Identification of a host 14-3-3 protein that interacts with *Xanthomonas* effector AvrRxv

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ABSTRACT

AvrRxv is a member of a family of pathogen effectors present in pathogens of both plant and mammalian species. *Xanthomonas campestris* pv. *vesicatoria* strains carrying AvrRxv induce a hypersensitive response (HR) in the tomato cultivar Hawaii 7998. Using a yeast two-hybrid screen, we identified a 14-3-3 protein from tomato that interacts with AvrRxv called *AvrRxv* interactor 1 (ARI1). The interaction was confirmed *in vitro* with affinity chromatography. Using mutagenesis, we identified a 14-3-3-binding domain in AvrRxv and demonstrated that a mutant in that domain showed concomitant loss of interaction with ARI1 and HR-inducing activity in tomato. These results demonstrate that the AvrRxv bacterial effector recruits 14-3-3 proteins for its function within host cells. AvrRxv homologues YopP and YopJ from *Yersinia* do not have AvrRxv-specific HR-inducing activity when delivered into tomato host cells by *Agrobacterium*. Although YopP itself cannot induce HR, its C-terminal domain containing the catalytic residues can replace that of AvrRxv in an AvrRxv-YopP chimera for HR-inducing activity. Phylogenetic analysis indicates that the sequences encoding the C-termini of family members are evolving independently from those encoding the N-termini. Our results support a model in which there are three functional domains in proteins of the family: translocation, interaction, and catalytic.

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1. Introduction

Xanthomonas campestris pv. *vesicatoria* (Xcv) (alternatively called *X. vesicatoria*, *X. axonopodis* pv. *vescicatoria* or *X. euvesicatoria*) is the causal agent of bacterial spot disease in both pepper and tomato [1,2]. Xcv strains carrying the avirulence gene *avrRxv* elicit resistance, characterized by localized necrosis referred to as the hypersensitive response (HR), in the tomato cultivar Hawaii 7998 [3]. AvrRxv-specific resistance in Hawaii 7998 is genetically complex [3,4]. It was found that *avrRxv* induces HR on a variety of non-host plants when expressed in *Xanthomonas* pathogens, including bean, soybean, cowpea, alfalfa, and cotton [5]. In contrast to AvrRxv-specific host resistance, the non-host resistance in bean is simply inherited. Although the plant HR response to *avrRxv* appears to be highly conserved, the genetic control of the resistance is not.

AvrRxv is a member of a family of pathogen effectors [6–8] that are translocated into host cells by the Type III secretion system (TTSS). AvrRxv exerts its effect intracellularly [9]. In addition to AvrRxv, there are three other AvrRxv family members present in Xcv. These are AvrBsT, XopJ and AvrXv4 [6,10,11], all of which are translocated to the host cell [8,12,13]. Genes with high levels of sequence similarity are also present in other plant pathogens [14–18], and in the plant symbiont *Rhizobium* [19]. Furthermore, AvrRxv-like sequences are found in mammalian pathogens, including YopJ and YopP from *Yersinia* spp., AvrA from *Salmonella*, and VopP from *Vibrio parahemeolyticus* [7,20–22]. Although there are nuclear localization signal sequences in many of these family members and PopP2 has been shown to localize to the nucleus, most AvrRxv family members, including AvrRxv, PopP1 and AvrXv4, apparently do not [6,13,17,23]. XopJ was shown to localize to the plasma membrane by virtue of an N-myristolylation motif, also essential for ability to induce HR on *Nicotiana* [24].

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Recent studies suggest that YopJ and VopP are acetyltransferases, which regulate signaling by interfering with phosphorylation [25]. Previously, it was hypothesized that family members were similar to cysteine proteases [8,26,27]. Apparently, the catalytic triad that is conserved in all members of the family may have acetyltransferase rather than protease activity [25]. Mutation of any amino acid in the triad results in the loss of function in YopJ, AvrBsT, AvrRxv and AvrXv4 [8,13,23]. It is not clear how to interpret analyses indicating that YopJ and AvrXv4 disrupt SUMO post-translation modifications in host cells [8,13], except possibly by affecting expression levels [25]. In mammalian hosts, YopJ exerts its effect on several known signal transduction pathways, thereby preventing an immune response [28–33]. Mukherjee and coworkers [29] showed that YopJ prevents activation of the mitogen-activated protein kinase (MAPK) and nuclear factor κB (NFκB) signaling pathways through its function as an acetyltransferase. Acetylation of MKKs by YopJ prevents activation. Similarly, VopA has also been shown to be an acetyltransferase that inhibits MAPK signaling by inactivating kinase activity [22]. This family of effectors directly manipulates host cell signaling.

Molecular genetic control of plant host responses specific to these effectors is under study. Resistance to AvrXv4, AvrBsT and PopP2 is controlled by single cognate plant genes [10,34,35]. It has been shown PopP2 physically interacts with the *Arabidopsis* resistance protein RRS1-R in yeast and co-localizes with RRS1 proteins in the nucleus [17]. The association between the putative transcription factor function of the TIR-NBS-LRR-WRKY RRS1 proteins and sumoylation or acetylation, if any, is not known. Resistance associated with AvrBsT in *Arabidopsis* is conferred by the lack of a serine hydrolase with carboxylesterase activity called SOBER1 [34]. SOBER1 preferentially works on molecules containing short chain acyl groups. It is possible that in the absence of SOBER1, AvrBsT modulates post-translational modification of a common substrate by affecting acylation [34]. The interactions of YopJ with members of mammalian signal transduction pathways are direct as demonstrated by Orth and coworkers [30] who isolated several MAPKs using YopJ as bait in a yeast two-hybrid screen. Because AvrRxv shares significant sequence identity with other members of the family, it is plausible that AvrRxv also directly interacts with and disrupts signaling pathways in host plants.

14-3-3 Proteins are ubiquitous acidic proteins that are involved in a great variety of cellular events mediated by protein–protein interactions [36–38]. Originally named for brain proteins based on electrophoretic mobility and column fractions, 14-3-3 proteins form homo- and/or heterodimers that serve as regulatory adapter proteins in diverse cellular functions, including metabolism, protein targeting, and signal transduction [39–41]. Most 14-3-3 proteins bind specifically to phospho-serine proteins. Analysis of known 14-3-3-binding sites and peptide libraries identified motifs that are bound by 14-3-3 proteins. The first motif discovered is RSXpSXP (X is any amino acid), where the serine is phosphorylated [36,38]. This motif is called the Raf motif because it was first identified in the Raf-1 protein kinase. In addition, there is a 14-3-3-binding motif that does not require phosphorylation called the R18 motif, found in an inhibitory peptide (DI/L/VE) [42]. Although the vast majority of 14-3-3 interactions requires phosphorylation, it is not required for binding of 14-3-3 proteins to several cellular proteins in mammals, most importantly, the ADP-ribosyltransferase effector ExoS of *Pseudomonas aeruginosa* [43–46]. Mutation of the key residues in ExoS for binding to the 14-3-3 inhibits virulence of *P. aeruginosa* in a cell infection model [47,48].

The involvement of some 14-3-3 proteins in plant host responses to pathogens is inferred by their transcriptional upregulation [49–52]. In addition, the ankyrin repeat-containing protein AKR2 that was shown to bind to a 14-3-3 protein controls level of resistance to a bacterial pathogen in *Arabidopsis* [53]. In mammalian cells, 14-3-3 proteins are involved in regulating apoptosis [54]. In addition, 14-3-3 proteins have been observed to be associated with kinases, often in large complexes [37]. Many of the mammalian protein kinases that associate with 14-3-3s lie in the same pathways (like the MAPK and ERK pathways) that are affected by the *Yersinia* effector proteins YopJ and YopP.

In this study we report the identification of a 14-3-3 protein, called ARI1, that interacts with AvrRxv. Xcv strains carrying AvrRxv mutants disrupted for 14-3-3-binding activity can no longer elicit an HR in tomato. We also analyzed the HR-inducing activity of AvrRxv homologues from *Yersinia*, when delivered by Xcv or expressed by the host. We show that although YopP itself cannot induce HR, its C-terminal domain containing the catalytic residues can replace that of AvrRxv for HR-inducing activity. These results demonstrate that the AvrRxv bacterial effector recruits 14-3-3 proteins for its function within host cells and that the catalytic residues can be swapped between diverse AvrRxv family members.

2. Materials and methods

2.1. Plant growth and inoculations

All tomatoes (*Solanum esculentum*) were grown from seed in 4-inch-diameter plastic pots with either UC or Sunshine potting mix in a greenhouse with ambient light. All plant inoculations were performed with either a plastic Pasteur pipet or a 1-cc syringe. Inoculated plants were maintained in Conviron E8 growth chambers (Controlled Environment, Inc., Winnipeg, Manitoba, Canada) with 16 h photoperiods. Plant inoculations of *Xanthomonas* stains at 5×10^8 CFU/ml in H_2O were performed on three to four different plants on the fourth to seventh leaves and experiments were done in triplicate. To assess the effect of gene expression *in planta*, *Agrobacterium*-mediated gene transfer was performed in tomato leaves as described [55,56].

2.2. Bacterial strains and molecular biology techniques

Bacterial strains used in this study were Xcv strains 89-1, 92-14, and 92-14 avrRxv:Ω [3,6]; *Eshcherichia coli* strains DH5α and TOP10 (Invitrogen, Carlsbad, CA, USA); *Agrobacterium tumefaciens* C5C81 ± vir [57]. Bacterial strains were grown as described by Morales et al. [55].

Standard molecular biology techniques were used for manipulating plasmids, ligation, transformation, and performing polymerase chain reaction (PCR) [58]. DNA for sequencing was isolated using Qiagen plasmid isolation kits (Valencia, CA, USA). Amplification products were cloned using Topo TA Cloning kit according to instructions (Invitrogen) and sequenced. For RNA isolations, *Xanthomonas* cells were lysed at 100 °C in 50 mM EDTA, 0.3 M Na-acetate, 0.625% SDS, 50 mM Tris HCl, pH 8.0. After two extractions each with phenol at 65 °C and chloroform, the nucleic acids were precipitated with isopropanol and then 4 M lithium acetate. The integrity of the RNA was determined by denaturing gel electrophoresis.

To determine the 5′-terminus of the transcription product of *avrRxv*, primer extension was performed [58]. Ten pmol each of four primers (MLP1, 5′-TCTAGGTTGCACTCTTATGGAGTC-3′; MLP3, 5′-TGAGCCGCCCACTCCTAATGAT-3′; MLP4, 5′-TGATGTTGAAAAC-GACTGCTGCTT-3′; DA1, 5′-CCGCCCACTCCTAATGATCTGA-3′) was labeled using T4 Polynucleotide Kinase (Promega, Madison, WI, USA) and (γ-^{32}P)ATP in T4 PKN buffer according to the manufacturer's instructions. Labeled primers were purified using Pharmacia Nick Column Sephadex G-50 (Pharmacia Biotech, Piscataway, NJ, USA), annealed to 15 μg denatured RNA at 65 °C for 3 h in first strand buffer (Promega), and extended at 42 °C with Superscript II Reverse Transcriptase (Invitrogen) and 0.1 M DTT, 7 μg actinomycin

D (Sigma–Aldrich, St. Louis, MO, USA), 35 U recombinant RNasin ribonuclease inhibitor (Promega) and 10 mM dNTPs. After extension, the samples were treated with RNAse A at 37 °C for 15 min, extracted with phenol, chloroform and iso-amylalcohol (25:24:1) and then precipitated with ethanol. An ^{35}S-dATP-labeled dideoxy sequence ladder was generated on pUC118RXV3 [3]. After fractionation, samples were visualized either by autoradiography or with a phosphoimager (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

2.3. Interaction screen and confirmation of interaction

A tomato cDNA library was made from the tomato cultivar Hawaii 7998, and cloned into the phagemid vector Hybrizap (Stratagene, La Jolla, CA, USA), containing the activation domain. The coding sequence of AvrRxv was cloned into the yeast two-hybrid vector pBD as a gene fusion with Gal4 DNA-binding domain (Stratagene), creating pBD-avrRxv. The pBD-avrRxv construct was transformed into the yeast strain PJ69-4a and tested for autoactivation of reporter genes. The yeast two-hybrid screen was carried out according to the manufacturer's instructions (Stratagene).

Full length *ARI1* was cloned with 5′ RACE. The *ARI1* open reading frame was tagged at its 3′-end with the c-myc epitope tag sequence using Tft9-Eco-For, 5′-GAATTCATGGCTTCTTCCAAAGAACG-3′ and 14-3-3-c-myc-Bam-rev, 5′-GGATCCTCACAAATCTTCTTCAGAAATCA ACTTTTGCTCTGCATCTTCACCTCCACCAGC-3′ into pCRII and then cloned into pAD and pRSETB on an *Eco*RI fragment from a partial digest, creating pAD-Ari1-myc, and p6XHis-Ari1-myc, respectively.

The interaction in the yeast two-hybrid system of tagged versions of the proteins was confirmed using pBD-avrRxv-Flag and pAD-Ari1-myc. The interaction of 6XHis-Ari1-Myc and AvrRxv-Flag was verified *in vitro* by co-immunoprecipitation. The MEGAscript T7 Transcription kit (Ambion, Austin, TX, USA) and the Flexi Rabbit Reticulocyte Lysate System (Promega) were used according to the manufacturer's instructions. *In vitro* translated ^{35}S-methionine labeled AvrRxv-Flag lysates were incubated alone and with excess unlabeled 6XHis-ARI1-Myc overnight, with Ni-NTA agarose according to the instructions (Qiagen, Valencia, CA, USA). Eluted proteins were size fractionated on a 4–20% Tris–glycine polyacrylamide gradient gel and immunoblotted as described below.

2.4. Cloning of AvrRxv variants, homologues and chimeras

PCR-mediated cloning was used to construct clones of *avrRxv* subclones, variants, homologues and chimeras incorporating restriction enzyme sites. After sequence verification of PCR products cloned into pCRII (Invitrogen), inserts were cloned behind the lac promoter in pVSP61 (DNA Plant Technology, Oakland, CA, USA) or pDSK519 [59] for testing in *Xanthomonas*, behind the Cm^R promoter in pTM100 for testing in *Xanthomonas* [60], or behind the 35S promoter in binary vectors pCB302 [61] or pMD1 [62] for testing in *Agrobacterium*.

The *Yersinia* homologue *yopJ* was cloned from pJ [28] into pVSP61 on a 1.8-kb *Sal*I/*Eco*RI fragment, creating pGPB3. The *yopJ* gene was cloned into pCRII and pMD1 using PCR primers yopJ-U34XbaI, 5′-TGCGTCTAGATGTCATACCGCTGTTAATTCCCTG-3′ and yopJ-L34XhoI, 5′-ATTCTCGAGGGGGTATTCCCATACTGGAGCAAGA-3′, incorporating *Xba*I and *Xho*I sites. *yopJ* was removed from pCRII on an *Eco*RI digest and cloned into pBD.

The *yopP* gene was cloned from *Y. enterolitica* plasmid JB580v using PCR and the following primers, yopP-fwd, 5′-GAATTCAT-GATTGGGCCAATATCAC-3′ and yopP-rev, 5′-GTCGACTTATACTTTGA GAAGTGT-3′, incorporating *Eco*RI and *Sal*I sites. *yopP* was removed from pCRII on an *Eco*RI fragment and cloned into pTM100 and pCB302, and on an *Eco*RI/*Sal*I fragment into pBD.

Chimeras were made between *yopP* and *avrRxv* by sequence overlap extension (SOE) as described in Ciesiolka et al. [6]. The chimeric coding sequence for N-AvrRxv$_{1-172}$-YopP$_{104-289}$-C was made with PCR using primers avrRxv/yopP-fwd, 5′-GAGCGG-TAGTGCGGCTGGGGGAGGGTGGAATACATTTC-3′ and avrRxv/yopP-rev, 5′-GAAATGTATTCCACCCTCCCCCAGCCGCACTACCGCTC-3′, along with F1-avrRxv, 5′-ATGTGCGACTCCATAAGAGTG-3′, and yopP-rev to make pCRII-RY4. For N-YopP$_{1-103}$-AvrRxv$_{173-373}$-C, primers yopP/avrRxv-fwd, 5′-CGCTTCATAATTAACATGGATGAAGACCCTAGGAG-3′ and yopP/avrRxv-rev, 5′-CTCCTAGGGTCTTCATCCATGTTAATTAT-GAAGCG-3′ were used with yopP-fwd and 3′-avrRxv-Sal, 5′-GTCGACATTGTCTCAGGATTGTAAGGC-3′ to make pCRII-YR3. The resulting chimeras were cloned into pTM100 and pCB302 on *Eco*RI fragments.

Mutations in two putative 14-3-3-binding sites in AvrRxv were made using SOE. For the AvrRxv-Raf mutant, primers AvrRXV-D 147A-fwd, 5′-CTGCGCAGCTTCGCCACTCCG-3′, and AvrRXV-D147A-rev, 5′-CTGCCCCGGAGTGGCGAAGCT-3′ incorporated the D147A mutant codon, and were used along with 5′avrRxv-Nco, 5′-GCCCAT GGATGTGCGACTCCATAAG-3′, and 3′avrRxv-BamHI, 3′-CGGATCCAT TGTCTCAGGATTGTAAGGC-3′ and ligated into pCRII, to make pCRII-avrRxv-Raf. An *Eco*RI fragment was cloned from that into pTM100, pCB302 and pBD. For the AvrRxv-R18 mutant, SOE primers MNIE-F, 5′-GAGTCACTTCGGCTGATGAACATTGAAAATCTC-3′, MNIE-R, 5′-CAGATGGGGGAGATTTTCAATGTTCATCAGCCG-3′, 5′avrRxv-Nco and 3′avrRxv-BamHI were used to incorporate the D123N mutation, and ligated into pCRII to make pCRII-avrRxv-D123N. pCRII-avrRxv-D123N was used as a template for SOE for the second mutation E125Q, and the product ligated into pCRII to make pCRII-avrRxv-R18. The insert was cloned into pTM100, pCB302 and pBD on an *Eco*RI fragment. To make a mutant in both the Raf and R18 domains, pCRII-avrRxv-R18 was used as a template for SOE with the Raf mutagenic primer sets, and cloned into pCRII to make pCRII-avrRxv-Raf/R18. The insert was cloned into pBD on an *Eco*RI fragment to make pBD-avrRxv-Raf/R18.

For interaction tests, the *avrRxv* open reading frame was tagged at the 3′-end using the FLAG epitope coding sequence [9] using primers avrRxv-BamHI, 5′-GGATCCATGTGCGACTCCATAAG-3′ and Xba-Flag-rev, 5′-TCTAGATCACTTATCATCATCATCCTTGTAATCGGATT CTAAGGCGTGACGGATCTTTCG-3′ and cloned into pCRII. The avrRxv-Flag insert was cloned into pCB302 and pBD on an *Eco*RI fragment.

Two *avrRxv* deletion constructs were made in the pBD bait vector. An N-terminal deletion AvrRxv$_{142-373}$ was constructed using PCR primers 5′M/avrRxv-EcoRI, 5′-GGGAATTCCTGCGCAGCTTCGA-CACTCCG-3′ and 3′-avrRxv-Sal. The PCR product was ligated into pCRII and then cloned on an *Eco*RI/*Sal*I fragment to make pBD-avrRxv-5′Δ. The C-terminal deletion AvrRxv$_{1-315}$ in pBD-avrRxv-3′Δ was constructed by cloning an *Eco*RI/*Xho*I fragment into pBD cut with *Eco*RI/*Sal*I.

Mutant *avrRxv* genes in the catalytic domain were made by SOE and are named by the amino acid codon that changed. Primers (Table 1) were used in the following combinations for wildtype-untagged, EG16/EG13; for wildtype-HA, EG4/EG10; for H180A-untagged, EG16/EG15, EG14/EG13, EG16/EG13; for H180A-HA, EG6/EG12, EG11/EG10, EG6/EG10; for E200A-untagged, EG16/IL, IR/EG13, EG16/EG13; for E200A-HA, EG4/IL, IR/EG10, EG4/EG10; for C244A-untagged, EG6/EG9, EG8/EG7, EG6/EG7; for C244A-HA, EG4/EG5, EG2/EG1, EG4/EG1; for E258A-untagged, EG16/EG18, EG17/EG13, EG16/EG13; and for E258-HA, EG6/EG18, EG17/EG10, EG6/EG10; to clone into pCRII and then into pDSK519 and pMD1 on *Xba*I/*Bam*HI fragments. An *Eco*RI sublcone from pCRII-avrRxv was cloned into pTM100, pCB302 and pBD. An *Eco*RI/*Hin*dIII fragment containing *avrRxv* from pUC118RXV3 [3] was cloned into pDSK519, creating pXVSC910, and into pVSP61, creating pLLM1.

Table 1
Primers used to construct mutant variants of *avrRxv* by sequence overlap extension

Primer name	Primer sequence, 5′ to 3′[a]
EG1	TGGATCCTCACGCATAGTCAGGCACATCGTAAGGGTAGGATTCTAAGGCGTGACG
EG2	GCGGAAGCACTTAAGTCAATCGGTGGG**GC**TGTCATATTTTCTCTTGATTATGC
EG4	TTCTAGATGTGCCGACTCCATAAGAGTGCAATTCAGATCCATACAAAAAATGGT
EG5	GCATAATCAAGAGAAAAATATGACA**G**CCCCACCGATTGACTTAAGTGCTTCC
EG6	TTCTAGATGTGCCGACTCCATAAGAGTGCAATTCAG
EG7	TGGATCCTCAGGATTCTAAGGCGTGACG
EG8	TCAATCGGTGGG**GC**TGTCATATTTTCTCTTGAT
EG9	AAAAATATGACA**GC**CCCCACCGATTGACTTAAG
EG10	TGGATCCTCACGCATAGTCAGGCACATCGTAAGGGTAGGATTCTAAGGCGTGACGG
EG11	GAAGACCCTAGGAGATGG**GC**TCGCGTCGCGTTCGACGTGCGCAAC
EG12	TCGAACGCGACGCGA**GC**CCATCTCCTAGGGTCTTC
EG13	TGGATCCTCAGGATTCTAAGGCGTGACGGGATCTTTCGAGCTCT
EG14	ACCCTAGGAGATGG**GC**TCGCGTCGCGTTCGACGTG
EG15	CACGTCGAACGCGACGCGA**GC**CCATCTCCTAGGGT
EG16	TGCCCTCTAGACGGCTGTGCCGACTCCATAAGAGTGCAATTCAG
E200A-IL	CAATGCGATAATCGTCGTGT
E200A-IR	GATTATCGCATTGG**CT**CCTG

[a] TCTAGA *Xba*I site; GGATCC *Bam*HI site; bold, altered nucleotide to change codon to alanine.

2.5. Immunoblotting

To assess the stability of mutant proteins, immunoblots were performed on the catalytic domain mutants. Xcv was grown on NYG medium and resuspended to an OD_{600} of 2.0 with 100 mM $MgCl_2$. A 20-μL aliquot was mixed 1:1 with $3\times$ Laemmli buffer, boiled for 5 min and centrifuged at $14,000 \times g$ for 5 min. Fifteen microliters of the supernatant was fractionated by 8–12.5% SDS-PAGE and transferred onto a nitrocellulose membrane (BioRad, Hercules, CA, USA). Immunoblotted membranes were incubated with mouse monoclonal antibodies that recognize HA (Covance, Berkeley, CA, USA) according to the manufacturer's instructions. Detection was performed using the EZ-ECL Chemiluminescence Detection kit for HRP (Biological Industries, Kibbutz Beit Haemek, Isreal) or SuperSignal West Pico (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's protocols.

2.6. AvrRxv family phylogeny

Homologues of AvrRxv, PopP1 and YopP were identified with BLASTP [63]. Amino acid and nucleotide sequences were aligned using ClustalX version 1.83.1 [64]. To optimize the nucleotide sequence alignments, nucleotide triplets were manually aligned using the amino acid alignments as a guide using McClade 4 (Sinauer Associates, Inc., Sunderland, MA, USA). Gaps were maintained. The 5′- and 3′-coding sequences were split at the gap in the amino acid alignment of family members that precedes D_{173} in AvrRxv. Phylogenetic relationships of coding sequences were inferred by using PAUP*Ver.4.0b10 (Sinauer Associated, Inc.) to implement likelihood analyses. Maximum likelihood models were generated using ModelTest version 3.6 [65]. A Jukes-Cantor neighbor-joining tree [66] was created to estimate its parameters based on the best-fit model from ModelTest. These initial parameter estimates were used to begin an iterative maximum likelihood analysis using a neighbor-joining heuristic search until the tree parameters converged [67]. To evaluate statistical support for the tree branches, a bootstrap analysis using a heuristic search of 100 random stepwise addition replicates was executed [68].

In addition, the two tree topologies based on either end of the gene were compared using the Shimodaira–Hasegawa test [69] to test for significant divergence in tree lengths. This test was performed using RELL with 1000 bootstrap replicates and the results evaluated as a one-tailed test. The Likelihood Ratio Test (LRT) indicated that $HKY + \Gamma$ was the most appropriate model for the 5′ dataset. This model allows for rate variation between transitions

and transversions (Ti/Tv = 0.922796), unequal base frequencies ($A = 0.248985$, $C = 0.289080$, $G = 0.271095$, $T = 0.190841$), and Γ distribution ($\alpha = 2.148966$ across four categories). The maximum likelihood search using this model resulted in one tree ($-\ln L = 13033.63456$). For the 3′ dataset, the Likelihood Ratio Test (LRT) indicated that $HKY + \Gamma$ was again the most appropriate model. This model allows for rate variation between transitions and transversions (Ti/Tv = 0.985939), unequal base frequencies ($A = 0.250419$, $C = 0.259215$, $G = 0.275177$, $T = 0.215188$), and Γ distribution ($\alpha = 1.470186$ across four categories). The maximum likelihood search using this model resulted in one tree ($-\ln L = 15691.99289$). All of the effector's N-termini and C-termini were also compared individually with BlastP to those of AvrRxv and YopP to further analyze the general evolution of the two termini. Results are expressed as % similarity, which takes into account conservative amino acid substitutions according to the BLOSUM62 algorithm [63].

3. Results

3.1. AvrRxv interacts with a 14-3-3 protein in the yeast two-hybrid system

To identify potential host factors that interact with AvrRxv, a cDNA library from tomato Hawaii 7998 was co-transformed with *avrRxv* in the yeast two-hybrid system system. The pBD-avrRxv construct did not autoactivate in yeast strain PJ69-4a, and the fusion protein was detected in yeast extracts by immunoblot analysis. The pBD-avrRxv bait construct was used to screen approximately 5×10^6 yeast cfu expressing clones of the Hawaii 7998 cDNA library for potential interactors. Several potential positives were identified in the primary screen and sequenced. More than half were ribosomal proteins that were not further characterized. The remaining potential interactors were re-transformed into yeast strain PJ69-4a containing the bait plasmid pBD-avrRxv and re-tested for interaction. One protein, ARI1 (AvrRxv interactor 1), continued to interact with AvrRxv in the secondary screens (Table 2). The ARI1 sequence is a 14-3-3 family member and is nearly identical to the previously cloned 14-3-3 protein from tomato, TFT9 (Genbank accession no. X98865) [51]. The pAD-Ari1 was tested for autoactivation by co-transforming it with either pBD or pBD-Lamin C. Neither the pBD and pAD-Ari1, nor the pBD-Lamin C and pAD-Ari1 plasmid pairs allowed growth of the yeast strain on selective media or induced β-galactosidase (Table 2). YopJ and YopP cloned as pBD fusions did not interact with ARI1. ARI1 was also tested for promiscuous interaction with the plant disease resistance gene product PTO, and a constitutively active mutant PTO Y207D [56]. No interaction was observed using PTO or PTO Y207D.

To localize the ARI1 interaction domain within the AvrRxv sequence, two *avrRxv* deletion constructs were tested for interaction. An N-terminal deletion of $AvrRxv_{142-373}$, contained in pBD-avrRxv 5′Δ, did not interact with ARI1 (Table 2). The first 141 amino acids of AvrRxv are essential for interaction with ARI1. On the other hand, the C-terminal deletion of $AvrRxv_{1-315}$ did interact. In fact, the β-galactosidase activity associated with the interaction of ARI1 with the C-terminal deletion $AvrRxv_{1-315}$ in pBD-avrRxv 3′Δ was twice that of the full-length construct (Table 2). The C-terminal 58 amino acids of AvrRxv, which are C-terminal to the protease domain, are not only dispensable for interaction with ARII, but may normally inhibit the interaction.

3.2. Mutational analysis of 14-3-3 protein-binding sequence motifs in AvrRxv

Analysis of the AvrRxv sequence indicated that there are two domains in AvrRxv that resemble known 14-3-3-binding motifs,

Table 2
Yeast two-hybrid screen for proteins that interact with AvrRxv

Interactors		Interaction analysis		
		Growth on medium[c]		Expression
Bait[a]	Target[b]	Adenine minus	Histidine minus	β-gal Activity[d]
pBD-p53	SV40	+	+	4.38 ± 0.02
pBD-Lamin C	SV40	−	−	0.83 ± 0.20
pBD-Lamin C	ARI1	−	−	0.00 ± 0.00
pBD	ARI1	−	−	0.84 ± 0.12
pBD-avrRxv	ARI1	++	++	61.44 ± 3
pBD-avrRxv 3′Δ	ARI1	++	++	122.33 ± 9
pBD-avrRxv 5′Δ	ARI1	−	−	0.94 ± 0.40
pBD-avrRxv:Raf	ARI1	++	++	55.82 ± 7
pBD-avrRxv:R18	ARI1	±	±	8.35 ± 4
pBD-avrRxv:Raf/R18	ARI1	±	±	17.57 ± 5

[a] Vector pBD contains the Gal4 DNA-binding domain.
[b] Target coding sequence constructs are in pAD, containing the activation domain.
[c] Bait and target constructs co-transformed into yeast strain pJ69-4a; no growth, −; some slow growth, ±; good growth, +; very good growth, ++.
[d] β-Galactosidase activity in Miller units.

RSFDTP (called Raf domain) and MDIE (called R18 domain) [42,45]. To test if either the Raf or R18 domain is important for the AvrRxv and ARI1 interaction, mutant versions of AvrRxv were assayed for interaction with ARI1 in yeast. The altered AvrRxv-Raf in which the aspartic acid in the putative Raf domain (RSF*D*TP) was changed to an alanine (D147A), behaved no differently than the full length AvrRxv (Table 2). Whereas, AvrRxv-R18 in which the two negatively charged amino acids aspartic acid and glutamic acid in the R18 domain M*DIE* were changed to the positively charged residues asparagine and glutamine (D123N/E125Q), interacted significantly less based on growth phenotype and β-galactosidase activity (14% of wildtype activity). These results are consistent with results from the N-terminal deletion mutant AvrRxv$_{142–373}$, which contains the Raf-like motif but lacks the R18 motif (Table 2). The AvrRxv Raf/R18 mutant containing all three mutations interacted less than wildtype, but more than the R18 mutant. Yeast strains containing the AvrRxv Raf/R18 mutant, compared to wildtype AvrRxv exhibited less growth on selective media and β-galactosidase activity (29% wildtype activity). Accordingly, neither AvrRxv-R18 nor AvrRxv–Raf/R18 induced HR when carried by Xcv or delivered by *Agrobacterium* (Table 3). However, AvrRxv-Raf was no different from wildtype AvrRxv. These results suggest that the ARI1 interaction, mediated by the R18 interaction domain in AvrRxv, is essential for AvrRxv's function *in planta*.

3.3. AvrRxv and ARI1 physically interact *in vitro*

To confirm the yeast two-hybrid results, the interaction of ARI1 and AvrRxv was tested using affinity chromatography and immunoprecipitation. The c-myc epitope-tagged and 6XHis-tagged ARI1 in the pAD vector maintained their interaction in yeast with AvrRxv and AvrRxv-Flag, encoded in pBD-avrRxv and pBD-avrRxv-Flag, respectively. The cloned avrRxv-Flag was shown to maintain HR-inducing activity in Xcv as shown previously [9]. *In vitro* translated ^{35}S-methionine labeled AvrRxv-Flag lysates were incubated with excess unlabeled 6XHis-ARI1-Myc and the mixture was affinity purified on Ni-NTA columns. AvrRxv-Flag proteins co-eluted with the 6XHis-ARI1-Myc fraction (Fig. 1). No AvrRxv-Flag was found in the eluant of the control column that lacked 6XHis-ARI1-Myc, although it was detected in the flow-through fraction of the control column. These results confirm the interaction of AvrRxv and ARI1.

An alternative approach to verify the interaction *in vitro* was used as well. Recombinant AvrRxv-Flag labeled with ^{35}S-methionine was mixed with equal amounts of recombinant ARI1-Myc or negative control luciferase and immunoprecipitated with anti-Myc antibody and Dynabeads Protein G. Although there was cross-reactivity of the AvrRxv-Flag with the Dynabeads Protein G, the intensity of the labeled protein band was four times greater in the AvrRxv-Flag and ARI1-Myc reaction, than in the negative controls AvrRxv-Flag alone or AvrRxv-Flag and luciferase reactions (data not shown). AvrRxv and ARI1 interact *in vitro*.

3.4. AvrRxv and YopP chimera has HR-inducing activity

AvrRxv homologues from *Yersina* were tested for activity in leaves of tomato lines Hawaii 7998 and Bonny Best (Table 4). AvrRxv induces a resistance, hypersensitive response (HR) on Hawaii and a susceptible, watersoaking (WS) response on Bonny Best, when carried by Xcv. Accordingly, when delivered by *Agrobacterium*, avrRxv induced HR on Hawaii, but not on Bonny Best. Both *Yersinia* homologues, YopJ and YopP, were not recognized by either plant line with both methods of delivery. However, when a chimera was made between the N-terminal 173 amino acids of AvrRxv and the C-terminal 185 amino acids of YopP, an HR was induced on Hawaii 7998 (Table 4; Fig. 2). In contrast, a chimera between the coding sequence for the N-terminal 103 amino acids of YopP and the C-terminal 200 amino acids of AvrRxv did not induce HR. It appears that the catalytic domain of AvrRxv family member

Table 3
Response of tomato lines to inoculation with *Xanthomonas* and *Agrobacterium* containing plasmid-borne AvrRxv and variants thereof

AvrRxv[b]	Tomato host response[a]					
	Xcv 89-1			*Agrobacterium*		
	Xcv plasmid[c]	Hawaii 7998	Bonny Best/Money-maker	Agro plasmid[d]	Hawaii 7998	Bonny Best/Money-maker
Wildtype	pTM100-avrRxv	HR	WS	pCB302-avrRxv	HR	NS
Raf[e]	pTM100-Raf	HR	WS	pCB302-Raf	HR	NS
R18[f]	pTM100-R18	WS	WS	pCB302-R18	NS	NS
Raf/R18	pTM100-Raf/R18	WS	WS	pCB302-Raf/R18	NS	NS
Wildtype	pXVSC910	HR	WS	pMD1-avrRxv	HR	NS
H180A	pDSK519-H180A	WS	WS	pMD1-H180A	NS	NS
E200A	pDSK519-E200A	WS	WS	pMD1-E200A	NS	NS
C244A	pDSK519-C244A	WS	WS	pMD1-C244A	NS	NS
E258A	pDSK519-E258A	HR	WS	pMD1-E258A	HR	NS

[a] Host responses are HR, hypersensitive response; WS, watersoaking; NS, no symptom.
[b] avrRxv wildtype gene and mutants, with an HA tag, induced same host response as untagged gene.
[c] Xcv carrying pTM100 or pDSK519 induced WS on Hawaii 7998 and Bonny Best.
[d] *Agrobacterium* carrying pCB302 or pMD1 induced NS on Hawaii 7998 and Bonny Best.
[e] Raf mutation in AvrRxv, D147A.
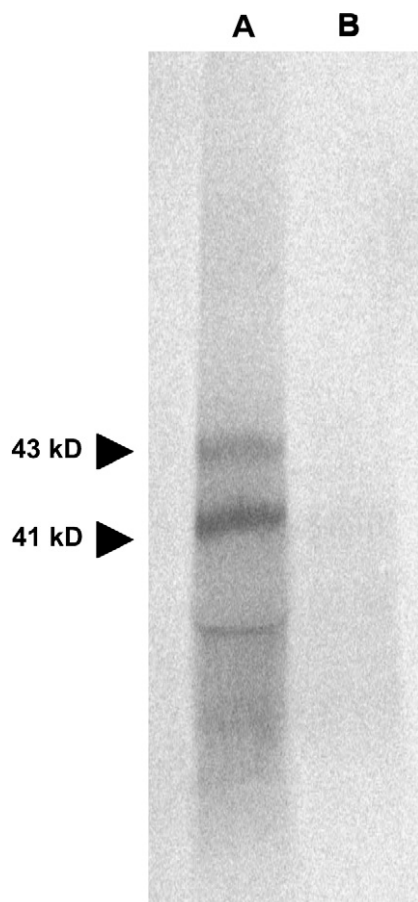[f] R18 mutation in AvrRxv, D123N/E125Q.

Fig. 1. Affinity purification of AvrRxv with ARI1. AvrRxv labeled with ^{35}S-methionine by *in vitro* translatation was affinity purified by interaction with ARI1-Myc containing an N-terminal 6XHis tag. Lane A: elution of ^{35}S-methionine labeled AvrRxv-Flag bound to 6XHis-ARI1-Myc from Ni-NTA column; Lane B: elution of ^{35}S-methionine labeled AvrRxv-Flag alone from Ni-NTA column. Arrows mark two AvrRxv translation products.

YopP functions to induce the host response associated with AvrRxv, when the appropriate AvrRxv N-terminal domain is present.

3.5. Phylogeny of coding sequence of C-terminus differs from that of N-terminus in the AvrRxv-family

Multiple alignments of AvrRxv family members indicate that the C-termini of the proteins have more overall similarity than the N-termini. When compared to AvrRxv, the plant pathogen effector C-termini are more similar (55% average similarity) than the N-termini (41% average similarity). Likewise the animal pathogen effector C-termini are also more similar to that of YopP (74% average similarity) than the N-termini (69% average similarity). Moreover, although the C-terminus of the plant pathogen effector AvrRxv has similarity to the C-termini of the animal effectors (43% average similarity) and the C-terminus of YopP has similarity to the C-termini of the plant effectors (30% average similarity), the N-termini in the cross-comparison do not (0–10% average similarity). The C-termini are more conserved than the N-termini.

Phylogenetic analyses were performed on the 5′- and 3′-coding sequences of family members. The Shimodaira–Hasegawa test showed that the maximum likelihood trees were very different ($P = 0.0001$) between the 5′- and the 3′-ends of the coding sequences of AvrRxv family members. These results show that the two parts of the gene are evolving independently and in very different ways (Fig. 3).

Table 4
Host responses to inoculation with *Xanthomonas* and *Agrobacterium* strains containing genes for AvrRxv, AvrRxv homologues and chimeras thereof

Protein termini		Tomato host response[a]					
		Xcv 89-1			*Agrobacterium*		
N	C	Xcv plasmid [b]	Hawaii 7998	Bonny Best	Agro plasmid[c]	Hawaii 7998	Bonny Best
AvrRxv	AvrRxv	pTM100-avrRxv	HR	WS	pCB302-avrRxv	HR	NS
YopP	YopP	pTM100-yopP	WS	WS	pCB302-yopP	NS	NS
AvrRxv	YopP	pTM100-RY4	HR	WS	pCB302-RY4	HR	NS
YopP	AvrRxv	pTM100-YR3	WS	WS	pCB302-YR3	NS	NS

[a] Host responses are HR, hypersensitive response; WS, watersoaking; NS, no symptom.
[b] Xcv 89-1 carrying pTM100 induced WS on Hawaii 7998 and Bonny Best.
[c] *Agrobacterium* carrying pCB302 induced NS on Hawaii 7998 and Bonny Best.

3.6. Catalytic triad mutants are inactive

Wildtype *avrRxv* induces an HR in leaves of tomato line Hawaii 7998 when carried by Xcv 89-1, or when delivered by *Agrobacterium*-mediated transformation (Table 3) [3,9]. When any one of the three active site amino acid residues is mutated as in H180A, E200A, and C244A, HR-inducing activity is lost when carried by Xcv 89-1 or delivered by *Agrobacterium* (Table 3). A mutation in an amino acid outside the active site has no effect on activity (E258A). The catalytic triad is essential for HR-inducing activity in our system.

3.7. Identification of the transcription initiation site

To identify the 5′-end of the *avrRxv* transcript, primer extension on RNA isolated from Xcv 89-1 (pXVSC910) was performed. The major extension products using MLP3, MLP4 and DA1 aligned with base 563 of the sequencing reaction (Fig. 4). There was no detectable extension product with primer MLP1. Minor start sites were also observed upstream of base 563 at 558, 557, 552, 546, and 530. No extension products were observed with RNA from the negative control strain Xcv 89-1 (pDSK519) or from Xcv 92-14 ± avrRxv. This transcription initiation site is 12 and 24 bases upstream of two start codons at 575 and 587, respectively, and 36 bases downstream of a −10 promoter motif (Fig. 4) [70].

4. Discussion

Xcv uses the TTSS to translocate effector proteins into host cells, where they affect cellular physiology, causing disease in tomato and pepper. Seventeen effectors have been confirmed in Xcv [71], with only a few characterized for molecular or biochemical function. Several members of the YopJ/AvrRxv family of effectors have been shown to use a catalytic triad to target host proteins [8,13,22,25]. In this study, we used the yeast two-hybrid interaction screen to identify the protein(s) with which AvrRxv physically interacts in tomato. We have shown that AvrRxv interacts with a 14-3-3 protein, which we call ARI1 for *AvrRxv interactor* (Table 2). This result was supported by binding of recombinant AvrRxv to ARI1 via *in vitro* affinity chromatography (Fig. 1). ARI1 is nearly identical to the previously cloned 14-3-3 from tomato, TFT9 [51]. TFT9 is one of at least ten 14-3-3 proteins in tomato. *Tft9* expression is induced in response to treatment with fusicoccin, a membrane depolarizing fungal toxin, but not in a resistance response to Avr9 [51]. Similar to ARI1 in this study, TFT9 is thought to play a role in defense signaling.

14-3-3 Proteins bind their ligands in a conserved amphipathic groove [36]. Ligands are thought to bind to 14-3-3 proteins using three basic mechanisms [47], including the phosphorylation-
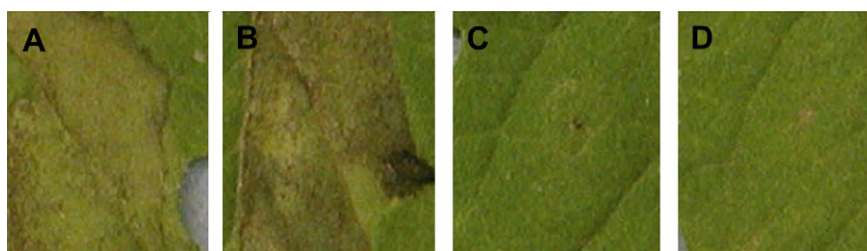
Fig. 2. Response of tomato line Hawaii 7998 to Xcv 89-1 carrying genes encoding (A) AvrRxv; (B) N-AvrRxv$_{1–172}$-YopP$_{104–289}$-C; (C) N-YopP$_{1–103}$-AvrRxv$_{173–373}$-C; and (D) vector alone, at 6 days post-inoculation.

independent manner via negatively charged residues coordinated by a basic cluster. The aspartate and glutamate residues of R18 peptide substitute for the phosphorylated residues in the phosphorylation-dependent mechanism. By mutagenesis of the coding sequence of AvrRxv, we demonstrated that although a putative Raf domain was not essential for both ARI1 binding or HR-inducing activity, a domain similar to the R18 domain was essential (Table 2). The AvrRxv-R18 mutant (D123N/E125Q), in which the acidic aspartate and glutamate residues were replaced by neutral amino acids, asparagine and glutamine, lost the ability to interact with ARI1 in yeast and lost HR-inducing activity, both when delivered by Xcv or *Agrobacterium* (Tables 2 and 3). Accordingly, an N-terminal deletion that removed the R18 domain (AvrRxv$_{142–373}$) did not interact with ARI1 in yeast (Table 2). These results further support the assertion that the interaction with ARI1 is specific to its function in the signaling pathway in tomato in response to AvrRxv. As mentioned above, studies of the structure of particular 14-3-3 proteins and their ligands have identified different binding mechanisms [47]. The fact that mutation of the acidic residues in the R18 domain of AvrRxv resulted in loss in ARI1 binding *in vitro* and loss of HR-inducing activity suggests that ARI1 may interact with AvrRxv by a basic cluster of amino acids within the amphipathic groove of ARI1. Similar to Zhang et al. [72], future experiments will include analysis of the impact on AvrRxv binding by mutagenesis of these basic residues in ARI1.

14-3-3 Proteins are involved in a wide variety of protein interaction-mediated functions in plants [38,40,41]. In *Arabidopsis*, there are up to 15 members of the 14-3-3 family [73]. They are
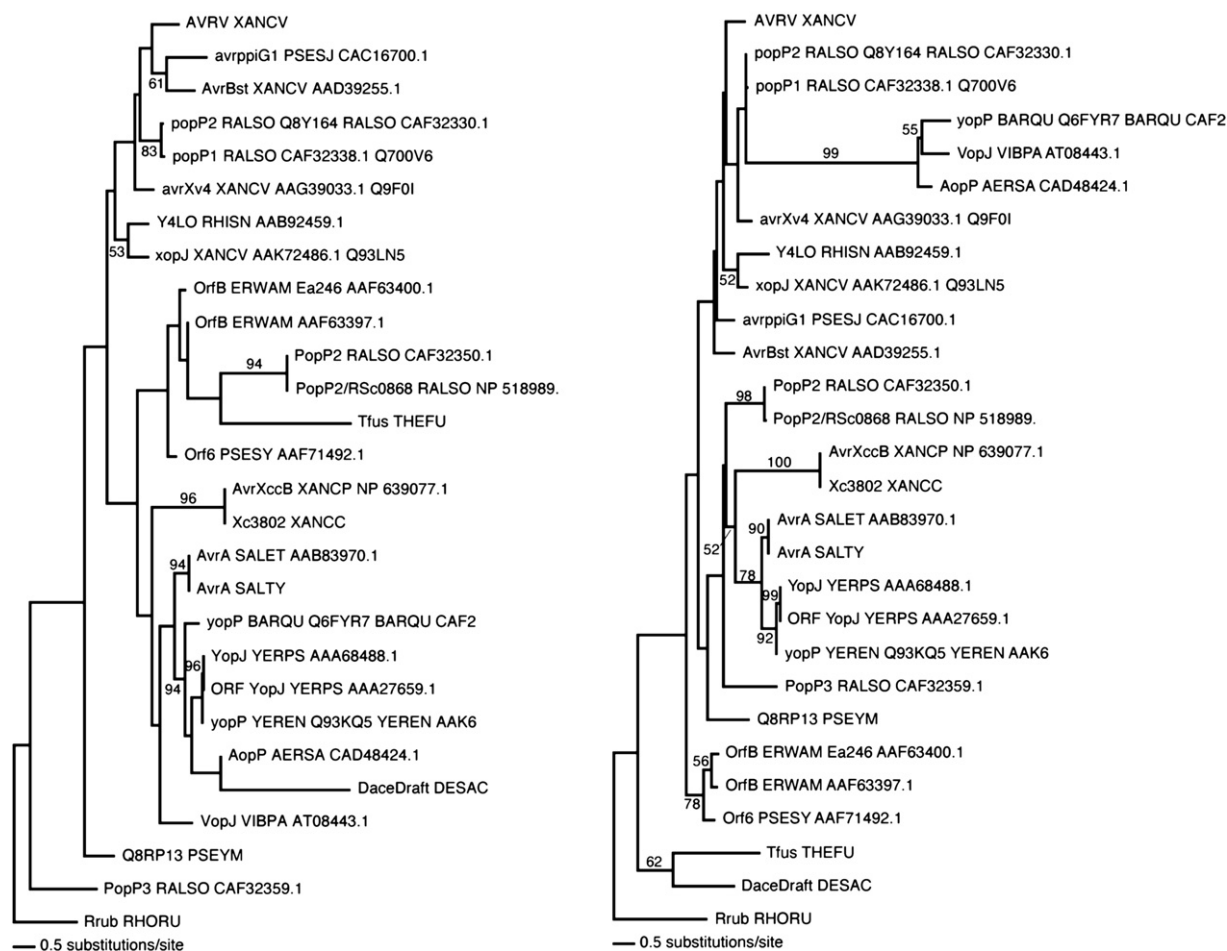

Fig. 3. Phylogenetic analysis of the 5′- and 3′-ends of the coding sequences of AvrRxv family members. Phylogenetic relationships were inferred and maximum likelihood models were generated with neighbor-joining tree estimations and heuristic searching until the tree parameters converged. Statistical support for the tree branches was evaluated by bootstrap analysis using a heuristic search of 100 random stepwise addition replicates. The two tree topologies were shown to be statistically different using the Shimodaira–Hasegawa test for significant divergence in tree lengths.
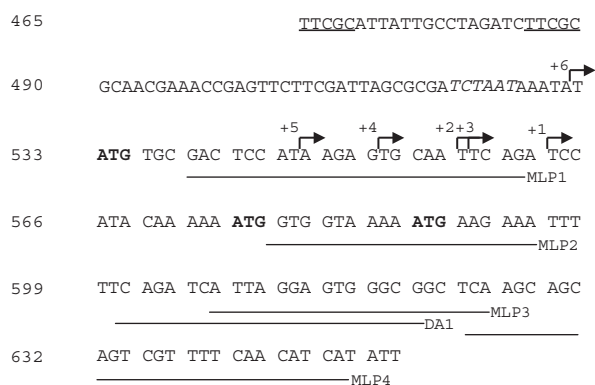
```
465                    TTCGCATTATTGCCTAGATCTTCGC

                                                          +6
490    GCAACGAAACCGAGTTCTTCGATTAGCGCGATCTAATAAATAT

                      +5        +4      +2+3       +1
533    ATG TGC GAC TCC ATA AGA GTG CAA TTC AGA TCC
           ─────────────────────────────────
                                       MLP1

566    ATA CAA AAA ATG GTG GTA AAA ATG AAG AAA TTT
                                               MLP2

599    TTC AGA TCA TTA GGA GTG GGC GGC TCA AGC AGC
               ──────────────────────── DA1    MLP3
       ──────────────────────────────

632    AGT CGT TTT CAA CAT CAT ATT
       ──────────────────── MLP4
```

Fig. 4. Sequence of region upstream of *avrRxv* with transcription initiation sites labeled. Primer extension was performed with primers MLP1, MLP2, DA1, MLP3 and MLP4 (indicated by horzontal lines). The 5′-end of extension products is indicated with arrows, with +1 being the major initiation site, and +2 through +6, progressively more minor. Potential translation start codons are indicated in bold. PIP-box sequence underlined; promoter −10 sequence italicized.

categorized by sequence into groups and isoforms. Based on its phylogenetic proximity to *Arabidopsis* protein general regulatory factor 9 (GRF9), ARI1 is a member of the μ isoform sub-family of the ε group (D group) of the phylogenetic tree [38]. Our study of ARI1's phylogeny, indicates that there are 18 members in ARI1's clade. The expression of five clade relatives is up-regulated in host responses to pathogens in tomato [51] and chickpea (Genbank accession no. ABQ95991) or to elicitors in poplar [50]. In barley, 14-3-3 proteins are up-regulated by powdery mildew [74]. Affinity purification of barley 14-3-3-binding partners indicated that the second largest class of interactors was involved in defense, including three different disease NBS-LRR resistance proteins [75]. This suggests that there is a direct role of 14-3-3 in resistance gene-mediated resistance signaling. To assess the impact of ARI1 on the AvrRxv-specific HR in tomato, ARI1 will be silenced. Interaction partners downstream of ARI1 will be identified.

Although there are quite a few close relatives of ARI1, not much is known about their specific function. ARI1's second nearest relative is a 14-3-3 protein D75 in tobacco that binds to and potentially regulates the RSG transcriptional activator involved in GA responses [76]. The closest *Arabidopsis* relative GRF9 (G14μ) binds to the signal peptide of a thylakoid-targeted chloroplast precursor protein and is found in the chloroplast stroma, possibly suggesting a role in protein import [77]. A soybean 14-3-3 was up-regulated upon infection by *Pseudomonas syringae* pv. *glycinea* [52]. The *Arabidopsis* relatives are localized in the chloroplast stroma and in the cytoplasm [77], and if that is generally true for all of ARI1's relatives, it rules out exclusive subcellular localization as a regulatory factor [38]. ARI1's precise role in the signaling response of tomato to the presence of AvrRxv remains to be discovered.

Interestingly, the C-terminal 58 amino acids of AvrRxv, which are C-terminal to the protease domain, are not only dispensable for interaction with ARI1, but may normally inhibit the interaction (Table 2). It is possible that the C-terminus plays a guarding or regulatory function. Additional AvrRxv mutagenesis experiments will allow dissection of this possibility.

Our result demonstrating that ARI1 directly interacts with AvrRxv is similar to the interaction of the effector ExoS from *P. aeruginosa* with a 14-3-3 protein [45]. ExoS requires the host factor 14-3-3 for activation and binds to it in a phosphorylation-independent manner [72]. It is possible that ARI1 also enhances the enzymatic activity of AvrRxv. In addition to the 14-3-3/ExoS interaction, other bacterial pathogens encode proteins that complex with mammalian 14-3-3s [78]. Our work provides additional evidence that bacterial effectors and proteins recruit 14-3-3

proteins for their function within host cells. Interestingly, a YopJ mutant (DVE53-55NAQ) that turns out to be in the equivalent R18 domain is inactive [79]. Perhaps, deeper analysis of YopJ host interactions may uncover a 14-3-3 protein for full level of activation. The elucidation of the role of these interactions may allow the rational design of inhibitory molecules that interfere with widely conserved mechanisms of virulence.

A chimera of AvrRxv and YopP has HR-inducing activity in leaves of Hawaii 7998 (Table 4; Fig. 2). That along with the results that both *Yersinia* homologues, YopJ and YopP and a YopP-AvrRxv chimera were not able to induce an HR, have important implications for our understanding of structure and function relationships in the family. Despite their divergent origins, the catalytic domain of YopP is interchangeable with that of AvrRxv. This suggests a strong conservation of function in the C-terminal domains of YopJ family members as was demonstrated by YopJ function in inhibition of yeast MAPK pathway [80,81]. The functional conservation of catalytic triad residues supports this as well (Table 3) [8,13,23]. We find this to be the case in our phylogenetic analyses of the family, where the C-termini containing the catalytic triad are significantly more conserved than the N-termini. Remarkably, the coding sequences for the two termini appear to be evolving independently (Fig. 3). Even when the YopP effector is delivered directly to the host cell via *Agrobacterium*-mediated expression, without the AvrRxv N-terminus, it cannot function (Table 4). Along these lines, the family member AvrA from *Salmonella* fails to complement YopJ activity [82]. In Xcv family members, TTSS signals are present in about the first 50 amino acids, comprising a translocation domain [12,34,83,84]. Based on our results, we predict that there is a third essential domain, the host-specific interaction domain that is C-terminal to the translocation domain and N-terminal to the protease domain. In AvrRxv the host-specific interaction domain contains the R18 motif, required for full level interaction with host factor ARI1 and HR-inducing activity. Analysis of the activity of other Xcv family members in tomato, and delineation of the translocation and host-specific interactions domains in them will allow testing of this prediction. The host-specific interaction domain may be highly specific to not only the host and particular target cells, but also to the protein target.

We have identified the transcription initiation site in *avrRxv* (Fig. 4), allowing more accurate prediction of the translation start codon. Based on the alignment of the amino acid sequence of AvrRxv with that of the closely related family member AvrBsT [6], and the distance between other *Xanthomonas* transcription initiation sites and start codons [70], it is most likely the ATG at base 587 that serves as the start codon. The PIP-box that is 31 bases upstream of the −10 promoter motif has not yet been shown to function in HrpX control of expression of *avrRxv* [6]. Analysis of the genome of Xcv revealed that there are PIP-boxes upstream of coding sequences for genes that have no obvious association with pathogenicity and also, HrpX controlled genes that do not contain this motif [11,12,85]. More work has to be done to understand the role of the PIP-box.

In conclusion, we have shown that the AvrRxv bacterial effector recruits a 14-3-3 protein for its function within host cells. An interaction domain in AvrRxv was essential for this recruitment and associated function in host cells. Different domains in this family of effector proteins were shown to have evolved differently, suggesting host-specific adaptations are ongoing.

Acknowledgements

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