Functional Characterization of Four *Xanthomonas euvesicatoria* Type III Effectors

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ABSTRACT

Pepper and tomato, as two common, popular, and important vegetables grown worldwide, provide human beings with high quality fruit of flavor and aroma, and a high concentration of vitamins and antioxidants. Pepper and tomato production is frequently affected by various pathogens, including nematodes, fungi, and bacteria. Among those phytopathogens, *Xanthomonas euvesicatoria* (*Xe*) causes a severe bacterial spot (BS) disease on pepper and tomato. The BS disease could cause a loss of approximately 10% of the total crop yield in the world. Breeding tomato and pepper cultivars with improved BS disease resistance is one of the most important breeding goals. A better understanding of the virulence mechanism of *Xe* could help breeders design new strategies for resistance breeding. In this dissertation, we characterized the virulence and avirulence functions of four *Xe* Type Three Secretion Effectors (T3Es): *Xe*-XopQ, *Xe*-XopX, *Xe*-XopN, and *Xe*-avrRxo1.

*Xe*-XopQ is a *Xe* T3E that functions as a determinant of host specificity. Here, we further explored the virulent and avirulent functions of *Xe*-XopQ. We identified another T3E *Xe*-XopX that could interact with XopQ and subsequently elicit the hypersensitive response in *N. benthamiana* in the *Agrobacterium*-mediated transient assay and *Xe*-mediated disease assay. The interaction is confirmed by bimolecular fluorescence complementation, co-immunoprecipitation and split luciferase assay. Intriguingly, we also revealed that XopX also interacts with multiple *Xe* T3Es including AvrBS2, XopN, XopB, and XopD in the co-IP assay. The virulent and avirulent functions of XopQ and AvrBS2 are compromised in the absence of *Xe*-XopX. Since XopX is conserved in diverse Xanthomonas spp., we speculate that *Xe*-XopX may have a general role required for the pathogenesis of *Xe*.

*Xe*-XopN has been reported to be a T3E with virulence function via targeting host defense-related proteins, including atypical receptor-like kinase named TARK1 and a 14-3-3 protein to suppress the PAMPs (pathogen-associated molecular patterns) triggered immunity upon *Xe* colonization of tomato. In this study, we revealed additional virulence
mechanisms of \textit{Xe}-XopN, where \textit{Xe}-XopN, is required for triggering the water-soaking symptom on \textit{Nicotiana benthamiana} and pepper plants infected with \textit{Xe}. In addition, we identified that XopN interacts with a transcription factor, NbVOZ, and represses the expression of \textit{NPR1}, a key component of the basal defense. Therefore, XopN has a role in maintaining a water-affluent environment for better replication of \textit{Xe}, and it can also interact with NbVOZ1/2 to regulate plant immunity.

AvrRxo1, a T3E of \textit{Xanthomonas oryzae} pv. \textit{oryzicola} (\textit{Xoc}), was previously identified to function as a NAD kinase. Here, we characterized a \textit{Xe} T3E, \textit{Xe} avrRxo1, that is a functional homologue of AvrRxo1, which is required for the full virulence of \textit{Xe} to colonize the pepper and \textit{N. benthamiana} plants. Overexpression of AvrRxo1 in bacterial or plant cells is toxic. Our group previously demonstrated AvrRxo1-ORF2 functions as an antitoxin that binds to AvrRxo1 to suppress its toxicity. In this study, we identified \textit{Xe}4429 as the homologue of AvrRxo1-ORF2, which could interact with \textit{Xe}-avrRxo1 to suppress its toxicity. We also revealed that \textit{Xe}4429 could bind to the promoter of \textit{Xe-avrRxo1} and suppress its transcription. Therefore, we found \textit{Xe}4429 encodes protein functions as an antitoxin and a transcription repressor in \textit{Xe} bacterial cells.
Peppers and tomatoes are two of the most important vegetables grown worldwide, providing humans with high quality of flavor and aroma, vitamins, and antioxidants. The pepper and tomato production is frequently threatened by various pathogens, including nematodes, fungi, and bacteria. Among those phytopathogens, Xanthomonas euvesicatoria (Xe) causes a severe bacterial spot (BS) disease on peppers and tomatoes. The BS disease can be easily identified due to the appearance of the dark, irregular, water-soaked areas on the leaf, which can cause approximately 10% loss of the total yield of peppers and tomatoes. Breeding tomato and pepper cultivars with improved BS disease resistance is one of the most critical breeding goals. A better understanding of the virulence mechanism of Xe could help breeders to design new strategies for resistance breeding. In my seminar, I will discuss the virulence and avirulence functions of Xe type three secretion (T3S) effectors: Xe XopN, Xe XopQ, and Xe XopX. In my study, I identified Xe XopN is a key factor that regulates the development of the water-soaking symptom on pepper plants infected with Xe. In addition, we revealed Xe XopN interacts with a transcription factor NbVOZ to regulate the expression of NbNPR1 and PR1 genes expression, which may also contribute to the development of water-soaking phenotype. In addition, I identified that Xe XopN could interact with a transcription factor, NbVOZ, and repress the expression of NbNPR1, a key component of the basal defense, and the pathogenesis-related gene PR1. Therefore, Xe XopN has a role in regulating a water-affluent environment to promote bacterial proliferation in the infected plant tissue. Xe XopQ is a Xe T3S effector that functions as a determinant of host specificity. In my study, I identified another T3S effector Xe XopX that could interact with Xe XopQ to trigger the defense response in Nicotiana benthamiana. I also confirmed Xe XopQ physically interacts with Xe XopX inside of plant cells by using bimolecular fluorescence complementation, co-immunoprecipitation and split luciferase assay. Intriguingly, Xe XopX could also interact with multiple Xe T3Es including AvrBS2 in a co-IP assay. The virulence and avirulent functions of Xe XopQ and AvrBS2 are compromised in the absence of Xe XopX.
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Chapter 1 Literature Review

Type III Secretion Effector: A Coin with Two-sides in the molecular plant-microbe interactions

Summary

During the co-evolution of plants and diverse phytopathogens, plants have evolved a sophisticated immune system with two-branched molecular mechanisms to protect their health and reproduction. The first branch of immune response recognizes the molecule patterns conserved in different classes of microbes, including the non-pathogens. The second branch of immune response recognizes specific pathogen virulence factors, either directly or indirectly. Almost all gram-negative bacterial pathogens possess the type III secretion system (T3S) that can inject a collection of bacterial effectors inside plant cells. These type three effectors (T3E) are required for the full bacterial pathogenesis. Many T3Es function as double-edged swords. For example, the T3Es can suppress plant immunity and promote bacterial proliferation on host plants that have no corresponding disease resistance ($R$) genes. On the other hand, if particular plant genotype carries specific $R$ genes that can recognize the corresponding T3E, a robust and quick immune response usually manifests with a localized programmed cell death or hypersensitive response (HR) at the pathogen infection sites. Characterization of every individual T3E for their molecular functions will allow us to understand the plant's immune system and may breed new crop cultivars to achieve sustainable production of food, vegetable, fiber, and biofuels. In my dissertation, I mainly studied the virulence functions of *Xanthomonas euvesicatoria* (*Xe*) type three effectors. Therefore, this review will highlight the recent progress on the effector-triggered immunity (ETI) and the effector-triggered susceptibility (ETS) in plant and microbe interactions, with a focus on *Xe* T3Es.

Part 1: Effector-triggered susceptibility
The increasing world population challenges the security of the global food supply. Global climate change has made this food crisis issue even worse in the past decades. Protecting crops from different plant pathogens has a critical role in meeting the growing demand for food quality and quantity [1]. Roughly, direct yield losses caused by pathogens, including viruses, bacteria, fungi, oomycetes, nematodes, and various insects are about 20-40% of global agricultural productivity [2].

Unlike animals, crops are not able to escape from the danger of pathogens via adequate movement [3-5]. On the one hand, as engaged in an everlasting biotic threat in the field, plants have established a sophisticated immune system to monitor and restrict the amplification of microbial. On the other hand, phytopathogens also evolve constantly to escape or suppress plant immunity to colonize plants. Recent studies revealed the complicated plant-pathogen interactions from the perspective of both organisms. It is of particular importance for agricultural practice to apply new knowledge and novel biotechnological approaches for crop protection. In the first part of the review, I will review phytopathogen infection strategies involving different T3Es that have been reported recently, in aiming to understand the ‘enemies’ of crops.

**Classic ‘zigzag’ model of plant immunity**

During the past two decades, scientists developed several profound working models to explain the molecular interactions between plants and phytopathogens. Here, in order to introduce T3Es and explain what roles they play in the replications of microbial and plant immunity, the classic ‘zigzag’ model is cited to illustrate the plant immune system (Figure 1-1) [6]. There are four phases in this model. Owing to perceived various kinds of microbial, transmembrane pattern recognition receptors (PRRs) are utilized to respond to pathogen-associated molecular patterns (PAMPs), which are conserved among diverse microbial and have evolved slowly [7]. In phase 1, the recognitions of conserved PAPMs by PRRs result in a PAMP-triggered immunity (PTI) that can limit further colonization. PTI is a moderate response, and usually, no
obvious symptom can be overserved. In phase 2, successful pathogens can employ effectors to interfere with PTI, resulting in the effector-triggered susceptibility (ETS). In phase 3, plants deploy specific nucleotide binding-leucine rich repeat (NLR) protein to recognize a given effector delivered by the phytopathogen, and the related immunity is named as effector-triggered immunity (ETI) [3]. ETI serves an accelerated and amplified PTI immunity response, protecting plants from the infection of phytopathogens by processing a suicide hypersensitive cell death response (HR) at the infected site. In phase 4, natural selection, on one hand, directs phytopathogens to escape ETI through secreting additional effectors to suppress ETI, shedding alternation of the recognized effector primary sequence or structure. On the other hand, natural selection addresses the evolution of new R proteins to recognize the diversified effectors and trigger ETI again. The two aspects above tangle together along with the co-evolution between plant hosts and phytopathogens.

**PTI: the first layer of plant immunity**

As the first layer of plant immunity, PTI conditions the basal and broad disease resistance to diverse phytopathogens [8]. One of the typical elicitors of PTI is the bacterial flagella, which is necessary for bacterial motility and pathogenicity in plants. Because of the importance of flagella, it is conserved in diverse bacterial species. The flagella functions as a PAMP that triggers defense responses in various plant species [9]. A synthetic 22-amino-acid peptide (flg22) from a conserved flagella domain has been used as the synthetic elicitor of PTI [7, 10, 11]. An Arabidopsis leucine-rich repeat (LRR)-receptor kinase FLS2 has been identified in a genetic screen by using flg22 [12]. FLS2 can be internalized by binding flg22, in which a receptor-mediated endocytic process is required [7, 13]. Mutant of fls2 exhibits enhanced susceptibility to spray application of pathogenic *Pseudomonas syringae* pv. *tomato* (Pst) *DC3000*, but not to straight infiltration of *Pst DC3000* into the leaf apoplast, implying that FLS2 mainly acts against early pathogen invasion [11, 14].

Bacterial elongation factor Tu (EF-Tu) activates similar defense responses like flg22 [15-17].
It can be recognized by an Arabidopsis LRR-kinase designated as EFR [17]. The mutant of efr allows higher efficiency of transient transformation with *Agrobacterium*, suggesting that PTI also limits *Agrobacterium* pathogenicity. Treatment with a conserved EF-Tu peptide, elf18, induces expression of a gene-set nearly identical to that induced by flg22 [17]. Furthermore, EFR transcription can be induced by flg22. Besides, both FLS2 and EFR belong to an atypical kinase family that might function similarly in the plant immune system. Thus, a common set of signaling pathways are shared by plant responses to different PAMPs. This convergence makes it possible for T3Es to target a limited number of genes and compromise PTI. By contrast, mutations in genes required for NLR function have no impact on responses of plants to flg22 [11]. Hence, NLR-dependent signaling and PAMP-mediated signaling own partially distinct components.

The molecules that can induce PTI are usually essential or critical for microbial growth, which can not be easily eliminated by the microbes, but variations exist in PAMPs in different microbes that can trigger a different response on different plant species. For instance, flagella of *Xanthomonas campestris* pv. *campestris* (Xcc) and *Agrobacterium tumefaciens* are less effective than that of *Pst DC3000* in triggering FLS2-mediated PTI in Arabidopsis [10]. Ef-Tu from *Pst DC3000* is much less active in eliciting PTI in Arabidopsis than Ef-Tu from *Agrobacterium* [16]. In terms of plant species, Arabidopsis accession Ws-0 carries a point mutation in FLS2, terminating its response to flg22 [9]. Individual plant species are not capable of recognizing all potential PAMPs [7]. Other unknown PAMPs and corresponding PRRs must exist. Twenty-eight of over 200 LRR-kinases in *Arabidopsis col-0* are induced within a treatment of flg22 for 30 min [11, 18]. Moreover, *Agrobacterium* extracts elicit PTI on a *fls2/efr*-1 double mutant [17]. Following the current model, additional uncovered PRRs or LRR kinases should be stimulated transcriptionally by engagement of appropriate PAMPs [19, 20]. Conversely, the irrelevance between PAMPs and PRRs will lead to natural selection.

*Suppression of PTI via Effectors*
One of the most critical functions for a collection of effectors in phytopathogens is to suppress PTI [8]. Although eukaryotic pathogens including oomycetes and fungi, deliver effectors to plants as well as bacterial pathogens, effectors of oomycetes and fungi are still poorly understood, so this review focuses on discussing plant cellular processes targeted by bacterial type III effectors. Plant disease-associated bacteria deliver 15–40 effectors into host cells using the type III secretion system (T3S). A Pst DC3000 strain mutated in the T3S triggers a faster and stronger transcriptional re-programming in hosts compared to the wild-type strain [21-24]. It can tell that the T3Es are necessary for colonization of bacterial pathogen in a manner mimicking or inhibiting eukaryotic cellular functions [25-28].

Here, I listed several classic cases of T3Es and their biochemical functions in regulating plant immunity [8, 29]. Two independent T3E effectors from Pst DC3000, AvrPto and AvrPtoB contribute to bacterial virulence by inhibiting upstream of MAPK, which is an early step in PTI [30]. AvrPtoB has two critical domains where the N terminus contributes to virulence function, while the C terminus blocks host cell death [31, 32]. A domain from the AvrPtoB C terminus forms an active E3 ligase and directs host protein degradation [33]. Another two unrelated T3Es from Pst DC3000, HopM, and AvrE, both target ARF-GEF protein, which is involved in host cell vesicle transport [28]. The redundant function of these two effectors suggests the importance of manipulating host vesicle transport in the successful bacterial colonization [34]. The Yersinia effector YopJ and its homologue in the avrRxv effector family have been reported to inhibit the turnover of MAP kinase cascades by acetylation of a MEK protein at its phosphorylation-regulated residues [35]. Although great achievements about the T3Es and their functions have been reached during the past two decades, the targets and biochemical activities of many bacterial type III effectors still await to be unraveled by plant pathologists.

_Virulence and disease symptoms associated with T3Es in Xanthomonas_

All _Xanthomonas_ strains own a T3S system, which is well studied and shows a critical function
in facilitating a favorable environment for the living and replication of *Xanthomonas*. It is common for the T3Es in a particular Xanthomonas strain to share redundancy of function [36]. However, regardless of being functional in suppressing elicitor-triggered immunity or promoting infection via modifying plant innate metabolism, an increasing number of T3 effectors of *Xanthomonas* have been characterized with aspects of enhanced virulence and development of disease symptom (Table 1-1).

As one of the first identified T3Es, AvrBs2 has been indicated to contribute to virulence as a result of its ability to support increased numbers of bacteria within host tissue [37, 38]. Four T3 effectors of *Xe*, AvrBs1-4, were all found to have contributions to pathogen fitness to varying extents in the field tests [39].

The TAL effector family consist of a large number of closely related T3Es, and many members of this family have a critical impact on virulence and disease symptom [40]. For example, two TAL effectors within a high homology, PthA from *Xanthomonas axonopodis pv. citri* (*Xac*) and pthXo1 from *Xanthomonas oryzae pv. oryzae* (*Xoo*) have a positive effect on the ability of the pathogens to spread as well as to elicit disease symptoms [41]. The phenotype can also be observed in plant expression of the gene. Besides, one benefit of TAL effectors to bacteria may be an establishment of an aqueous apoplastic environment in the infected sites of leaf tissues. A period of high atmospheric relative humidity after rainfall, has repeatedly been shown to promote disease outbreaks in crops, implying that the availability of water is imperative for pathogenesis. The TAL effectors of *Xanthomonas campestris pv. malvacearum* (*Xcm*) and *Xoo* are associated with the increased appearance of water soaking, suggesting TAL effector can alter the water status in the apoplast for better colonization of bacterial pathogens in plants. As a consequence, a high water availability in the apoplast can in turn direct the success of potentially pathogenic microbes.

Another function of TAL effectors might be the promotion of bacteria release from infected tissue to uninfected tissue. *Xcm-avrB6* and *Xe-avrBS3* have been reported to increase populations of bacteria in the uninfected areas of cotton and pepper leaves, respectively [42,
The lesions, chlorotic and desiccated leaf tissues are severely limited in the absence of the above mentioned two major TAL effectors in field tests [44-46], suggesting that enhanced release could have a fitness benefit for *Xanthomonas* colonization in a more spreading scale [39]. The function of *Xanthomonas* TAL effector can be interfered by R protein [47]. For example, the R gene xa13 specifically recognizes and subsequently interferes with the function of the TAL effector PthXo1, implying a practical application providing resistance against rice bacterial blight disease [41].

Chlorosis is a prevalent symptom in plants infected by *Xanthomonas*, in which a conserved T3E, early chlorosis factor (ECF), also named as XopAA, is reported to contribute this category of symptom [48]. ECF was identified by a complementation approach, in particular, the presence of ECF render *Xanthomonas* strains a greater ability to induce chlorosis but ECF did not show a detectable influence on bacterial populations. XopD and XopN of *Xe* have both been shown to contribute to virulence and disease symptoms in tomato. Plants infected with *Xe* strains impaired of XopD present a more significant leaf tissue necrosis, indicating that XopD can suppress innate plant immunity [45]. XopN is a T3E gene widely distributed in *Xanthomonas*. *Xe* Strains without XopN possess a smaller bacterial population and less necrosis and senescence in the plants after infection [46]. Impairment of the XopN homologue in *Xcc* also results in reduced disease in radish [49]. Other candidate T3 effectors have been characterized to impact virulence and disease symptoms, including XopJ or AvrXv4 [50], XopX [51], XopAE or HpaF [52] and XopAH or AvrXccC [53].

Biochemical functions of type three effectors in *Xanthomonas*

After reviewing the virulence function and symptom induced by *Xanthomonas* T3Es, here we will go further to discuss putative structural motifs and biochemical activities of some *Xanthomonas* T3Es underlying the previously mentioned virulence mechanism (Table 1-1). Structural motifs of 15 *Xanthomonas* T3Es have been linked to the function of those effectors. Twelve of the 15 indicate they may have enzymatic activities. Recent progress on specific
functions of individual T3 effectors in Xanthomonas gives an insight into how Xanthomonas effectors interact with host plants via altering plant innate metabolism. In the part of the present review, we focus on highlighting the biological functions of five Xanthomonas T3Es.

AvrBs3

The AvrBs3 group is a large family that consists of closely related TAL T3 effectors of Xanthomonas. Structurally, the N-terminal and C-terminal portions of TAL effectors are highly conserved. The conserved C-terminal portion of TAL effectors contains a strong nuclear localization signal (NLS) motif, as well as a robust acidic transcription activation domain (AD). Both of them are associated with biological effects such as pathogen virulence, inducement of host disease symptoms and facilitating pathogen spread [54-58]. A variety of TAL effectors are associated with biological effects, including virulence of the pathogen and disease symptoms.

The most distinguishing feature of overall TAL effectors is their particular repetitive region containing varying numbers of near-identical repeats of 34 or 35 amino acids, which are able to determine DNA binding specificity. Thus, different TAL effectors bind to unique DNA elements in diverse host genomes. TAL effectors can function as transcription factors that bind to specific DNA elements of targeted host genes and regulate their transcriptions in the host nucleus [59].

As a typical TAL, AvrBs3 has 17.5 repeats and encodes a basic helix-loop-helix transcription factor. It has been reported that, specifically, it binds to the DNA sequence in several genes such as upa20 (up-regulated by AvrBs3 20), which is responsible for the hypertrophy of the host cells. The same sequence is also found in the promoter of the resistant gene, BS3, resulting in the expression of BS3 in the presence of AvrBs3 [60]. Changes in the TAL effector binding domain can eliminate the response to TAL effector.

TAL effector, pthXo6 in Xoo, has been shown to promote virulence upon the infection of Xoo to rice. PthXo6 can upregulate the expression of a rice gene OsTFX1, encoding a plant transcription factor in bZip family [61]. A mutant in the DNA binding domain of pthXo6 led
to a reduction of virulence, indicating the importance of DNA binding domain for the complete function of pthXo6. In turn, the ectopic overexpression of OsTFX1 in rice restores the virulence function of pthXo6 [61]. As a member in bZip transcription factor family, OsTFX1 might induce or repress multiple genes that are involved in host immunity. All strains of Xoo were proved to be capable to induce the expression of OsTFX1, supporting that OsTFX1 is essential for the bacterial fitness of Xoo and its fission to rice. Furthermore, it might be a result of natural selection and long co-evolution between Xoo strains and rice. However, transgenic plants ectopic overexpressing of OsTFX1 did not cause any phenotypic abnormalities, implying post-translational regulating mechanism may exist upon infection of Xoo to rice [61].

**XopD**

XopD is a conserved T3E in diverse *Xanthomonas* strains, and it has multiple functions in the infection of *Xanthomonas* to various hosts (Table 1-1). Apart from the ability to suppress defense gene expression, the most significant function of XopD is its small ubiquitin-like modifier (SUMO) activity. It has a SUMO protease domain and is also a member of the C48 protease family and has been proven to be able to release SUMO from SUMO-modified plant proteins [62, 63]. The SUMOylation of host proteins regulates stress and developmental responses by subtly managing protein stability [64-66]. Specific alterations to the predicted catalytic sites of XopD also caused compromised virulence consistent with delayed necrosis [45].

XopD is localized at the nucleus and has a DNA binding activity [45, 62]. Sequence analysis revealed that the helix-loop-helix region of XopD at amino acids 113–131 is the key domain for its binding ability. The site-directed mutation on V118 of XopD reduced its contribution to virulence along with delayed development of necrosis in the infected tissues. It suggests the DNA binding domain in XopD protein is required for its complete functions. Two ethylene response factor-associated amphiphilic repression (EAR) motifs [L/FDLNL/F(x)P] were also characterized in XopD at amino acids 244–249 and 284–289. EAR motifs are found in host
transcription factors that repress salicylic acid- and jasmine acid-induced defense gene transcription in planta, but the exact mechanism remains unknown. Mutations in the EAR motifs led to a reduction in virulence and delayed necrosis, but did not influence the localization and catalytic activity of XopD.

Therefore, the functions of XopD can be split into two aspects. The first one is transcriptional regulation, that is directed to related defense or senescence transcription factors, either through DNA binding or the EAR motifs or both; the second one is post-translational modification, that is addressed to destabilize host defense proteins by way of deSUMOlaytion, which is the catalytic activity of XopD [45, 66].

**XopJ**

The T3Es in XopJ family are present in diverse phytopathogenic bacteria [67]. Since a high identity from the C55 group of cysteine protease, members in this family have been proposed to regulate the stability and activity of host proteins via SUMOlaytion (Table 1-1). For example, AvrXv4 likely functions as SUMO protease. Additionally, other members of the XopJ group are also found to function as transacetylases [50].

YopJ, the first identified member in this large family, has been previously revealed to target on trans-acetylating threonine and serine residues in several mitogen-activated kinases (MAPs). It can prevent the phosphorylation of MAP kinase, which is an important behavior to initiate innate immunity signal transduction in response to the attack of phytopathogens [68]. XopJ is also shown to interfere with the host callose deposition, which is a common PTI signaling event [69]. The myristoylation motif at the N-terminus can partially determine the localization of XopJ and it is usually targeting the protein at the host cell membranes [69, 70]. However, the direct host targets of XopJ have not been identified.

Another important member of this family is the AvrBsT in Xcc. Like YopJ, AvrBsT also possesses a transacetylase activity, but it fails to trigger resistant response in most ecotypes of Arabidopsis because its biochemical activity can be inhibited by the carboxylesterase activity
of SOBER (suppressor of AvrBsT elicited resistance) [71]. Consistently, accession Pi-0 carrying a null mutation in SOBER shows a strong HR in an AvrBsT-dependent manner. AvrRxv is another member belonging to the XopJ group. Previous reports suggest that the C-terminus and N-terminus of AvrRxv collaborate to execute its biochemical function that is required for the pathogenesis of Xcc. XopJ can induce HR in the tomato cultivar Hawaii 7998, which depends on several conserved catalytic residues at the C-terminal of XopJ [67, 72]. Interestingly, the ability of AvrRxv to elicit an HR remained after the replacement with the C-terminal domain of YopP. As the C-terminal domain of YopP has previously been characterized as a trans-acetylate MAP kinase, implying the putative transacetylation activity of AvrRxv. Besides, AvrRxv binds a tomato 14-3-3 protein mediated by the N-terminal segment of AvrRxv (1–141 amino acids) [67]. The 14-3-3 proteins have been extensively studied to function as chaperons in a variety of cellular complexes, like MAP kinase-related signal transduction pathways. AvrRxv fulfills its transacetylase activity and represses host innate immunity by binding to 14-3-3 protein that is engaged in the MAP kinase signal transduction pathway.

XopN
XopN has been characterized to interact with a variety of proteins including OsVOZ and OsXNP in rice and an atypical (phosphorylation-deficient) receptor-like kinase named TARK1, as well as 14-3-3 proteins, such as TFT1, TFT3, TFT5 and TFT6 in tomato (Table 1-1) [46, 73-75]. TARK1, similar to other RLK proteins, presents two membrane-spanning regions including an LRR domain outside the cell and a nonfunctional kinase domain in the cytoplasm. The interaction with TARK1 has been shown to require an “LXXLL” sequence in the cytoplasmic portion of TARK1. Intriguingly, an “LXXLL” motif in the N-terminal portion of XopN is also required for its interaction with TARK1. A site mutation in XopN lost the ability to bind to TARK1, which resulted in a reduction in its virulence effect but not its suppression of callose deposition [46]. Therefore, the crosstalk between TARK1 and XopN contributes to the virulence effects of XopN during Xe infection to tomato. Furthermore, suppression of
TARK1 expression in tomato also caused a partial suppression of XopN-mediated susceptibility [46]. These evidences comprehensively demonstrate that XopN interferes with TARK1-dependent signaling events in tomatoes invaded by Xe. However, as an atypical kinase without intrinsic catalytic activity, the mechanism of TART1 engaged in host defense response is unknown yet, which may be distinct from signal transduction pathways mediated by other RLKs [76]. Also, the involvement of XopN was shown to interact with 14-3-3 proteins which are known to be involved in diverse signal pathways in plants and animals. It is perhaps a consequence of several anti-parallel a-helical repeats and HEAT (huntingtin, elongation factor 3, PR65/A, TOR1) repeats in XopN [44].

**XopAC and XopAH**

Two T3Es of *Xanthomonas*, XopAC, and XopAH, are taken together to be reviewed as a result of their sequences related to the Fido domain, which is derived from the fic [filamentation induced by cyclic adenosine monophosphate (cAMP)] and doc (death on curing) domains [77]. A fido domain is present in the T3E from the animal pathogen *Vibrio parahaemolyticus*, which was recently found to cause host cell cytotoxicity and can covalently modify Rho GTPases with adenosine monophosphate moieties (Table 1-1). This modification has been named as an AMPylation reaction and the central motif sequence in this domain is HPFx(D/E)GN(G/K)R, in which histidine facilitates AMPylation. This modification has been proposed to alter signaling functions of the target proteins, similar to ribosylation, acetylation and phosphorylation modifications [78, 79]. Structural comparisons showed T3 effector AvrB of *P. syringae*, XopAC of *Xcc* and XopAH of *Xcc* all contain Fido-domain-like segments [80]. Although they do not have the histidine residue, other amino acid residues structurally related to histidine have been proposed to provide the same function [77].

**Part 2: Effector triggered immunity**

*The gene-for-gene model*
Over 50 years ago, H.H. Flor, working on flax and the flax rust fungus, gave a genetic definition for the interaction between plants and phytopathogens, which presented as the gene-for-gene hypothesis/model [55, 81-83]. In this hypothesis, plant resistance is activated when a specific Avr (avirulence) protein from the pathogen is recognized by a specific R (resistance) protein from the plant. A receptor-ligand model was used to interpret the biochemical nature of the gene-for-gene hypothesis. As explained in this model, plants can activate defense mechanisms upon R-protein-mediated recognition of pathogen-delivered Avr products. A ‘matched set’ of R and Avr alleles is necessary for the induction of resistance. If the two components do not ‘fit’ then resistance is not activated, and the plant is susceptible to the pathogen infection.

When pathogens infect plants that are lacking corresponding R proteins, the Avr products might function as virulence factors, which can repress plant immunity by reprogramming host transcriptome and altering the cellular metabolisms to favorite pathogen proliferation. The Avr products can directly or indirectly interact with plant pathogenesis targets. In order to suppress the pathogen infection, plants evolved a collection of R proteins to monitor and recognize the Avr products. The R-protein-mediated recognition of Avr products activates the plant defense responses, which are frequently indicated by an outburst of superoxide and nitric oxide, calcium fluxes and results in localized cell death. Pathogens can evade the R protein-mediated recognition when the Avr proteins are lost or mutated. Both pathogens and plants have therefore continually evolved in this battle to secure their survival and propagation.

Direct NLR-Effectector Interactions

The core concept in the gene-for-gene model is that the crosstalk in a match pair of proteins, one from the host plants and another one from the phytopathogens, leads to the disease resistance. After half a century of research, the miracle of how a specific Avr (avirulence) protein and a specific R (resistance) protein interact with each has been gradually unraveled. On the one hand, the typical plant R protein usually presents in a nucleotide-binding/leucine-rich-repeat (NLR) scheme. On the other hand, the Avr protein is named as the effector, which
is usually delivered by type three secretion system in bacterial pathogen. In general, in terms of their recognition, an NLR protein is held in an ADP-bound “OFF” state when there is no infection of phytopathogens or a particular pathogen effector is absent. For instance, the activation of flax L6 protein and its recognition of flax rust (*Melampsora lini*) effector, AvrL567, requires the assistance of the Toll-like interleukin-1 receptor (TIR) domain at N-terminal. NLR protein turns to ‘ON’ state after recognizing a pathogen effector. NLR protein functions as a receptor and ligand to recognize a pathogen effector, a series of conformational changes complex causes the exchange of ADP to ATP at its nucleotide-binding (NB) domain. Eventually, the interaction between NLR protein and pathogen effector initiates ETI signaling events and plant cell death at the infection site.

Yeast two-hybrid and other *in vitro* interaction assays support the function of several direct NLR-effector interactions in plant resistance [36, 84-88]. A reverse genetic screen identified an NLR protein, Roq1, with a TIR domain, that can recognize XopQ to trigger the immune response of *N. benthamiana* [90]. Impairment of the LRR domain in Roq1 eliminates its ability to recognize XopQ and also abolish the subsequent cell death as well as *Xe* disease resistance in *N. benthamiana*. NLR mutational and domain swap experiments in the other R protein pairs also confirm the determinant role of variable LRR domains in the recognizing of specific pathogen effectors, where the mechanism remains unclear [88, 89].

*Indirect NLR Surveillance of Effector Activities*

Compared with the high diversity of direct NLR-effector recognitions, indirect NLR recognition of various pathogen effectors converges to a limited set of host proteins that are involved in the resistance signaling network. Beneficially, this strategy might help NLR mediated recognition catch up with the rapid pathogen evolution. Heretofore, there are two sub-models that have been developed to explain the indirect NLR surveillance system.

One indirect recognition model is named a guard model [3, 6, 91]. In this model, the receptor serves as a guard binding to a host protein which can be modified by a pathogen effector. Hence,
the host immunity can be initiated when the targeted host protein is modified. For instance, two Arabidopsis plasma membrane NLRs, RPS2 (resistance to *Pseudomonas syringae* 2) and RPM1 (resistance to *Pseudomonas syringae* pv. *maculicola* 1) constitutively guard a host protein, RIN4 (RPM1-interacting protein 4) [92-94]. RIN4 can be interfered by diverse *P. syringae* effectors AvrRpm1, AvrB and AvrRpt2 through divergent modifications [92-94]. For example, the isomerization of RIN4 induced by AvrB-initiated phosphorylation can be perceived by RPM1 to activate the immune response [95, 96]. In the absence of RPS2 and RPM1, RIN4 is targeted for manipulation by above bacterial effectors owing to downregulating basal resistance response activities [97-99].

Another indirect recognition model is named as a decoy model. In this model, the host factor with no resistance activity protects structurally related basal defense components from pathogen effectors’ targeting and modification. This kind of host factor is defined as decoy, which can trap foreign effectors and thereby trigger ETI [100, 101]. For instance, tomato NLR Prf (*Pseudomonas* resistance and fenthion sensitivity) confers resistance to AvrPto from *P. syringae*. Prf is held in a locked form as a consequence of its binding to protein kinase Pto [102, 103]. Like PBS-1, the role of Pto in basal immunity has not been reported yet. However, the kinase domain of Pto is essential for resembling the complex consisting of FLS2, EFR and their coreceptor BAK1 (BRI1-associated receptor kinase 1), which are all targeted by AvrPto during infection [104, 105]. Besides, the Arabidopsis plasma membrane NLR RPS5 is another R protein that fits the decoy model. PRS5 is in an off state as a result of the interaction with the intact protein kinase PBS1 (AvrPphB susceptible 1) via its N-terminal CC domain, whereas PRS5 switches to an ON state after PBS1 is cleaved by the effector AvrPphB [106]. Several PBS1-like kinases have been proven critical in the plant surveillance mechanism, such as the soluble kinase BIK1 (Botrytis-induced kinase 1), which is a key component of PRR signaling in PTI [106-108]. In order to escape the immune system, *P. syringae* deploys AvrPphB, a key virulence effector, to cleave PBS1-like kinases including BIK1 [109]. Although PBS1 has no obvious basal resistance function, it can initiate RPS5 after cleavage, indicating the decoy
model has been applied to counter immunity and trigger susceptibility [106]. Hence, in theory, a family of structure-related host defense proteins or a complex of host defense components can be shielded by a single molecular decoy associated with one or more R proteins.

**Effector recognizes specific promoter DNA sequence of host genes**

Different from the recognition at the protein level, another kind of effector recognition occurs with the host DNAs. A specific class of transcription activator-like (TAL) effectors in *Xanthomonas* and *Ralstonia* bacterial species can be straightforwardly delivered to plant nuclei, where they bind to the promoters of host defense-related genes to regulate their expressions and enhance susceptibility [110, 111]. TAL effector has a unique series of amino acid repeats that can specifically bind to the promoter elements of their host targets to reprogramme the defense-related transcriptional network [112, 113].

In order to counter TAL, rice, and pepper plants have naturally developed corresponding R (resistance) genes, whose promoters can bind to TAL and initiate their expressions. Typical immune responses, including an outburst of superoxide and nitric oxide, calcium fluxes and HR, can be induced to stop pathogens from further spreading when those R genes are expressed driven by TALs [114, 115].

There is no conserved feature for the products of these R genes. In pepper, the binding of *Xe* TAL effector AvrBs3 to the resistance gene, BS3, can activate a homolog of a flavin-dependent monooxygenase enzyme and subsequent HR at the infection sites [116]. In addition, the *Xe* TAL effector AvrBs4 can induce the expression of the resistant gene, Bs4c-R, to elicit HR in pepper. In tomato, the TAL effector actives a TIR-NLR protein, Bs4, and results in disease resistance [115, 117]. In rice, the recognition between resistant gene *Xa10* gene and *Xoo* TAL effector AvrXa10 induces endoplasmic reticulum Ca2+ release and subsequent HR [118]. Another *Xoo* TAL effector, AvrXa27, can upregulate the expression of the rice resistance gene *Xa27*, whose protein can be secreted outside the plant cell to the apoplast and block the further bacterial growth there [114, 119].
As shown above, in order to preserve intracellular immunity, different plant species have evolved various mechanisms to defend against TAL, implying a high diversity on the TALs and the novelty of this pathogenicity. It is still not known whether NLRs are involved in the binding of the TAL effector to corresponding R genes. TAL effector-induced resistance occurring on host DNA provides the new possibility for innovative defense adaptations in plant defense pathways.

*Necessary ETI signaling components, NDR1 and EDS1*

Activation of the NLR signaling pathway requires the assistance of other components. Two central regulators for the NLR signaling transduction have been identified via the genetic screening. One component is the plasma membrane-anchored integrin-like protein NDR1 (non-race-specific disease resistance 1) while another one is the nucleocytoplasmic lipase-like protein EDS1 (enhanced disease susceptibility 1) [120-122]. As mentioned above, NLRs can be divided into two classes depending on the structure on the N-terminal TIR domain or coiled-coil (CC) domain. The corresponding NLRs are named as CC-NLR and TIR-NLR, respectively. In the plant immunity, NDR1 and EDS1 are recruited by CC-NLR and TIR-NLR sensor receptors to elicit ETI [122]. Moreover, EDS1 signals in all TIR-NLR-related defense events, including SA accumulation, HR and defense transcriptional reprogramming [122-124]. By contrast, NDR1 plays an essential role in the SA biosynthetic pathway, elicited by several CC-NLRs [125].

The mechanisms underlying the functions of NDR1 and EDS1 proteins in ETI are different. Arabidopsis NDR1 anchors RIN4 (guarded by the CC-NLRs RPS2 and RPM1) at the plasma membrane [120, 121]. This interaction was also found to be substantial for soybean CC-NLR resistance [126-128]. By contrast, Arabidopsis EDS1 forms nuclear signaling complexes with SAG101 (senescence-associated gene 101) and soluble nucleocytoplasmic with PAD4 (phytoalexin-deficient 4), respectively [129, 130]. Crystal structure analysis has shown that PAD4 and SAG101 compete for the same EDS1 interface. It is consistent with the fact that
PAD4-EDS1 and SAG101-EDS1 complexes have distinct immunity functions in ETI, which might be a mechanism of counterbalancing [130, 131]. Although EDS1 does not show a catalytic mechanism for TIR-NLR or basal resistance signaling, it resembles a plant lipase-like molecular switch as a consequence of its structural similarity to the lipase enzyme at N-terminal [131, 132]. Intriguingly, EDS1 was found to associate with Arabidopsis TIR-NLR receptor RPS4 and *P. syringae* effector AvrRps4 (recognized by the Arabidopsis RRS1/RPS4 TIR-NLR pair), implying the function of EDS1 in the recognition between R protein and T3E [133-135]. Thus, EDS1 seems to serve as a molecular hub not only for TIR-NLR effector activation but also for downstream defense pathways.

*The cognate R genes recognize Xanthomonas T3Es*

After discussing types of interactions between R proteins and T3Es, and the related signal transduction pathways, we would like to give a specific review on cognate R genes to T3Es in terms of *Xanthomonas* bacteria. Since *Xanthomonas* is one of the most important bacterial pathogens that causes a significant yield loss on the production of assorted vegetables, fruits, and crops, including *Solanaceae, Poaceae, Brassicaceae*, and *Rosaceae* for human beings. The ultimate goal for understanding the T3E function is to translate the knowledge to the breeding of durable and broad resistance [136]. Aim at this resistant breeding goal, during the past two decades, a variety of *R* genes against *Xanthomonas* species and their T3E have been cloned and characterized (Table 1-2). Most of them belong to the two largest and well-studied classes of R genes, RLK and NLR. *Bs2, Bs4, Xa1*, and *Rxo1* encode proteins in the NLR family while *Xa21* and *Xa26* are the members of the RLK class.

In addition to the major classes of *R* genes, studies of *Xoo*-caused disease in rice have identified a variety of novel *R* genes, particularly against TAL effectors, indicating that the battle continues in the host nucleus [29]. The host transcriptional machinery can be altered under the attack of *Xanthomonas*, in which XopD and the various TAL effectors play a critical role in regulating the expression of host genes. Under selective pressure, owing to termination of the
infection, hosts have to develop alternative strategies to abrogate the function of TAL effectors. Xa27 and Bs3 have been both reported to have the function of repressing self-inflicted cell damage and host lesion. Although they do not have a high similarity on the primary sequence and subcellular localizations, the mechanism underlying their function in plant immunity and resistance seems analogous. More concisely, Xa27 and BS3 can modify the gene-promoter combinations, which are the targets of TAL effectors. In addition, the discoveries of another two recessive resistance genes, xa5 and xa13, provide new insights to host adaptations for resistance against Xoo and its TAL effectors [41, 137-139]. Xa5 and xa13 interfere with TAL effector function via different mechanisms. Specifically, Xa13 interferes with PthXo1 and blocks its action as a TAL effector. By contrast, Xa5 serves as a component in the transcription complex that can interfere with the general TAL effector target on the host transcription machinery [41].
Figure 1-1 A zigzag model illustrates the quantitative output of the plant immune system [6].
<table>
<thead>
<tr>
<th>T3E (group)</th>
<th>Biochemical and biological functions</th>
<th>Host target(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AvrBs2</td>
<td>Glycerolphosphoryl diester phosphodiesterase</td>
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<td>Kearney and Staskawicicz, 1990; Swords et al., 1996</td>
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<td>AvrBs3/PthA</td>
<td>Site-specific DNA binding; nuclear localization; transcription activation domain</td>
<td>Os8N3(Os); OsTFX1; upa20 (Ca)</td>
<td>Zhu et al., 1998; Yang et al., 2006; Romer et al., 2007; Sugio et al., 2007; Kay et al., 2007;</td>
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<td>XopC</td>
<td>Haloacid dehalogenase hydrolase and phosphoribosyl transferase domain</td>
<td></td>
<td>Noel et al., 2003</td>
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<td>XopD</td>
<td>Cysteine SUMO C48 protease; DNA binding; nuclear localization; EAR motif</td>
<td></td>
<td>Noel et al., 2002; Hotson et al., 2003; Chosed et al., 2007; Kim et al., 2008;</td>
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<tr>
<td>XopE (HopX)</td>
<td>Transglutaminase family</td>
<td></td>
<td>da Silva et al., 2002; Nimchuk et al., 2007; Thieme et al., 2007</td>
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<td>XopG (HopH1 and HopAP1)</td>
<td>M27 zinc protease (clostridial toxin)</td>
<td></td>
<td>DaSilva et al., 2002; Ochiai et al., 2005; Salzberg et al., 2008</td>
</tr>
<tr>
<td>XopJ (HopJ1) [AvrBsT, AvrXv4, AvrRxv]</td>
<td>Acetyltransferase, C55 cysteine ubiquitin-like protease</td>
<td>14-3-3 protein (SI)</td>
<td>Noel et al., 2003; Roden et al., 2004b; Thieme et al., 2007; Whalen et al., 2008; Bartetzko et al., 2009;</td>
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<tr>
<td>XopH (HopAO1)</td>
<td>Tyrosine phosphatase</td>
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<td>Bretz et al., 2003; Espinosa et al., 2003</td>
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<td>XopN (HopAU1)</td>
<td>ARM/HEAT repeat</td>
<td>VOZ2 and XNP (Os), TARK1, 14-3-3 proteins</td>
<td>Roden et al., 2004a; Kim et al., 2009; Taylor et al., 2012; Cheong et al., 2013;</td>
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<td>XopQ (HopQ)</td>
<td>Nucleoside hydrolase</td>
<td>14-3-3 protein TFT4 (Nb, Ca and Sl); 14-3-3 proteins including TFT1 and TFT5 (At); MAPKKKa/MEK2/SIPK (Nb)</td>
<td>Wei et al., 2007; Li et al., 2013; Teper et al., 2014</td>
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<td>XopAA (HopAE1)</td>
<td>Colicin Ia C8 and C9 domains; ECF</td>
<td>Morales et al., 2005</td>
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<td>XopAC</td>
<td>FIDO domain (AMPylation) PF02661; LRR, VHR</td>
<td>Xu et al., 2008; Kinch et al., 2009</td>
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<td>FIDO domain (AMPylation) PF02661</td>
<td>Wang et al., 2007; Kinch et al., 2009</td>
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<td>XopAl (HopO1)</td>
<td>VIP2; ADP-ribosyltransferase</td>
<td>Silva et al., 2002</td>
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<tr>
<td>XopAJ [AvrRxo1]</td>
<td>Thiol protease, ATP/GTP binding, phosphorylation of NAD to 3'-NADP</td>
<td>Zhao et al., 2004; Shidore et al., 2017</td>
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</table>

The note for the name of T3E (group): Analogue T3Es from Pseudomonas syringae in a certain T3E group are shown in parentheses while those from Xanthomonas in the same group are present in square brackets.

Abbreviations for Biochemical and biological functions: ARM/HEAT, armadillo/Huntington, elongation factor 3, PR65/A, TOR domain; EAR, ethylene receptor factor-associated amphiphilic repression; ECF, early chlorosis factor; FIDO, fic, doc, AvrB domain; LRR, leucine-rich repeat; SUMO, small ubiquitin modifier protein; VHR, vascular hypersensitivity in Arabidopsis landrace Col-0.

Abbreviations for host targets: (Ca), pepper; (Os), rice; TARK, tomato atypical receptor kinase; (Sl), tomato; (Nb) Nicotiana Benthamiana (tobacco); Arabidopsis thaliana (At).
Table 1-2 Cloned R genes for resistance to *Xanthomonas* species

<table>
<thead>
<tr>
<th>Gene</th>
<th>Plant</th>
<th>Type</th>
<th>Comments</th>
<th>Cognate T3 effector</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Bs2</td>
<td>Pepper</td>
<td>NLR</td>
<td></td>
<td>AvrBs2</td>
<td>Tai et al., 1999</td>
</tr>
<tr>
<td>Bs3</td>
<td>Pepper</td>
<td>Inducible</td>
<td>Flavin oxygenase-related protein</td>
<td>AvrBs3</td>
<td>Romer et al., 2007</td>
</tr>
<tr>
<td>Bs3-E</td>
<td>Pepper</td>
<td>Inducible</td>
<td>Promoter deletion of Bs3</td>
<td>AvrBs3Δ16</td>
<td>Romer et al., 2009</td>
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<td>Bs4</td>
<td>Tomato</td>
<td>NLR</td>
<td>Cytoplasm</td>
<td>AvrBs4</td>
<td>Schormack et al., 2004</td>
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<td>Rxo1</td>
<td>Maize</td>
<td>NLR</td>
<td>Cytoplasm; Condition rice resistant ability to <em>Xoc</em></td>
<td>AvrRxo1</td>
<td>Zhao et al., 2005</td>
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<td>Xa1</td>
<td>Rice</td>
<td>NLR</td>
<td>Cytoplasm</td>
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<td>xa13</td>
<td>Rice</td>
<td>Membrane protein; unresponsive S gene to TAL effector PthXo1</td>
<td>PthXo1</td>
<td>Chu et al., 2006; Yang et al., 2006</td>
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<tr>
<td>Xa21</td>
<td>Rice</td>
<td>RLK</td>
<td>Extracellular receptor, broad resistance</td>
<td>None</td>
<td>Song et al., 1995</td>
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</tbody>
</table>
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Chapter 2 Xanthomonas euvesicatoria type III effector XopQ interacts with another effector, XopX, to elicit the defense response in Nicotiana benthamiana

Summary

Xanthomonas euvesicatoria (Xe), a gram-negative bacterium, can infect pepper and tomato plants and causes bacterial leaf spot disease. In contrast, Xe is unable to infect the nonhost plant Nicotiana benthamiana (N. benthamiana; a relative of the tobacco plant). The nonhost resistance in N. benthamiana is conditioned by a disease resistant gene NbRoq1 that recognizes a type III effector Xe-XopQ expressed by Xe. However, the Agrobacterium-mediated transient expression of Xe-XopQ alone in N. benthamiana failed to trigger programmed cell death (PCD). It was speculated that an unknown T3E is required for enhancing the PCD triggered by Xe-XopQ/Roq1. In this study, we identified that Xe-XopX is the T3E, which is required for triggering the strong PCD response from Xe-XopQ/NbRoq1 in N. benthamiana. We confirmed that Xe-XopQ physically interacts with Xe-XopX in vivo by using bimolecular fluorescence complementation (BiFc), co-immunoprecipitation (co-IP), and split luciferase assays. Interestingly, we also discovered that the core domain of Xe-XopX interacts with multiple T3Es, including AvrBS2, AvrPphD1, Xe1298, XopB, XopN, Xe-AvrRxo1, and XopJ1 in plant cells. The deletion of Xe-XopX in Xe compromised the NbRoq1/Xe-XopQ-mediated disease resistance in N. benthamiana and the Bs2/AvrBs2-mediated disease resistance in pepper. Compared to the wild type Xe strain, the XeΔXopX mutant strain has a reduced growth ability on pepper plants lacking BS2 and on N. benthamiana lacking EDSI. Therefore, we speculate that Xe-XopX confers avirulence function by helping the translocation of other type T3Es including Xe-XopQ and AvrBs2.

Introduction
*Xanthomonas euvesicatoria* (*Xe*) causes bacterial spot disease on cultivated pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum*) and are the most devastating to crops grown in warm, humid climates such as in southeastern Asia and the southeastern/midwestern United States [1]. As a model plant also in the *Solanaceae* family and genetically closely related to tomato and pepper, *N. benthamiana* could be a useful tool for research on the mechanisms underlying the interactions between *Xanthomonas* pathovars and hosts [2, 3]. However, *N. benthamiana* is not a natural host of *Xe*. Recently, a large-scale screening of 21 different *Xe* type three secretion (T3S) effectors among 86 *Solanaceae* lines revealed that XopQ is the key effector determining the host range for many *Nicotiana* species [4, 5]. Specifically, wild-type *Xe* induces a strong hypersensitive response (HR), while the deletion mutant *XeΔXopQ* grows to a high titer and produces the appearance of water soaking and disease lesions in *N. benthamiana* [4]. This new finding confers that it is feasible to take advantage of the abundant genetic resources and efficient transformation system of *N. benthamiana* for studying the biological and biochemical functions of type III effectors (T3Es) upon the infection of *Xe* to *N. benthamiana*.

Research indicates that *NbEDS1* is necessary for nonhost resistance against *Xe* and XopQ in *N. benthamiana*; this reported correlation implies that a Toll-like interleukin-1 receptor (TIR) domain-containing resistant (R) protein must present in many *Nicotiana* lines to specifically recognize XopQ [5, 6]. More recently, a resistant gene, *Recognition of XopQ 1* (*Roq1*), has been identified from *N. benthamiana* using a reverse genetic screening approach [7]. Similar to many other typical disease-resistant proteins, *Roq1* has a nucleotide-binding leucine-rich repeat (NLR) domain, while the TIR domain determines its dependence on EDS1-mediated signal pathways in a plant’s immunity response. *Roq1* and its orthologs mediate the recognition of XopQ alleles from various *Xanthomonas* species, as well as HopQ1 from *Pseudomonas* pathovars in the *Nicotiana* genus [8]. Another gene, *N. benthamiana* N requirement gene 1 (*NRG1*), is reported to mediate XopQ-triggered immunity through associating various EDS1 signaling events, such as (a) XopQ elicited HR by the recognition of *Roq1* and (b) XopQ-
induced transcriptional changes in *N. benthamiana* [9]. Thus, in the classic gene-for-gene model, a new member consisting of *Xe*-XopQ, *Nb*Roq1, *Nb*NRG1, and *Nb*EDS1 is characterized and might serve as a candidate that may dramatically improve disease resistance among crops, since XopQ has conserved homologues in diverse bacterial pathogens including pathovars of *Xanthomonas, Pseudomonas,* and *Acidovorax* [8]. However, the *Agrobacterium*-mediated transient expression of *Xe*-XopQ alone in *N. benthamiana* failed to trigger programmed cell death (PCD). It was speculated that an unknown component or mechanism is required for inducing PCD triggered by *Xe*-XopQ/Roq1. Intriguingly, *Xanthomonas campestris* pv. *campestris* (*Xcc*), the causal agent for black rot and the most important disease affecting Brassica vegetable crops worldwide, can cause neither a strong immunity response like HR nor disease symptoms in *N. benthamiana* with the presence of its own *Xcc*-XopQ. Another T3E, XopX, identified in *Xe* can condition *Xcc* strain 8004 to have the ability to cause HR in *N. benthamiana* [10]. Unexpectedly, the characterization of XopX reveals that there is no XopX activation involved in nonhost resistance [10, 11]. Thus, we hypothesize that XopX can interact with XopQ and the two T3Es together influence the immunity response in *N. benthamiana*.

In the present study, we confirmed *Xe*-XopX is required for triggering strong PCD by *Xe*-XopQ/NbRoq1 in *N. benthamiana*. The physical interaction between *Xe*-XopQ and *Xe*-XopX *in vivo* is affirmed by bimolecular fluorescence complementation (BiFc), co-immunoprecipitation (co-IP), and split luciferase assay. Furthermore, we revealed that the core domain of *Xe*-XopX interacts with multiple T3Es, including AvrBS2, AvrPphD1, Xe1298, XopB, XopN, *Xe*-AvrRxo1, and XopJ1 in plant cells. The deletion of *Xe*-XopX in *Xe* compromised the *Nb*Roq1/Xe-XopQ-mediated disease resistance in *N. benthamiana* as well as the Bs2/AvrBs2-mediated disease resistance in pepper. By contrast, compared to the wild type *Xe* strain, the *Xe ΔXopX* mutant strain has reduced growth ability on pepper plants lacking Bs2 and *N. benthamiana* lacking EDS1. Therefore, in considering these various findings, we speculate that XopX may contribute to bacterial virulence via helping the translocation of other
type T3Es, which facilitates an enhanced immunity response in *N. benthamiana* and pepper infected by a high titer inoculation of *Xe*.

**Results**

*Deletion of XopQ and its homologues in diverse bacterial pathogens impart their ability to infect N. benthamiana*

Bacterial spot disease (BS) caused by *Xe* is one of the most important bacterial diseases that threatens pepper production. However, pepper tends to be recalcitrant for tissue culture and transformation, which hinders the characterization of molecular interaction between *Xe* and pepper plants. The model plant *N. benthamiana* is genetically related to pepper, and they both belong to the *Solanaceae* family. One of the most significant advantages of analyzing *N. benthamiana* instead of pepper is that *N. benthamiana* can be easily transformed. However, *N. benthamiana* is not a natural host of *Xe*. In order to take advantage of the abundant genetic resources in *N. benthamiana* that enables the study of the biological and biochemical functions of the type III effectors (T3Es) of *Xe*, we developed a mutant *Xe* strain that is pathogenic on *N. benthamiana*. We performed marker-exchange mutagenesis to delete XopQ from *Xe*. We were successful in infecting *N. benthamiana* plants with the mutant strain *XeΔXopQ* (Figure 2-1 A and C). Furthermore, when *Xe*-XopQ homologues were eliminated from *Xcc* strain *Xcc8004*, Arabidopsis pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) *DC3000*, and the watermelon pathogen *Acidovorax citruli* *AAC00-1*, all three bacteria phytopathogens become pathogenetic in *N. benthamiana* (Figure 2-1 A and C). This outcome is consistent with previous research suggesting that XopQ plays a critical role in determining host specificity. The important function of *Xe*-XopQ and its homologues are dependent on *NbEDS1* (Figure 2-1 B). Wild-type strains of *Xe*, *Pst DC3000*, and *AAC00-1* all exhibited a significantly weaker response in *eds1* mutant of *N. benthamiana* compared with analogous findings for wild-type *N. benthamiana*. Consistently, the transient complementation of *NbEDS1* restored the XopQ/Roq1-mediated resistance response (Figure S2-1). Accordingly, we developed a host-microbe system
consisting of \textit{Xe} mutant strains and \textit{N. benthamiana} in order to investigate the virulence and avirulence mechanisms of \textit{Xe} with respect to infection in selected plants in the \textit{Solanaceae} plant family.

\textit{Co-expression of XopQ and XopX elicits a programmed cell death in \textit{N. benthamiana} in Agrobacterium-mediated transient assay}

Despite the fact that \textit{Xe}, \textit{Pst DC3000}, and \textit{AAC0-1} containing \textit{Xe}-XopQ or its homologues can cause strong cell death in \textit{N. benthamiana}, this was not the case in \textit{Agrobacterium}-mediated transient assays where we observed that all XopQ homologues only elicited a mild chlorotic response. Even when all the XopQ homologues were fused with the strong and constitutive CaMV35S promoter, strong cell death was not observed at 4 day post inoculation (dpi) (Figure 2-2 A). We hypothesize that XopQ alone was insufficient to trigger programmed cell death in \textit{N. benthamiana}.

Another T3E in \textit{Xe}, \textit{Xe}-XopX, sparked our interest as a due to its ability to confer \textit{Xcc}-eliciting cell death in \textit{N. benthamiana} (conversely, \textit{Xcc}, \textit{per se}, does not trigger a hypersensitive response in \textit{N. benthamiana})[10]. We speculated that XopQ may require XopX to elicit a strong HR in \textit{Agrobacterium}-mediated transient assays. Accordingly, we co-infiltrated XopQ and XopX to \textit{N. benthamiana} and a very rapid hypersensitive response occurred (Figure 2-2 B). To confirm these findings, additional tests were conducted. We then inoculated \textit{Xe}-XopX along with other XopQ homologues from \textit{Pst DC3000}, \textit{Xcc}, and \textit{AAC00-1}; in response, cell death was observed in \textit{N. benthamiana} in all cases at 3 dpi (Figure 2-2 B). This outcome demonstrates the function of XopQ as being determinant in the host range, depending on the function of XopX, which has not been previously reported.

\textit{Mutant Xe and Xcc strains with deletion of either XopQ or XopX was unable to trigger HR in \textit{N. benthamiana}}

As noted earlier, \textit{N. benthamiana} exhibits strong cell death upon infection with wild-type \textit{Xcc}
harboring Xe-XopQ or with the co-expression of Xcc-XopQ and Xe-XopX via Agrobacterium-mediated transient assay. Therefore, we attempted to exclude the possibility that Xe-XopX alone would be sufficient to trigger cell death; thus, a mutant strain of XccΔXopQ carrying a plasmid-borne Xe-XopX, was created using marker exchange and complementary approaches. The Xcc strain was inoculated into N. benthamiana and no cell death was observed (Figure 2-3 A). Taken together, we concluded that Xcc is capable of triggering cell death only when Xcc-XopQ and Xe-XopX are both present in the bacterial cells.

Compared with Xcc, Xe is able to trigger cell death in N. benthamiana when XopQ plays a dominant role in triggering the immune response. However, in the current study, XopX also appears to play a role in triggering an immune response. Accordingly, we investigated whether a single T3E, XopQ or XopX in Xe, would be sufficient to trigger a hypersensitive response in N. benthamiana. As shown in Figure 2-3 B, mutant Xe strains with deletions of either T3E, XopQ or XopX, were unable to trigger cell death in N. benthamiana. The double mutant Xe XopQ-/XopX- was similarly unable to trigger cell death with complementation of either XopQ or XopX (Figure 2-3 B). These results are consistent with findings from Agrobacterium-mediated transient assays, further substantiating that XopQ requires the presence of XopX to determine host specificity.

The virulent and avirulent functions of XopX varies with the presence or absence of XopQ

The evidence that emerged from this investigation confirms that Xe-XopX has an avirulent-like function, where the co-expression of Xe- XopX with Xe-XopQ or its homologs in N. benthamiana triggered HR-like cell death. However, previous reports suggest that XopX has a virulent function for bacterial pathogenesis, a finding that augments its functional complexity [10, 11]. The contradiction led us to hypothesize that XopX demonstrates a virulent role in the absence of XopQ or its homologues; whereas XopX is avirulent in the presence of XopQ or its homologues. To test this hypothesis, we developed two groups of growth curve assays with Xe strains inoculated in both N. benthamiana and pepper plants. In the first group, wild type Xe
has a reduced growth population-level in comparison to \( Xe\Delta XopX \) in \( N. benthamiana \) (Figure 2-4 A). This outcome suggests that XopX has an avirulent function when paired with XopQ and a cognate \( R \) gene \( NbRoq1 \) in \( N. benthamiana \). As shown in Figure 2-4B, when the same pairs of strains, \( Xe \) and \( Xe\Delta XopX \), were inoculated on pepper plants that are without the \( Roq1 \) gene, wild type \( Xe \) grew to a higher population level than that of \( Xe\Delta XopX \) in pepper plants, which suggests that XopX has a virulent function. In another growth curve assay, \( Xe\Delta XopQ \) grew to a higher population level than that of the \( Xe\Delta XopQ \Delta XopX \) in both tobacco and pepper (Figure 2-4 C and D), which again supports the idea that XopX has a virulent function in the absence of XopQ, even in the presence of the cognate \( R \) gene \( NbRoq1 \). These observations support our hypothesis that \( Xe\)-XopX plays a dual role in the host and pathogen interactions. XopQ and XopX are both conserved T3Es present in diverse bacterial phytopathogens and they also have high a high frequency of co-occurrence in different \( Xanthomonas \) species (Figure S2-2).

**Essential residues of XopQ are required for its interaction with XopX**

To understand the interaction between XopX and XopQ and their ability to induce an immune response in \( N. benthamiana \), we reviewed prior findings on the biochemical and biological functioning of the two T3Es. Previous results show that XopQ has a putative nucleotide hydrolase (NH) catalytic site [12-15]. Here, we introduced mutations on the putative NH catalytic site of \( Xe\)-XopQ to investigate the essential amino acid residues required for its interplay with XopX to trigger the cell death phenotype in \( N. benthamiana \).

In a disease assay conducted on \( N. benthamiana \), \( Xe\Delta XopQ \) (\( pXopQ-Y279A \)) was found to trigger a faster cell death than that of wild-type \( Xe \); in contrast, \( Xe\Delta XopQ \) (\( pXopQ-S65A \)), \( Xe\Delta XopQ \) (\( pXopQ-D116A \)) and \( Xe\Delta XopQ \) (\( pXopQ-DDD120_122_123AAA \)) induced similar symptoms as did \( Xe\Delta XopQ \) (Figure 2-5 A). This outcome illustrates that the presence of D116, DDD120_122_123, and Y279 might all be required for XopQ to trigger Rop1-mediated HR in \( N. benthamiana \). Interestingly, the mutation at S65 could induce a stronger immune response,
suggesting that XopQ-S65A has improved protein stability or protein activity, which needs to be further investigated. In *Agrobacterium*-mediated transient assays, XopQ-D116A lost its ability to elicit cell death after being co-infiltrated with *Xe*-XopX in *N. benthamiana*, while other mutations including XopQ-S65A, XopQ-DDD120_122_123AAA, and XopQ-Y279A all mimicked the wild-type XopQ by triggering cell death in *N. benthamiana* (Figure 2-5 B). Thus, we conclude that D116 is the probable interaction site or catalytic site required for XopQ to fulfill its biological function.

The core domain of XopX physically interacts with multiple T3Es within plant cells
Since the co-infiltration of XopQ and XopX could trigger cell death in *N. benthamiana*, we tried to determine if the two effector proteins interact inside plant cells. To this end, we employed three methodologies including Co-immunoprecipitation (co-IP), bimolecular fluorescence complementation (BiFC), and split luciferase assays to validate the interactions between XopQ and XopX. As the full-length XopX was not expressed very well in our transient assay, we used a truncated version of XopX, designated as XopX_{112-573} for all further assays. As shown in Figure 2-6A, B and C, XopQ indeed physically interacts with XopX_{112-573} in vivo. The BiFC result also confirms that the interaction occurred in the cytosol and plasma membrane of the transformed plant cells (Figure 2-6 B). The strong XopQ-XopX interaction observed inside the plant cells prompted us to investigate whether XopX also interacts with other T3Es. Surprisingly, in our co-IP assay, XopX_{112-573} was able to interact with AvrBS2, AvrPphD1, Xe1298, XopB, XopN, Xe4428 (a homologue of AvrRxo1) and XopJ effector proteins (Figure 2-6 A). This finding led us to speculate whether or not XopX might have a role in assisting other T3Es to fulfill their function of promoting *Xe* infection. To our knowledge, this is the first report showing that a T3E could interact with numerous other T3Es within plant cells. Additional studies, however, are needed to identify the key residue in XopX that interacts with other effectors, as well as to characterize the interaction mechanisms among those effectors.
The deletion of XopX compromises both the virulent and avirulent functions of AvrBS2

Since XopX interacts with AvrBS2, the first identified virulent T3E in Xe, we decided to test the influence of XopX on the virulent and avirulent functions of AvrBS2. As shown in Figure 2-7 A, XeΔXopQ, the XopQ deletion mutant of Xe, grew much more efficiently than the double mutant strain XeΔXopQΔavrBS2. This finding is consistent with the fact that AvrBS2 plays a significant virulent role in enhancing Xe infection in pepper. We further investigated the virulence function of AvrBS2 in mutant Xe strains where both XopQ and XopX have been deleted. The Xe strain XeΔXopQΔXopX still grew to a higher population level than that of XeΔXopQΔXopXΔAvrBS2 (Figure 2-7 A and B); however, the difference between the two bacterial populations was reduced, and the two strains triggered similar disease symptoms (Figure 2-7 A and B). This discovery is consistent with an earlier report showing that AvrBS2 had a reduced virulence function in the absence of XopX at the early infection stage on susceptible pepper plants [10]. In addition, when Xe strains with or without XopX were inoculated on pepper plants carrying the Bs2 gene (ECW-R20), XeΔXopX triggered a weaker and slower HR than that of the wild type Xe strain (Figure 2-8A). The growth curve assay also confirmed that XeΔXopX grew to higher population levels than that of wild type Xe on ECW-R20 plants (Figure 2-8B). Therefore, the avirulence function of AvrBS2 was also partially compromised in the absence of XopX. Taken together, we speculate that XopX might facilitate the translocation of AvrBS2 and other T3Es at the early stages of Xe colonization, which needs to be further investigated in the future.

Discussion

In this study, we revealed that the Xe T3E XopX have functions by interacting with other T3Es inside of plant cells, which can modulate not only PTI, but also the ETI (effector-triggered immunity) and ETS (effector-triggered susceptibility), especially for XopQ and AvrBS2 [10, 11]. Although XopX could enable Xcc strain Xcc 8004 to trigger HR in N.
benthamiana, XopX alone delivered by bacteria is not able to elicit HR on N. benthamiana in the absence of XopQ, nor on BS2 pepper plants (ECW-R20) in the absence of AvrBS2. There is also no previous report of XopX-triggered nonhost resistance in any plant species. In the tomato and pepper plant disease assays, XopX is required for the full virulent functioning of Xe strain GM98-38 [10]. Transgenic N. benthamiana expressing XopX is viable and also shows more susceptibility to wild type Xanthomonas and Pseudomonas pathovars, but not to T3S mutant strain (Figure S2-3). Stock et al. (2015) suggest that the primary virulent role of XopX is associated with its ability to modulate PTI [16]. However, the authors also demonstrated that Xe-XopX could also promote the accumulation of pattern-triggered immunity (PTI) gene transcripts [11]. These results suggest XopX may have both virulent and avirulent functions [17]. Based on our new finding, we suggest that XopX might function as a helper T3E in the crosstalk between susceptible hosts and pathogenic bacterial pathogens. The virulent or avirulent function of XopX might depend on the interaction of other T3Es and the cognate plant R proteins that may only present in specific plant genotypes.

Given that XopX is cytotoxic when expressed in yeast, it is also likely that XopX targets a broadly conserved eukaryotic cell process in which other T3Es are also involved [18]. Since the T3Es that interact with XopX share very low sequence homology to each other, the interactions between these T3Es and XopX might be via the T3S signal translocation peptide at their N terminus, which may have a conserved secondary structure being recognized by XopX. Binformatic analysis also revealed that XopX has a novel methionine-rich domain that is nearly ubiquitous in Xe [11], which may enable it to form a flexible protein structure and interact with diverse proteins.

Although XopX is required for the full virulent and avirulent functions of at least two effectors, XoPQ and AvrBs2, the deletion of XopX can only delay, but not completely abolish, the AvrBS2-mediated cell death on BS2 pepper plants in our tested conditions. We therefore speculate that XopX is not part of the T3S system; instead it might function as an non-essential facilitator of the T3S translocation during Xe infection in hosts. XopX is conserved among
almost all *Xanthomonas* pathovars except *Xanthomonas axonopodis* pv. *anacardii*, the causal agent of cashew bacterial leaf spot disease in Brazil [19]. This cashew disease usually breaks out during the rainy season in tropical areas. We speculate that XopX could be essential for the colonization of *Xe* in specific environmental conditions, such as in times of cold temperatures or drought conditions. It also encourages *Xe* to compete with other phytopathogens for more nutrients and water in a commensal microbial community on the phyllosphere. Therefore, the exact function of XopX still needs to be further investigated.

XopQ and its homologues are also known to have a virulent function in host plants apart from their avirulent function to trigger disease resistance in nonhost plants. During the infection of *Xe* in Solanaceous plants, XopQ shows its ability to inhibit cell death induced by avirulent *Xe* in disease-resistanot pepper, and also enhances the growth of *Xe* in resistant pepper and tomato [12, 15, 20, 21]. The structural and functional studies on XopQ and its homologues suggest that it might also function as nucleoside hydrolases [8, 12-15]. However, purified proteins of neither *Xoo*-XopQ nor *Xe*-XopQ exhibit any expected nucleoside hydrolase activity *in vivo* [14]. Furthermore, the AvrBs2 protein was predicted to be a glycerolphosphodiesterase (GDE) but GDE enzymatic activity could not be confirmed *in vivo* [22]. As the interactor of both XopQ and AvrBS2, it would be worthy to test whether or not XopX is required for XopQ and AvrBS2 to fulfill their predicted biochemical functions.

**Methods and materials**

*Plant material and inoculation experiments*

Seeds from *N. benthamiana* and *Capsicum annuum* (*C.annuum*, cultivar *ECW*) were germinated in soil at room temperature under a 8h/16h light/dark cycle at 25°C/20°C. After germination, the plants were grown under a 14h/10h light/dark cycle at 25°C/20°C. Ultimately, 6-week-old plants were used for experiments.

*Bacterial growth*
*Escherichia coli* (*E. coli*) DH5α and Rho5 were grown on Luria agar medium at 37°C. *Agrobacterium tumefaciens* (*A. tumefaciens*) strain GV2260 and AAC00-1 were grown at 28°C on a Luria agar medium. Two *Xanthomonas* pathovars including *Xe* and *Xcc* were grown on NYGA medium while *Pst DC3000* was grown on PA medium at 28°C. The following concentrations of antibiotic selection were utilized in this study: 50 μg/ml kanamycin (Km), 100 μg/ml spectinomycin (Sp), and 30 μg/ml gentamycin (Gm). Note that *AAC00-1, Xcc, Xe, Pst* and *A. tumefaciens* strain GV2260 are all intrinsically resistant to rifampicin (Rif) in concentrations of 100 μg/ml.

*Phytopathogen strains and mutant strains generated by marker exchange approach*

Four different phytopathogens were used in this study: *AAC00-1, Xcc, Xe, and Pst DC3000*. XopQ and three of its homologues (Aave3626, *Xcc*-XopQ and HopQ) were eliminated from the four named phytopathogens, respectively, using marker exchange [23]. A general marker exchange construct scheme is shown in the supplemental information. Kanamycin was used as the marker for selecting the mutant strain. Upstream and downstream sequences flanking XopQ and its homologues were amplified using genome DNAs of *AAC00-1, Xcc, Xe, and Pst DC3000* as a template. In terms of the primers we utilized, *Xe*-XopQ upstream was amplified with primers “*Xe*-XopQ upstream SwaI For” and “*Xe*-XopQ downstream SwaI Rev,” while *Xe*-XopQ downstream was amplified with primers “*Xe*-XopQ upstream pmeI For” and “*Xe*-XopQ downstream pmeI Rev.” For primers used to amplify the other three homologues of XopQ, they follow the above naming rule and can be found in the primer list in the supplemental table provided. The upstream sequence of XopQ was cloned to the PCR8 vector using Gibson assembly (New England Biolabs); the downstream sequence of XopQ was cloned to the construct during a final step - again using Gibson assembly. We refer to the latter as *PCR8*-XopQ upstream-Km~XopQ downstream, which was subcloned to vector PLVC18L via LR Gateway cloning (Invitrogen) to generate PLVC18L-XopQ upstream-Kmr-XopQ downstream. The plasmid DNA was transfected to Rho5 for further conjugation. Note that Rho5 already
carried the helper plasmid DNA. 

*Xe* and *Rho5* carrying the construct were co-cultured for 4 hours in liquid medium with diaminopimelate (DAP), an important cell wall constituent. They were then placed on a nutrient broth medium for 48 hours for co-cultivation. The *XeΔXopQ* mutant was selected for by rifamycin and kanamycin in NYGA medium, plus. Other mutants of *AAC00-1, Xcc, and Pst DC3000* were created using the same protocol.

In order to eliminate another T3E gene (*XopX*) from the *XeΔXopQ* background, we needed to remove the selection marker introduced to *Xe* during the last step. The plasmid DNA of *pEDV6-flippase-Gm–SacB/R* is able to recognize the flanking sequence of kanamycin and the gene is removed using flippase. After conjugating the above-named plasmid DNA to *XeΔXopN (Km*)*, the *Xe* clone carrying the vector was screened on selection medium containing gentamycin. We then tested the material to ensure that the kanamycin had been removed, but it remained sensitive to this antibiotic. Due to the fact that the SacB/R gene is toxic in the presence of sucrose, the supplementation of sucrose in the medium is typically used to eliminate the pEDV6 construct. The *Xe* clone sensitive to both gentamycin and kanamycin can be used to neutralize another T3E. This process represents the typical procedure for fabricating mutant strains of phytopathogens impaired with multiple T3Es. Using this approach, we synthesized 5 mutants: *XeΔXopQ, XeΔXopQΔXopX, AAC00-1ΔAave3626, XccΔXopQ,* and *PstDC3000ΔHopQ*, which we then applied to this project. All primers used for marker exchange can be found in supplemental table S2-1.

*Gene cloning, site-directed mutagenesis, and plasmid construction*

Four open reading frames (ORF) of XopQ and its homologues, along with the ORF of *Xe-XopX*, were cloned to pEarley101 vector with a 35S promoter. Since we used the same procedure for cloning each, we show *Xe-XopQ* as the example, as follows. The *Xe-XopQ* ORF was amplified from the genome DNA of *Xe* using primers “*Xe-XopQ ORF For*” and “*Xe-XopQ ORF Rev*,” and then cloned into the pDonr207 vector (donor vector) using BP Gateway cloning
(Invitrogen). The sequences for the other primers can be found in the supplementary table. The vector or \textit{pDonr207-Xe-XopQ-ORF} was subcloned to a pEarley101 vector in \textit{GV2260} using the LR Gateway cloning kit as previously described.

The vector \textit{pDonr207-Xe-XopQ-ORF} was used to generate 4 mutants \textit{Xe-XopQ-D116A}, \textit{Xe-XopQ-DDD120_122_123AAA}, \textit{Xe-XopQ-Y279A}, and \textit{Xe-XopQ-S65A} by site-directed mutagenesis using primers “\textit{Xe-XopQ-ORF-D116A For}” and “\textit{Xe-XopQ-ORF-D116A Rev},” and others listed in Table S2-1. The vector \textit{pDonr207-Xe-XopQ-ORF-D116A} along with the other three XopQ mutants in the donor vectors, were subcloned into the pEarleyGate101 vector in \textit{GV2260} using modified LR Gateway cloning approach as above.

\textit{Bacterial growth curve assay}

Bacterial proliferation in inoculated \textit{N. benthamiana} and \textit{C. annuum} plants were measured by standard growth curve assays [24, 25]. The infiltration inoculation method was used in this study. In brief, the bacteria were cultivated on respective medium supplemented with appropriate antibiotics at 28 °C for 48 hours; the bacterial cells were suspended in 10 mM MgCl$_2$ and diluted to 1X10$^5$ CFU/ml. The bacterial inoculum was infiltrated into the backside of the plant leaf using a blunt-end needleless syringe. The inoculated plants were maintained under 14 h light/10 h dark conditions at room temperature for 6 days; then the leaf discs (990 mm$^2$) were randomly sampled from inoculated leaves at Day 0 and Day 6 for growth curve assay. The sampled leaf discs were ground in 990 μL of 10mM MgCl$_2$, and then vortexed for one minute before dilution and plated on respective media supplemented with proper selection antibiotics. The plates were cultivated at 28°C until the bacterial colonies could be measured. The bacterial colony numbers were used to calculate the bacterial proliferation ratio ($\log_{10}$ CFU/cm$^2$). All growth curves underwent three biological repeats with three technical replicates.

\textit{Agrobacterium-mediated transient assay, western blot, and co-immunoprecipitation}
*Agrobacterium* infiltration, plant protein isolation, and co-IP were performed as previously described [26]. Different *Agrobacterium*-carrying constructs were infiltrated to the mesophyll tissues using blunt-end needleless syringes within a concentration of OD600 of 0.4. Lead disks (1 cm²) were collected at 2 dpi and ground in a 100 µl 1X Laemmli SDS-PAGE buffer. Then, 25 µl protein extract samples were loaded into a 10% SDS-PAGE gel. The protein samples were blotted to PVDF membrane and hybridized with appropriate antibodies [anti-HA-HRP (1: 2,000), anti-T7-3Flag (1: 2,000)]. The western blot signal was detected by using an ECL kit (Thermo Scientific, USA). Following western blot detection, the PVDF membrane was stained with 0.5 % Ponceau S solution to detect the Rubisco protein as the equal loading control. For co-IP, the Flag-tagged fusion protein was immunoprecipitated with anti-3Flag and the co-IP samples were detected with anti-HA-HRP. Prior to immunoprecipitation, 25 µl of the samples were retained as the input control. The immunoprecipitated proteins and input controls were loaded onto a 10% SDS-PAGE gel, blotted to a nitrocellulose membrane, and probed with either anti-Flag followed by a secondary antibody or anti-HA-HRP.

**Bimolecular fluorescence complementation (BiFC)**

BiFC analyses were performed using source materials as previously described [27, 28], which features an optimized split-mVenus yellow fluorescent protein displaying a much-reduced background in planta. The vector pDOE-01 was acquired from the Arabidopsis Biological Resource Center, Stock no. CD3-1901.

The XopQ-ORF amplicon was amplified using pDonr207-XopQ as the template with primers “XopQ-ORF BiFC For” and “XopQ-ORF BiFC Rev” (Table S2-1). This amplicon was then cloned into pDOE01 MCS upstream of the N-terminal of mVenus (35S:X-NmVenus210) using a Gibson Assembly Kit (New England Biolabs). This vector also served as the negative control for BiFC experiments because the C-terminal YFP at MCS upstream remained isolated, but is still in-frame to allow for translation of the C-terminal YFP portion. A truncated amplicon of *XopX*112-573 was amplified out using pEntry-XopX112-573 as the template with primers “XopX112-
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BiFC For” and “XopX112-573 BiFC Rev” (Table S2-1). This material was then cloned into the pDOE01-35S:X-CVenus210 portion of above parent vector harboring XopQ.

*N. benthamiana* leaves were infiltrated with *Agrobacterium GV2260* cultures within a concentration of OD600 of 0.2 and incubated for 48 h post-inoculation. Imaging of BiFC results were performed using a Zeiss LSM880 fluorescent microscope with a 505–550-nm band-pass emission filter and a 488-nm argon laser.

**Split Luciferase construct generation and assay**

*Xe*-XopQ and truncated *Xe*-XopX112-573 in donor vectors generated above were subcloned to the luciferase expression vector via LR cloning. Specifically, *Xe*-XopQ was cloned to the vector harboring N-terminal luciferase (NLUC), while *Xe*-XopX112-573 was cloned to the vector harboring C-terminal luciferase (CLUC); both were then transfected into *Agrobacterium GV2260*. *GV2260* cultures carrying above two constructs were co-infiltrated to *N. benthamiana* leaves within OD600 0.4 (0.2 + 0.2). After 48 hours, luciferin (0.25 g/L) was infiltrated to the same spot carrying the *Agrobacterium* and incubated for an additional 10 min. The positive controls, NLUC-RAR and CLUC-SGT1, along with the negative control, NLUC-GUS and CLUC-GUS, were used to validate the efficacy of this approach. Prior to detecting the luciferase signal, *N. benthamiana* leaves were placed in the dark for 10 min owing to quenching auto-fluorescence signals. Chemical fluorescence signals were detected using the CCD camera of a Gel DocTM XR+ System (Bio-Rad).

**RNA isolation, RT-PCR and real-time PCR**

For RT-PCR, total RNA was extracted from *N. benthamiana* leaf tissues using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. DNA contamination was eliminated by treating total RNAs with UltraPure DNase I (Invitrogen). The integrity of the total RNA were verified by running products through a 0.8% agarose gel and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA
synthesis was performed using the SuperScript III First-Strand System for RT-PCR kit (Invitrogen) with an oligo-dT primer. Real-time PCR was carried out on the cDNA, which was diluted 20 times, using the Quantitect SYBR Green PCR kit (Qiagen) and gene-specific primers in a LightCycler (Roche) according to the manufacturer’s instruction. Gene-specific primers for real-time PCR were synthesized by IDT (Integrated DNA Technologies) and designated as “NbActin real-time PCR For,” “NbActin real-time PCR Rev,” etc. (Supplemental Table 2-1).

Trypan blue staining

The trypan blue staining assay was conducted according to prior reports [29, 30]. *N. benthamiana* leaves inoculated by *Agrobacteria* were boiled for approximately 1 min in 0.025% trypan blue staining solution (lactic acid:glycerol:water-saturated phenol:water=1:1:1:1) and were incubated overnight at room temperature. Then, samples were decolorized in a 0.25% (w/v) chloral hydrate destaining buffer for 24 hours.
Figure 2-1 XopQ and its homologues in diverse bacterial pathogens triggered NbEDS1-dependent nonhost disease resistance in *N. benthamiana*.

Four phytopathogens including *Xe*, *Xcc*, *AAC00-1*, *Pst DC3000*, and their XopQ knock-out mutants with a concentration of OD600 at 0.4 were inoculated to wild-type *N. benthamiana* (A) and *EDS1* mutant of *N. benthamiana* (B). Pictures were obtained 2 days post-inoculation. (C) *In planta* growth of these four bacterial pathogens and their XopQ deletion mutants measured at Days 0 and 6 in *N. benthamiana* leaves infiltrated with a starting inoculum of $1 \times 10^5$ CFU/mL. Star indicates a statistically significant difference. Experiments were replicated three times with comparable results.
Figure 2- 2 Co-expression of XopQ and XopX elicited programmed cell death in *N. benthamiana* in *Agrobacterium*-mediated transient assays.

(A) XopX and XopQ homologues fused with a 35S overexpression promoter were individually
inoculated to wild-type *N. benthamiana* within a concentration of OD600 at 0.4. Picture was taken at 2 days post-inoculation. Trypan blue staining was subsequently processed using the same leaf. (B) Four XopQ homologues and *Xe*-XopX were co-inoculated to wild-type *N. benthamiana* within a concentration of OD600 at 0.4 + 0.4. Picture was taken at 2 days post-inoculation. Trypan blue staining was subsequently processed using the same leaf. (C) Real-time PCR was used to check expression levels of pathogenetic related genes in *N. benthamiana* treated with mock (MgCl₂), *Xe*-XopQ only, *Xe*-XopX only, and a combination of *Xe*-XopQ and *Xe*-XopX.
Figure 2- 3 Mutant Xe or Xcc strains lacking either XopQ or XopX failed to trigger programmed cell death in *N. benthamiana*.

(A) *N. benthamiana* leaf inoculated by Xcc, *Xcc* ∆*XopQ*, a combination of *Xcc* ∆*XopQ* and *Xe*-XopX (*Agrobacteria*), and a combination of *Xcc* and *Xe*-XopX (*Agrobacteria*) with a concentration of OD600 at 0.4 (single inoculation) , or at 0.4 (0.2 + 0.2) (co-inoculation). Picture was taken at 2 days post-inoculation. (B) *N. benthamiana* leaf inoculated by *Xe* ∆*XopQ* ∆*XopX*, *Xe* ∆*XopX*, *Xe* ∆*XopQ*, a combination of *Xe* ∆*XopQ* ∆*XopX* and *Xe*-XopX (*Agrobacteria*), a combination of *Xe* ∆*XopQ* ∆*XopX* and *Xe*-XopX (*Agrobacteria*), and a combination of *Xe*-XopQ and *Xe*-XopX (*Agrobacteria*) with a concentration of OD600 at 0.4 (single inoculation) , or at 0.4 (0.2 + 0.2) (co-inoculation). Picture was taken at 2 days post-inoculation. Trypan blue staining was subsequently processed using the same leaf.
Figure 2- 4 XopX displayed a virulent function in the presence of XopQ, while XopX displayed an avirulent function in the absence of XopQ.

*In planta* growth comparing two pairs of *Xe* strains: *Xe* with *XeΔXopX* (A and C), and *XeΔXopQ* with *XeΔXopQΔXopX* (B and D) measured at Days 0 and 6 in *N. benthamiana* and pepper leaves infiltrated with a starting inoculum of $1 \times 10^5$ CFU/mL. Star indicates statistically significant differences. Experiments were replicated three times with comparable results.
Figure 2- 5 Mutations in the predicted active site of Xe-XopQ affected avirulence in N. benthamiana.

(A) N. benthamiana leaf was inoculated by mock (MgCl₂) as a control, and 7 Xe strains including wild-type Xe, XeΔXopQ, XeΔXopQ pXopQ, XeΔXopQ pXopQ-D116A, XeΔXopQ pXopQ-DDD120_122_123AAA, XeΔXopQ pXopQ-S65A, and XeΔXopQ pXopQ-Y279A within a concentration at OD₆₀₀ 0.4. Picture was taken at 2 days post-inoculation. (B) N. benthamiana leaf was inoculated by mock (MgCl₂) as a control and five Agrobacteria combinations, including Xe-XopX along with XopQ and its four XopQ site mutations at a concentration of OD₆₀₀ 0.4 (0.2 +0.2). Picture was taken at 2 days post-inoculation.
Figure 2- 6 The core domain of XopX physically interacted with multiple T3Es within plant cells.

(A) Co-immunoprecipitation of truncated XopX and multiple T3Es. XopX112-573-3xFlag, was transiently co-expressed with XopQ, avrBS2, avrpphd1, Xe1298, XopB, XopN-GFP, avrRxo1-GFP, XopJ1-GFP, and GFP—all fused with a HA tag in N. benthamiana leaves. The proteins
pulled down by anti-3Flag beads were detected via western blotting using a-HA-HRP primary antibody. Two western blots of the input protein extracts (prior to precipitation) are shown in the middle and bottom. (B) Split luciferase assay results for XopQ with XopX and XopX112-573. Negative control is a combination of GUS-LucN and LucC-GUS, while positive control is a combination of RAR1-LucN and LucC-SGT1. (C) BiFC results for XopQ with XopX112-573. Negative control and positive control are from Dr. Eric Beers lab (507 Latham Hall, 220 Ag Quad Ln. Blacksburg, VA). Scale bars are 50 μm in all panels.
Figure 2-7 Impairment of XopX partially eliminated the virulence function of AvrBS2.

(A) In planta growth comparing two pairs of Xe strains: XeΔXopQ with XeΔXopQΔXopX, and XeΔXopQΔXopX with XeΔXopQΔXopXΔavrBS2 at days 0 and 8 in N. benthamiana leaves infiltrated with a starting inoculum of $1 \times 10^5$ CFU/mL. The letters suggest statistically significant differences. (B) Corresponding disease symptoms from (A). Experiments were repeated three times with comparable results.
Figure 2-8 Impairment of XopX partially eliminated the avirulent function of AvrBS2 in BS2 pepper.

(A) Xe and Xe△XopX at OD600 0.4 were inoculated to BS2 pepper to visualize cell death phenotypes. Pictures of the front side of the leaf (left) and the back side of the leaf (right) were obtained at 48 hpi. (B) Ion leakage of N. benthamiana leaves inoculated by Xe and Xe△XopX were measured at 4 time intervals: 0 h, 4 h, 8 h, and 12 h. Experiments were repeated three times with comparable results.
Supplementary information

Figure S2- 1 Transient expression of NbEDS1 restored the ability of *N. benthamiana* to recognize *Xe* and induced cell death.

Four treatments including *XeΔXopQ* with a concentration of OD600 at 0.4 (A), Wild-type *Xe* at OD600 0.4 (B), the combination of 35S:NbEDS1 with *Xe* at OD600 0.4 + 0.4 (C), and the combination of 35S:NbEDS1 with *XeΔXopQ* at OD600 0.4 + 0.4 (D) were inoculated to *eds1* mutant of *N. benthamiana*. 
Figure S2- 2 XopX is conserved in Xanthomonas and its cooccurrence with a core set T3Es.

The information utilized in this figure was obtained from the software of String (Search Tool for the Retrieval of Interacting Genes/Proteins).
Figure S2- 3 *In planta* growth of \( Xe^{\Delta XopQ} \) in transgenic *N. benthamiana* inducibly expressing XopX.

The strain of \( Xe^{\Delta XopQ} \) was infiltrated to XopX transgenic *N. benthamiana* leaves infiltrated with a starting inoculum of \( 1 \times 10^5 \) CFU/mL. The leaves were pre-treated with dexamethasone (DEX) and mock. The population of \( Xe^{\Delta XopQ} \) was measured at Days 0 and 6 post inoculation. Experiments were replicated three times with comparable results.
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References

15. Gupta, M.K., et al., Mutations in the Predicted Active Site of Xanthomonas oryzae pv.
Chapter 3 Xanthomonas euvesicatoria type III effector XopN interacts with transcription factor NbVOZ to promote an aqueous environment and enhance bacterial proliferation

Summary

Bacterial spot (BS) disease is one of the most common and destructive diseases that infect both tomatoes and peppers. BS can be easily identified due to the appearance of dark, irregular, water-soaked areas on the leaf. While a number of studies have indicated that an aqueous environment is essential for bacterial proliferation, it remains unclear which component of Xanthomonas euvesicatoria (Xe) contributes principally to the water-soaked symptoms of BS in affected pepper and tomato plants. In this study, we report that a Xe type III secreted effector, Xe-XopN, is required for triggering the water-soaking symptom on Nicotiana benthamiana (N. benthamiana) and pepper plants infected with Xe. In addition, we revealed that XopN interacts with two members in a transcription factor, NbVOZ, and represses the expression of NPR1, a key component of basal defense. Therefore, XopN has a role in maintaining a water-affluent environment for better replication of Xe, and it can also interact with NbVOZ1/2 to regulate plant immunity.

Introduction

Because of climate change and the demand for a more sustainable food supply, the concerns of various plant diseases that can severely threaten world crop production are becoming a more urgent problem than ever. In field conditions, plants can be infected by diverse microorganisms throughout their lifetimes. Certain genetically-adapted microorganisms have the ability to colonize and proliferate in host plants and cause disease problems. It is well known that the environment plays a crucial role in the battle between host plants and phytopathogens [1-3]. The classic concept of the “disease triangle” demonstrates that disease can only occur under specific environmental conditions. Notably, among environmental factors involving water
availability such as drought conditions and heavy rainfall events are well-known to have a significant impact on the occurrence and/or proliferation of plant disease [4]. Specifically, under drought conditions plants are usually more resistant to disease in comparison to plants exposed to high humidity. These findings reinforce the notion that global water wars that support human life may also be reflected in the important battle between plants and pathogens. Recent longitudinal studies show that pathogenic bacteria develop virulent proteins to promote an aqueous apoplast, which supports the successful colonization of pathogenic microbes [5]. For example, Xanthomonas gardneri (X. gardneri) recruits a transcription activator-like (TAL) effector (TALE), AvrHah1 to induce water-soaked lesions in fruits and leaves of infected tomato plants. With the function of AvrHah1, water can be drawn into the apoplast of the infected area by X. gardneri. The pull of water can facilitate the entry of additional bacteria and consequently causes more severe disease. Two T3S (Type III Secretion) effectors in Pseudomonas syringae, HopM1 and AvrE, have been also reported to be responsible for the establishment of the aqueous apoplast [1, 6-8].

Bacterial spot (BS) disease caused by Xanthomonas euvesicatoria (Xe), is a primary concern for producers of bell peppers and tomatoes [9]. Xe, as a phyllosphere bacterial pathogen, proliferates mainly into the airfilled apoplast, which is connected directly to openair through epidermal pores called stomata. The water status within the apoplast could, therefore, be influenced by localized humidity levels during pathogen infection. In pepper and tomato fields, phyllosphere bacterial disease outbreaks typically occur after rainfall and/or during periods of high humidity, consistent with the “disease triangle” dogma in plant pathology. In addition, one of the earliest and most common symptoms of phyllosphere bacterial disease is the appearance of so-called “water-soaked” lesions in infected tissues. Although a highly recognizable feature of BS, it remains unclear the degree to which water soaking plays an active role in pathogen pathogenesis. Thus far, no molecular components have been identified as regulators of water-soaking symptoms in either Xe or its host plant candidate.
NPR1 is a well-studied master regulator for the expression of pathogenesis-related (PR) genes including PR1, PR2, and PR5 in plant response to various biotic challenges [10-14]. NPR1 also serves as a bridge connecting the expressions of PR genes and salicylic acid (SA) levels for triggering systemic acquired resistance (SAR), which is a very important plant immune system providing a broad-spectrum resistance effectively against a wide range of phytopathogens [15, 16]. Despite an extensive study on the role of NPR1 in plant immunity, few studies report the mechanism of transcriptional regulation on NPR1, and in particular whether and how the T3E influences NPR1 expression.

In this study, we identified XopN, a type three secretion (T3S) effector, as a functional contributor to the development of water-soaking in infected plant tissues. Furthermore, our results showed that both a glycerol uptake facilitator (glpF) and the type two secretion system of Xe are also partially responsible for the development of the water-soaking phenotype. We also demonstrated that XopN plays a role in suppressing the expression of NPR1 (nonexpressor of PR1) perhaps via the modification of the vascular one zinc transcription factor (VOZ) in *Nicotiana benthamiana* (*N. benthamiana*).

**Results**

*Increased humidity promotes the bacterial proliferation of Xe in infected tobacco and pepper plants*

To investigate if and how humidity impacts pathogen proliferation in plants infected with *Xe*, we setup disease assays under four different relative humidity (RH) levels: 40%, 60%, 80% and 90% RH, to mimick humidity levels in crop fields after different rainfall amounts. We monitored the growing populations of wild type *Xe* and various *Xe* isogenic strains, which were inoculated on *N. benthamiana* and pepper plants. As shown in Figure 3-1 A and C, *Xe* infected on *N. benthamiana* and pepper plants have increased population levels in response to increased humidity levels. However, the increased humidity did not appear to boost the proliferation of *XeΔHrcV* (a mutant with an impaired type III secretion system) (Figure 3-1). The *Xe*
inoculated *N. benthamiana* and pepper plant leaves developed more obvious water-soaking symptoms when the plants were incubated under higher humidity (90 % RH) compared to those that were incubated under lower relative humidity (40 % RH). This finding indicates that some T3Es might be associated with the increased growth of *Xe* in *N. benthamiana* and pepper under higher humidity conditions.

*XopN is a key contributor to the water-soaking phenotype in Xe-infected tobacco and pepper plants*

Since the mutant *XeΔHrcV* strain was unable to trigger water-soaking symptoms in either tobacco or pepper under any experimental conditions (Figure 3-1 B and D), we speculate that some T3E(s) must contribute to the development of a watery environment in the apoplast. Here, we attempted to investigate whether the T3E XopN in *Xe* could contribute to the water-soaking symptoms in both *N. benthamiana* and pepper leaves infected by *Xe* bacteria. Our previous work showed that the mutant *Xe* strain *XeΔXopQ* could infect *N. benthamiana* plants (Chapter 2). In this study, we further deleted either *XopN*, *XopX* or *Xe4428* (*Xe avrRxo1*, a homologue of *Xoc avrRxo1*), in the *XeΔXopQ* background. These isogenic strains were inoculated on *N. benthamiana* plants. The infected plants were maintained under different RH conditions. As shown in Figure 3-2A, when *XopN* was deleted from *Xe*, the water-soaking phenotype was dramatically reduced; in contrast, the deletion of *XopX* or *Xe-AvrRxo1* did not to significantly compromise the water-soaking phenotype. We also performed a growth curve assay to monitor the bacterial growth on both pepper and *N. benthamiana* plants grown under 40% RH or 90% RH. As shown in Figure 3-2 B and C, under 40% RH, the *XeΔXopQΔXopN* strain grows to a reduced population level as compared to the *XeΔXopQ* strain, which suggests XopN has a significant virulent function. However, under 90% RH, *XeΔXopQΔXopN* and *XeΔXopQ* grew to a similar sized population level on both *N. benthamiana* and pepper plants. The mutant, *XeΔHrcC* grew to a similar population level under either 40% or 90% RH on either *N. benthamiana* or pepper plants.
To test if the XopN triggered water-soaking phenotype is related to increased water content in the apoplast, we directly measured the apoplast water content in N. benthamiana leaves infected with Xe strains with or without XopN. As shown in Figure 3-2D, the apoplast water in N. benthamiana leaves infected with XeΔXopQ reached 11.6 ± 2.2 mg/cm², which is significantly higher than the leaves infected with XeΔXopQΔXopN (4.4 ± 0.8 mg/cm²). In control plants, there is no significant difference in the total relative water content of N. benthamiana leaves infected with the two Xe strains (Figure 3-2E).

In addition, we performed an Agrobacterium-mediated transient assay to express XopN, XopX, Xe avrRxo1, and XopQ in N. benthamiana. The overexpression of XopN resulted in a water-soaking like cell-death in N. benthamiana and pepper at 2 days post-inoculation (dpi) (Figure 3-3), while the overexpression of XopX, XopQ or Xe AvrRxo1 resulted in dry and yellowish cell death (Figure 3-3). Interestingly, this particular phenotype can only be induced by the expression of XopN in the cytosol but not in the nucleus (Supplemental Figure S3-1). Taken together, our results demonstrate that XopN, expressed and delivered either by Xe or Agrobacterium tumefaciens into plant cells, is able to elicit water-soaking symptoms.

Different water-soaking phenotypes in the presence or absence of XopN is not caused by the variation in levels of bacterial populations in the infected plant leaves

XopN has a significant virulent function as the Xe strain carrying XopN would grow to a higher bacterial population as compared to plant leaves infected by the Xe strain when XopN was deleted. Therefore, we hypothesized that XeΔXopN mutants elicited a reduced water-soaking phenotype than the Xe strains carrying XopN, because the XeΔXopN mutants proliferate to a reduced bacterial population level in comparison to Xe strains expressing XopN. To test this possibility, the starting inoculum of the XeΔXopQΔXopN mutant was adjusted to 2x10⁸ CFU/ml⁻¹, while XeΔXopQ was adjusted to 1x10⁸ CFU/ml⁻¹. After infiltrating the bacterial inoculums into pepper and N. benthamiana leaves, the bacterial populations were measured at 4 dpi. Because the starting inoculum of XeΔXopQΔXopN is 2-fold higher than that of
Expression of XopN could increase stomatal conductance

For plant leaves infected with Xe, the bacterial cells propagate in the apoplast, which is directly connected to the external environment through the stomata. We, therefore, also measured if XopN could regulate the stomatal aperture and subsequently impact the exchange of water between the apoplast and the open air. In this study, we employed a portable photosynthesis system LI-COR-6400XT to measure the stomatal conductance of N. benthamiana leaves infiltrated by Xe mutant strains, XeΔXopQ, XeΔXopQΔXopX, XeΔXopQΔAvrBs2, and XeΔXopQΔXopN. As illustrated in Figure 3-5, the plant leaves infiltrated with XeΔXopQΔXopN have the lowest stomatal conductance rate (mol H2O m-2 s-1), suggesting the infected leaves have a reduced stomata aperture. While the leaves infected with XeΔXopQ, XeΔXopQΔXopX, and XeΔXopQΔAvrBs2 have similar stomatal conductance rates, which are all higher than that of XeΔXopQΔXopN. The higher stomatal conductance rate also reflects a relatively higher apoplast water potential [17, 18]. Therefore, this result is consistent with our previous observation that XopN, but not XopX or AvrBs2, could increase water content in the apoplast, which might subsequently increase the stomatal conductance rate.

Expression of XopN enhanced the growth of a nonpathogenic Xe strain on tobacco and pepper leaves

Since XopN could induce a water-soaking phenotype, and an increased water content in the apoplast, we also investigated whether the expression of XopN could promote the growth of the co-colonized bacterial cells. We first co-inoculated an Agrobacterium tumefaciens strain

XeΔXopQ, the two bacterial populations at 4 dpi reached a similar level (Figure 3-4 A and B). However, XeΔXopQ that carries XopN still triggered a more obvious water-soaking phenotype than that of XeΔXopQΔXopN in both N. benthamiana and pepper plants (Figure 3-4 C and D). This result negates the possibility that water-soaking triggered by XopN resulted from an increased bacterial population in the infected leaves.
expressing XopN along with \( Xe\triangle HrcV \) on \( N. benthamiana \) (Figure 3-6A). Expression of XopN along with the inoculation of \( Xe\triangle HrcV \) triggered a much stronger water-soaking phenotype than the expression of XopN alone, while \( Xe\triangle HrcV \) alone or MgCl\(_2\) could not trigger any water-soaking phenotype (Figure 3-6A).

We also evaluated the impact of XopN on the \textit{in planta} growth of \( Xe\triangle HrcV \). In this experiment, we first infiltrated either \( Xe\triangle XopQ \) or \( Xe\triangle XopQ\triangle XopN \) (4x10^8 cfu/ml) into the whole leaves of \( N. benthamiana \) and pepper plants. At 48 hrs post-inoculation (hpi), the pre-infected leaves were spray-inoculated with the mutant strain \( Xe\triangle HrcV (Km^-) \) (1x10^5 CFU/ml). Another 48 hours later, the bacterial populations of \( Xe\triangle HrcV (Km^-) \) were measured by plating the recovered bacteria on culture media with or without the supplementation of kanamycin. Only the nonpathogenic \( Xe\triangle HrcV (Km^-) \) strain carrying a kanamycin resistance gene allows growth on the media supplemented with kanamycin. As shown in Figure 3-6 B and C, inoculated leaves co-infected with \( Xe\triangle HrcV (Km^-) \) and \( Xe\triangle XopQ \) grew to higher population levels than leaves co-infected with \( Xe\triangle XopQ\triangle XopN \). As controls, we also measured the bacterial populations of \( Xe\triangle XopQ \) or \( Xe\triangle XopQ\triangle XopN \) in the inoculated plant leaves at 0 hpi and 48 hpi (before the spray inoculation with \( Xe\triangle HrcV (Km^-) \), where no significant growth difference was detected between the two strains on either \( N. benthamiana \) or pepper plants. Therefore, we speculate that the pre-inoculation of \( Xe\triangle XopQ \) (carrying XopN) created a favorable environment for enhancing \( Xe\triangle HrcV (Km^-) \), colonization on tobacco and pepper plants. In contrast, the nonpathogenic strain \( Xe\triangle HrcV (Km^-) \) has a relatively lower growth rate on either \( N. benthamiana \) or pepper plants pre-inoculated by \( Xe\triangle XopQ\triangle XopN \) that is lacking XopN.

\textit{XopN interacts with NbVOZ1 and NbVOZ2 and regulates the expression of NPR1 in \( N. benthamiana \)}

To investigate whether XopN could regulate the expression of plant defense-related genes, we employed real-time PCR to monitor the expression levels of the pathogenesis-related (PR)
genes NbPR1 and the SAR regulator NbNPR1. The *N. benthamiana* plants were sprayed with either MgCl$_2$ (mock) or either of the *Xe* mutant strains (*XeΔXopQ* or *XeΔXopQΔXopN*). The inoculated leaf tissue was collected at 24 hours post-inoculation. The expression of NbNPR1 and NbPR1 were detected by using gene-specific primers (Supplemental information Table S1). Interestingly, inoculation with *XeΔXopQΔXopN* induced about 50 to 100 fold higher expression levels of NbNPR1 and NbPR1 than inoculation with *XeΔXopQ* (Figure 3-7 A). Therefore, the expression of XopN may repress the expression of *NPR1* and *PR1* in *N. benthamiana*.

A previous report suggests that *Xoo*-XopN from the rice pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) interacts with a rice plant transcription factor, OsVOZ2 *in vivo* [19]. Therefore, we attempted to test if *Xe*-XopN could interact with the VOZ protein in *N. benthamiana* plant cells. As illustrated in Figure 3-7 B and C, we confirmed that XopN indeed interacts with both NbVOZ1 and NbVOZ2 via our results of the split-luciferase assay (Figure 3-7 B) and co-IP assay (Figure 3-7 C). In addition, the co-expression of NbVOZ1 or NbVOZ2 with *Xe*-XopN could localize *Xe*-XopN-YFP to the nucleus, while the expression of *Xe*-XopN-YFP alone predominately localizes in the cytosol of transformed plant cells (Supplemental Figure S3-2). Since the VOZ1 and VOZ2 encoding transcription factors are conserved in all plant species, we hypothesized that XopN might regulate the expression of NbNPR1 and NbPR1 by interacting with the NbVOZ transcription factor. To test this hypothesis, we cloned the NbNPR1 promoter in front of a GUS (β-glucuronidase) reporter gene. The NbNPR1 promoter:GUS was co-expressed with either NbVOZ1, NbVOZ2 or GFP by using an *Agrobacterium*-mediated transient assay. Surprisingly, NbVOZ1 or NbVOZ2 co-expressed with the NbNPR1 promoter:GUS fusion resulted in a stronger GUS staining signal than that of co-expression with the GFP gene. We also attempted to co-express *Xe*-XopN with the NbNPR1 promoter:GUS in combination with NbVOZ on *N. benthamiana* plant leaves. However, no conclusive data was obtained (data not shown).

To our knowledge, this is the first report that the expression of *NPR1* could be regulated by
the VOZ transcription factor. However, further studies are needed to determine the molecular interactions between XopN, VOZ, and NPR1, which may also help us understand if NPR1 or other targets of VOZ could contribute to the water-soaking phenotype triggered by Xe-XopN.

Discussion

The Bacterial spot disease caused by Xanthomonas euvesicatoria (Xe) can be easily identified due to the appearance of dark, irregular, water-soaked areas on the leaf. While a number of studies have indicated that an aqueous environment is essential for bacterial proliferation, it remains unclear which component of Xe contributes to the development of water-soaked symptoms in infected pepper and tomato plants. In this study, we identified that the T3E Xe-XopN has a key role in promoting the water-soaking phenotype in Xe infected plant leaves.

The virulence function of XopN has been previously characterized. For example, Xoo-XopN expressed in Xanthomonas oryzae pv. oryzae (Xoo), a xylem pathogen causing bacterial blight in rice, can suppress LipA-induced callose deposition [19, 20]. The XopN expressed by Xanthomonas euvesicatoria can interact with host defense-related proteins including the atypical receptor-like kinase named TARK1 and a 14-3-3 protein to suppress the PAMPs (pathogen-associated molecular patterns) involved in plant triggered immunity (PTI) upon Xe colonization in tomato. Xe-XopN and Xoo-XopN may share conserved functions to suppress PTI in different plant species [21, 22]. In this study, we characterized a new virulence function of XopN, that promotes the establishment of a water-enriched apoplast and enhanced colonization and proliferation of Xe bacterial cells in leaf tissue. However, are there any connections between the previously characterized virulence function and the XopN-mediated water-soaking phenotype? Is that possible that PTI-related events such as callose deposition can block water from being transported from the plant cell to the apoplast space? Answers to these questions will help gain a deep understanding of effector-trigger susceptibility.

Several residues of Xe-XopN have been reported to be essential for the full virulent function
of *Xe*-XopN. Specifically, *Xe*-XopN interacts with the tomato 14-3-3 isoform TFT1 in a phosphorylation manner at S688 along with two leucine residues (L64, L65) [21]. Moreover, mutations of these key residues in XopN prevents its binding to TFT1 in plant extracts [21]. When expressed in *Xe*, these XopN mutants also have reduced virulent function [21]. The residues L64 and L65 are required for XopN to bind to the Atypical Receptor-Like Kinase1 (TARK1) in tomato. In our preliminary data (data not shown), the mutation of S688 along with L64 and L65 did not alter the ability of XopN to trigger water-soaking phenotype. Therefore, the XopN triggered water-soaking phenotype may be independent of TFT1 and TARK1.

Although *Xe*-XopN is the primary component regulating the development of the water-soaking phenotype triggered by *Xe*, deletion of XopN did not completely abolish the water-soaking phenotype (Figure 3-2A). As shown in supplemental Figure S3-3A, we also demonstrated that the deletion of the type II secretion system (T2SS) in *Xe* reduced the water-soaking phenotype triggered by *Xe*, while the deletion of both XopN and the T2S almost completely blocked the water-soaking symptom. In Gram-negative bacteria various enzymes like the lipolytic enzyme are generally secreted via T2S [23]. It was proposed that the T2SS could enhance the establishment of T3S (Type Three Secretion System) during the *Xe* infection [24]. Thus, T2SS per se might not have the role of controlling the water-soaking phenotype, but by contrast, its function might be to help other T3Es including XopN to be translocated into plant cells. We also identified that the deletion of *glnF*, a glycerol transporter gene in *Xe*, partially compromised the water-soaking phenotype when the mutant strain was inoculated on *N. benthamiana* (Figure S3-3B). The *glnF* has been reported to participate in the synthesis of exopolysaccharides in *Xe*, where the exopolysaccharide produced by *Xe* may also contribute to the development of the water-soaking phenotype [5, 25]. However, the exact function of *glnF* and how it contributes to the water-soaking symptom is still in need of further investigation.

Apart from its contribution to the induction of the water-soaking phenotype, XopN was found
to be able to suppress the expression of NPR1 and PR1 in *N. benthamiana*. As NPR1 functions as a master regulator of the salicylic acid (SA)-signaling pathway and is a key component of plant basal immunity, it will be intriguing to investigate further whether or not XopN can suppress the SA-mediated SAR by down-regulating the expression of NPR1. To test this possibility, we also generated transgenic *N. benthamiana* plants to induce the expression of XopN (data not shown). These transgenic plants will allow us to further characterize the molecular functions of XopN in the future.

Previous research on the NPR1-mediated signal transduction pathway mainly focuses on how NPR1 regulates its downstream signaling events. For example, NPR1 induces the expression of the SA-dependent defense gene and disease resistance via its interaction with the transcription factors TCP and TGA in the nucleus. Several post-translational modifications of NPR1 proteins, including those that effect phosphorylation, S-nitrosylation and sumoylation, are essential for the regulation of NPR1 [26, 27]. However, few studies elucidate the regulation expression of NPR1 by upstream components. A recent study revealed the presence of W-box sequences in the promoter of the AtNPR1 genes. The expression of AtNPR1 can be regulated by WRKY6 and WRKY18 in a SA-dependent way [28-31]. SA also promotes the formation of a protein complex that includes NPR1, CDK8 (CYCLIN-DEPENDENT KINASE8) and WRKY18 in *Arabidopsis* plant cells. The complex can bind to the W-box in the NPR1 promoter region and regulate AtNPR1 expression [29].

In this study, we identified another transcription factor *NbVOZ* that might be targeted by XopN to regulate the transcriptional capacity of *NbNPR1*. *VOZ* is a plant transcription factor family with two members, *VOZ1* and *VOZ2*, that are conserved in diverse plant species [32-36]. Interestingly, a VOZ family member in rice, *OsVOZ2*, was identified as a target of *Xoo*-XopN in rice plant cells [19]. Consistently, *Xe*-XopN can interact with two members of *NbVOZ*, *NbVOZ1* and *NbVOZ2*, in *N. benthamiana* plant cells (Figure 3-7 B and C), which may also
regulate the transcriptional activity of NbNPR1 in a GUS-reporter assay (Figure 3-7 D). In the future, electrophoretic mobility shift assay and a chromatin immunoprecipitation (ChIP)-PCR will be performed to validate whether NbVOZ1 and NbVOZ2, can bind to the promoter of NbNPR1. Transgenic *N. benthamiana* plants with the overexpression of NbVOZ1 and NbVOZ2, and mutant *N. benthamiana* plants carrying VOZ1/VOZ2 deletions, will be also generated for evaluating the function of NbVOZ1/NbVOZ2 in response to the Xe infection.

**Methods and materials**

*Plant material and growth conditions*
Seeds from *N. benthamiana* and pepper (*Capsicum annuum, Ca*) were germinated in soil at room temperature under 8h/16h light/dark cycle at 25°C/20°C. After germination, plants were grown under 14h/10h light/dark cycle at 25°C/20°C. For the experiments described herein, 6-week-old tobacco and pepper plants were used.

*Bacteria growth*
*Escherichia coli* (*E. coli*) DH5α and Rho5 were grown on Luria agar medium at 37°C. *Agrobacterium tumefaciens* (*A. tumefaciens*) strain GV2260 and Xe were grown on a Luria agar medium and an NYGA medium at 28°C. The concentrations of antibiotic selections in this study were as follows: 50 μg/ml kanamycin (Km), 100 μg/ml Spectinomycin (Sp), and 30 μg/ml gentamycin (Gm). Xe and *A. tumefaciens* strain GV2260 were intrinsically both resistant to rifampicin (Rif) with a concentration of 100 μg/ml.

*Phytopathogen strains and mutant strains generated by the marker-exchange approach*
Xe and its various mutants were utilized for the investigation in this study. The T3Es were knocked out from Xe genome one-by-one. The general procedure we used included two steps: (a) marker exchange to remove the gene, and (b) application of flippase to remove kanamycin, which is the marker introduced during the last step in order to identify the mutant strain [37].
Upstream and downstream sequences flanking XopN were amplified using the genome DNA of Xe as the template. In terms of the primers we utilized, Xe-XopN upstream was amplified with primers “Xe-XopN upstream SwaI For” and “Xe-XopN downstream SwaI Rev,” while Xe-XopN downstream was amplified with primers “Xe-XopN upstream pmeI For” and “Xe-XopN downstream pmeI Rev”. The upstream sequence of XopN was cloned to the PCR8 vector using the Gibson assembly kit (New England Biolabs), while the downstream sequence of XopN was cloned to the construct from the final step, again via Gibson assembly. Finally, the construct was named as PCR8-XopN upstream-Km r-XopN downstream, which we then subcloned to vector PLVC18L by LR Gateway cloning (Invitrogen) to generate pLVC18L-XopN upstream-Km r-XopN downstream-SacB/R.

The plasmid DNA was transfected to Rho5 for further conjugation. Note that Rho5 already carries the helper plasmid DNA for conjugation. Xe and Rho5 carrying the construct were co-cultured for 4 hours in a liquid medium with diaminopimelic acid. After conjugation, the mix of bacteria was placed on the medium without supplementation of antibiotic to co-culture for another 48 hours. The XeΔXopN mutant was selected from the NYGA medium supplemented with Rifamycin and Kanamycin.

In order to remove another T3E gene from XeΔXopN profile, we needed to remove the selection marker introduced to Xe during the final step. A plasmid DNA pEDV6-flippase-Gentamycin–SacB/R can recognize the flanking sequence of Kanamycin and cut off the Kanamycin gene using the flippase. After conjugating the above-described plasmid DNA to XeΔXopN (Km r), we then screen the Xe clone carrying the vector based on the selection of gentamycin, after which we determined whether kanamycin had been removed, given that it is sensitive to this antibiotic. Since the SacB/R gene is toxic when combined with sucrose, the supplementation of sucrose in the medium was used to eliminate the pEDV6 construct. The Xe clone sensitive to both gentamycin and kanamycin can be used to knock out the other T3Es.

In general, the description provided above represents the typical procedure for producing Xe mutant strains impaired by multiple T3Es. For this investigation, we prepared the following 11
Xe mutants using this approach: $Xe \Delta XopQ$, $Xe \Delta HrcV$, $Xe \Delta HrcV$ (Km), $Xe \Delta XopQ \Delta XopN$, $Xe \Delta XopQ \Delta XopX$, $Xe \Delta XopQ \Delta avrBS2$, $Xe \Delta XopQ \Delta avrRxo1$, $Xe \Delta XopQ \Delta glpF$, $Xe \Delta XopQ \Delta T2SS$, $Xe \Delta XopQ \Delta T2SS \Delta XopN$, and $Xe \Delta XopQ \Delta glpF \Delta T2SS$. All primers used for marker exchange in this chapter can be found in the supplemental table S3-1.

Gene cloning, site-directed mutagenesis, and plasmid construction

The ORF of XopN and the ORFs of NbVOZ1, NB, and VOZ2 were cloned to two destination vectors, pEarley101 and pEarley102 vectors, both carrying a 35S promoter. These two vectors, pEarley101 and pEarley102, displayed HA-tag and 3Flag-tag at C terminals, respectively. The Xe-XopN ORF was amplified from the genome DNA of Xe using primers “Xe-XopN ORF For” and “Xe-XopN ORF Rev”, and cloned into the pDonr207 vector (donor vector) using BP Gateway cloning (Invitrogen). The same procedure was followed for cloning the ORFs of NbVOZ1 and NbVOZ2 to the pDonr207 vector. The sequences of primers are listed in the supplementary table S3-1. The above donor vectors were subcloned to pEarley101 or pEarley102, in GV2260 using modified LR Gateway cloning kit as described [38].

Bacterial growth curve assay

The bacterial proliferation in inoculated *N. benthamiana* and *C. annuum* plants was assessed via standard growth curve assays [39, 40]. Different humidity settings were achieved by covering an acrylamide fiberglass box, in which a humidifier and a humidity/temperature Data Logger (Lascar) were placed. The humidity and temperature were recorded over the period of disease assay.

Two inoculation methods (infiltration and spray) were used in this study. The bacteria were cultivated on a medium supplemented with appropriate antibiotics at 28°C for two days; the bacterial cells were then suspended in 10 mM MgCl₂ and diluted to 1X10⁵ CFU/ml. The bacterial inoculum was infiltrated into the backside of the plant leaf using a blunt-end needleless syringe. For the subsequent inoculation, $Xe \Delta HrcV$ with Kanamycin resistance was
spray-inoculated to *N. benthamiana* leaves pre-treated by *XeΔXopQ* and *XeΔXopQΔXopN*. The bacterial cells were then collected and suspended in 10mM MgCl₂ with 0.02% Silwet L77 and diluted to OD600 0.1. The plants were covered and kept at 100% moisture levels for one day prior to inoculation. The inoculated plants were maintained under 14 h light/10 h dark at room temperature for 6 days; leaf discs (990 mm²) were randomly sampled from the inoculated leaves at Day 0 and Day 6 for growth curve assays. The sampled leaf discs were grounded in 990 μL of 10 mM MgCl₂, and vortexed for one minute before dilution and plating on respective media supplemented with proper antibiotics. The plates were cultivated at 28°C until the bacteria colonies could be counted. These bacteria colony numbers were then used to calculate the bacteria proliferation ratio (Log₁₀ CFU/cm²). All growth curves represent three biological repeats with replicates.

*Agrobacterium*-mediated transient assay, western blot, and co-immunoprecipitation

The agrobacteria infiltration, plant protein isolation, and co-IP were performed as described by Krasileva KV et al. (2010) [41]. *Agrobacterium* strains carrying different constructs were adjusted to OD600 0.4 and infiltrated to the mesophyll tissue using blunt-end syringes without needles. Lead disks (1 cm²) were collected at 2 dpi and ground in a 100 μl 1X Laemmli SDS-PAGE buffer. Then, 25 μL protein extract samples were loaded into 10% SDS-PAGE gel. The protein samples were blotted to PVDF membrane and hybridized with the appropriate antibody [anti-HA-HRP (1: 2,000), anti-T7-3Flag (1: 2,000)]. The western blot signal was detected by using an ECL kit (Thermo Scientific, USA). After western blotting detection, the PVDF membrane with stained with 0.5 % Ponceau S solution to detect the Rubisco protein as the equal loading control. For co-IP, the Flag-tagged fusion protein was immunoprecipitated with anti-3Flag and the co-IP samples were detected with anti-HA-HRP. Prior to immunoprecipitation, 25 μL of samples were saved as the input control. The immunoprecipitated proteins and input controls were loaded on 10% SDS-PAGE gel, blotted with a nitrocellulose membrane, and probed with either anti-Flag followed by a secondary
antibody or anti-HA-HRP.

**RNA isolation, RT-PCR and real-time PCR**

For RT-PCR, total RNA was isolated from *N. benthamiana* leaf tissues using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. DNA contamination was eliminated by treating total RNA with UltraPure DNase I (Invitrogen). The integrity and quantity of total RNA were ascertained by running it through 0.8% agarose gel using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Then, cDNA synthesis was performed using the SuperScript III First-Strand System for RT-PCR kit (Invitrogen) with an oligo-dT primer based on the manufacturer’s instructions. Real-time PCR was carried out with a specimen of cDNA diluted 20-times using the Qiagen SYBR Green PCR kit in accordance with the manufacturer’s protocol. Gene-specific primers for real-time PCR were synthesized by IDT (Integrated DNA Technologies) and referred to as “NbActin real-time PCR For,” “NbActin real-time PCR Rev,” etc.

**GUS Assay—Construction of Reporter Plasmids and Transgenic Plants**

The promoter region of NbNPR1 was amplified via PCR using *N. benthamiana* genome DNA as the template. We then cloned it to pDonor207 vector, which was subsequently introduced to the plasmid DNA pKgwfs7.0 carrying a GUS gene [42]. The final construct is referred to as pKgwfs7.0-NbNPR1promoter:GUS. After being transfected to *GV2260*, it was used for the GUS reporter assay. NbVOZ1 and NbVOZ2 driven by the 35S promoter were then used to determine if they could regulate the transcriptional activity of the NbNPR1 promoter. Two combinations were utilized: 1) 35S:NbVOZ1 and NbNPR1promoter:GUS, and 2) 35S:NbVOZ1 and NbNPR1promoter:GUS. Another combination, 35S:GFP and NbNPR1promoter:GUS, served as the control. *N. benthamiana* leaves were infiltrated with *GV2260* cultures within OD600 0.4 (0.2 + 0.2).

According to the methods described by Jefferson et al. (1987) [43, 44], after incubation for 48
hours, we determined the GUS enzyme activity in the tested plants by staining with X-glucuronide (Life Technologies/GibcoBRL) as a substrate. After distaining with 70% ethanol overnight, we were able to evaluate the GUS assay capacity using Image J.

Split Luciferase constructs generation and assay
The ORFs of NbVOZ1, NbVOZ2, and XopN in donor vectors generated above were cloned to luciferase expression vectors via LR cloning. Specifically, NbVOZ1 and NbVOZ2 were cloned to the vector harboring N-terminal luciferase (NLUC), while XopN was cloned to the vector harboring C-terminal luciferase (CLUC); all were then transfected into Agrobacterium GV2260. Two combinations were utilized: 1) NLUC-NbVOZ1 and CLUC-XopN, and 2) NLUC-NbVOZ2 and CLUC-XopN. A positive control consists of NLUC-Rar1 and CLUC-Sgt1 along with a negative control consists of two empty vectors that were also used to validate the appropriateness of this approach. N. benthamiana leaves were infiltrated with GV2260 cultures within OD600 0.4 (0.2 + 0.2). After incubation for 48 hours, luciferin (0.25 g/L) was infiltrated to the same spot carrying the Agrobacterium and incubated for an additional 10 min. Prior to detecting the luciferase signal, the N. benthamiana leaves were placed in the dark for 10 min owing to quenching auto-fluorescence signals. Chemical fluorescence signals were detected using the CCD camera of the Gel DocTM XR+ System (Bio-Rad).

Stomatal conductance
A portable photosynthesis system (LI-6400T, Li-Cor Inc., USA) with a 6400-02B light source (blue and red diode) was used to measure the following photosynthetic gas exchange parameters of the N. benthamiana leaves in vivo: net photosynthetic rate ($P_{n}$), intercellular CO$_2$ concentration ($C_i$), transpiration rate ($E$), and stomatal conductance ($G_s$). Before the measurement, N. benthamiana leaves were infiltrated by four isogenic Xe strains including $Xe\triangle XopQ$, $Xe\triangle XopQ\triangle XopX$, $Xe\triangle XopQ\triangle AvrBs2$, and $Xe\triangle XopQ\triangle XopN$. After the measurement, the data of $E$ and $G_s$ were utilized for further analysis. Measurements were...
obtained under an artificial irradiance of 1000 μmol (photons) m$^{-2}$ s$^{-1}$ at a temperature of 25°C using the fifth completely expanded leaf from the top of each plant. CO$_2$ concentration and ambient water-vapor pressure were maintained at 385 μmol mol$^{-1}$ and 1.30 ± 0.15 kPa, respectively.
Figure 3-1 The amplification of Xe increases with elevated humidity in both tobacco and pepper.

Bacterial populations of $Xe\Delta XopQ$ and $Xe\Delta HrcV$ grown on (A) and pepper (C) leaves under 40% humidity including (RH), 60% RH, 80% RH and 90% RH. The populations were measured at 6 days post-inoculation (dpi) with a starting inoculum of $1 \times 10^5$ cfu ml$^{-1}$. Two-way ANOVA with Tukey’s test (significance set at $P \leq 0.05$) was performed. Significant differences are indicated by different letters. $n = 3$ technical replicates; data are shown as mean ± s.e. The phenotypes of Xe infection, under 40% RH and 90% RH, of N. benthamiana (B) and pepper (D) leaves that were inoculated by $Xe\Delta XopQ$ and $Xe\Delta HrcV$, respectively. The left half of the leaf was inoculated by $Xe\Delta HrcV$, while right half was inoculated by $Xe\Delta XopQ$. The pictures of visualized symptom were taken 6 dpi with a starting inoculum of $1 \times 10^5$ cfu ml$^{-1}$. Experiments were repeated three times with similar results.
Figure 3- 2 XopN is required for developing of the water-soaking phenotype in the Xe infected tobacco and pepper plants, but this function can be impacted by high humidity.

Four Xe isogenic strains including $Xe \triangle XopQ$, $Xe \triangle XopQ \triangle XopX$, $Xe \triangle XopQ \triangle avrRxo1$, and $Xe \triangle XopQ \triangle XopN$ within a concentration OD600 at 0.4 were inoculated to wild type $N. benthamiana$ (A). Pictures were obtained at 2 days post-inoculation. In planta growth of three $Xe$ strains including $Xe \triangle XopQ$, $Xe \triangle XopQ$, and $Xe \triangle HrcV$ measured at Day 6 with a starting inoculum of $10^4$ CFU/mL after infiltration of leaves of $N. benthamiana$ (B) and pepper (C) under normal humidity and 90% relative humidity. Two-way ANOVA with Tukey’s test (significance set at $P \leq 0.05$) was performed. $n = 3$ biological replicates; data are shown as mean ± s.e. Different letters indicate statistically significant differences. Leaf relative water content and apoplastic water content were measured in $N. benthamiana$ leaves inoculated by
$Xe\triangle Xo^{\text{op}}Q$ and $Xe\triangle Xo^{\text{op}}Q\triangle Xo^{\text{op}}N$ as shown in (D) and (E), respectively. Star indicates statistically significant differences. Experiments were repeated three times with similar results.
Figure 3- 3 Transient expression of XopN induces water-soaking symptoms in both tobacco and pepper.

*N. benthamiana* and pepper leaves were inoculated by Agrobacteria harboring Xe-XopN, Xe-XopX, Xe-XopQ and Xe-avrRxo1 driven by 35S promoter at a concentration at OD600 0.4. Pictures were obtained 2 days post-inoculation. Experiments were repeated three times with similar results.
Figure 3- 4 Different water-soaking phenotypes in presence or absence of XopN is not caused by the variation of bacterial populations in the infected plant leaves.

*In planta* growth of *Xe ΔXopQ* and *Xe ΔXopQ ΔXopN* measured at 4 Day post infiltration to leaves of *N. benthamiana* (A) and pepper (C). The concentrations of starting inoculum of *Xe ΔXopQ* and *Xe ΔXopQ ΔXopN* were, respectively, $1 \times 10^8$ cfu ml$^{-1}$ and $2 \times 10^8$ cfu ml$^{-1}$. Visualized water-soaking phenotypes infected by *Xe* strains are shown in (B), *N. benthamiana*, and (D), pepper, respectively. On both leaves, the left spot indicates the inoculation of *Xe ΔXopQ ΔXopN*, while the right spot denotes the inoculation of *Xe ΔXopQ*. Experiments were repeated three times with similar results.
Figure 3- 5 XopN may up regulate stomatal aperture.

Stomatal conductance of *N. benthamiana* leaves were measured after inoculation by four *Xanthomonas* isogenic strains including *XeΔXopQ, XeΔXopQ ΔXopX, XeΔXopQ ΔavrBS2, XeΔXopQ ΔXopN*. The measurements were conducted 2 days post-inoculation and the starting inoculum concentration was $1 \times 10^5$ cfu ml$^{-1}$. The data are presented in a box plot. Experiments were repeated three times with similar results.
A nonpathogenic \textit{Xe} strain, \textit{Xe}Δ\textit{HrcV}, can trigger weak water soaking with
the co-expression of XopN and can be introduced by water soaking induced by XopN.

(A) *N. benthamiana* leaf was inoculated by MgCl₂, *Xe*-XopN (Agrobacteria), *Xe*Δ*HrcV*, a combination of *Xe*-XopN (Agrobacteria) and *Xe*Δ*HrcV* with a concentration of OD600 at 0.4 (single inoculation), or a concentration of OD600 at 0.4 (0.2 + 0.2, co-inoculation). The image was taken 2 days post-inoculation. For (B) and (C), the inoculation of *Xe*Δ*HrcV* followed the inoculation of *Xe*Δ*XopQ* and *Xe*Δ*XopQ*Δ*XopN* 48 hours later. The population of *Xe*Δ*HrcV* mutant strain with kanamycin-resistance was obtained at another 48 hours post-inoculation with a starting inoculum at 1 × 10⁵ cfu ml⁻¹ in *N. benthamiana* (B) and pepper (C). Two-way ANOVA with Tukey’s test (significance set at P ≤ 0.05) was performed. Significant differences are indicated by asterisk. n = 3 biological replicates; data are shown as mean ± s.e. Experiments were replicated three times with similar results.
Figure 3- 7 XopN can regulate NbNPR1 expression through its interaction with NbVOZ1 and NbVOZ2.

(A) The relative expression levels of NbNPR1 and NbPR1 in leaves infected by XeΔXopQ and XeΔXopQΔXopN 24 hours post-inoculation with an inoculum concentration of $1 \times 10^5$ cfu ml$^{-1}$. (B) Split luciferase results for XopN with NbVOZ1 or NbVOZ2. A combination of GUS-LucN and LucC-GUS was used as the negative control; a combination of RAR1-LucN and LucC-SGT1 was used as the positive control. (C) Co-immunoprecipitation of XopN fused with a 3×Flag tag and NbVOZ1 or NbVOZ2 fused with a HA tag in N. benthamiana leaves. The proteins pulled down by anti-3Flag beads were detected using western blotting with a-HA-HRP primary antibody. Two Western blots of the input protein extract (prior to precipitation) are shown in the middle and bottom. (D) Semi-quantitative evaluation of the impact of NbVOZ1 and NbVOZ2 on the activity of NbNPR1 promoter fused with a GUS reporter. The co-inoculation of Agrobacteria carrying NbNPR1 promoter:GUS along with Agrobacteria carrying NbVOZ1, NbVOZ2, or GFP with a concentration of OD600 at 0.4 (0.2 + 0.2). The staining was conducted 2 days post-inoculation and the picture was taken after destaining.
Figure S3- 1 Transient expression of XopN can trigger water-soaking cell death only localized at cytosol.

(A) Agrobacteria strain GV2260 carrying 35S:XopN-GFP and 35S:XopN-NLS-GFP (Nuclear Localization Signal, NLS) at OD600 0.4 were inoculated to N. benthamiana. The fluorescent signals of 35S:XopN-GFP and 35S:XopN-NLS-GFP displayed their cellular localizations as shown respectively in (B) and (C). The image of the leaf in (A) was taken at 48 hours post-inoculation simultaneously with the observation of fluorescent signals. Experiments were repeated three times with similar results.
Figure S3- 2 The localization of XopN can be changed upon co-expression with NbVOZ1 and NbVOZ2.

Co-expression of 3 sets of proteins including 35S:XopN-GFP with empty vector (A), 35S:XopN-GFP with 35S:NbVOZ1-3Flag (B), and 35S:XopN-GFP with 35S:NbVOZ2-3Flag (C) at OD600 0.8 (0.4 + 0.4) were inoculated to N. benthamiana. The fluorescent signals were observed at 48 hours post-inoculation. Experiments were repeated three times with similar results.
Figure S3- 3 Type 2 secretion system (T2SS) and glycerol uptake facilitator (glpF) are also involved in instigating water-soaking symptoms.

(A) Four Xe isogenic strains, including $Xe\triangle XopQ$, $Xe\triangle T2SS\triangle XopQ$, $Xe\triangle XopQ\triangle XopN$ and $Xe\triangle T2SS\triangle XopQ\triangle XopN$ within a concentration OD600 at 0.4 were inoculated to wild-type *N. benthamiana*. (B) Four Xe isogenic strains including $Xe\triangle XopQ$, $Xe\triangle glpF \triangle XopQ$, $Xe\triangle XopQ\triangle T2SS$ and $Xe\triangle T2SS\triangle XopQ\triangle glpF$ within a concentration OD600 at 0.4 were inoculated to wild-type *N. benthamiana*. Pictures were obtained at 2 days post-inoculation. Experiments were repeated three times with similar results.
Table S3- 1 Sequences of primers utilized in this chapter

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References


35. Koguchi, M., et al., *Vascular plant one-zinc-finger protein 2* is localized both to the nucleus


Chapter 4 *Xanthomonas euvesicatoria* Gene Xe4429 Encodes an Antitoxin and also Functions as a Transcription Repressor

**Summary**

*Xanthomonas euvesicatoria* (*Xe*) is the causal agent of bacterial spot (BS) disease in pepper and tomato. We identified a *Xe* type three-secretion effector (T3E), Xe4428, which is a homologue of *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) T3E AvrRxo1 that functions as a NAD kinase. Xe4428 is required for the full virulence of *Xe* to infect pepper plants. Overexpression of AvrRxo1 or Xe4428 alone in bacterial or plant cells is toxic. We previously demonstrated that AvrRxo1-ORF2 functions as an antitoxin capable of binding to AvrRxo1 to suppress its toxicity. However, the detailed biochemical and biological functions of AvrRxo1-ORF2 remain unclear. In this study, we identified Xe4429 as the homologue of AvrRxo1-ORF2, which interacts with Xe4428 to suppress its toxicity in *Xe* bacterial cells. We also revealed that Xe4429 could bind to the promoter region of *Xe4428* and regulates its transcription. Therefore, we conclude that Xe4429 functions as an antitoxin and a transcription repressor in the *Xe* bacterial cells.

**Introduction**

Rice bacterial leaf streak (BLS) disease, caused by *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) is one of the most important bacterial diseases in rice fields. Thus far, no major resistance genes have ever been identified from any rice germplasm. A maize resistant gene *Rxo1* was isolated that can specifically recognize an effector gene *avrRxo1* to trigger disease resistance to BLS in transgenic rice plants [1-3]. The *Rxo1* gene encodes a typical NB-LRR type disease resistant protein, while the *avrRxo1* gene encodes a type III effector (T3E) protein [3]. In the genome of *Xoc*, *avrRxo1-orf1* is adjacent to another gene, *avrRxo1-orf2*, which encodes an atypical molecular chaperone of AvrRxo1-ORF1 [4, 5]. Overexpression of AvrRxo1-ORF1 in yeast, *E. coli*, and *N. benthamiana* plant cells is toxic, where the toxicity can be blocked by
the co-expression of AvrRxo1-ORF2 [4]. AvrRxo1-ORF1 and ORF2 can be co-crystallized, which showed AvrRxo1-ORF2 physically binds to AvrRxo1-ORF1, thereby serving as an antitoxin to eliminate the toxicity of AvrRxo1-ORF1 [4]. AvrRxo1-ORF1 has an ATP binding domain and a kinase domain, which can phosphorylate NAD, a central metabolite and signaling molecule in biosystems, to form 3’-NADP in both bacterial and yeast cells [5]. Agrobacterium-mediated transient expression of AvrRxo1 in N. benthamiana or infected rice leaves with Xoc carrying avrRxo1 could trigger the accumulation of 3’-NADP, implying that AvrRxo1 is capable of manipulating the primary metabolic pathway in planta.

A point mutation on aspartic acid D193A in putative substrate-binding site of AvrRxo1 could inhibit its toxicity to bacterial, yeast, and plant cells, suppress its ability to trigger Rxo1-mediated disease resistance, and impair the capacity of suppression of the flg22-triggered ROS accumulation [5]. Another point mutation on the threonine T167N in the ATP-binding motif could also suppress the toxicity of AvrRxo1, but not completely abolish its NAD enzyme activity and the full virulence function [5-7]. An AvrRxo1 homologue, Xe4428, was identified in the genome of Xanthomonas euvesicatoria (Xe), which is the causal agent of bacterial spot (BS) disease in pepper plants. Xe4428 is adjacent to Xe4429, which is a homologue of Xoc-AvrRxo1-orf2. However, the detailed biological and biochemical functions of Xe4428 and Xe4429 has never been characterized.

In this study, we confirmed that Xe4428 is a functional homologue of AvrRxo1, which can trigger the Rxo1-mediated defense responses in N. benthamiana. Therefore, we re-named Xe4428 as Xe-AvrRxo1. Xe-AvrRxo1 also has a virulence function that can promote the Xe bacterial proliferation on both pepper and N. benthamiana plants. We then revealed that Xe4429 encodes an antitoxin that can suppress the toxicity of Xe-AvrRxo1. In addition, we identified that Xe4429 can bind to the promoter region of Xe-AvrRxo1 to suppress its expression in Xe bacterial cells. Hence, Xe4429 functions as an antitoxin and a bacterial transcription repressor.
Results and discussions

*Xe4428 is a homologue of Xoc-avrRxo1 that can trigger the Rxo1-mediated defense response in N. benthamiana plants*

Xoc-avrRxo1 has been previously shown to be able to trigger the Rxo1-mediated disease resistance in rice and *N. benthamiana* plants. Here, we investigated whether Rxo1 could also recognize *Xe*-avrRxo1 to trigger a defense response in *N. benthamiana* plants. As shown in Figure 4-1A and Figure S4-1, *Agrobacterium*-mediated transient expression of Rxo1 and *Xe*-avrRxo1 triggered strong cell death in *N. benthamiana*, tomato and pepper leaves. As a control, the mutant of Rxo1, Rxo1-D291E, and the mutant of *Xe*-avrRxo1, *Xe*-avrRxo1-D193T, failed to trigger cell death in *N. benthamiana*, tomato, and pepper leaves (Figure 4-1A and Figure S4-1). The inoculated *N. benthamiana* leaf was stained with DAB to detect the accumulation of H$_2$O$_2$. As shown in Figure 4-1B, the co-expression of *Rxo1* and *Xe*-avrRxo1 resulted in an increased accumulation of H$_2$O$_2$.

We also monitored the expression of defense-related genes by RT-qPCR. As shown in Figure 4-1C, the expression level of Nb*NPR1* and Nb*PR4* with the co-infiltration of *Rxo1* and *Xe*-avrRxo1 increased dramatically, while the expression of Nb*PR10* is significantly downregulated in comparison with the controls. Therefore, we confirmed that *Xe*-avrRxo1 is a functional homologue of Xoc-AvrRxo1 that can trigger Rxo1-mediated defense response in *N. benthamiana* plants, which may involve the salicylic acid-associated signaling pathway.

*Xe4428 possesses a significant virulence function that can enhance Xe proliferation via inducing stomatal opening in both pepper and tobacco plants*

Our previous result showed the deletion of *Xe* effector XopQ would alter the host specificity of *Xe*, which allows it to infect *N. benthamiana* plants. In order to characterize the virulence function of *Xe*-avrRxo1, we generated isogenic *Xe* mutant strains that carry a deletion of XopQ alone or the double deletion of both *Xe-XopQ* and *Xe-avrRxo1*. The isogenic *Xe* strains
(XeΔXopQ and XeΔXopQΔXe-avrRxo1) were able to infect *N. benthamiana*, pepper, and tomato plants. However, inoculated XeΔXopQΔXe-avrRxo1 strain grows to significantly lower population levels than that of XeΔXopQ on both *N. benthamiana* and pepper plants (Figure 4-2). Therefore, we conclude that Xe-avrRxo1 possesses a significant virulence function that can promote bacterial proliferation on the inoculated plants.

Our previous result suggests that the expression of Xoc-avrRxo1 could increase the stomatal aperture in Arabidopsis. Therefore, we attempted to test whether Xe-avrRxo1 also has a similar role in regulating stomatal aperture. In this study, we choose to measure the stomatal conductance of pepper leaves infiltrated by wild-type Xe and mutant strain XeΔavrRxo1 using a portable photosynthesis system (LI-COR 6400XT). As illustrated in Figure 4-3, upon infection with XeΔavrRxo1, the pepper leaves have reduced CO₂ conductance in stomatal cells in comparison to the pepper leaves inoculated with wild type Xe strain. This result indicates that pepper leaves inoculated with wild type Xe might have increased stomata aperture that resulted in a higher rate of CO₂ conductance than that of the mutant XeΔavrRxo1 strain. Therefore, we speculate that Xe-avrRxo1 also has the role of increasing the stomatal aperture, which still needs to be further validated by directly observing the stomatal aperture size under a microscope.

*Expression of Xe-avrRxo1 is toxic to Xe and E. coli, and the toxicity can be blocked by the co-expression of Xe4429*

To confirm if Xe-avrRxo1 (designated as Xe-avrRxo1 ORF1) is toxic to bacterial cells, and if Xe4429 (designated as Xe-avrRxo1-ORF2) can block the toxicity of Xe4428, we employed an arabinosis-inducible expression system to express wild type Xe-avrRxo1 or mutant Xe-avrRxo1-D193T with or without the Xe4429 (Xe-avrRxo1-ORF2) in *E. coli* and *Xe* cells. As shown in Figure 4-4A, the expression of Xe-avrRxo1 but not Xe-avrRxo1-D193T, or Xe-avrRxo1 plus Xe4429 (Xe-avrRxo1-ORF2) could inhibit the bacterial growth. As the control, when the bacterial strains were grown in LB medium without the supplement of arabinose, all
bacterial strains, except the strain carrying \( Xe\text{-avrRxo1} \) alone, have a similar growth rate. We speculate that a leaking expression of \( Xe\text{-avrRxo1} \) might reduce the bacterial growth rate. Nevertheless, we conclude that \( Xe\text{-avrRxo1} \) also possesses toxicity to \( E.\ coli \), and the co-expression with \( Xe4429 \) (\( Xe\text{-avrRxo1-ORF2} \)) could block its toxicity. The point mutation in the putative substrate-binding site D193T also abolished the toxicity of \( Xe\text{-avrRxo1} \).

The same set of expression constructs were also transformed into a mutant \( Xe \) strain where both \( Xe\text{-avrRxo1} \) and \( Xe4429 \) (\( Xe\text{ΔavrRxo1ΔXe4429} \)) were deleted. The transformed \( Xe \) strains were used for a growth curve assay. As shown in Figure 4-4B, inducible expression of wild type \( Xe\text{-avrRxo1} \), but not the \( Xe\text{ avrRxo1-D193T} \), could strongly inhibit the bacterial cell proliferation. Interestingly, the co-expression of \( Xe\text{-avrRxo1} \) with \( Xe4429 \) (\( Xe\text{-avrRxo1-ORF2} \)) still had a much slower growth rate than the other controls (Figure 4-4B). We speculate that the expressed \( Xe\text{-AvrRxo1} \) protein might not completely bind with \( Xe4429 \) (\( Xe\text{ avrRxo1-ORF2} \)).

We originally cloned the open reading frames of \( Xe\text{-avrRxo1} \) and \( Xe4429 \), each in a single DNA fragment. The DNA fragment containing two gene open reading frames was subcloned behind the arabinose inducible promoter. Therefore, we speculate that \( Xe\text{-avrRxo1} \) has a higher expression rate than that of \( Xe4429 \), resulting in more \( Xe\text{-AvrRxo1} \) proteins than \( Xe4429 \) proteins. The free \( Xe\text{-avrRxo1} \) proteins without binding of \( Xe4428 \) would be toxic to \( Xe \) cells. However, if we know the wild type \( Xe \) strain also expresses \( Xe\text{ avrRxo1} \) and \( Xe4429 \) from a single operon, where \( Xe\text{-avrRxo1} \) is located in front of \( Xe4429 \), then why we did not see \( Xe\text{-avrRxo1} \) have any toxicity to the wild type \( Xe \) cells? Previous reports about the toxin-antitoxin pairs suggest that some antitoxins also function as the transcriptional repressor, where it can bind to the promoter sequence of the toxin gene [8, 9].

To test if \( Xe4429 \) (\( Xe\text{-avrRxo1-ORF2} \)) also has a similar function, we amplified out the \( Xe \) genomic DNA fragment that contains the native promoter and the open reading frames of \( Xe\text{-avrRxo1} \) and \( Xe4429 \). The DNA fragment was cloned to a broad-host spectrum vector pVSP61 [10] to generate a complementary construct p\( Xe\text{ avrRxo1-ORF1:ORF2} \). We also performed site-directed mutagenesis to introduce a point mutation D193T into p\( Xe\text{ avrRxo1-ORF1:ORF2} \),
which resulted in pXe-avrRxo1-ORF1-D193T:ORF2. The two complementary constructs were transformed into mutant XeΔavrRxo1ΔXe4429 strain. The wild type Xe, XeΔavrRxo1ΔXe4429, XeΔavrRxo1ΔXe4429 (pXe avrRxo1-ORF1:ORF2), and XeΔavrRxo1ΔXe4429 (pXe avrRxo1-ORF1-D193T:ORF2) strains were used for growth curve assay. As shown in Figure 4-4C, all strains have an almost identical growth rate. As a control, we also confirmed that the arabinose supplement did not alter the growth rate of these Xe strains. Therefore, we conclude that when Xe-avrRxo1 and Xe4429 (Xe-avrRxo1 ORF2) are co-expressed by the native promoter in Xe, the toxicity of Xe-avrRxo1 could be completely blocked by Xe4429.

**Purified ORF2 (Xe4429) protein binds to the promoter of Xe4428 in electrophoretic mobility shift assay**

As previously described, we speculate that Xe4429 could bind to the promoter region of Xe-avrRxo1 and repress the transcription of Xe-avrRxo1, which would result in a tight regulation of Xe-avrRxo1. To test this hypothesis, we purified either Xe4429 protein alone or Xe-AvrRxo1:Xe4429 protein complex for an electrophoretic mobility shift assay (EMSA). The purified proteins were incubated with a DNA fragment containing the promoter of Xe-avrRxo1 before they were separated in agarose gel. As shown in Figure 4-5, the mobility of the DNA fragment containing the Xe-avrRxo1 promoter region altered in the DNA electrophoresis gel when it was mixed with purified Xe4429 (Xe-avrRxo1-ORF2) recombinant protein. The shifting DNA band could be inhibited by supplementing large amounts of unlabeled competitor DNA that contains the promoter of Xe-avrRxo1. In contrast, Xe-avrRxo1: Xe4429 protein complex did not alter the mobility of the DNA fragment. Therefore, our EMSA suggests Xe4429, which is the homologue of Xoc-avrRxo1-ORF2 binds to the promoter region of Xe-avrRxo1 in vitro.

*Xe4429 functions as a transcriptional repressor in Xe cells that were grown on nutrient broth medium but not in the plant leaf tissue*
Since Xe4429 is able to bind to the promoter of \(Xe-avrRxo1\) (Figure 4-5), we also attempt to investigate if Xe4429 can regulate the expression of \(Xe-avrRxo1\) in \(Xe\) bacterial cells. To this end, we first cloned the native promoter of \(Xe-avrRxo1\) and fused it with the Nanoluc gene [11]. This DNA fragment was further cloned into a pBMTBX vector that carries either Xe4429 alone, or \(Xe-avrRxo1: Xe4429\), or a GFP gene as a control. As a control, we also cloned the Nanoluc gene into the pBMTBX vector. Detailed schemes of tested constructs are illustrated in Figure 4-5A. These expression constructs were transformed into the mutant \(Xe\Delta Xe-avrRxo1\Delta Xe4429\) strain, and grown on the medium supplemented with or without 0.4 % arabinose (expression inducer). As shown in Figure 4-5B (panel 2), the expression of Xe4429 repressed the expression of \(Xe-avrRxo1\) promoter:Nanoluc. In contrast, the expression of GFP could not repress the expression of \(Xe-avrRxo1\) promoter:Nanoluc (Figure 4-5 (panel 4)). Interestingly, the expression of \(Xe-avrRxo1: Xe4429\) complex also repressed the expression of \(Xe-avrRxo1\) promoter:Nanoluc (Figure 4-5 (panel 3)). This result is inconsistent with the EMSA result shown in Figure 4-4, where the \(Xe-avrRxo1: Xe4429\) complex could not bind to the promoter of \(Xe-avrRxo1\). We speculate that in the \(Xe\) cells, the \(Xe-avrRxo1: Xe4429\) complex may have association and dissociation dynamics allowing some free Xe4429 that could bind to the promoter region of \(Xe-avRxo1\). An alternative explanation is that the association and dissociation dynamics also result in free \(Xe-AvrRxo1\) that could be toxic to \(Xe\) bacterial cells, and therefore, suppress the expression of Nanoluc. This possibility is indeed consistent with the growth curve data shown in Figure 4-4B, where the co-expression \(Xe-avrRxo1\) and Xe4429 controlled by the same arabinose inducible promoter still has some toxicity to \(Xe\) bacterial cells.

We also investigated if Xe4429 could repress the expression \(Xe-avrRxo1\) when the \(Xe\) bacterial cells were grown in plant tissues. The \(Xe\) strains, as listed in Figure 4-5A, were infiltrated into pepper leaves, supplemented with either arabinose (expression inducer) or glucose (non-inducer as the control). Strong Nanoluc enzyme activities were observed in all \(Xe\) strains (Figure 4-6). Therefore, when the \(Xe\) bacterial cells were grown in the pepper leaf tissues, Xe4429 could not repress the expression of \(Xe-avrRxo1\).
To summarize, in this study, we characterized the biological and biochemical functions of $Xe$-avrRxo1 and Xe4429. Our results revealed that $Xe$-avrRxo1 is a functional homologue of $Xoc$-AvrRxo1, while Xe4429 encodes an antitoxin and also functions as a bacterial transcription repressor. Therefore, Xe4429 can regulate the toxicity of Xe-avrRxo1 by direct protein-protein interaction, and also by protein-DNA interaction to suppress the transcription of Xe-avrRxo1.

**Methods and materials**

*Plant material and growth conditions*

Seeds from *N.benthamiana*, pepper (*Capsicum annuum*, Ca), and tomato (*Solanum lycopersicum*, Sl, cultivar: Rio Grande) were germinated in soil at room temperature under 12h/12h light/dark cycle at 25°C/20°C. After germination, plants were grown under 14h/10h light/dark cycle at 25°C/20°C. The 6-week-old tobacco, pepper, and tomato plants were used for the experiments detailed herein.

*Bacterial growth*

*Escherichia coli* (*E. coli*) strains DH5α and Rho5 were grown on Luria agar medium at 37 °C. *Agrobacterium tumefaciens* (*A. tumefaciens*) GV2260 strain and Xe were grown on Luria agar medium and NYGA medium at 28 °C, respectively. *E. coli* and Rho5 antibiotic selections used in this study were as follows: 50 μg/ml kanamycin, 100 μg/ml carbenicillin, 100 μg/ml spectinomycin, chloramphenicol 34 μg/ml, and 50 μg/ml gentamycin. Xe and *A. tumefaciens* antibiotic selection were 100 μg/ml rifampicin, and/or 50 μg/ml kanamycin.

*Gene cloning, site-directed mutagenesis, plasmid construction, and Agrobacterium-mediated transient assay*

Two ORFs of Xe-avrRxo1 and its corresponding resistant gene, Rxo1, were ultimately cloned to the pEarley101 vector with a 35S promoter using identical procedures for each. The Xe-avrRxo1 was amplified from the genomic DNA of Xe using primers “Xe-avrRxo1 ORF For”
and “Xe-avrRxo1 ORF Rev,” while Rxo1 was amplified from the genomic DNA of maize using primers “Rxo1 ORF For” and “Rxo1 ORF Rev,” which were both subsequently cloned into the pDonr207 vector (donor vector) using BP Gateway cloning (Invitrogen). The sequences of the other primers used in this study are provided in Supplementary Table 4-1. The donor vectors noted above were subcloned to the pEarley101 vector in GV2260 using modified LR Gateway cloning kit as previously described [12].

The vector of pDonr207-Xe-avrRxo1-ORF was used to generate its mutant, Xe-avrRxo1-D193T, using primers “Xe-avrRxo1-ORF-D193T For” and “Xe-avrRxo1-ORF-D193T Rev”; a similar approach was used for the mutant Rxo1-D291E. The various primer sequences are listed in Table S4-1. The vectors of pDonr207-Xe-avrRxo1-ORF-D193T and pDonr207-Rxo1-ORF-D291E were subcloned to the pEarley101 vector in GV2260 using a modified LR Gateway cloning kit as noted above. Using blunt-end syringes, the leaf mesophyll tissues were infiltrated by Agrobacterium strains harboring different constructs within the concentration of OD600 0.4 [13].

RNA isolation, RT-PCR and real-time PCR

For RT-PCR, the total RNA was extracted from N.benthamiana leaves using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Any DNA residue was eliminated by treating with UltraPure DNase I (Invitrogen). The integrity and quantity of total RNA were determined by electrophoresis in 1% agarose gel and a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA synthesis was performed using the SuperScript III First-Strand RT-PCR Kit (Invitrogen) with an oligo-dT primer based on the manufacturer’s instructions. Real-time PCR was conducted with cDNA 20 times diluted and specific primers (Supplemental Table 4-1) using the Quantitect SYBR Green PCR kit (Qiagen) according to the manufacturer’s protocols.

Reactive oxygen species (ROS) detection
The reactive oxygen species were visualized in leaf tissues by staining with DAB (Sigma-Aldrich), as described previously [14, 15]. *N.benthamiana* leaves were detached and vacuum-infiltrated with the DAB solution. The leaf samples were then placed in a petri dish with the DAB solution for 4 hours at room temperature. Afterwards, the leaves were fixed with a solution containing 60% [vol/vol] ethanol, 20% [vol/vol] lactic acid, and 20% [vol/vol] glycerol. The fixed leaves were decolorized in a 2.5 g chloral hydrate per milliliter water solution overnight and were visualized using a white-light microscope.

**Bacterial growth curve assay**

Bacterial proliferation on the inoculated *N.benthamiana, C.anuum, and S.lycopersicum* plants was assessed via standard growth curve assays [16, 17]. During the spray inoculation, the bacterial strains were cultivated on their respective medium plate at 28°C for two days, after which the bacterial cells were collected and suspended in 10 mM MgCl₂ with 0.02% Silwet L77 and diluted to OD600 at 0.2. The plants were covered and maintained at 100% moisture levels for one day prior to inoculation. The bacterial inoculum was sprayed on the plant leaves using a spray bottle. The inoculated plants were covered, sealed, and maintained under 100% moisture overnight, and then cultivated under 14 h light/10 h dark at room temperature for an additional 5 days. Leaf discs (990 mm²) were randomly sampled from inoculated leaves on the fifth day for growth curve assay. The sampled leaf discs were ground in 588 μL of 10 mM MgCl₂, and vortexed evenly prior to dilution and plating on respective medium supplemented with proper antibiotics. The plates were cultivated at 28°C until the bacteria colonies could be counted; these numbers were then used to calculate the bacteria proliferation ratio (Log_{10} CFU/cm²). All growth curves underwent three biological repeats with technical replicates.

**Monitoring the growth rates of E. coli and Xe strains expressing AvrRxo1 in enriched medium**

The *Xe-AvrRxo1-ORF1, Xe-AvrRxo1-ORF1:ORF2, Xe-AvrRxo1-ORF1-D193T, and nanoluc (negative control)* were cloned into the Gateway-compatible vector, pBMTBX (from Addgene).
and expressed in *E. coli* strain C41. Strains were grown at 37°C in LB liquid medium supplemented with 100 μg/ml ampicillin. The bacterial culture was diluted to OD600 0.01, and the expression of the protein was induced by adding 0.4 mM arabinose. Bacterial cultures with a mock inducer, glucose, served as the control. Four *Xe* strains, (a) wild-type *Xe*, (b) *XeΔavrRxo1*, (c) *XeΔavrRxo1 (pavrRxo1-wild type)*, and (d) *XeΔavrRxo1 (pavrRxo1-D193T)* were also grown in a NYG medium for evaluating their toxicity to *Xe*. The bacterial growth was monitored by a spectrometer.

**Cloning, expression, and purification of AvrRxo1-ORF1:ORF2 and avrRxo1-ORF2**

DNA fragments containing AvrRxo1-ORF1:ORF2 and AvrRxo1-ORF2 genes were amplified from the genomic DNA of *Xe* and cloned into a pENTR-D-TOPO vector (Invitrogen, Carlsbad, CA) [1]. They were then subcloned into a modified gateway compatible pGEX4T-1 destination vector via LR cloning (Invitrogen). A TEV protease cleavage site was introduced between the GST and the targeted protein for removal of the GST tag. The plasmids were transformed into *E. coli* C41 cells (Lucigen, Middleton, WI) and grown overnight in 5 ml LB medium containing 100 mg/L ampicillin at 37°C. The culture was transferred into 1 liter LB medium containing ampicillin to reach a concentration of OD600 at 0.6. Then, 1 mM Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to the bacterial culture and then incubated at 220 rpm at 28°C for another 8 hours. The bacterial cells were harvested and lysed by incubating with 1 μg/ml lysozyme on ice for 30 min, followed by sonication on ice. The lysate was centrifuged at 13,000 g for 15 min at 4°C and the supernatant was collected, which was then used for subsequent purification. Glutathione Sepharose 4B (GenScript, Piscataway, NJ) affinity resin and Q-sepharose ion-exchange column were used to purify the proteins of AvrRxo1-ORF1:ORF2 and AvrRxo1-ORF2. The purity of the proteins was evaluated by 12% SDS–PAGE. The protein concentration was determined by a protein assay kit (Bio-Rad) using bovine serum albumin as the standard [4].
Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay (EMSA) was performed as previously reported [18, 19]. The Xe-avrRxo1 promoter DNA was amplified from the pDonr207-Xe-avrRxo1 using FAM labeled “M13For” and “M13Rev” primers. The unlabeled competitor DNA of the Xe-avrRxo1 promoter was amplified with regular M13 For/Rev primers. In brief, the FAM-labeled DNA fragment of the Xe-avrRxo1 promoter was mixed with both avrRxo1-ORF1:ORF2 and avrRxo1-ORF2 protein, along with different amounts of the unlabeled competitor DNA fragment in a reaction buffer (10 mM Tris, 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, 5 %v/v glycerol, 0.01 mg/mL BSA, pH 7.5) at room temperature for 20 min. The reactions were processed and separated on 1% agarose gel in the TAE buffer. The fluorescence signal was captured by a gel scanner, Typhoon FLA7000 equipped with a 635 nm laser filter (GE Healthcare, Piscataway, NJ).

Xe-avrRxo1 ORF2 transcription regulating trial

Based on the XeΔavrRxo1 mutant background, an additional five Xe strains were generated. For this phase of the investigation, a pVSP61 backbone carrying a constitutive expression promoter served as the positive control; in contrast, pBMTBX-Nanoluc served as the blank or negative control [11]. The avrRxo1-ORF2, avrRxo1-ORF1:ORF2, and GFP were cloned to the same vector with the avrRxo1 promoter expressing Nanoluc. Accordingly, five constructs were then transformed to XeΔavrRxo1: (a) pBMTBX-Nanoluc, (b) pBMTBX-Xe4428-ORF2-Xe4428pro-Nanoluc, (c) pVSP61-Xe4428pro-Nanoluc, (d) pBMTBX-Xe4428-ORF1:ORF2-Xe4428pro-Nanoluc, and (e) pBMTBX-GFP-Xe4428pro-Nanoluc. The bacteria were cultured on the LB medium with the antibiotic and 0.4 mM arabinose. Note that bacterial cultures grown on a medium supplemented with 0.4 mM glucose served as controls. All bacteria were collected after incubation for 48 hours at 28°C. The bacteria was adjusted to OD600 0.1 with 10 mM MgCl₂, for following Nanoluc reporter assay in vitro as well as infiltration to pepper leaves. Additionally, 1 mM substrate was added to the bacterial cultures, or infiltrated to pepper leaves.
After incubation for 10 mins, chemical fluorescence signals were detected using the CCD camera of a Gel DocTM XR+ System (Bio-Rad).

**Stomatal conductance**

A portable photosynthesis system LI-6400T (Li-Cor Inc., USA) with a 6400-02B light source (blue and red diode) was utilized to measure the following photosynthetic gas exchange parameters: net photosynthetic rate \( (P_n) \), transpiration rate \( (E) \), stomatal conductance \( (G_s) \), and the intercellular \( \text{CO}_2 \) concentration \( (C_i) \) of *pepper* leaves infiltrated by \( Xe \) and \( Xe\triangle avocado \) *in vivo* as reported \cite{20}. \( G_s \) was used for further analysis. Measurements were obtained under an artificial irradiance of 1000 \( \mu \text{mol} \) (photons) \( \text{m}^{-2} \) \( \text{s}^{-1} \) at 25°C using a completely expanded leaf in the same position from each plant. The \( \text{CO}_2 \) concentration and ambient water vapor pressure were set at 385 \( \mu \text{mol} \) mol\(^{-1} \) and \( 1.30 \pm 0.15 \) kPa, respectively.
Xe4428 is a homologue of Xoc-avrRx1 that can trigger the Rxo1-mediated defense response in *N. benthamiana*.

(A) Transient assay in *N. benthamiana* leaf by *Agrobacteria*. The *agrobacteria* harboring the following constructs were used as inoculants: MgCl₂, 35S:Rxo1, 35S:Xe-avrRx1, a combination of 35S:Rxo1 and 35S:avrRx1, 35S:Xe-avrRx1-D193T, a combination of 35S:Rxo1 and 35S:Xe-avrRx1-D193T, 35S:Rxo1-D291E, and a combination of 35S:Rxo1-D291E and 35S:Xe-avrRx1 within OD600 0.4 (single inoculation) or OD600 0.4 + 0.4 (co-inoculation). Picture was obtained 2 days post-inoculation. Experiments were replicated three times within similar results. (B) DAB staining of *N. benthamiana* leaf from (A). (C) The relative expression levels of *NbNPR1, NbPR4* and *NbPR10* in *N. benthamiana* leaves infected as above for 24 hours. Experiments were repeated three times with similar results and data are shown as mean ± s.e.
Figure 4- 2 Xe4428 possesses a remarkable virulence function that can enhance X. euvesicatoria proliferation on both pepper and tobacco.

In planta growth of XeΔXopQ, XeΔXopQΔavrRxo1, XeΔXopQΔavrRxo1 (pavrRxo1-D193T), and XeΔXopQΔavrRxo1 (pavrRxo1-wild type) measured at Day 6 after infiltration in leaves of pepper (A and B) and N. benthamiana (C, D and E). The concentration of the starting inoculum of Xe strains was 1 × 10⁵ cfu ml⁻¹. Visualized disease symptom phenotypes infected by Xe strains are shown in (A) pepper and (C) N. benthamiana. (D) DAB staining of N. benthamiana leaves from (C). Experiments were repeated three times with similar results and data are shown as mean ± s.e.
Figure 4- 3 Expression of Xe-avrRxo1 in Xe could increase the stomatal conductance in pepper leaves.

Stomatal conductance of pepper leaves was measured by using a portable photosynthesis system (LI-COR 6400) at two days post inoculation with the Xe isogenic strains, Xe and XeΔavrRxo1, respectively. The measurements were conducted at two days post-inoculation, and the starting inoculum concentration was adjusted to $1 \times 10^5$ cfu ml$^{-1}$. Experiments were replicated three times with similar results, and data are shown as mean ± s.e.
Figure 4- 4 Expression of *AvrRxo1-ORF1* (*Xe4428*) is toxic to *Xe* and *E. coli*, and its toxicity can be suppressed by *AvrRxo1-ORF2* (*Xe4429*).

Growth of four *E. coli* strains (A) and four *XeΔavrRxo1* strains (B) harboring pBMTBX-*Xe* avrRxo1-ORF1, pBMTBX-*Xe* avrRxo1-ORF1-ORF2, pBMTBX-*Xe* avrRxo1-ORF1-D193T, and pBMTBX-Nanoluc, respectively, with and without 0.4% arabinose (inducer) at 37°C and 28°C. Growth of four *Xe* strains (C) including wild type *Xe*, *XeΔavrRxo1*, *XeΔavrRxo1* (*pavrRxo1-D193T*), and *Xe ΔavrRxo1* (*pavrRxo1-wild type*) with and without 0.4% arabinose (the expression inducer) at 28°C. Experiments were replicated three times with similar results and data are shown as mean ± s.e.
Figure 4- 5 Purified Xe-avrRxo1-ORF2 (Xe4429) protein binds to the promoter of Xe-avrRxo1 (Xe4428).

Labelled Xe-avrRxo1 promoter DNA and unlabeled Xe-avrRxo1 promoter DNA combined with purified proteins, Xe-avrRxo1-ORF1-ORF2 and Xe-avrRxo1-ORF2, respectively, were processed via an electrophoretic mobility shift assay. Experiments were repeated three times with similar results.

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Experiments were repeated three times with similar results.
Figure 4-6 Nanoluc reporter assay to test the transcription activity driven by Xe-avrRxo1 promoter in vitro.

1. \textit{Xe}^{avrRxo1-ORF1:ORF2}
2. \textit{Xe}^{avrRxo1-ORF1:ORF2} (pBMTBX-Xcv4428-ORF2-Xcv4428pro-Nanoluc)
3. \textit{Xe}^{avrRxo1-ORF1:ORF2} (pBMTBX-Xcv4428-ORF1-ORF2-Xcv4428pro-Nanoluc)
4. \textit{Xe}^{avrRxo1-ORF1:ORF2} (pBMTBX-GFP-Xcv4428pro-Nanoluc)
5. \textit{Xe}^{avrRxo1-ORF1:ORF2} (pBMTBX-Nanoluc)

(A) Scheme of the different constructs used for the nanoluc activity assay. (B) The following six \textit{Xe} strains were grown on a nutrient broth medium containing arabinose or glucose (the control): 1) \textit{Xe}^{avrRxo1}, 2) \textit{Xe}^{avrRxo1} (pBMTBX-avrRxo1-ORF2-avrRxo1pro-Nanoluc),
3) $Xe\Delta avrRxo1$ ($pBMTBX-avrRxo1$-ORF1-ORF2-$avrRxo1$pro-Nanoluc), 4) $Xe\Delta avrRxo1$ ($pVSP61$-$avrRxo1$pro-Nanoluc), 5) $Xe\Delta avrRxo1$ ($pBMTBX$-Nanoluc), and 6) $Xe\Delta avrRxo1$ ($pBMTBX$-GFP-$avrRxo1$pro-Nanoluc). Experiments were repeated three times with similar results.
1. *Xe*\(^{avrRxo1}\)
2. *Xe*\(^{avrRxo1}\) (pBMTBX-avrRxo1-ORF2-avrRxo1pro-Nanoluc)
3. *Xe*\(^{avrRxo1}\) (pBMTBX-avrRxo1-ORF1-ORF2-avrRxo1pro-Nanoluc)
4. *Xe*\(^{avrRxo1}\) (pVSP61-avrRxo1pro-Nanoluc)
5. *Xe*\(^{avrRxo1}\) (pBMTBX-Nanoluc)
6. *Xe*\(^{avrRxo1}\) (pBMTBX-GFP-avrRxo1pro-Nanoluc)

**Figure 4- 7 Nanoluc reporter assay to test the transcription activity driven by *Xe-avrRxo1* promoter *in vivo*.**

Six *Xe* strains (listed above) were infiltrated into pepper leaf with either 0.4% arabinose or 0.4% glucose (the negative control). Chemical fluorescence signals in the pepper leaf were detected using the CCD camera of a Gel DocTM XR+ System (Bio-Rad). Experiments were conducted for three times within similar results.
Supplementary information

Figure S4- 1 Xe4428 is a homologue of Xoc-avrRxo1 that can trigger the Rxo1-mediated defense response in pepper and tomato.

Transient assay in leaves of pepper (*Capsicum annuum*, Ca, cultivar: Early Calwonder) and tomato (*Solanum lycopersicum*, Sl, cultivar: Money Maker) by Agrobacteria. The agrobacteria harboring the following constructs were used as inoculants: MgCl₂, 35S:Rxo1, 35S:Xe-avrRxo1, a combination of 35S:Rxo1 and 35S:avrRxo1, 35S:Xe-avrRxo1-D193T, a combination of 35S:Rxo1 and 35S:Xe-avrRxo1-D193T, 35S:Rxo1-D291E, and a combination of 35S:Rxo1-D291E and 35S:Xe-avrRxo1 with OD600 0.4 (single inoculation) or OD600 0.4 + 0.4 (co-inoculation). Picture was obtained 2 days post-inoculation. Experiments were replicated three times within similar results.
Table S4-1 Sequences of primers utilized in this chapter

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<tr>
<td>Rxo1 For</td>
<td>ATGTATCCGTATGCACGtTCTGGTTCCGAACACTG</td>
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<tr>
<td>Rxo1 Rev</td>
<td>CAGTTCGCAACCAGAACGTCGACATACGGATACAT</td>
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<td>Rxo1_D291E For</td>
<td>TCTGATTATCCCTGGATGAA GTTGGATGGACCAAC</td>
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<td>GTTGCCCATCCACCTTTCTCCAGGATAATCAGA</td>
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<td>Rxo1_D5081 For</td>
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<td>Xe-avrRxo1 upstream SwaI For</td>
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<tr>
<td>Xe-avrRxo1 upstream SwaI Rev</td>
<td>aagctTaatattggatecatttACGAAATTCCTCTCCACCTA</td>
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<tr>
<td>Xe-avrRxo1 upstream Pmel For</td>
<td>aagctTaatattggatecatttACGAAATTCCTCTCCACCTA</td>
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<tr>
<td>Xe-avrRxo1 upstream Pmel Rev</td>
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<tr>
<td>Xe-XopQ upstream SwaI For</td>
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<td>Xe-XopQ upstream SwaI Rev</td>
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<td>Xe-XopQ upstream Pmel For</td>
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<td>Xe-XopQ upstream Pmel Rev</td>
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<td>Xe-avrRxo1-ORF1 For</td>
<td>caccGATCCATGGCATCGCCCGCGCATTTCGTTG</td>
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<td>Xe-avrRxo1-ORF1 Rev</td>
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<td>Xe-avrRxo1-ORF2 For</td>
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<td>AAAGGATCCCTGCAGTTATGACCAAGGAAAGGTGCTCAA</td>
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<td>Xe-avrRxo1-promoter For</td>
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<td>Xe4428_D193T For</td>
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<td>Xe4428_D193T Rev</td>
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References