New Phytologist

Research

Using forward genetics in *Nicotiana benthamiana* to uncover the immune signaling pathway mediating recognition of the *Xanthomonas perforans* effector XopJ4

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Received: *5 May 2018*
Accepted: *23 July 2018*

New Phytologist (2019) **221:** 1001–1009
doi: 10.1111/nph.15411

Key words: JIM2, *Nicotiana benthamiana*, nonhost resistance, plant immunity, plant pathogen, *Xanthomonas perforans*, XopJ4, ZAR1.

Summary

- The immune pathway responsible for perception of the *Xanthomonas perforans* effector XopJ4 was identified in the plant *Nicotiana benthamiana*. This pathogen causes significant yield loss in commercial tomato cultivation.
- Genetic mapping and viral-induced gene silencing were used to identify immune signaling components of the XopJ4 perception pathway in *N. benthamiana*. Transient complementation assays were performed to determine the functionality of gene variants and co-immunoprecipitation assays were used to gain insight into the molecular mechanism of the pathway.
- Two *N. benthamiana* ethyl methanesulfonate (EMS) mutants deficient for XopJ4 perception were identified as having loss-of-function mutations in the gene encoding the nucleotide binding, leucine-rich repeat (NLR) protein NbZAR1. Silencing of a receptor-like cytoplasmic kinase family XII gene, subsequently named XOPJ4 IMMUNITY 2 (JIM2), blocks perception of XopJ4.
- This study demonstrates the feasibility of conducting mutant screens in *N. benthamiana* to investigate the genetic basis of the plant immune system and other processes. The identification of NbZAR1 and JIM2 as mediating XopJ4 perception in *N. benthamiana* supports the model of ZAR1 being involved in the perception of many different pathogen effector proteins with specificity dictated by associated receptor-like cytoplasmic kinases.

Introduction

Bacterial phytopathogens in the genus *Xanthomonas* use a Type III Secretion System to deliver effector proteins into the plant cell. Effector proteins can function to inhibit plant immunity or manipulate the metabolism of the host to favor growth of the bacteria (Gürlebeck *et al.*, 2006). If the plant is able to detect the presence of an effector protein, a strong immune response may be induced which prevents pathogen proliferation and restricts host range (Alfano & Collmer, 2004; Castañeda *et al.*, 2005; Wei *et al.*, 2007; Schwartz *et al.*, 2015). This strong immune response often results in localized cell death known as the hypersensitive response. Identifying the pathways responsible for mediating effector protein recognition can enable efforts to engineer disease resistance in susceptible crop species (Wulff *et al.*, 2011).

The perception of intracellular pathogen effector proteins in plants is frequently mediated by proteins from the nucleotide binding, leucine-rich repeat (NLR) protein family (Jones *et al.*, 2016). NLR recognition of an effector protein can occur through a physical interaction between the NLR and the cognate effector or by an indirect mechanism in which the NLR protein is 'guarding' another component which is modified by the effector (Khan *et al.*, 2016). While plants have additional receptor pathways independent from the NLRs, such as receptor-like kinases (Macho & Zipfel, 2014), NLR proteins have been demonstrated to mediate many resistance responses against a broad range of pathogens including bacteria, fungi, oomycetes, viruses and nematodes (Dodds & Rathjen, 2010).

Tomato is susceptible to *Xanthomonas perforans*, which causes the disease Bacterial Leaf Spot. This disease can result in significant yield losses in commercial tomato production and there is limited genetic resistance available within commercial cultivars (Stall *et al.*, 2009; Kim *et al.*, 2015). The plant *Nicotiana benthamiana* is resistant to *X. perforans* due to the presence of several pathways capable of perceiving effector proteins found in this pathogen including AvrBsT, XopQ and XopJ4/AvrXv4 (Roden *et al.*, 2004; Schwartz *et al.*, 2015). The XopJ4 effector is widely conserved among strains of *X. perforans* and has therefore been proposed as a good target for identifying genetic mechanisms of disease resistance against this pathogen (Timilsina *et al.*, 2016).

XopJ4 is part of the YopJ family of effector proteins. These effectors are distributed across many species of bacterial

pathogens of both plants and animals and are thought to be acetyltransferases that disrupt the function of proteins inside the host cell (Ma & Ma, 2016). The YopJ effector protein HopZ1a from *Pseudomonas syringae* is recognized in *Arabidopsis thaliana* and triggers a hypersensitive cell death response that depends on the NLR protein ZAR1 and the Receptor-Like Cytoplasmic Kinase (RLCK) family XII protein ZED1 (Lewis *et al.*, 2010, 2013). HopZ1a has been reported to directly acetylate ZED1, which interacts with ZAR1 to initiate an immune response. ZAR1 is also required for the perception of several non-YopJ effectors in *Arabidopsis* including the *Xanthomonas* AvrAC (Wang *et al.*, 2015) and the *Pseudomonas syringae* HopF2a (Seto *et al.*, 2017). In each case, a different RLCK XII family member is required for the immune response and for AvrAC an additional RLCK VII protein is also required.

The YopJ effector PopP2 from *Ralstonia solanacearum* is recognized in *A. thaliana* by a ZAR1-independent pathway (Deslandes *et al.*, 2002). PopP2 acetylates a WRKY domain on the NLR protein RRS1 to trigger immune activation (Sarris *et al.*, 2015). The NLR protein RPS4 is required for RRS1-mediated perception of PopP2 and is thought to form a complex with RRS1 (Narusaka *et al.*, 2009; Williams *et al.*, 2014). The widespread distribution of YopJ-family effector proteins and the existence of two evolutionarily independent mechanisms for the perception of YopJ effectors highlights the importance of these proteins in pathogenesis.

Four YopJ effector proteins have been identified in various *Xanthomonas* species, although not all *Xanthomonas* have YopJ effectors. These include XopJ4/AvrRx4, XopJ, AvrRxv and AvrBsT. AvrRxv, XopJ4 and AvrBsT have all been associated with avirulence responses in various Solanaceous plant species, although the recognition mechanisms for the perception of these effectors are not well understood (Minsavage *et al.*, 1990; Whalen *et al.*, 1993; Astua-Monge *et al.*, 2000). The AvrBsT effector triggers a strong avirulence response on pepper and *N. benthamiana*. The recognition of AvrBsT in pepper has been reported to be dependent on SGT1 and PIK1 (Kim *et al.*, 2014). AvrRxv triggers an avirulence response on tomato line Hawaii 7998, the basis for which is multigenic based on segregation analysis (Whalen *et al.*, 1993; Yu *et al.*, 1995). XopJ4 triggers an avirulence response on *Solanum pennellii* accession LA716. While efforts have been made to map the resistance genes for AvrRxv and XopJ4 (Yu *et al.*, 1995; Astua-Monge *et al.*, 2000; Sharlach *et al.*, 2013), the genes involved have not been conclusively identified.

In this work, we used a forward genetic screen to identify components of the XopJ4 perception pathway in the model plant *N. benthamiana*. This resulted in the identification of an NLR protein, NbZAR1, which is a homolog of the *A. thaliana* protein ZAR1 (AtZAR1) and is required for XopJ4 perception. To our knowledge this represents the first reported example of mapping an ethyl methanesulfonate (EMS) mutant in *N. benthamiana*, which is a widely used model plant but is challenging to use for forward genetics due to it being an allotetraploid with a large (*c.* 3.1 Gb) and incomplete reference genome (Naim *et al.*, 2012). A subsequent reverse genetic screen identified an RLCK XII gene also required for the perception of XopJ4 which was named XOPJ4 IMMUNITY 2 (JIM2). The identification of these components enables future work to engineer resistance against *Xanthomonas* in tomato and other crop species.

Materials and Methods

Generation of *N. benthamiana* mutant population

Seeds of *N. benthamiana* 16c (line expressing green fluorescent protein) (Ruiz *et al.*, 1998) were chemically mutagenized with EMS (Weigel & Glazebrook, 2006). Briefly, the seeds were presoaked at room temperature in potassium phosphate buffer (PPB, 100 mM, pH 7.5) for 2 h, then transferred to 100 mM PPB supplemented with 0.4% EMS and incubated for 15 h at 28°C with gentle shaking at 60 rpm. The treated seeds were washed 20 times with sterile water and dried on filter paper. Approximately 450 mutagenized individual plants were grown and selfed to create the M_2 mutant population.

Genetic mapping in *N. benthamiana* using high-throughput sequencing

The *N. benthamiana zar1-1* mutant was backcrossed to the wild-type and the F_1 progeny were selfed to create an F_2 mapping population. F_2 plants were phenotyped by transient expression of XopJ4 using *Agrobacterium* and placed into two separate pools, based on the presence or absence of a cell death response, before genomic DNA extraction. Illumina DNA sequencing was performed using one HiSeqX lane with 150 bp paired-end reads for each pool. The reads were mapped to the *N. benthamiana* reference genome (Naim *et al.*, 2012) and single nucleotide polymorphisms (SNPs) were identified using the GATK toolkit (DePristo *et al.*, 2011). The SNPs were filtered for mapping quality, possibility of being caused by EMS, and having a large difference in frequency between the mutant and wild-type pools (> 0.25).

Transient expression

Agrobacterium tumefaciens strain GV3101 was used for transient expression. The binary plasmids pE1776 (with OCS promoter and UAS for strong expression) (Ni *et al.*, 1995) and pORE E4 (Coutu *et al.*, 2007) were used as expression vectors for the desired genes. The primer sequences used for cloning are listed in Supporting Information Table S1. To construct the viral-induced gene silencing (VIGS) resistant version of JIM2, the codon usage of the region targeted by the JIM2 VIGS construct was altered while conserving the predicted amino acid sequence (see Fig. S7). This sequence was subsequently fused to the rest of the JIM2 coding sequence and cloned into a vector for transient expression. The plasmids were transformed into *Agrobacterium* and cultures were grown overnight in LB media (1% tryptone, 0.5% yeast extract, 1% NaCl) with appropriate selection (rifampicin 100 μg ml^{-1}, gentamycin 25 μg ml^{-1}, kanamycin 50 μg ml^{-1}). The cultures were centrifuged, suspended in infiltration buffer (10 mM $MgCl_2$, 10 mM 2-(N-morpholino)ethanesulfonic acid

(MES) pH 5.6), diluted to the appropriate OD_{600} and infiltrated into leaf tissue using a needleless syringe.

Xanthomonas gene knockout and complementation

For the knockout of XopJ4 in *X. perforans* 4B, 1046 bp upstream and 1127 bp downstream of XopJ4 was cloned into the pLVC18 plasmid containing a SacB counter-selectable marker (Lindgren *et al.*, 1986). This plasmid was conjugated into *X. perforans* already lacking the XopQ and AvrBsT genes (Schwartz *et al.*, 2015) and selected on NYG (0.5% peptone, 0.3% yeast extract, 2% glycerol) plates containing tetracycline (10 μg ml^{-1}). Colonies were screened for a single crossover event at the target locus by PCR. Positive colonies were grown overnight and plated on NYG plates with 5% sucrose to select for a second crossover event. Colonies were again screened by PCR to obtain XopJ4 deletion strains. For complementation, the XopJ4 gene including the promoter and terminator was cloned onto the plasmid pVSP61 (obtained from William Tucker, DNA Plant Technology, Oakland, CA). This plasmid, which can replicate in *Xanthomonas*, was conjugated into *X. perforans* and selected for with 25 μg ml^{-1} kanamycin. The primers used for construction of the knockout and complementation plasmids are listed in Table S1.

Bacterial growth assays and visible immune responses

Xanthomonas liquid cultures were grown in NYG media with selection overnight. Cells were collected by centrifugation, washed once and suspended in 10 mM $MgCl_2$ to an OD_{600} of 0.0001. Plant leaves were infiltrated by needleless syringe. For the growth assay, punches were collected from infiltrated leaf tissue 0 and 6 d after infiltration, homogenized in 10 mM $MgCl_2$ and serially diluted before plating on NYG plates with selection.

Viral-induced gene silencing

For VIGS, *c.* 300 bp of the target gene was cloned into the TRV2 vector (Liu *et al.*, 2002). This vector was transformed into *A. tumefaciens* GV3101. The resulting *Agrobacterium* strain was grown overnight and coinfiltrated with another *Agrobacterium* strain harboring the TRV1 vector at an OD_{600} of 0.2 each by needleless syringe. Plants were infiltrated at *c.* 4 wk old and used for transient assays 2–4 wk after infiltration.

Coimmunoprecipitation

The desired proteins were coexpressed in *N. benthamiana* leaf tissue using *Agrobacterium*-mediated transient expression. The tissue was frozen in liquid nitrogen and ground using a mortar and pestle. The samples were suspended in immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.3% Igepal, 10 mM 1,4-dithiothreitol, 1× protease inhibitor cocktail) and centrifuged (20 min, 21 000 ***g***, 4°C). The supernatant was incubated with α-Flag beads (10 μl, A2220 Sigma) at 4°C for 3 h. The samples were centrifuged (2 min, 1000 ***g***) and washed three times with immunoprecipitation buffer, before elution by boiling with Laemmli buffer. The α-Flag western blots were performed using α-Flag antibody (F7425, Sigma) as the primary antibody and α-Rabbit IgG-Peroxidase (A0545, Sigma) as the secondary antibody. A single α-HA-Peroxidase antibody (3F10, Roche) was used for the α-HA western blots.

Results

Identification of two allelic *N. benthamiana* mutants impaired in XopJ4 recognition

We conducted a forward genetic screen of 2000 M_2 plants from an EMS-mutagenized population of *N. benthamiana* for individuals lacking a cell death response to transiently expressed XopJ4. Two mutants were identified that failed to respond to XopJ4 (Fig. 1). These mutants were crossed to each other and to wild-type plants and the resulting F_1 progenies were phenotyped by transient expression of XopJ4 (Fig. S1). A cell death response was observed in the backcrossed F_1 progeny but not in the F_1 progeny of the two mutants, indicating that both are recessive and allelic.

Mutations in NbZAR1 are causative for the loss of XopJ4 recognition

An F_2 mapping population generated from one of the backcrossed plants was used for identification of the mutation responsible for the loss of XopJ4 recognition. Forty-three individuals lacking a response to XopJ4 were identified out of 188 F_2 plants. These mutant individuals were pooled for genomic DNA extraction along with a separate pool of F_2 plants from the same population that were capable of responding to XopJ4. These pools were subjected to Illumina sequencing yielding *c.* 30× genome coverage. SNPs were annotated by comparison to a reference genome (Naim *et al.*, 2012) and filtered by mapping quality,

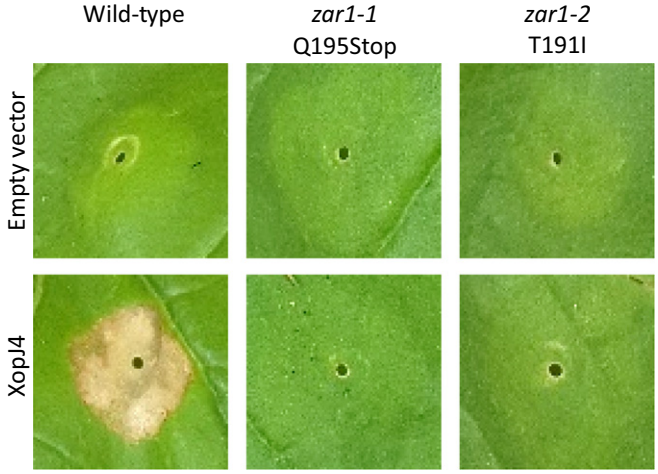

Fig. 1 Phenotype of the *zar1-1* and *zar1-2* mutants. *Agrobacterium* was used to transiently express XopJ4 in leaf tissue of wild-type and *zar1* mutant *Nicotiana benthamiana* plants. *Agrobacterium* harboring an empty vector construct was used as a control. The plants were infiltrated at an OD_{600} of 0.5 and imaged at 3 d post-infiltration.

possibly of being induced by EMS, and showing a differing absolute abundance of at least 25% between the mutant pool and wild-type pool. Out of 2994 SNPs passing these filters, 33 were predicted to occur within gene sequences and three were predicted to result in an amino acid change (Table 1). One of these was a mutation introducing a stop codon at position 195 (Q195Stop) in the predicted protein sequence of the gene *Nbv5tr6207061*, named NbZAR1 after its Arabidopsis homolog. The sequence of NbZAR1 was confirmed by Sanger sequencing and deposited into the NCBI database (accession MH532570). Cosegregation of the mutant phenotype with this mutation was confirmed by Sanger sequencing of this SNP in 43 mutant F_2 plants, which were all homozygous for the mutation (Fig. S2). Sequencing of NbZAR1 in the second allelic mutant revealed the presence of a single nucleotide change resulting in the substitution of threonine 191 with an isoleucine in the predicted protein sequence. Together with the allelism test, these data indicate that both mutants, subsequently named *zar1-1* and *zar1*-2, are loss-of-function mutations in NbZAR1 that prevent XopJ4 recognition in *N. benthamiana*. Both mutations are present within the predicted NB-ARC domain of NbZAR1, with T191I occurring within the conserved P-loop motif (Fig. S3).

N. benthamiana zar1-1 and *zar1-2* are deficient for resistance against *X. perforans* expressing XopJ4

To test whether the avirulence activity of XopJ4 was compromised in the *zar1* mutants, the XopJ4 gene was knocked out in an *X. perforans* (Xp) strain already deficient for XopQ and AvrBsT, as these two effectors trigger avirulence responses in *N. benthamiana* (Schwartz *et al.*, 2015). This knockout strain, along with parental and complemented strains, was infiltrated into *N. benthamiana* leaves at a low inoculum concentration and bacterial growth was assayed by measuring colony forming units 6 d after infiltration. Growth of Xp ΔAvrBst ΔXopQ ΔXopJ4 was found to be *c.* 100-fold greater in wild-type *N. benthamiana* leaf tissue compared to Xp ΔAvrBst ΔXopQ and the complemented strain Xp ΔAvrBst ΔXopQ ΔXopJ4 + XopJ4 (Fig. 2). This indicates that XopJ4 triggers an avirulence response on wild-type *N. benthamiana*. No avirulence effect of XopJ4 was observed on the *zar1-1* and *zar1-2* mutants as a similar high level of bacterial growth was observed regardless of the presence of XopJ4 (Fig. 2). Consistent with the growth phenotypes, a visible immune response was observed in wild-type *N. benthamiana* plants infiltrated with Xp expressing XopJ4 (Fig. 2). This response was not observed in the *zar1-1* and *zar1-2* mutants.

Identification of JIM2, a kinase required for XopJ4 perception

The ZAR1 protein from *A. thaliana* interacts with several RLCK XII proteins which are required for the recognition of specific bacterial effectors including ZED1 (HopZ1a recognition) (Lewis *et al.*, 2013), RKS1 (AvrAC recognition) (Wang *et al.*, 2015) and ZRK3 (HopF2a recognition) (Seto *et al.*, 2017). We therefore hypothesized that an RLCK XII protein may be involved in the ZAR1-mediated recognition of XopJ4. Four RLCK XII genes were identified in the genome of *N. benthamiana* and targeted for silencing by VIGS (Fig. S5). The silencing of one particular RLCK XII, hereafter named XOPJ4 IMMUNITY 2 (JIM2), compromised the ability of the plant to recognize XopJ4 (Fig. S6). The sequence of JIM2 was deposited in the NCBI database (accession MH532571). A codon-altered version of JIM2, JIM2_VR, was designed to evade silencing by VIGS (Fig. S7). Transient expression of JIM2_VR in JIM2-silenced leaves rescued the cell death response triggered by XopJ4 (Fig. 3), demonstrating that JIM2 is required for recognition of XopJ4 in *N. benthamiana*.

Differential recognition of XopJ4 in *N. benthamiana*

In contrast to the results shown in Fig. 2, Xp ΔAvrBst ΔXopQ has previously been shown to grow and cause apparent disease symptoms on wild-type *N. benthamiana* (Schwartz *et al.*, 2015). We obtained the *N. benthamiana* used in the Schwartz *et al.* study and compared it with the 16c line used to generate the EMS population used for the screen. Transient expression of XopJ4 in the wild-type from the Schwartz *et al.* study did not result in a visible cell death response (Fig. S8). Additionally, a visible immune response to Xp ΔAvrBst ΔXopQ was not observed (Fig. S8). This explains why an avirulence effect of XopJ4 in *N. benthamiana* was not reported in the Schwartz *et al.* study and suggests that there may be genetic differences between various wild-type *N. benthamiana* used in different research laboratories. To test whether the lack of XopJ4 recognition in the *N. benthamiana*

Table 1 Candidate mutations in the *zar1-1* mutant.

				Mutant pool		WT pool				
Contig	Position	Ref[1]	Alt[2]	Coverage	Alt (%)[3]	Coverage	Alt (%)	Gene model	Closest *A.t.* hit[4]	Amino acid change
951	6091	C	T	48	100%	47	51%	Nbv5tr6207061	ZAR1	Q195Stop
3627	90609	G	A	39	77%	45	44%	Nbv5tr6202333	ATCKX6	G229R
2070	226450	C	T	32	88%	30	60%	Nbv5tr6200350		A18V

Illumina sequencing of pooled mutant and wild-type (WT) plants revealed three predicted candidate mutations for the phenotype of the *zar1-1 Nicotiana benthamiana* mutant.
[1]Nucleotide present in the reference genome.
[2]Alternative nucleotide detected.
[3]Percentage of sequencing reads supporting the alternative nucleotide.
[4]The annotated name for the most similar Arabidopsis protein.

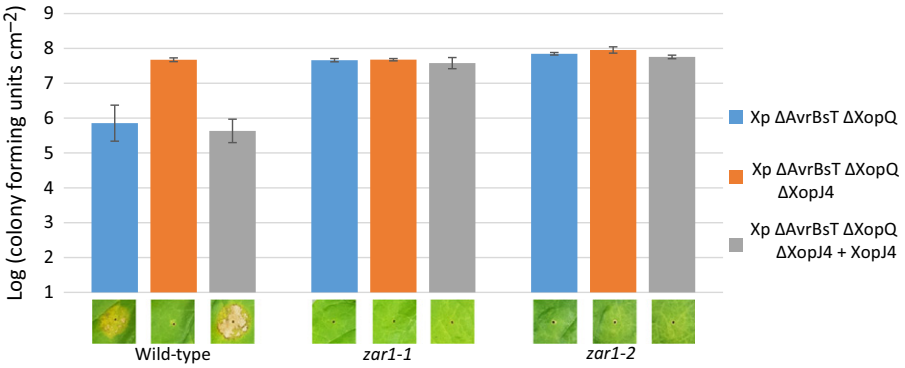

Fig. 2 Bacterial growth and visible immune response to *Xanthomonas perforans*. *Nicotiana benthamiana* wild-type and *zar1* mutants were infiltrated with the indicated genotype of *X. perforans* at an OD_{600} of 0.0001. Bacterial growth was assayed at 6 d post-infiltration and the visible immune response was photographed at 7 d post-infiltration. Error bars indicate ± SD from three biological replicates. Bacterial abundance at 0 d post-infiltration is presented in Supporting Information Fig. S4.

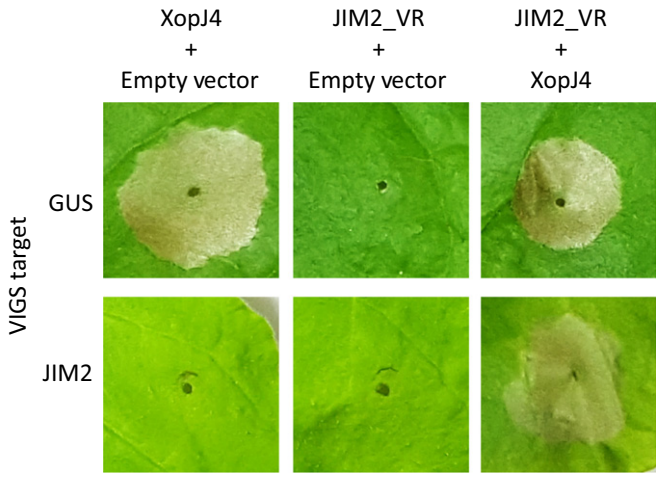

Fig. 3 Immune response to XopJ4 in JIM2 VIGS plants. The GUS gene (as a negative control) and JIM2 were targeted for gene silencing using VIGS in *Nicotiana benthamiana*. XopJ4 and a VIGS-resistant codon-altered version of JIM2 (JIM2_VR) were transiently expressed separately and together using *Agrobacterium*. The plants were infiltrated at an OD_{600} of 0.5 total and imaged at 3 d post-infiltration.

from the Schwartz *et al.* study could be complemented by transient expression of either NbZAR1 or JIM2, these proteins were expressed with and without XopJ4 in leaf tissue (Fig. S9). Expression of JIM2 but not NbZAR1 along with XopJ4 was observed to give a strong cell death response in this *N. benthamiana* variety (Fig. S9), suggesting that a defect in JIM2 may be responsible for the differential recognition of XopJ4. However, sequencing of the JIM2 gene did not reveal any polymorphisms between these two *N. benthamiana* lines and the basis for the differential response to XopJ4 therefore remains unknown. With the exception of the indicated images in Figs S8 and S9, all wild-type *N. benthamiana* plants used in this paper are from the 16c line used to make the mutant population.

AtZAR1 and SlZAR1 fail to complement the *N. benthamiana zar1-1* mutant

To gain insight into the evolution history of ZAR1, a phylogenetic tree was reconstructed using homologous protein sequences obtained from NCBI and the One Thousand Plant Genomes project (Matasci *et al.*, 2014) (Fig. S10). This analysis revealed that most plants have a single putative ortholog of ZAR1. To test whether AtZAR1 is functionally equivalent to NbZAR1, AtZAR1 was transiently expressed in the *zar1-1* mutant along with JIM2 and XopJ4. Whereas transient expression of NbZAR1 was sufficient to restore XopJ4 recognition in the *zar1-1* mutant, expression of AtZAR1 was not (Fig. 4). By contrast, transient expression of AtZAR1 in *zar1-1* was able to complement the immune response triggered by coexpression of ZED1 and HopZ1a, which is consistent with previously reported VIGS results (Baudin *et al.*, 2017). The inability of AtZAR1 to complement the XopJ4 perception defect in *zar1-1* plants indicates a partial functional divergence between NbZAR1 and AtZAR1.

Tomato (*Solanum lycopersicum*) contains a putative ZAR1 ortholog but is unable to perceive the XopJ4 effector protein (Astua-Monge *et al.*, 2000). SlZAR1 (Solyc02g084890) was cloned and transiently expressed in the *zar1-1* mutant to test if this gene can functionally complement NbZAR1 for XopJ4 perception. Transient expression of SlZAR1, JIM2 and XopJ4 failed to trigger a visible immune response in the *zar1-1* mutant (Fig. 5). A multiple sequence alignment of ZAR1 proteins from various plant species revealed several missense mutations at conserved sites in the SlZAR1 protein which may make the protein nonfunctional (Figs S3, S11).

JIM2 coimmunoprecipitates with NbZAR1 but not XopJ4

AtZAR1 has been shown to physically interact with the RLCK XII family proteins ZED1, RKS1 and ZRK3, and these interactions are believed to be important for effector perception (Lewis *et al.*, 2013; Wang *et al.*, 2015). We therefore hypothesized that there may be physical interactions between NbZAR1 and JIM2 and performed coimmunoprecipitation experiments to test for this. The Arabidopsis NLR protein RPP1 was included along with its cognate effector ATR1 as controls (Krasileva *et al.*, 2010). To avoid triggering a hypersensitive response, the experiments were performed in the *zar1-1 N. benthamiana* mutant using the T191I variant of NbZAR1 (the nonfunctional variant present in *zar1-2*). JIM2-6xHA was observed to be pulled down

Fig. 4 AtZAR1 complementation of *zar1-1*. *Agrobacterium* was used to transiently express the indicated genes in leaf tissue of wild-type *Nicotiana benthamiana* and the *zar1-1* mutant. The *Agrobacterium* was infiltrated at an OD_{600} of 0.3 for each construct and the plants were imaged at 2 d post-infiltration.

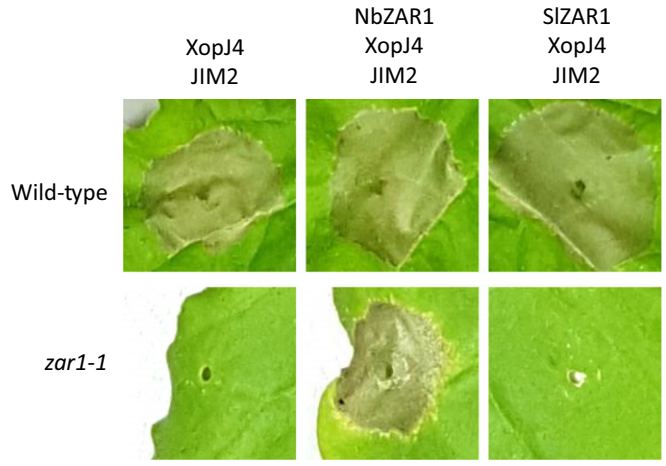

Fig. 5 Functional complementation testing of SlZAR1. The indicated genes were transiently expressed using *Agrobacterium* in *Nicotiana benthamiana* wild-type and the *zar1-1* mutant. The plants were infiltrated at an OD_{600} of 0.3 for each construct and imaged at 3 d post-infiltration.

with NbZar1-T191I-3xFlag (Fig. 6). JIM2-6xHA was not pulled down by RPP1-3xFlag. Interactions were not observed between XopJ4-6xHA and NbZar1-3xFlag or XopJ4-6xHA and JIM2-3xFlag.

Discussion

Mechanism of XopJ4 perception

Recognition of XopJ4 in *N. benthamiana* is dependent on the putative active site of these proteins (Roden *et al.*, 2004), suggesting that they are modifying a host protein, possibly by acetylation, which then triggers an immune response. The YopJ protein HopZ1a from *Pseudomonas* has been reported to acetylate the RLCK XII protein ZED1 in *Arabidopsis* (Lewis *et al.*, 2013), a paralog of JIM2. In contrast to recognition of HopZ1a, the ZAR1-mediated recognition of AvrAC involves not only an RLCK XII protein (RKS1) but an RLCK VII protein as well (PBL2) (Wang *et al.*, 2015). AvrAC directly interacts with and modifies PBL2 but not RKS1. JIM2 was found to interact with NbZAR1 but not XopJ4 by coimmunoprecipitation (Fig. 6). It remains unclear whether NbZAR1 and JIM2 are sufficient for XopJ4 recognition or if there is an additional component of the perception pathway. Although part of the RLCK superfamily, ZED1 lacks a conserved 'HRD' motif believed to be essential for kinase activity and is believed to be a pseudokinase (Lewis *et al.*, 2013). JIM2 is also missing the HRD motif and is therefore likely to also lack kinase activity (Fig. S12).

Evolutionary history of ZAR1

A single putative ortholog of ZAR1 was observed in most plant genomes, indicating that this gene has not undergone extensive duplication or divergence as observed for some other NLR genes (Fig. S10). This may be explained by the model that ZAR1 utilizes other proteins such as the RLCK XII's for specificity and does not directly interact with effector proteins (Innes, 2015). In this model, the RLCK XII's and other interacting proteins act as sensors that are under varying evolutionary pressures whereas ZAR1 acts as a conserved switch to activate the immune system. Some NLR proteins believed to be 'helper' NLRs, which are required for the activation of immune pathways but not directly perceiving a specific effector protein, have been observed to have a similar pattern of evolution as ZAR1 in which most plant species have one or a few copies of the gene which have limited sequence divergence. The helper NLR proteins NRG1 and ADR1 follow this model (Collier *et al.*, 2011). The ZAR1 phylogenetic tree supports the hypothesis that NbZAR1 and AtZAR1 are orthologs but a long branch to the Brassicales sequences (including AtZAR1) is a consequence of divergence of the ZAR1-

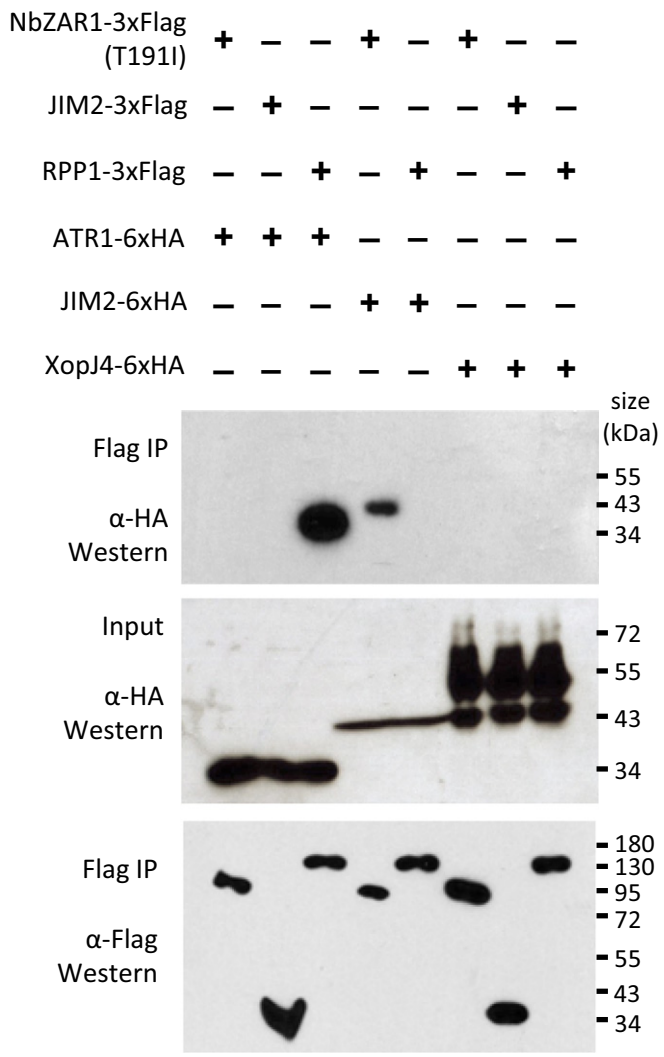

Fig. 6 Coimmunoprecipitation of NbZAR1, JIM2 and XopJ4. *Agrobacterium* was used to transiently express the indicated proteins in *zar1* mutant *Nicotiana benthamiana* plants. Protein was extracted and a coimmunoprecipitation experiment was performed using anti-Flag beads. Western blots were performed using the indicated primary antibodies of the input protein as well as the precipitated fraction. The Arabidopsis NLR protein RPP1 and its cognate effector ATR1 were included as controls. Expected molecular weights: NbZAR1-3xFlag 100 kDa, JIM2-3xFlag 42 kDa, RPP1-3xFlag 139 kDa, ATR1-6xHA 39 kDa, JIM2-6xHA 48 kDa, XopJ4-6xHA 49 kDa.

like proteins present in this plant order (Fig. S10). This suggests that AtZAR1 and related proteins from the Brassicales may have an altered functionality compared to the rest of the ZAR1-like proteins and may explain the inability of AtZAR1 to complement the *zar1-1* mutant for perception of XopJ4.

Utilization of NbZAR1 and JIM2 for engineering disease resistance into other crop species

The identification of NbZAR1 and JIM2 may facilitate engineering resistance against *Xanthomonas* in crop species. While putative orthologs of NbZAR1 are widely distributed among dicot species (Fig. S10), these genes may not be functionally interchangeable with NbZAR1 (as observed for AtZAR1, Fig. 4). Alternatively, these putative ZAR1 orthologs may have mutations that disrupt function, as appears to be the case for the tomato ZAR1 (Figs 5, S11). The inability of SlZAR1 to complement the *zar1-1* mutant is notable given that tomato and *N. benthamiana* are closely related and both belong to the Solanaceae family. Using the *N. benthamiana zar1* mutants and JIM2 VIGS plants allows for functional testing of homologous genes from target crop species. YopJ-family effector proteins are found in a diverse set of plant bacterial pathogens besides *Xanthomonas* including *Ralstonia*, *Pseudomonas*, *Acidovorax* and *Erwinia* (Lewis *et al.*, 2011). The role of NbZAR1 and JIM2 in providing resistance against pathogens with other YopJ effector proteins remains to be investigated.

Forward genetics in *N. benthamiana*

N. benthamiana is commonly used in plant biology research due to its amenability to transient expression of heterologous proteins and gene silencing. This plant has been used previously to identify components of the plant immune system using reverse genetics (Peart *et al.*, 2005; Schultink *et al.*, 2017; Wang *et al.*, 2018) but we are not aware of a previous example of EMS mutagenesis being used for forward genetics in this species. Although *N. benthamiana* has a large genome (*c.* 3.1 Gb) (Naim *et al.*, 2012) and is an allotetraploid with 19 chromosomes (Chase *et al.*, 2003), we demonstrated that it can be readily used for forward genetics. Despite the currently available reference genome for *N. benthamiana* being fragmented, causative mutations can be identified with only modest (30×) sequencing coverage of pooled mutant genomic DNA from a mapping population. This highlights the feasibility of doing forward genetics in nonmodel plants lacking a contiguous reference genome. The generation time of *N. benthamiana*, *c.* 3.5 months, is longer than that of the commonly used model Arabidopsis, but the ability to rapidly test candidate genes by transient expression or VIGS is a significant advantage. The potential to use transient expression as an assay in *N. benthamiana* for a desired process, including to test immune perception as demonstrated here, enables screens that are not possible in other model plants and allows for conducting independent screens on the same plant. We anticipate greater use of forward genetic mutant screens in *N. benthamiana* and other plants with complex genomes in the future to investigate processes that are not present in traditional genetic model plants.

Acknowledgements

We thank the laboratory of Peter Waterhouse for supplying EMS-mutagenized *N. benthamiana* seeds. We also thank Amanda McRae and Arielle Lee for technical assistance and Laurent Deslandes for comments on the manuscript. This material is based upon work that is supported by the National Institute of Food and Agriculture, US Department of Agriculture, under award number 2016-67012-25106. T.Q. is supported by the Tang Distinguished Scholarship at the University of California, Berkeley. This project was also supported by the Two Blades Foundation.

Author contributions

T.Q. performed the coimmunoprecipitation experiments. J.B. generated the mutant *N. benthamiana* population. A.S. performed the other experiments, designed the project and wrote the manuscript. B.S. designed and supervised the project.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

Fig. S1 Allelism test of *zar1-1* and *zar1-2*.

Fig. S2 Segregation of the Nbv5tr6207061 candidate mutation.

Fig. S3 Multiple protein align for AtZAR1, SlZAR1 and NbZAR1.

Fig. S4 Day zero growth assay.

Fig. S5 Phylogenetic tree of RLCK XII proteins.

Fig. S6 Silencing of RLCK XII genes.

Fig. S7 Sequence of the codon-altered, VIGS-resistant JIM2 construct.

Fig. S8 Differential XopJ4 response among wild type *Nicotiana benthamiana* varieties.

Fig. S9 Differential XopJ4 response complemented by expression of JIM2.

Fig. S10 Phylogenetic analysis of ZAR1.

Fig. S11 Multiple protein alignment for SlZAR1.

Fig. S12 Multiple protein alignment for RLCK XII proteins.

Table S1 Primer sequences.

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