strains bearing GFP and GFP:XopX constructs were mixed to a final concentration of 2×10^8 CFU ml⁻¹ with *A. tumefaciens* GV2260 pER8(NtMEK2^{DD}). Estradiol was applied by spraying 24 h after infiltration. At 10 h after estradiol application, prior to the onset of macroscopic tissue collapse, infiltrated leaf tissue was vacuum infiltrated with 3,3'-diaminobenzidine solution. Leaf tissue was destained overnight in ethanol and imaged under a dissecting microscope. ROS accumulation is indicated by dark brown staining. **B**, *A. tumefaciens* strains bearing binary vectors for GFP or GFP:XopX expression were co-inoculated into *N. benthamiana* leaves with strains bearing binary vectors for expression of MAPK signaling components. Final *A. tumefaciens* concentrations were 2×10^8 CFU ml⁻¹ (GFP, GFP:XopX, SIMPK1, and empty) or 1×10^8 CFU ml⁻¹ (SIMAPKKKa and SIMKK2^{DD}). MAPK component constructs were driven by estradiol-inducible promoters and estradiol was applied by spraying 24 h postinoculation. Leaf tissue was harvested 10 h after estradiol treatment for protein extraction. Protein from leaf extracts was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and subjected to immunoblotting with anti-hemagglutinin (HA) or anti-GFP primary antibodies followed by horseradish-peroxidase-conjugated secondary antibodies. Asterisks at right of blots indicate protein band corresponding to the labeled protein in each lane.

alters the steady state levels of immune-related or highly expressed proteins specifically.

XopX does not contribute to ETI suppression in resistant tomato.

Because XopX interfered with the activity of a broad array of PCD elicitors in *N. benthamiana*, we hypothesized that XopX might interfere with ETI elicited by *X. euvesicatoria* in resistant tomato. Therefore, we tested whether resistant tomato exhibited increased ETI in response to *X. euvesicatoria* lacking *xopX*. The tomato 'Hawaii 7998' is resistant to WT and this resistance is triggered by the T3E AvrRxv (Whalen et al. 1993). We generated unmarked deletions of *avrRxv* in WT and $\Delta xopX$ ($\Delta avrRxv$ and $\Delta xopX\Delta avrRxv$, respectively). Then, we monitored growth of these *X. euvesicatoria* strains in 'Hawaii 7998' and the susceptible tomato 'Hawaii 7981'. $\Delta xopX$ growth was slightly reduced compared with WT in 'Hawaii 7998' leaves at 3 dpi but this defect was transient and not significant (Fig. 6A). By contrast, $\Delta xopX$ growth was significantly lower than WT in Hawaii 7981 leaves (Fig. 6A). $\Delta avrRxv$ growth was higher than WT in 'Hawaii 7998' but lower in Hawaii 7981 (Fig. 6A), suggesting a virulence role for AvrRxv that has not been previously reported (Bonshtien et al. 2005; Whalen et al. 1993). $\Delta xopX\Delta avrRxv$ growth was significantly lower than $\Delta avrRxv$ in both 'Hawaii 7998' and '7981', further demon-

strating that XopX virulence activity is independent of AvrRxv in both genetic backgrounds (Fig. 6A).

When inoculated into 'Hawaii 7998' leaves at high titer, *X. euvesicatoria* triggers rapid PCD, which is characteristic of ETI (Whalen et al. 1993). We inoculated WT, $\Delta xopX$, or $\Delta hrcV$ into 'Hawaii 7998' leaves at 2×10^8 CFU ml^{-1} and quantified electrolyte leakage from the leaves (Fig. 6B). Leaves inoculated with WT or $\Delta xopX$ had similar levels of electrolyte leakage, which were significantly higher than $\Delta hrcV$ (Fig. 6B). This suggests that XopX is not able to suppress AvrRxv-elicited ETI in tomato. To confirm that the lack of ETI suppression by XopX was not specific to AvrRxv ETI, we repeated this experiment in transgenic tomato 'VF36' expressing the pepper resistance (*R*) gene *Bs2*, which recognizes *avrBs2* from WT (Tai et al. 1999). As in 'Hawaii 7998', WT elicited high levels of electrolyte leakage (significantly higher than $\Delta hrcV$) when inoculated at high titer into tomato 'VF36' *Bs2* leaves (Fig. 6B). Electrolyte leakage from $\Delta xopX$ -inoculated leaves was similar to that from WT-inoculated leaves at 24 hpi and slightly lower at 48 hpi (Fig. 6B), indicating that XopX does not suppress AvrBs2 ETI and may, in fact, contribute to PCD associated with AvrBs2 ETI. These data suggest that, although transient expression of XopX in *N. benthamiana* interferes with PCD, *X. euvesicatoria*-delivered XopX is not a general suppressor of ETI in tomato.

Fig. 6. Analysis of XopX effect on AvrRxv- and AvrBs2-dependent effector-triggered immunity (ETI) responses in tomato. **A**, Hawaii 7998 (resistant; top) or Hawaii 7981 (susceptible; bottom) tomato leaves were inoculated with *Xanthomonas euvesicatoria* wild type (WT), $\Delta xopX$, $\Delta avrRxv$, or $\Delta xopX\Delta avrRxv$ at 2×10^4 CFU ml^{-1} . Leaf bacteria were enumerated for each treatment 0, 3, 6, and 9 days postinoculation (dpi). Values are mean leaf bacteria (CFU cm^{-2} leaf) $\pm$ standard deviation (SD) ($n = 3$). **B**, Hawaii 7998 (top) or VF36 *Bs2* (bottom) tomato leaves were inoculated with *X. euvesicatoria* WT, $\Delta xopX$, or $\Delta hrcV$ at 2×10^8 CFU ml^{-1} . Four leaf disks were harvested per sample at 24 and 48 h postinoculation (hpi), rinsed, and floated on 3 ml of distilled water for 2 h with gentle shaking, and the conductivity of the bathing water was measured. Values are mean percent electrolyte leakage ($\mu\text{S mm}^{-2}$ leaf mL^{-1} bathing water) $\pm$ SD ($n = 3$ plants). Different letters above bars indicate statistically significant differences within each time point as determined by two-way analysis of variance and Tukey's honestly significant difference, $P < 0.05$.

DISCUSSION

We have identified a role for the *Xanthomonas* core T3E XopX in modulating plant immune signaling, including suppression of flagellin-induced ROS production and interference with rapid PCD elicited by Pto detection of AvrPto, activation of MAPK signaling, and the general cell death elicitor, Bax. However, we also found that XopX itself promotes plant defense gene transcription, ET production, and PCD during *X. euvesicatoria* infection of tomato and when transiently expressed in *N. benthamiana*. This complex combination of suppression and activation of immunity-related plant responses raises intriguing parallels with well-studied *P. syringae* pv. *tomato* T3Es, especially the core T3Es AvrE1 and HopM1 (Badel et al. 2006) and potent PTI suppressors AvrPto and AvrPtoB (Cohn and Martin 2005). Therefore, our results provide a useful starting point for contextualizing XopX function.

The plant immune system features multiple nodes that positively regulate immune signaling but negatively regulate each other; thus, perturbation of one node by a T3E may lead to hyperactivation of another (Sato et al. 2010). As recently noted (Kadota et al. 2014), null mutations in certain *A. thaliana* PTI signaling components display overactive immune responses, including increased PTI gene expression (He et al. 2007; Kadota et al. 2014; Kemmerling et al. 2007; Nishimura et al. 2003; Petersen et al. 2000; Zhang et al. 2010), leading to the hypothesis that severe perturbation of specific PTI signaling components is detected by the plant (Segonzac and Zipfel 2011).

Suppression of flg22-elicited ROS by XopX suggests that it interferes with plant immune signaling at an early stage. However, XopX promotes—rather than suppresses—PTI-elicited gene expression, indicating that it does not suppress PTI signaling to all downstream outputs. It is known that flg22-elicited PTI gene expression is independent of NbRbohD-mediated ROS production in *N. benthamiana* (Segonzac et al. 2011). Thus, our data is consistent with a model whereby XopX functions to target a specific node of plant immunity (i.e., NbRbohD-produced ROS). This targeting would not result in the suppression of PTI gene expression. Rather, perturbation of the node targeted by XopX would result in increased PTI gene transcription.

The idea that T3E perturbation of plant immunity itself leads to a plant defense response was put forward as a model to explain why the functionally redundant *P. syringae* pv. *tomato* T3Es AvrE1 and HopM1 suppress immunity but also cause R-gene-independent PCD across a range of plant species (Badel et al. 2006). In this default to death and defense model (Lindeberg et al. 2012), AvrE1 and HopM1 suppress plant immunity by interfering with a critical plant cell process: vesicle trafficking (Ham et al. 2009; Nomura et al. 2006). The plant cell responds to this perturbation of a critical cell process by initiating, in this case, a PCD response. The fact that AvrE1 and HopM1 elicit PCD in many plants suggests that this process is not mediated by R protein detection of a T3E but, instead, is a general response to T3E virulence activity (Badel et al. 2006; Lindeberg et al. 2012). XopX activation of PTI gene transcription, ET production, and PCD is consistent with this model. Notably, XopX is also cytotoxic in yeast (Salomon et al. 2011), which suggests that—although the cellular pathways leading to yeast cell death and PCD may differ—XopX may indeed target a basic cell process common among eukaryotes.

Whether it is suppressed by the pathogen or just ineffective in halting pathogen growth in susceptible plants, the default to death and defense model implies that the PCD that underlies symptom development may, in fact, be a plant defense response to T3Es. Consistent with plant regulation of PCD in susceptible interactions (i.e., through the phytohormones ET and SA), SIMAPKKK α (a positive regulator of ETI) is also required for

PCD during *P. syringae* pv. *tomato* infection of tomato (del Pozo et al. 2004). Previously, *P. syringae* pv. *tomato* T3Es AvrPto and AvrPtoB were shown to promote ET biosynthesis and subsequent PCD during *P. syringae* pv. *tomato* infection of tomato (Cohn and Martin 2005), although the direct mechanism by which these T3Es contribute to bacterial virulence appears to be through the inactivation of plant receptor-like kinases (Gimenez-Ibanez et al. 2009; Gohre et al. 2008; Xiang et al. 2008). Elicitation of defense-associated phytohormone signaling and PCD may then be a side effect of AvrPto/AvrPtoB virulence activity—perhaps another example of the plant default to death and defense response—which hemibiotrophic pathogens must control in order to achieve maximum virulence. Indeed, *X. euvesicatoria* T3Es XopD and XopJ suppress ET- or SA-dependent symptom development during late stages of infection to increase bacterial pathogenesis (Kim et al. 2008, 2013; Ustun et al. 2013).

We cannot rule out the possibility that XopX-elicited PCD is due to weak detection by an R protein, although this seems unlikely given the correlation between PCD and bacterial virulence. XopX homologs are found in almost every sequenced *Xanthomonas* strain, and variation among XopX homologs at the amino acid sequence level correlates with *Xanthomonas* phylogeny. This suggests that XopX is an ancient *Xanthomonas* T3E and that it has evolved along with host-specific traits that distinguish *Xanthomonas* strains. It is not clear why XopX from *X. euvesicatoria* confers PCD-eliciting activity on *N. benthamiana* leaves in response to *X. campestris* pv. *campestris* (Metz et al. 2005), the genome of which encodes two XopX homologs. Future comparative studies of XopX homologs will help to distinguish whether variation in XopX is due to evolved virulence function or escape from host detection.

Because transient expression of XopX itself elicits PCD, we were surprised to find that XopX interfered with ETI and PCD elicited by Pto-AvrPto, MAPK signaling components, and Bax. Antagonism between PCD-eliciting T3Es has been previously described. For example, AvrRpt2-elicited ETI was shown to interfere with AvrRpm1-elicited ETI in *A. thaliana* (Reuber and Ausubel 1996; Ritter and Dangl 1996). This genetic relationship was later explained by the fact that prior AvrRpt2 proteolysis of guard cell protein RIN4 prevents phosphorylation of RIN4 by AvrRpm1 (Axtell and Staskawicz 2003; Kim et al. 2005; Mackey et al. 2002, 2003). XopX antagonism of a wide range of PCD elicitors is consistent with XopX targeting of a basic cell process required by the PCD pathways tested here. However, our results suggest that XopX is not a general suppressor of known ETI pathways when delivered by *X. euvesicatoria* in tomato (Fig. 6). These findings suggest that suppression of plant immunity by transient expression of T3Es should be interpreted with caution, because results may be affected by different levels of protein expression, biological differences between plant species (i.e., *N. benthamiana* and tomato), or the relative strength of different immune signaling pathways.

In conclusion, the study of highly conserved, core T3Es sheds light on the principal virulence strategies used by plant pathogens. Our results add XopX to the class of T3Es (along with AvrE1 and HopM1) that activate the plant default to death and defense response. Further elucidating the function of XopX and other core T3Es in this emerging T3E class will be important for uncovering the mechanisms that regulate both plant defense and symptom development during plant-microbe interactions.

MATERIALS AND METHODS

Bacterial culture and transformation.

Detailed descriptions of all bacterial strains and plasmids used are provided in Table 1. *Escherichia coli* strains were cul-

tured with lysogeny broth (LB) (pH 7.0, 1% tryptone, 0.5% yeast extract, and 171 mM NaCl) at 37°C, *X. euvesicatoria* 85-10 (and derivative strains) with nutrient yeast glycerol (NYG) (pH 7.0, 0.5% peptone, 0.3% yeast extract, and 2% glycerol.) (Turner et al. 1984), or *hrp*-inducing medium (XVM2) (pH 6.7, 20 mM NaCl, 10 mM (NH₄)₂SO₄, 5 mM MgSO₄, 1 mM CaCl₂, 0.16 mM KH₂PO₄, 0.32 mM K₂HPO₄, 0.01 mM FeSO₄, 10 mM fructose, 10 mM sucrose, and 0.03% casamino acid) (Wengelnik et al. 1996) at 28°C, *P. syringae* pv. *tomato* DC3000 Δ *hrcU* with NYG at 28°C, and *Agrobacterium tumefaciens* strains with LB at 28°C, with appropriate antibiotics. LB and NYG were supplemented with 1.5% (wt/vol) agar (LA and NYGA, respectively) for bacterial growth on plates. Antibiotics were used at the following final concentrations: carbenicillin (50 µg ml⁻¹), chloramphenicol (25 µg ml⁻¹), cyclohexamide (50 µg ml⁻¹), kanamycin (50 µg ml⁻¹), rifampicin (100 µg ml⁻¹), spectinomycin (50 µg ml⁻¹), and tetracycline (5 µg ml⁻¹ for *A. tumefaciens* and 10 µg ml⁻¹ for *X. euvesicatoria*). Plasmids were introduced into *E. coli* and *A. tumefaciens* by heat shock transformation and into *X. euvesicatoria* by triparental mating using *E. coli* HB101, pRK600 (Kessler et al. 1992).

Plant materials.

Seed from *N. benthamiana* and tomato (*S. lycopersicum*) 'VF36', 'Hawaii 7998', 'Hawaii 7981', and 'VF36' Bs2 were germinated in soil at room temperature (RT) under 16 h of light. After germination, tomato plants were grown in a greenhouse (16 h of light, 25 to 28°C) and *N. benthamiana* in a growth chamber (16 h of light, 25°C). For experiments, 4- to 6-week-old tomato and 6- to 8-week-old *N. benthamiana* plants were used.

Genetic complementation of Δ *xopX*.

The *xopX* locus from *X. euvesicatoria* 85-10 (including 387 bp upstream and 355 bp downstream of the *xopX* coding sequence) was cloned by PCR using the primer pair RK2-1/WS2-12 (Table 2) and ligated into pCR8 (Life Technologies, Carlsbad, CA, U.S.A.), creating pCR8(*xopX*₂₆₂₃). The *xopX*

locus from pCR8(*xopX*₂₆₂₃) was then subcloned into pDSK519 by digesting pCR8(*xopX*₂₆₂₃) with *Eco*RI to obtain the *xopX* fragment; then, ligating this fragment into the *Eco*RI site of pDSK519 created pDSK519(*xopX*₂₆₂₃). pDSK519(*xopX*₂₆₂₃) was then introduced into Δ *xopX* by triparental mating and selection of exconjugates with kanamycin.

X. euvesicatoria growth in tomato leaves.

X. euvesicatoria strains grown for 48 h on NYGA were resuspended in 1 mM MgCl₂ and diluted to 1 × 10⁵ CFU ml⁻¹ (for low-titer inoculation in tomato 'VF36'), 2 × 10⁸ CFU ml⁻¹ (for high-titer inoculation in tomato 'VF36'), or 2 × 10⁴ CFU ml⁻¹ (for low-titer inoculation in tomato 'Hawaii 7998' and 'Hawaii 7981'). *X. euvesicatoria* suspensions were infiltrated into the apoplastic space of tomato leaves using a needleless syringe. To quantify *X. euvesicatoria* bacterial population in leaf tissue, 0.5 cm² of leaf tissue was harvested at 0, 3, 6, and 9 dpi, ground completely in 1 ml of 1 mM MgCl₂, serially diluted in 1 mM MgCl₂, and plated on NYGA with appropriate antibiotics. *X. euvesicatoria* CFUs on plates were counted after 48 h of incubation at 28°C.

Preparation of *X. euvesicatoria* culture for protein expression.

Individual *X. euvesicatoria* colonies were isolated on NYGA and cultured in NYG for 24 h at 28°C with shaking. Cellular pellets were collected by centrifugation (8,000 × g, 5 min, RT), resuspended in 1 mM MgCl₂, collected again by centrifugation, and resuspended in NYG or XVM2 at a final titer of 4 × 10⁸ CFU ml⁻¹. Cultures were incubated for 2 to 4 h at 28°C with shaking. The optical density at 600 nm (OD₆₀₀) of final cultures was measured using a spectrophotometer and cellular pellets were collected by centrifugation (16,000 × g, 2 min, RT).

Agrobacterium-mediated transient protein expression in *N. benthamiana*.

A. tumefaciens grown for 24 to 48 h on LA plates was resuspended in LB. Pellets were collected by centrifugation

Table 2. Primers used

Target	Primer name and sequence ^a	Reference
For cloning <i>xopX</i>	CA359: ATGGAGATCAAGAAACAGCAAACC	This work
	CA360: GGACGAAGGCACAGTGCTGGCTGCGGCCT	
<i>xopX</i> ₂₆₂₃	RK2-1: CAATGCGCTGCAACGACGCCTG	This work
	WS2-12: ACAAGGTCAAGGAAGGCAGCGG	
For deleting <i>avrRxv</i>		
<i>avrRxv</i> upstream	WS1-40: TTGCCCTTGATGCCGCCGTT	This work
	JG767: <u>GAAGCTTAAGGATCC</u> ATATTTATTAGATCGCGCTAATCG	
<i>avrRxv</i> downstream	JG768: <u>GGATCCG</u> ACAATTACCAAATATTATTACTTTCC	This work
	WS1-41: <u>GAAGCTT</u> ATTGCCGATGCCGATGCTGG	
For quantitative polymerase chain reaction		
<i>SI</i> ACTIN	JG234: GAGCGTGGTTACTCGTTCA	Kim et al. 2008
	JG136: CTAATATCCACGTCACATTTTCAT	
<i>SI</i> Pti5	CA305: ATTCGCGATTTCGGCTAGACATGGT	Kim et al. 2009; Nguyen et al. 2010
	CA306: AGTAGTGCCCTTAGCACCTCGCATT	
<i>SILRR22</i>	CA293: AAGATTGGAGGTTGCCATTGGAGC	Kim et al. 2009; Nguyen et al. 2010
	CA294: ATCGCGATGAATGATCGGTGGAGT	
<i>SIGRAS2</i>	CA295: TAATCCAAGGGATGAGCTTCT	Kim et al. 2009; Nguyen et al. 2010
	CA296: CCACCAACGTGACCACCTT	
<i>SI</i> WRKY28	CA301: ACAGATGCAGCTACCTCATCTCTCA	Kim et al. 2009; Nguyen et al. 2010
	CA302: GTGCTCAAAGCCTCATGGTTCTTG	
<i>NbPP2A</i>	WS252: GACCCTGATGTTGATGTTTCGCT	Nguyen et al. 2010
	WS253: GAGGGATTTGAAGAGAGATTTTC	
<i>NbAcre31</i>	WS258: AATTCGGCCATCGTGATCTTGGTC	Nguyen et al. 2010
	WS259: GAGAACTGGGATTGCCTGAAGGA	
<i>NbPti5</i>	WS256: CCTCCAAGTTTGAGCTCGGATAGT	Nguyen et al. 2010
	WS257: CCAAGAAATTCTCCATGCACTCTGTG	

^a Restriction sites are underlined.

(8,000 × g, 2 min, RT) and resuspended after removing supernatant in *Agrobacterium* induction medium (10 mM MES [pH 5.6], 10 mM MgCl₂, and 150 μM acetosyringone). For expression of GFP or GFP:XopX alone, *A. tumefaciens* was resuspended at a final titer of 4 × 10⁸ CFU ml⁻¹. Final *A. tumefaciens* concentrations in Pto-AvrPto co-inoculations were 2 × 10⁸ CFU ml⁻¹ for all strains. Final *A. tumefaciens* concentrations in MAPK component co-inoculations were 2 × 10⁸ CFU ml⁻¹ (GFP, GFP:XopX, SIMPK1, empty) or 1 × 10⁸ CFU ml⁻¹ (SIMAPKKKα, SIMKK2^{DD}). Final *A. tumefaciens* concentrations in Bax co-inoculations were 4 × 10⁸ CFU ml⁻¹ for all strains. Descriptions and references for all strains and plasmids used are provided in Table 1. Briefly, GFP and GFP:XopX constructs were driven by the 35S promoter and borne on binary vector pGWB6 in *A. tumefaciens* C58C1(pCH32). Pto-AvrPto was driven by the 35S promoter and borne on pBTEx in *A. tumefaciens* EHA105. MAPK components have a C-terminal HA tag and were driven by an estradiol-inducible promoter and borne on pER8 in *A. tumefaciens* GV2260. Bax was driven by the 35S promoter and borne on pBTEx in *A. tumefaciens* GV2260.

Final *A. tumefaciens* suspensions were syringe infiltrated into *N. benthamiana* leaves using a needleless syringe. For estradiol-inducible constructs, 5 μM 17-β estradiol with 0.05% Tween-20 was applied by spraying leaves 24 h after initial *A. tumefaciens* infiltration. Infiltrated plants were placed under continuous light at RT for the duration of all experiments.

Analysis of protein expression.

Protein levels from *X. euvesicatoria* cultures and transient expression in *N. benthamiana* were analyzed by immunoblot, essentially as previously described (Mudgett and Staskawicz 1999). For *X. euvesicatoria* cultures, collected cellular pellets were denatured in urea sample buffer (8 M urea, 15% β-mercaptoethanol, 60 mM Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS], 11.8% glycerol, 0.0013% bromophenol blue, and protease inhibitor cocktail at 25 nl ml⁻¹) (Sigma-Aldrich, St. Louis) using 100 μl per 1 OD₆₀₀ unit (1 ml of *X. euvesicatoria* culture at 1 OD₆₀₀ = 1 OD₆₀₀ unit). For *N. benthamiana*, leaf tissue was completely disrupted in urea sample buffer. Total protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane. Protein was detected by ECL Plus (GE Healthcare, Buckinghamshire, United Kingdom) after blotting membranes with rabbit anti-XopX antisera (Covance, Denver, PA, U.S.A.), mouse anti-HA antibody (Covance, Berkeley, CA, U.S.A.), or mouse anti-GFP antibody (Clontech, Mountain View, CA, U.S.A.) followed by horseradish-peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA, U.S.A.). The anti-XopX antisera were generated against recombinant, full-length XopX with an N-terminal hexa-histidine tag expressed in *E. coli* off of pDEST17(*xopX*), batch purified with nickel-NTA resin under denaturing conditions (100 mM NaH₂PO₄, 10 mM Tris HCl, 8 M urea [pH 8.0 for lysis, pH 6.3 for wash, and pH 4.5 for elution], 10 mM imidazole for lysis, and 20 mM imidazole for wash or elution), and separated by SDS-PAGE.

Trypan blue staining for PCD.

Sample preparation prior to trypan blue staining in *N. benthamiana* and tomato experiments differed slightly. For *N. benthamiana*, transient expression of GFP or GFP:XopX was performed in different regions of the same leaf using *A. tumefaciens* at 4 × 10⁸ CFU ml⁻¹. The whole leaf was harvested at 3 dpi, photographed, and subjected to trypan blue staining. For tomato, *X. euvesicatoria* WT and Δ*xopX* were inoculated into different regions of the same leaf at 2 × 10⁸ CFU ml⁻¹. Whole leaves were harvest at 1, 2, and 3 dpi, photographed, and sub-

jected to trypan blue staining. Trypan blue staining was performed as described (van Wees 2008), with slight modifications. Whole leaves were completely submerged in 50 ml of staining solution (67% ethanol, 8.3% lactic acid, 8.3% phenol, 8.3% glycerol, and trypan blue at 2.5 mg ml⁻¹), boiled for 5 min, and incubated overnight (O/N) at RT. Leaves were transferred to destaining solution (67% ethanol, 8.3% lactic acid, 8.3% phenol, and 8.3% glycerol) O/N at RT with gentle shaking. Leaves were washed in ethanol, dried briefly, and photographed. Regions of dark blue indicate uptake of trypan blue by dead and dying cells.

Electrolyte leakage quantification.

Electrolyte leakage quantification in *N. benthamiana* and tomato experiments differed slightly. For *N. benthamiana*, transient expression of GFP or GFP:XopX was performed in different regions of the same leaf using *A. tumefaciens* at 4 × 10⁸ CFU ml⁻¹. Four leaf disks (12 mm in diameter) were sampled from each inoculation region at 20 hpi, rinsed in distilled water, and floated on 5 ml of distilled water in a six-well tissue plate under continuous light. At 24 h after initial *A. tumefaciens* inoculation, conductivity of the bathing water was measured using a conductivity meter, and repeated every subsequent 24 h. Mean conductivity ± standard deviation (SD) from three plants is reported for each treatment. For tomato, *X. euvesicatoria* strains were inoculated into different leaflets of the same leaf at 2 × 10⁸ CFU ml⁻¹. Leaf disks (4 by 12 mm in diameter for 'VF36', 5 by 8 mm in diameter for 'Hawaii 7998' and 'VF36' Bs2) were sampled at indicated times (24, 48, and 72 hpi for 'VF36'; 16 and 24 hpi for 'Hawaii 7998' and 'VF36' Bs2), rinsed in distilled water, and floated on 5 ml (VF36) or 3 ml ('Hawaii 7998' and 'VF36' Bs2) of distilled water for 4 h with gentle shaking. Conductivity of the bathing water was then measured. Mean conductivity ± SD from three plants is reported for each treatment.

ET quantification.

Sample preparation prior to ET quantification in *N. benthamiana* and tomato experiments differed slightly. For *N. benthamiana*, transient expression of GFP or GFP:XopX was performed on two halves of the same leaf, divided by the midvein, using *A. tumefaciens* at 4 × 10⁸ CFU ml⁻¹. Whole leaves were excised and GFP- and GFP:XopX-expressing halves were separated by cutting along the midvein. For tomato, *X. euvesicatoria* strains were inoculated into different leaflets of the same leaf at 2 × 10⁸ CFU ml⁻¹. Leaf halves (*N. benthamiana*) or leaflets (tomato) were weighed, placed in a glass tube capped with a Suba-Seal septa stopper (Sigma-Aldrich), and incubated for 1 h at 25°C. A 1-ml gas sample was injected into a gas chromatograph and ET levels were quantified. Mean ET ± SD from three (*N. benthamiana*) or four (tomato) plants is reported for each treatment.

SA quantification.

SA quantification was performed as described (Defraia et al. 2008), with slight modifications. Sample preparation prior to SA quantification in *N. benthamiana* and tomato experiments differed slightly. For *N. benthamiana*, transient expression of GFP or GFP:XopX was performed on different regions of the same leaf using *A. tumefaciens* at 4 × 10⁸ CFU ml⁻¹. For tomato, *X. euvesicatoria* strains were inoculated into different leaflets of the same leaf at 2 × 10⁸ CFU ml⁻¹. Inoculated leaf tissue (50 mg) was excised and homogenized in 500 μl of 0.1 M acetate buffer, pH 5.6. Samples were centrifuged (16,000 × g, 15 min, 4°C), 100 μl of supernatant was transferred to a new tube for free SA quantification, and 50 μl of supernatant was incubated with 5 μl of β-glucosidase (Sigma-Aldrich) at 0.4 U

μl^{-1} for 90 min at 37°C for total SA quantification. In all, 60 μl of LB, 20 μl of extract, and 50 μl of *Acinetobacter* sp. ADPWH_{lux} (Huang et al. 2005) were added to each well of a black 96-well plate at 4×10^8 CFU ml^{-1} . The plate was incubated at 37°C for 60 min; then, luminescence was measured using a plate reader with luminometer function. To generate a standard curve, 1 μl of SA standard solution was diluted 10-fold in untreated leaf extract from *N. benthamiana* or tomato 'MoneyMaker' *nahG* (Brading et al. 2000). In total, 5 μl of each standard was added to 60 μl of LB and 50 μl of *Acinetobacter* sp. ADPWH_{lux} (4×10^8 CFU ml^{-1}) and luminescence was measured at the same time as experimental samples. Mean SA (free or total) $\pm$ SD from three (*N. benthamiana*) or four (tomato) plants is reported for each treatment.

Quantification of plant gene transcript abundance by reverse-transcriptase qPCR.

Sample preparation prior to transcript quantification in *N. benthamiana* and tomato experiments differed slightly. For *N. benthamiana*, transient expression of GFP or GFP:XopX was performed on different regions of the same leaf using *A. tumefaciens* at 4×10^8 CFU ml^{-1} . At 24 hpi, GFP- and GFP:XopX-expressing regions were inoculated with 1 mM MgCl_2 (mock) or *P. syringae* pv. *tomato* ΔhrcU at 2×10^8 CFU ml^{-1} . For tomato, *X. euvesicatoria* strains were inoculated into different leaflets of the same leaf at 2×10^8 CFU ml^{-1} . Leaf tissue was excised and total RNA was extracted using TRI Reagent (Life Technologies). RNA (5 μg) was used for cDNA synthesis with poly-T primers and Maxima Reverse Transcriptase (Thermo Scientific, Waltham, MA, U.S.A.).

qPCR of cDNA was performed on MJ Opticon 2 (Bio-Rad) using SYBR Green reporter (Thermo Scientific) with gene-specific primers (Table 2). Relative fold changes in gene transcript abundance were determined by the comparative cycle threshold (C_T) method (Livak and Schmittgen 2001). C_T values were determined by the average of two technical replicates per gene per sample. Relative fold change for each gene was calibrated to the average ΔC_T values from mock (1 mM MgCl_2) treatments with *NbPP2A* or *SIACTIN* used as the internal control. Mean $2^{-\Delta\Delta C_T}$ values $\pm$ SD for four plants are reported.

Quantification of flg22-elicited ROS.

Transient expression of GFP or GFP:XopX was performed on different regions of the same leaf using *A. tumefaciens* at 4×10^8 CFU ml^{-1} . At 24 hpi, three leaf disks (4 mm in diameter) were excised from each inoculated region and floated in 200 μl of sterile distilled water O/N in a 96-well plate. To initiate the assay, water was removed and 100 μl of solution (luminol at 34 μg ml^{-1} and horseradish peroxidase at 20 μg ml^{-1}) with 200 nM flg22 (flg22) or without flg22 (mock) was added to each well. Using an automatic plate reader with luminometer function, luminescence from individual leaf disks was measured as relative light units (RLU) and recorded at regular intervals for approximately 90 min starting immediately after addition of solution. The time interval between plate reads was approximately 50 s. For each sample, RLU measured at the indicated time point were averaged for three technical replicates from each biological replicate. Mean RLU $\pm$ SD for four plants is reported.

Scoring PCD phenotype in *N. benthamiana* after XopX coexpression with PCD elicitors.

N. benthamiana leaves were co-inoculated with two *A. tumefaciens* strains to express GFP or GFP:XopX with PCD elicitors Pto-AvrPto, SIMAPKK α , SIMKK2^{DD}, SIMPK1, Bax, or appropriate empty vectors. For each experiment, co-inoculations were performed with the first three fully expanded leaves from

each of six plants. For estradiol-inducible MAPK component constructs, 5 μM 17- β estradiol with 0.05% Tween-20 was applied by spraying leaves 24 h after initial *A. tumefaciens* infiltration. Inoculated leaf tissue was monitored for 3 to 5 days and each inoculated region was qualitatively scored for the amount of tissue collapse (0 to 10% collapse = none or limited, 10 to 85% collapse = partial, and >85% collapse = full). The percentage of co-inoculated regions with each level of tissue collapse at 3 dpi (Pto-AvrPto or Bax) or 5 dpi (MAPK components) was compared between GFP and GFP:XopX treatments for each PCD elicitor.

DAB staining of *N. benthamiana* leaves.

N. benthamiana leaves were co-inoculated with two *A. tumefaciens* strains to express GFP or GFP:XopX with NtMEK2^{DD}. To induce NtMEK2^{DD} expression, 5 μM 17- β estradiol with 0.05% Tween-20 was applied by spraying leaves 24 h after initial *A. tumefaciens* infiltration. At 10 h after estradiol treatment, inoculated regions were excised, submerged in staining solution (DAB at 1 mg ml^{-1} , 10 mM K_2HPO_4 , and 0.05% Tween-20), subjected to vacuum for 5 min, and incubated in the dark at RT for 4 h. Leaf chlorophyll was cleared by floating the leaf in ethanol with gentle shaking for several days. Cellular accumulation of H_2O_2 was imaged using a light microscope under $\times 10$ magnification.

Genomic deletion of *avrRxv* in *X. euvesicatoria* 85-10 and ΔxopX .

The genomic region (1,567 bp) directly upstream of the *avrRxv* coding sequence was amplified by PCR with a 3' *Bam*HI::HindIII extension (primer pair WS01-40/JG767) and ligated into pCR8 (Life Technologies). The genomic region (1,594 bp) directly downstream of *avrRxv* was amplified as a *Bam*HI-HindIII fragment (primer pair JG768/WS01-41) and blunt-end ligated into pJET1.2 (Thermo Scientific). The *Bam*HI-HindIII downstream fragment was then subcloned into the *Bam*HI::HindIII site of the pCR8 vector containing the upstream fragment, generating the ΔavrRxv construct. The ΔavrRxv construct was then recombined using LR Clonase II (Life Technologies) into a Gateway-compatible version of the suicide vector pLVC18-Rfc, generating pLVC18-Rfc(ΔavrRxv). pLVC18-Rfc(ΔavrRxv) was then introduced into *X. euvesicatoria* 85-10 and ΔxopX by triparental mating. Single-crossover exconjugates were first selected on tetracycline NYGA and then grown in NYG without tetracycline selection, with several subculturing steps. Cultures were diluted and plated on NYGA without tetracycline. Individual colonies were replica plated with and without tetracycline selection to identify tetracycline-sensitive double-crossover ΔavrRxv mutants. Deletion of the *avrRxv* coding sequence in individual mutants was confirmed by colony PCR and by loss of the ability of mutants to induce the hypersensitive response in 'Hawaii 7998' tomato.

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