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Roq1 mediates recognition of the Xanthomonas and Pseudomonas effector proteins XopQ and HopQ1

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SUMMARY

Xanthomonas spp. are phytopathogenic bacteria that can cause disease on a wide variety of plant species resulting in significant impacts on crop yields. Limited genetic resistance is available in most crop species and current control methods are often inadequate, particularly when environmental conditions favor disease. The plant *Nicotiana benthamiana* has been shown to be resistant to Xanthomonas and Pseudomonas due to an immune response triggered by the bacterial effector proteins XopQ and HopQ1, respectively. We used a reverse genetic screen to identify *Recognition of XopQ 1* (Roq1), a nucleotide-binding leucine-rich repeat (NLR) protein with a Toll-like interleukin-1 receptor (TIR) domain, which mediates XopQ recognition in *N. benthamiana*. Roq1 orthologs appear to be present only in the Nicotiana genus. Expression of Roq1 was found to be sufficient for XopQ recognition in both the closely-related *Nicotiana sylvestris* and the distantly-related beet plant (*Beta vulgaris*). Roq1 was found to co-immunoprecipitate with XopQ, suggesting a physical association between the two proteins. Roq1 is able to recognize XopQ alleles from various Xanthomonas species, as well as HopQ1 from Pseudomonas, demonstrating widespread potential application in protecting crop plants from these pathogens.

Keywords: plant immunity, plant pathogens, plant disease resistance, *Nicotiana benthamiana*, Xanthomonas, Pseudomonas, XopQ, HopQ1, NLR, non-host resistance.

INTRODUCTION

Plant pathogens are a significant problem for agriculture, resulting in an estimated 10% decrease in crop yields despite control measures (Oerke, 2005). Utilizing genetic resistance to protect plants is generally preferable over chemical methods, which can be more expensive and pose risks to human or environmental health (Jones *et al.*, 2014; Vincelli, 2016); however, in many cases genetic resistance is not available. The identification of the genetic basis of disease resistance pathways can allow for the creation of resistant crop varieties either through breeding or transgenic approaches (Dangl *et al.*, 2013; Rodriguez-Moreno *et al.*, 2017). As pathogens may evolve to overcome resistance mediated by a single resistance gene, it is desirable to utilize several independent resistance pathways against the same pathogen to confer durable resistance. The identification of resistance genes, particularly those that have broad specificity across many pathogen species, therefore remains a significant focus of molecular plant pathology research.

Plant bacterial pathogens in the genus Xanthomonas contain a Type III Secretion System, which is used to deliver effector proteins into the plant cell (Rossier *et al.*, 1999). These effector proteins can function to suppress the immune system of the plant or to manipulate the metabolism of the host to promote pathogenesis (Gürlebeck *et al.*, 2006; Block *et al.*, 2008; Toruño *et al.*, 2016). While effector proteins are typically beneficial to the pathogen, if the plant contains a perception pathway capable of detecting a particular effector protein a strong immune response can be triggered known as effector-triggered immunity (ETI; Alfano and Collmer, 2004; Chisholm *et al.*, 2006; Jones and Dangl, 2006). An ETI response is often associated with a visible cell death response known as the hypersensitive response. Identification of the genes responsible for specific ETI pathways can allow for engineering disease resistance into susceptible crop varieties.

Many ETI responses in plants are mediated by a member of the nucleotide-binding leucine-rich repeat (NLR) protein family, with the typical plant genome encoding between 100 and 600 of these proteins (Jones *et al.*, 2016). There are two general models for the activation of an NLR protein by an effector (Jones and Dangl, 2006). The first is

through physical interaction between the effector protein and the cognate NLR receptor involving the formation of a protein complex. This mechanism of activation depends on the physical structure of the effector protein but not on its enzymatic activity. Alternatively, NLR recognition can be based on the activity of an effector. In the 'guard hypothesis' (Dangl and Jones, 2001), an NLR protein is activated when it detects perturbations (such as acetylation, cleavage or uridylation) to a specific host factor, typically a protein. Because effector perception in the guard model is dependent on the activity and not the physical structure of the effector protein, this model is generally thought to convey a resistance that is more difficult to overcome without a loss of effector virulence function.

NLR proteins can be divided into groups based on their domain architecture and genetic dependencies required for their function. The two N-terminal domains common on plant NLR proteins are the Toll-like interleukin-1 receptor (TIR) domain on TIR-NLRs (TLRs) and the coiled coil domain on CC-NLRs. All NLR proteins appear to depend on the protein SGT1 for function, whereas the TLRs require a functional EDS1 protein and a subset of the CC-NLRs in the Solanaceae require the NRC proteins (Wiermer *et al.*, 2005; Shirasu, 2009; Wu *et al.*, 2016). These genetic dependencies can be used to help determine which family or clade of genes may be responsible for mediating the perception of a particular effector protein.

The effector protein XopQ from Xanthomonas and the close-homolog HopQ1 from Pseudomonas are widely distributed and highly conserved among various species in these genera. XopQ and HopQ1 have been shown to suppress the immune system of the plant and promote pathogen virulence (Li *et al.*, 2013b; Sinha *et al.*, 2013; Hann *et al.*, 2014; Teper *et al.*, 2014; Gupta *et al.*, 2015). The mechanism by which XopQ suppresses immunity is not fully understood and is complicated by the observation that XopQ recognition in *Nicotiana benthamiana* can suppress visible cell death responses induced by the expression of other pathogen effectors independently from the virulence activity of XopQ (Adlung and Bonas, 2017). XopQ has homology to nucleoside hydrolases and a structural study suggested that it can hydrolyze a molecule with a ribosyl group (Yu *et al.*, 2014), as demonstrated by *in vitro* hydrolase activity on the substrate 4-nitrophyenyl β-D-ribofuranoside (Gupta *et al.*, 2015). A recent study demonstrated that HopQ1 can hydrolyze the cytokinin precursor iP-riboside 5′-monophosphate (iPRMP) *in vitro* and activate cytokinin signaling *in vivo*, suggesting this as the mechanism for immune suppression (Hann *et al.*, 2014). An alternative hypothesis is that XopQ/HopQ1 virulence function occurs through direct targeting of 14-3-3 proteins. Both HopQ1 and XopQ have been shown to interact with 14-3-3 proteins *in vivo* following phosphorylation of a 14-3-3 binding site and that this interaction is important for the virulence function (Li *et al.*, 2013b; Teper *et al.*, 2014). As 14-3-3 proteins have a role in the plant immune system (Oh *et al.*, 2010), it has been proposed that one or more of the 14-3-3s are targets of XopQ/HopQ1 and that the virulence function is achieved through modification, degradation or sequestration of these proteins (Teper *et al.*, 2014).

Both XopQ and HopQ1 are recognized in the plants *N. benthamiana* and *Nicotiana tabacum* and trigger an ETI response (Wei *et al.*, 2007; Ferrante *et al.*, 2009; Schwartz *et al.*, 2015). The recognition of XopQ/HopQ1 in Nicotiana species is not dependent on interaction with host 14-3-3 proteins, suggesting that 14-3-3 proteins are not involved in the recognition pathway (Li *et al.*, 2013b). Perception of XopQ/HopQ1 in *N. tabacum* is independent of the putative active site of the protein (Li *et al.*, 2013a; Adlung and Bonas, 2017). The recognition of XopQ and HopQ1 was recently reported to be dependent on the *EDS1* gene, suggesting a TLR may be involved in the pathway (Adlung *et al.*, 2016). In this study we investigated the perception pathway of XopQ in *N. benthamiana* and identified a TLR protein, subsequently named *Recognition of XopQ 1* (Roq1), required for perception of XopQ/HopQ1.

RESULTS

Generation of stable *Nicotiana benthamiana eds1* mutants

Having a stable knockout of *EDS1* in *N. benthamiana* can aid in determining whether an unknown immune perception pathway may be mediated by a TLR and can also be useful for experiments investigating the mechanism of TLR activation. For these reasons we used CRISPR/CAS9 (Jinek *et al.*, 2012) to induce mutations in the *EDS1* gene in *N. benthamiana.* Three guide RNAs were designed and tested *in vitro* to determine if they could mediate CAS9 cleavage of the target *EDS1* gene. All three guides were active *in vitro*, and two of the guides were active when expressed transiently from a binary plasmid delivered by Agrobacterium into *N. benthamiana* (Figure S1). These constructs were used to create stable *N. benthamiana* transformants. Heterozygous mutants were obtained in the T0 population, which were selfed to allow isolation of homozygous lines. Two independent frameshift deletion alleles, one for each guide sequence, were obtained and named *eds1-1* and *eds1-2* (Figure S1).

Perception of XopQ depends on *EDS1*

The Xanthomonas effector protein XopQ and the close homolog HopQ1 from Pseudomonas have previously been shown to trigger an ETI response in *N. benthamiana*, indicating the presence of an immune perception pathway capable of detecting XopQ. To test if this pathway is dependent on *EDS1*, we performed a growth assay using wild-type *Xanthomonas euvesicatoria* and the XopQ knockout on both wild-type and *eds1* mutant plants. Wild-type

X. euvesicatoria grew approximately 100-fold more on the *eds1* mutant than the wild-type plants, whereas the XopQ knockout grew to similarly high levels on both the wild-type and *eds1* plant (Figure 1). This result is consistent with the findings of Adlung *et al.* (2016). Similar but less pronounced results were observed for *Xanthomonas gardneri*, which grew 10-fold more in the absence of either *EDS1* or XopQ (Figure 1). Associated with increased bacterial growth was the development of necrosis or water soaking at the infiltration site interpreted as disease symptoms (Figure 1).

Development of a fluorescence-based assay for XopQ perception

Unlike some recognized effector proteins that give a strong visible cell death response when transiently expressed, expression of XopQ in wild-type *N. benthamiana* typically gives a mild chlorotic phenotype not well suited for a screen (Figure 2). The disease symptoms observed associated with a loss of XopQ recognition do provide a visible response (Figure 1), but this assay was found to be inconsistent in plants silenced for *EDS1* using viral-induced gene silencing (VIGS), possibly due to only a partial knockdown of the target gene. An alternative assay was designed in which the XopQ protein was transiently expressed along with a Tobacco Mosaic Virus replicon containing a green fluorescent protein (GFP) gene (Marillonnet *et al.*, 2005). In wild-type plants, XopQ perception resulted in immune activation and a lack of visible GFP from the viral replicon (Figure 2). Lack of XopQ perception in the *eds1* mutant allowed for strong GFP fluorescence to be observed using a handheld long-wave UV light. This phenotype was very robust in the *eds1* mutants and consistent, though weaker, in *EDS1* VIGS plants. This assay was employed to conduct a reverse genetic screen of the TLR genes in *N. benthamiana* to identify the immune recognition receptor for XopQ.

Identification of a TLR required for XopQ perception

Viral-induced gene silencing constructs were designed to target all TLR genes in *N. benthamiana*. Nine VIGS constructs were cloned with fragments of up to four TLRs each. In this initial screen one of the nine constructs, targeting three distinct TLRs, was found to consistently prevent perception of XopQ and allow for expression of the GFP reporter. Individual VIGS constructs were made for these three candidate genes and one was found to prevent XopQ perception (Figure 3). The gene targeted by this construct was named Roq1. Putative orthologs of Roq1 were identified in several other Nicotiana species including *N. tabacum* (XP_009615050), *Nicotiana tomentosiformis* (XP_009615050) and *Nicotiana attenuata* (XP_019226668), but were absent in *Solanum lycopersicum*, *Solanum tuberosum*, *Capsicum annuum* and all other non-Nicotiana species examined.

Roq1 is sufficient to allow perception of XopQ in *Nicotiana sylvestris* and *Beta vulgaris*

Nicotiana sylvestris was found to contain an aberrant copy of Roq1 missing part of the first and second exons, and containing a stop codon in a conserved part of the second exon (Figure S2). Disruption of the endogenous Roq1 gene in *N. sylvestris* is consistent with a previous report that this

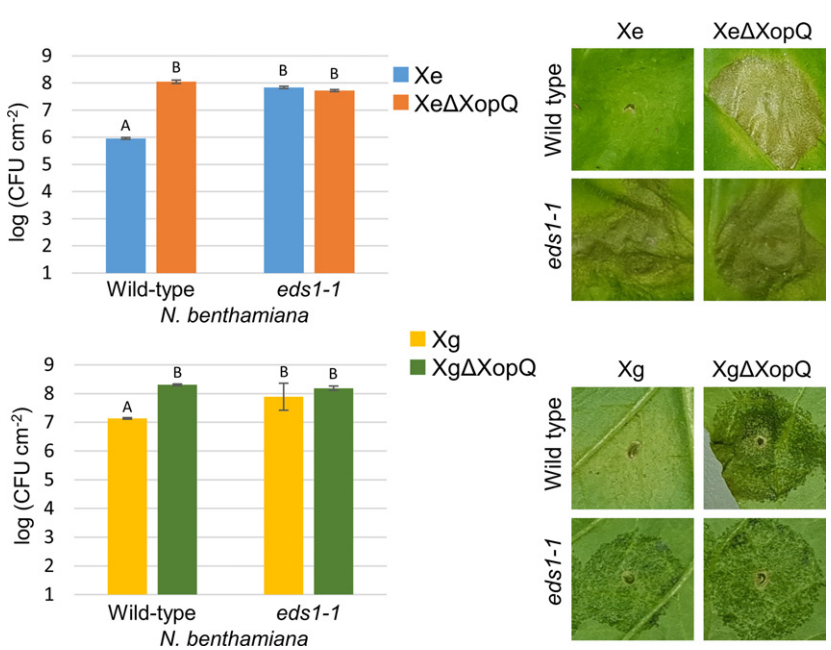

Figure 1. Effect of XopQ perception on bacterial growth and disease symptoms.
Xanthomonas euvesicatoria (Xe) and *Xanthomonas gardneri* (Xg) wild-type and XopQ knockout strains were infiltrated into *Nicotiana benthamiana* leaves at low inoculum (OD_{600} = 0.0001). Bacterial growth was assayed at 6 days post-infiltration, and disease symptoms were imaged at 13 and 9 days post-infiltration for Xe and Xg, respectively. Error bars indicate standard deviation from three replicates. Letters indicate statistically significant differences between samples based on Student's *t*-test, with a *P*-value < 0.01 for pairs of samples with differing letters. CFU, colony forming units. These growth assays and disease symptom experiments were completed three times with similar results.

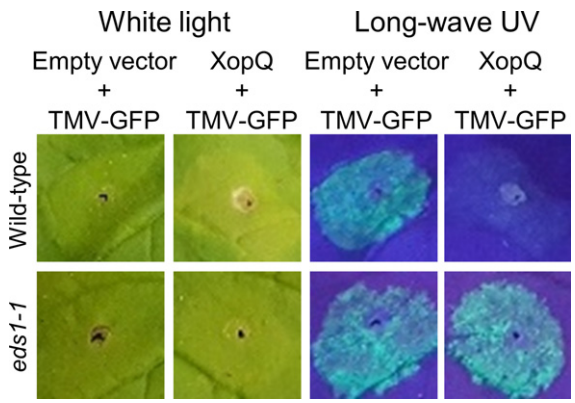

Figure 2. Green fluorescent protein (GFP)-based reporter for immune activation.
GFP-TMV was co-expressed with an empty vector or XopQ transiently using Agrobacterium in wild-type and *eds1-1* mutant *Nicotiana benthamiana* leaves. The leaves were imaged 3 days post-infiltration under white light (left) or long-wave UV (right) using a handheld digital camera. The images shown here are representative of many independent experiments.

species is unable to perceive XopQ (Adlung *et al.*, 2016). Transient expression of the *N. benthamiana* Roq1 gene along with XopQ in *N. sylvestris* resulted in a visible hypersensitive response that was not present when either Roq1 or XopQ were expressed alone (Figure 4). To determine if Roq1 can be used to enable perception of XopQ in plants outside of the Nicotiana genus, Roq1 was co-expressed with XopQ in *Beta vulgaris*. This resulted in a chlorotic effect similar to that observed when XopQ is expressed in wild-type *N. benthamiana* leaves (Figure 4).

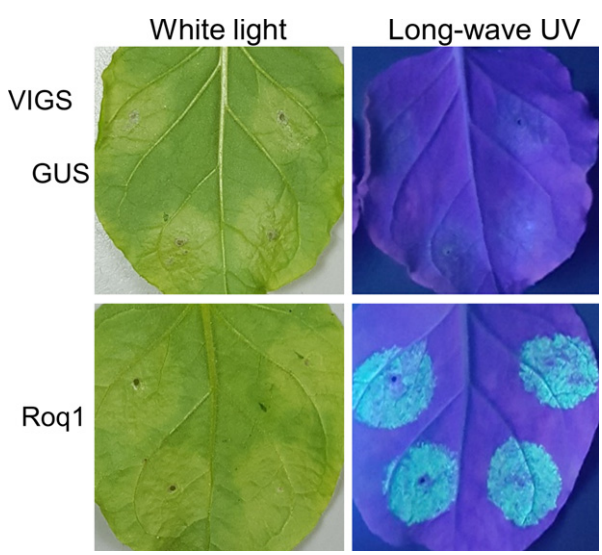

Figure 3. Green fluorescent protein (GFP)-TMV reporter in viral-induced gene silencing (VIGS) leaves of *Nicotiana benthamiana*.
VIGS was used to downregulate either the GUS gene (as a negative control, top) or Roq1 (bottom). XopQ was transiently expressed along with the TMV-GFP reporter using Agrobacterium, and the plants were imaged at 4 days post-infiltration under white light (left) or long-wave UV (right). This result was observed in three independent gene silencing experiments.

Roq1 co-immunoprecipitates with XopQ

The recognition of HopQ1 in *N. tabacum* has previously been shown to be independent of HopQ1 activity (Li *et al.*, 2013a). We therefore hypothesized that Roq1 may physically interact with XopQ and HopQ1 instead of 'guarding' a protein or molecule that is modified by this effector. To test this, we performed a series of co-immunoprecipitation experiments in *eds1-1 N. benthamiana*. Roq1-6xHA was found to be pulled down by both XopQ-3xFlag and HopQ1-3xFlag, but not by ATR1-3xFlag, an effector known to interact with the TLR RPP1 (Figure 5; Krasileva *et al.*, 2010). ATR1-3xFlag was found to pull down RPP1-6xHA, consistent with previous results, but not Roq1-6xHA. Two dominant bands were observed on the Western blot for XopQ and HopQ1, which is consistent with previous blots of HopQ1 expression in *N. benthamiana* and may represent full-length and processed forms of the peptides (Li *et al.*, 2013b).

Roq1 can mediate the perception of diverse XopQ and HopQ1 alleles

Alleles of XopQ and HopQ1 are widely distributed among Xanthomonas and Pseudomonas species that are pathogenic on various crops. This suggests that Roq1 may be useful to engineer resistance into these crops species, but depends on Roq1 being able to recognize the different XopQ and HopQ1 alleles. To sample the diversity of XopQ/HopQ1 effector proteins, we generated a phylogenetic tree

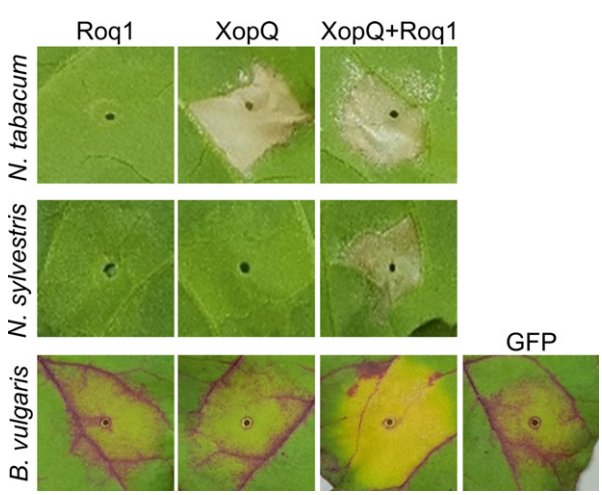

Figure 4. Transient expression of Roq1 and XopQ.
Roq1 and XopQ were transiently expressed using Agrobacterium in leaf tissue of *Nicotiana tabacum*, *Nicotiana sylvestris and Beta vulgaris*. The Agrobacterium was infiltrated at a total OD_{600} of 0.5 and the plants were imaged at 6 days post-infiltration for *N. tabacum* and *N. sylvestris*, and 9 days post-infiltration for *B. vulgaris*. These images are representative of more than 10 infiltrations from three independent experiments.

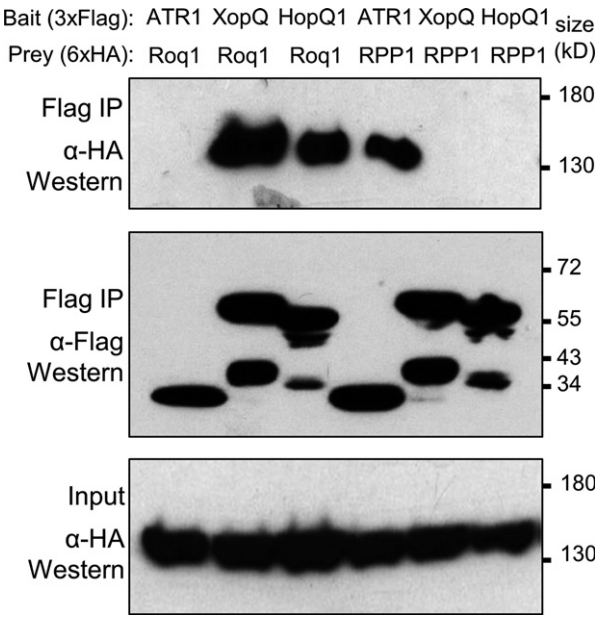

Figure 5. Co-immunoprecipitation of XopQ and Roq1.
XopQ-3xFlag, ATR1-3xFlag and HopQ1-3xFlag were transiently co-expressed with either Rpp1-6xHA or Roq1-6xHA in the *Nicotiana benthamiana eds1* mutant. Western blots with α-HA primary antibody (top) and α-Flag primary antibody (middle) show proteins pulled down with anti-Flag beads for each combination. A Western blot of the input protein extract (prior to precipitation) is shown on the bottom. The Arabidopsis NLR protein RPP1 and its cognate effector ATR1 were included as controls. Predicted molecular weights are as follows: XopQ-3xFlag 53 kD, HopQ1-3xFlag 52 kD, ATR1-3xFlag 33 kD, RPP1-6xHA 145 kD and Roq1-6xHA 159 kD. The blots shown here are representative of the CoIP results from three independent experiments.

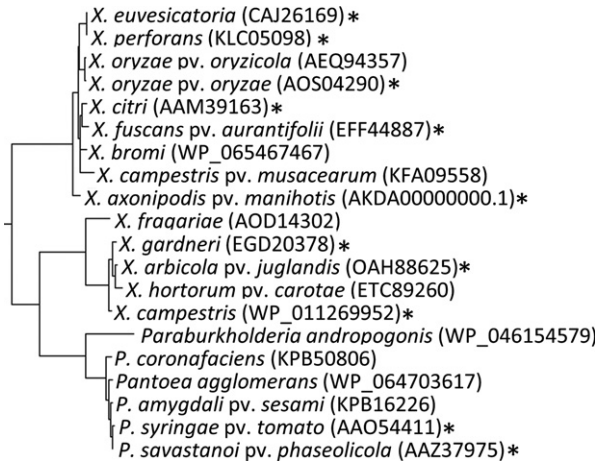

Figure 6. XopQ phylogenetic tree.
XopQ homologs were identified by BLAST search and used to generate a phylogenetic tree. Sequences identified with * were cloned for transient expression in *Nicotiana sylvestris*. These sequences all elicited an immune response in *N. sylvestris* when co-expressed with Roq1 (Figure 7).

and cloned select alleles for transient expression in *N. sylvestris* (Figure 6). Out of 11 XopQ/HopQ1 alleles cloned, all elicited a recognition response in *N. sylvestris* when co-expressed with Roq1 as observed by suppression of the GFP-TMV reporter (Figure 7). An alignment of the tested XopQ/HopQ1 protein sequences is shown in Figure S3.

DISCUSSION

Mechanism of Roq1 recognition of XopQ

Co-immunoprecipitation experiments showed that Roq1 can physically associate with XopQ/HopQ1 as determined by immunoprecipitation after transient co-expression in *N. benthamiana* (Figure 5). Roq1 did not interact with ATR1 (an effector from *Hyaloperonospora arabidopsidis*), indicating that there is specificity and that Roq1 is not interacting with the beads during the procedure. Furthermore, XopQ and HopQ1 did not interact with the Arabidopsis TLR protein RPP1, indicating these proteins have some specificity to Roq1 and are not able to pull down all TLR proteins. These data are consistent with the model that Roq1 specifically associates with XopQ during activation. While the interaction between Roq1 and XopQ may be direct, the possibility of an indirect interaction mediated by an unknown protein cannot be excluded. While the various XopQ/HopQ1 alleles have significant amino acid variation, especially at the N-terminus, there are highly conserved regions that could mediate interaction with Roq1 (Figure S3). *Xanthomonas euvesicatoria* XopQ and *Pseudomonas syringae* pv. *tomato* HopQ1 share 65% amino acid identity with the variable N-terminal region removed. While the EDS1 protein is required for perception of XopQ, it is not required for interaction between Roq1 and XopQ or RPP1 and ATR1. These data support a model in which EDS1 is involved in the signaling process downstream of the TLR proteins and is not required for expression or proper folding of TLRs.

Distribution and specificity of Roq1

The Roq1 gene is highly conserved in several Nicotiana species but was not detected in any species outside this genus. This suggests that Roq1 evolved within the Nicotiana lineage and is consistent with the previous observation that XopQ failed to elicit an immune response when expressed in non-Nicotiana species (Adlung *et al.*, 2016). The wide distribution and high conservation of XopQ/HopQ1 in Xanthomonas and Pseudomonas pathogens may be a consequence of the narrow distribution of the Roq1 gene. Although XopQ/HopQ1 is highly conserved, there are amino acid differences in the alleles of different Xanthomonas and Pseudomonas species. The observation that co-expression of Roq1 with XopQ/HopQ1 alleles from 11 different species can elicit an immune response in *N. sylvestris* indicates that Roq1 is able to recognize the

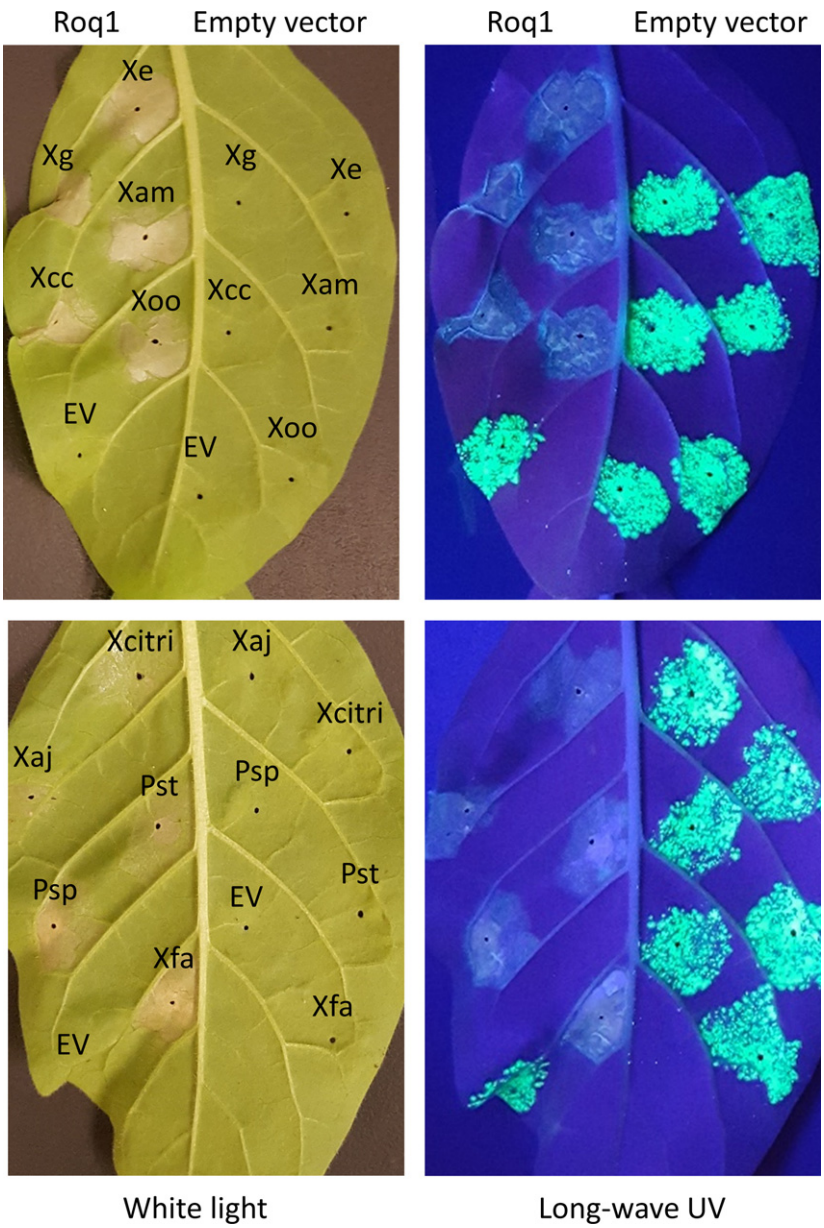

Figure 7. Roq1 perception of XopQ/HopQ1 alleles. The indicated XopQ/HopQ1 alleles were cloned and transiently expressed along with the green fluorescent protein (GFP)-TMV reporter and either Roq1 (left half of leaf) or an empty vector (right half of leaf) in *Nicotiana sylvestris*. The leaves were imaged at 6 days post-infiltration under white light and long-wave UV to view the visible HR response and viral GFP expression. Agrobacterium harboring the expression constructs was infiltrated at an OD_{600} of 0.05 for each of the three TMV-GFP constructs and 0.3 for the elicitor and receptor constructs. Xe, *Xanthomonas euvsicatoria*; Xg, *Xanthomonas gardneri*; Xam, *Xanthomonas axonipodis* pv. *manihotis*; Xcc, *Xanthomonas campestris* pv. *campestris*; Xoo, *Xanthomonas oryzae* pv. *oryzae*; EV, empty vector; Xaj, *Xanthomonas axonopodis* pv. *juglandis*; Xcitri, *Xanthomonas citri*; Psp, *Pseudomonas savastanoi* pv. *phaseolicola*; Pst, *Pseudomonas syringae* pv. *tomato*; *Xfa*, *Xanthomonas fuscans* subsp. *aurantifolii*. The results shown here are representative of three independent experiments.

different alleles despite the various amino acid differences (Figures 7 and S3).

Use of Roq1 to confer resistance against Xanthomonas and Pseudomonas

Because Roq1 is able to recognize diverse XopQ/HopQ1 alleles and this effector protein is present in many Xanthomonas and Pseudomonas species (Figure 7), Roq1 has the potential to be useful for engineering resistance against these pathogens in many crop species. Some resistance genes do not function when moved into other plant species, particularly when those species are not closely related to the original plant. In some cases this is due to additional components of the perception pathway that are absent in the target plant species (Ade *et al.*, 2007). If Roq1 directly interacts with XopQ, Roq1 may be able to function in a distantly related plant species without the requirement for the transfer of additional genes (Wulff *et al.*, 2011). This is supported by the observation that co-expression of Roq1 and XopQ triggers a visible reaction, possibly an immune response, in *B. vulgaris* (Figure 4). The chlorotic response triggered by co-expression of Roq1 and XopQ was not observed when either Roq1 or XopQ were expressed in *B. vulgaris* alone, despite the same total concentration of Agrobacterium. Roq1 activity does depend on the downstream signaling components, such as EDS1 (Figures 1

and 2). EDS1 is present in many plant species, and therefore it may be that only Roq1 is needed to confer resistance against pathogens containing XopQ. Notably the grasses do not appear to have full-length TLRs (Meyers *et al.*, 2003; Sarris *et al.*, 2016), and it may be necessary to add EDS1 and other signaling components required for TLR activity to allow Roq1 to function in grasses.

Widespread use of the Roq1 resistance gene in crops to protect them from Xanthomonas and Pseudomonas will place a large selection pressure on pathogens either to evolve a variant of XopQ/HopQ1 that evades recognition or to lose the effector. *Pseudomonas syringae* pv. *tabaci*, which can cause disease on *N. tabacum* plants containing a functional copy of Roq1, lacks the HopQ1 effector presumably as an adaptation to this host. Complementation of *P. syringae* pv. *tabaci* with a copy of HopQ1 was found to increase disease symptoms in bean (*Phaseolus vulgaris*), but decrease them in *N. tabacum* (Ferrante *et al.*, 2009). This appears to be an example of a pathogen overcoming Roq1-mediated host resistance by losing HopQ1, but having a resulting virulence defect on plants unable to perceive this effector. The virulence contribution of XopQ/HopQ1 appears to be specific to the particular host/pathogen interaction, as virulence defects were not observed for the *X. euvesicatoria* and *X. gardneri* XopQ mutants on *eds1 N. benthamiana* (Figure 1). It would be prudent to deploy Roq1 into crop species only in combination with other resistance genes with activity against the same pathogen to achieve a more durable resistance, although a loss of XopQ/HopQ1 to overcome Roq1-mediated resistance may reduce disease severity.

EXPERIMENTAL PROCEDURES

CAS9 mediated knockout of *EDS1*

Guides to target the *N. benthamiana EDS1* gene were designed and tested *in vitro* using purified CAS9 protein (M0386S, New England Biolabs), polymerase chain reaction (PCR)-amplified target DNA, and T7 transcription to produce the guides (E2040S, New England Biolabs). The primers used for making the T7 templates are listed in Table S1. The digestions were incubated at 37°C for 1 h with 30 nM RNA, 20 nM target DNA and 30 nM CAS9 enzyme. Following digestion, the reactions were incubated at 80°C for 10 min prior to DNA gel electrophoresis. The selected guide sequences were cloned into a pDONR221-based entry plasmid containing the CAS9 gene driven by a 35S promoter and the *Arabidopsis thaliana* U6-26 promoter to drive guide expression. Gateway LR reactions (Life Technologies) were performed to move the guide and CAS9 cassette into the pFast-G04 binary vector (Shimada *et al.*, 2010), which was used for stable transformation into *N. benthamiana* by Agrobacterium co-cultivation. Transformed plants were genotyped by PCR and Sanger sequencing. The primer sequences used for genotyping are listed in Table S1.

Vigs

Target TLR genes from *N. benthamiana* were identified using a BLAST search of a transcript database (Nakasugi *et al.*, 2013). The predicted protein sequences of identified genes were aligned and manually curated to remove gene fragments and pseudogenes. Candidate TLRs were targeted for silencing by cloning approximately 300 bp into the TRV2 VIGS vector (Liu *et al.*, 2002) using restriction ligation cloning with the enzyme BsaI. The sequences of the primers and amplified targets are listed in Table S1, with up to four target sequences included per vector. The plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 and infiltrated into *N. benthamiana* plants along with TRV1 at an OD_{600} of 0.2 each. The plants were infiltrated at 4–6 weeks of age and phenotyped 2–4 weeks after infiltration.

GFP-reporter assay for XopQ perception

Agrobacterium harboring the vectors pICH2011 (containing the first part of the viral replicon), pICH14011 (containing the C31 integrase for replicon assembly) and pICH7410 (containing the second part of the viral replicon and the GFP-coding sequence; Marillonnet *et al.*, 2005) was mixed to a final OD_{600} of 0.05 each along with an Agrobacterium strain for transient expression of XopQ at an OD_{600} of 0.3. The binary plasmids pE1776 and pORE E4 (Coutu *et al.*, 2007) were used for transient expression of XopQ with similar results. The Agrobacterium mixture was infiltrated into leaf tissue and imaged under long-wave UV light 3–5 days post-infiltration to visualize GFP expression.

Growth assay

To assay bacterial growth, Xanthomonas was grown overnight in NYG (0.5% peptone, 0.3% yeast extract, 2% glycerol) with 100 μg mL^{-1} rifampicin on a shaker at 30°C. The cultures were spun down at 10 000 ***g***, washed once with 10 mM $MgCl_2$, and infiltrated into leaf tissue at OD_{600} = 0.0001. Leaf punches were collected at 6 days post-infiltration, homogenized and serially diluted before plating on NYG agar plates with 100 μg mL^{-1} rifampicin and 50 μg mL^{-1} cycloheximide. Colonies were counted 3–4 days after plating.

Transient expression using *Agrobacterium tumefaciens*

Agrobacterium tumefaciens cultures were grown overnight in LB with selection at 30°C on a shaker. Cells were pelleted by centrifugation at 10 000 ***g*** and resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.6). The cells were diluted to the appropriate OD_{600} and infiltrated into leaf tissue using a needleless syringe.

Plant material and bacteria strains

Nicotiana sylvestris was obtained from Select Seeds in Union, Connecticut, USA ('Woodland Tobacco 346'). *Nicotiana tabacum* and *B. vulgaris* 'Detroit Dark Red, Morse's Strain' (Plantation Products, MA, USA) were used for transient expression. *Xanthomonas euvesicatoria* 85-10 and *X. gardneri* 153 were used for pathogen assays. The *X. euvesicatoria* 85-10 ΔXopQ knockout was constructed previously (Schwartz *et al.*, 2015). *Agrobacterium tumefaciens* strains GV3101 and C58C1 were used for transient expression and VIGS.

XopQ knockout in *Xanthomonas gardneri*

Regions (1000 bp) upstream and downstream of the XopQ coding sequence were cloned into the pLVC18 plasmid (Lindgren *et al.*, 1986) containing a SacB counter-selectable marker. This plasmid was conjugated into *X. gardneri* and single crossover events were selected for by tetracycline resistance (10 μg mL^{-1}) and PCR. Colonies were plated onto NYG media containing 5% sucrose to select for a second crossover event and screened by PCR to identify

XopQ deletion strains. The primers used for cloning the knockout construct and genotyping are listed in Table S1.

Co-immunoprecipitation assay

The coding sequences were first cloned into entry vectors with 3xFlag or 6xHA fusions, and then a Gateway LR reaction (Life Technologies) was used to move the desired sequence into the binary plasmid pE1776. This plasmid contains the mannopine synthase promoter and three octopine synthase upstream activators (Ni *et al.*, 1995) to enable strong expression of the gene of interest. The constructs were transformed into Agrobacterium strain GV3101, which was used for transient expression in the leaf tissue of *eds1 N. benthamiana* plants. Tissue (0.5 g per sample) was collected 2 days post-infiltration and ground with a mortar and pestle to a homogeneous powder in liquid nitrogen. The samples were suspended in 1 mL of the protein extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 0.2% Nonidet P-40, 6 mM β-mercaptoethanol, 1 × protease inhibitor cocktail). Samples were centrifuged twice (10 min, 21,000 ***g***, 4°C). The supernatant was then transferred to a new tube with 10 μL Flag beads (A2220, Sigma) and incubated at 4°C for 3 h. The samples were centrifuged (1 min, 1000 ***g***), then washed three times with 1 mL of the protein extraction buffer. The protein was eluted by boiling for 5 min in 50 μL of 3 × Laemmli buffer, centrifuged and loaded for Western blot. For the anti-Flag Western, the primary antibody was F7425 (Sigma) and the secondary antibody was A0545 (Sigma).

ACCESSION NUMBERS

The sequence of the Roq1 gene from *N. benthamiana* is available from the GenBank database with the accession number MF773579.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. *EDS1* guides and mutant alleles.

Figure S2. Roq1 gene models.

Figure S3. Protein alignment of XopQ/HopQ1 alleles.

Table S1. Primer sequences.

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