Conserved RxLR Effectors From Oomycetes *Hyaloperonospora arabidopsidis* and *Phytophthora sojae* Suppress PAMP- and Effector-Triggered Immunity in Diverse Plants

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Accepted 31 October 2017.

Effector proteins are exported to the interior of host cells by diverse plant pathogens. Many oomycete pathogens maintain large families of candidate effector genes, encoding proteins with a secretory leader followed by an RxLR motif. Although most of these genes are very divergent between oomycete species, several genes are conserved between *Phytophthora* species and *Hyaloperonospora arabidopsidis*, suggesting that they play important roles in pathogenicity. We describe a pair of conserved effector candidates, *HaRxL23* and *PsAvh73*, from *H. arabidopsidis* and *P. sojae* respectively. We show that *HaRxL23* is expressed early during infection of *Arabidopsis*. *HaRxL23* triggers an ecotype-specific defense response in *Arabidopsis*, suggesting that it is recognized by a host surveillance protein. *HaRxL23* and *PsAvh73* can suppress pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) in *Nicotiana benthamiana* and effector-triggered immunity (ETI) in soybean. Transgenic *Arabidopsis* constitutively expressing *HaRxL23* or *PsAvh73* exhibit suppression of PTI and enhancement of bacterial and oomycete virulence. Together, our experiments demonstrate that these conserved oomycete RxLR effectors suppress PTI and ETI across diverse plant species.

Oomycete pathogens have evolved sophisticated mechanisms to avoid or suppress host immune responses (Jiang and Tyler 2012; Kamoun et al. 2015). One of the most destructive genera of oomycetes is *Phytophthora*, which is composed of over 120 species that cause important diseases on many dicot crops (Erwin and Ribiero 1996). For example, *Phytophthora infestans*, the causative agent of potato late blight disease, triggered the Great Irish Famine from 1845 to 1849 and remains one of the most destructive pathogens of potato and tomato today. Downy mildew pathogens comprise a sister group to the *Phytophthora* genus (Thines 2014), and some analyses actually place them within the genus (Ye et al. 2016). Downy mildews are obligate parasites and cause destructive diseases on grape, lettuce, cucurbits, and other crops (Gisi 2002; Lucas et al. 1995). Overall, it is difficult to control oomycete diseases because they rapidly evolve to overcome fungicides or genetic resistance in the host (Fry 2008).

Plants maintain a robust, multilayered immune system that enables them to resist most pathogens (Dodds and Rathjen 2010). The primary layer of plant immunity is activated by the recognition of conserved microbial molecules, referred to as pathogen (or microbe)-associated molecular patterns (PAMPs or MAMPs). Such PAMPs or MAMPs are recognized by plant pattern recognition receptors, thereby activating PAMP-triggered immunity (PTI) (Chisholm et al. 2006; Jones and Dangl 2006). Outputs of PTI include production of reactive oxygen species, callose deposition, activation of MAPK signaling cascades, and induction of defense genes (Boller and Felix 2009; Couto and Zipfel 2016; Macho and Zipfel 2014).

Pathogens deliver effector proteins to the exterior and interior of plant cells to interfere with immune responses (Hogenhout et al. 2009; Toruño et al. 2016). Effectors have been extensively studied in bacterial phytopathogens, which use type III secretion (T3S) to deliver 20 to 40 effector proteins to the interior of plant cells (Chang et al. 2014; Feng and Zhou 2012). Some pathogen effectors are recognized by host resistance (R) proteins directly or indirectly, activating effector-triggered immunity (ETI) (Chisholm et al. 2006; Cui et al. 2015; Jones and Dangl 2006; van der Hooft and Kamoun 2008). ETI often culminates in plant cell death at the site of infection (often termed the hypersensitive response [HR]), accompanied by production of reactive oxygen species, antimicrobial metabolites, and induction of defense genes (Dodds and Rathjen 2010). Pathogens can counteract ETI through deletion or silencing of Avr/effector genes to avoid R protein recognition or by suppressing downstream signaling events (Feng and Zhou 2012).

Phytophthora species and downy mildew pathogens are thought to secrete effectors from intracellular feeding structures called haustoria as well as from intercellular hyphae (Torto et al. 2003; Wang et al. 2017; Whisson et al. 2007). Based on their localization in the host, effectors are broadly categorized as apoplastic or cytoplasmic (Birch et al. 2006; Kamoun 2006; Qutob et al. 2006). Bioinformatic approaches have led to the identification of many effector candidates in the genomes of oomycetes (Adhikari et al. 2013; Baxter et al. 2010; Fabro et al. 2011; Haas et al. 2009; Jiang et al. 2008; Lamour et al. 2012; Lévesque et al. 2010; Savory et al. 2012; Tian et al. 2011; Tyler et al. 2006; Win et al. 2012). Some of the most widely studied oomycete effector proteins are defined by the presence of a signal peptide (SP) in the N terminus followed by the amino acid motifs RxLR (arginine [Arg]-any amino acid-leucine-Arg) and EER (glutamate [Glu]-Glu-Arg) within the first 100 or so residues of the N terminus (Rehmany et al. 2005; Whisson et al. 2007). The SP presumably enables secretion of the

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*The e-Xtra logo stands for “electronic extra” and indicates that eight supplementary figures and two supplementary tables are published online.

effectors outside the pathogen. The RxLR motif has been proposed to facilitate translocation of the effectors to the interior of host cells and this is a subject of active investigation (Jiang and Tyler 2012; Petre and Kamoun 2014). The SP and RxLR are typically followed by a C-terminal domain that mediates the specific function of the effector protein inside the host cell (e.g., interaction with a host protein). Many RxLR effectors also contain conserved C-terminal motifs, called W, Y, or L, based on canonical tryptophan (W), tyrosine (Y), and leucine (L) residues. These motifs are generally arranged as repeats and have been shown to be required for defense suppression activity or recognition by R proteins (Dou et al. 2008; Jiang et al. 2008). Structural studies have demonstrated that these domains form a core α -helical fold that can accommodate structural changes (mutation of surface residues, repeat copy number variation, indels) that can facilitate coevolution with the host (Boutemy et al. 2011; Win et al. 2012).

Phytophthora spp. and downy mildew pathogens maintain large superfamilies of predicted RxLR proteins, ranging in size from over 100 members (e.g., *Hyaloperonospora arabidopsidis*) to over 500 (e.g., *P. infestans*) (Jiang and Tyler 2012). Recent studies have revealed that many RxLR effectors are capable of suppressing plant immunity (Anderson et al. 2015). Specific targets of RxLR proteins are also coming into focus. For example, *P. infestans* RxLR effector Avr3a interacts with the *Nicotiana benthamiana* U-box protein CMPG1, and this interaction appears to promote virulence by stabilizing CMPG1 (Bos et al. 2010; González-Lamothe et al. 2006). *P. infestans* PexRD2 interferes with mitogen-activated protein kinase (MAPK) signaling. *P. infestans* Pi03192 targets NAC transcription factors (McLellan et al. 2013). *H. arabidopsidis* HaRxL44 targets a subunit of the mediator transcriptional regulatory complex to manipulate plant hormone crosstalk (Caillaud et al. 2012). These and other studies suggest that RxLR effectors can target a variety of plant regulatory proteins; this point is underscored by the identification of plant-pathogen protein interactomes, which suggest that effectors from oomycetes, fungi, and bacteria converge onto highly connected hubs in the plant immune network (Mukhtar et al. 2011; Weßling et al. 2014). However, these studies are only the first steps toward a full understanding of the mechanisms and evolution of host manipulation via RxLR proteins.

Hyaloperonospora arabidopsidis is an obligate biotroph and is the causative agent of *Arabidopsis* downy mildew (Coates and Beynon 2010; Holub 2008). The genome of the *H. arabidopsidis* isolate Emoy2 contains at least 134 RxLR candidate effector genes (HaRxL genes) (Baxter et al. 2010; McDowell 2014).

Very few of the *H. arabidopsidis* effectors have recognizable homologs in *Phytophthora* species (Baxter et al. 2010). However, a few candidate effector genes are syntenic and conserved between these distantly related oomycetes (e.g., *HaRxL96* [Anderson et al. 2012]). We anticipate that analysis of conserved effectors will reveal virulence functions that are important for *Phytophthora* and downy mildew plant pathogens, leading to the identification of conserved plant targets.

In this study, we characterize a conserved, orthologous pair of effectors from *H. arabidopsidis* and *Phytophthora sojae*, *HaRxL23* and *PsAvh73*. *HaRxL23* is expressed early during infection and triggers an ecotype-specific defense response in *Arabidopsis*, suggesting that it is a bona fide effector protein. Functional comparisons of *HaRxL23* and *PsAvh73* proteins revealed that both effectors have similar functional capabilities and can suppress immunity in diverse plant species.

RESULTS

HaRxL23 and *PsAvh73* are syntenic and share conserved functional domains.

HaRxL23 is one of four high-confidence *H. arabidopsidis* RxLR candidate effector genes for which a homolog is present at syntenic loci in *Phytophthora* genomes (Baxter et al. 2010). Figure 1 illustrates the primary sequence conservation between *HaRxL23* and homologous genes from *P. sojae* and *P. infestans*. All three genes contain a predicted SP at their N termini. The SP is followed by RLLR and RALR motifs in *HaRxL23* and *PsAvh73*, respectively, followed by a short, acidic dEER-like motif. *HaRxL23* and *PsAvh73* each encode a single copy of the W and Y motifs that are found in many RxLR effectors and comprise a conserved protein fold (Win et al. 2012). W and Y motifs are typically highly variable but are unusually well-conserved among the members of the *HaRxL23* and *PsAvh73* family of orthologs (Boutemy et al. 2011; Jiang et al. 2008). No other motifs or subcellular localization signals could be discerned in the sequences of these effectors.

To assess allelic diversity at the *HaRxL23* locus, we sequenced this gene in two additional isolates (Emco5 and Hiks1). In both isolates, the nucleotide sequence is identical to the reference sequence from the genome of *H. arabidopsidis* Emoy2. Similarly, it has been reported that the sequence of *PsAvh73* is monomorphic among four races that encompass the pathotype diversity of *P. sojae* in the United States (Förster et al. 1994; Wang et al. 2011). Thus, *HaRxL23* and *PsAvh73* are likely conserved within their respective species.

Fig. 1. Alignment of amino acid sequences from *HaRxL23*, *PsAvh73*, and *Phytophthora infestans* PITG_00707.1. The predicted signal peptide (SP), RxLR, and W-Y motifs are indicated.

HaRxL23 is induced early during pathogen infection.

We tested whether *HaRxL23* was induced during infection by virulent *H. arabidopsidis*. Four-week old *Arabidopsis* ecotype Oystese (Oy-1) plants were inoculated with a spore suspension from the virulent *H. arabidopsidis* isolate Emoy2. RNA was isolated from infected plant tissue over a time course, and the relative abundance of *HaRxL23* transcripts was assayed using quantitative real-time polymerase chain reaction (qRT-PCR). The abundance of *HaRxL23* transcripts peaked at around 12 h postinoculation (hpi) (Fig. 2) and declined during the later time-points, to a level lower than that observed in spores immediately after inoculation.

HaRxL23 is recognized by *Arabidopsis* in an ecotype-specific manner.

As another functional test for *HaRxL23*, we assayed whether it induces ETI in *Arabidopsis*. We screened 48 *Arabidopsis* accessions using the effector detector vector (EDV) system, in which oomycete effectors are delivered by *Pseudomonas* bacteria via T3S to the interior of *Arabidopsis* cells (Sohn et al. 2007). We used the *Pseudomonas fluorescens* strain EtHAN (effector to host analyzer), which is a nonpathogen of *Arabidopsis*, lacks discernable T3S effectors and contains a genetically engineered T3S system to deliver effectors of interest (Thomas et al. 2009). This approach ensured the exclusive delivery of our effector into the host, without complications from endogenous bacterial effectors. Equally important, EtHAN does not trigger disease symptoms in *Arabidopsis*, reducing complications of visual HR interpretation.

As a standard of comparison for a typical HR, we used *Pseudomonas syringae* pv. *tomato* DC3000 carrying *AvrRpt2* in the resistant *Arabidopsis* ecotype Col-0 (Fig. 3A and B) (Sohn et al. 2007). We also used the *H. arabidopsidis* avirulence effector *Atr13*, which has been previously shown to trigger HR due to recognition of this effector by the RPP13 resistance protein (Allen et al. 2004). When delivered via the EtHAN/EDV system, *Atr13* produced a typical leaf collapse phenotype, albeit weaker than that of *AvrRpt2* delivered from *P. syringae* pv. *tomato* DC3000 (Fig. 3A and B) (Sohn et al. 2007).

Fig. 2. *HaRxL23* is induced at an early timepoint in *Hyaloperonospora arabidopsidis* infection. *Arabidopsis* Oy-1 plants were challenged with 5×10^4 spores of the virulent *H. arabidopsidis* isolate Emoy2 per milliliter. *HaRxL23* expression was assayed using quantitative, real-time polymerase chain reaction with primers specific for *HaRxL23* over a time course, using the cDNA obtained at timepoints shown on the x axis. Transcript abundance of *HaRxL23* was measured relative to *H. arabidopsidis* *Actin* and is shown normalized to *HaRxL23* abundance at the 0-h timepoint.

We inoculated *P. fluorescens* EtHAN expressing *HaRxL23* onto 48 *Arabidopsis* ecotypes (Supplementary Table S2). We observed leaf collapse symptoms comparable to the two controls in the *Arabidopsis* ecotype Ei-4 (Fig. 3A and B). Two other *Arabidopsis* ecotypes, Ob-0 and Pla-1, also showed partial leaf collapse phenotypes in response to EtHAN (*HaRxL23*), indicative of a weak HR (Supplementary Fig. S2). We then examined the growth in ecotype Ei-4 of *P. syringae* pv. *tomato* DC3000 expressing *HaRxL23* compared with *P. syringae* pv. *tomato* DC3000 containing an empty vector. We observed a fourfold reduction in bacterial growth in DC3000(*HaRxL23*) compared with DC3000 in Ei-4 (Fig. 3C). When ecotype Ei-4 was inoculated with Emoy2, the source of the allele of *HaRxL23* used in these experiments, it proved resistant (Supplementary Figs. S5 and S6) consistent with the possibility of an R protein in Ei-4 that recognizes *HaRxL23*. These experiments indicate that *HaRxL23* is recognized by the *Arabidopsis* immune system in an ecotype-specific manner, suggestive of gene-for-gene resistance and consistent with the hypothesis that *HaRxL23* is an effector.

To test whether *PsAvh73* has avirulence activity in *Arabidopsis* similar to *HaRxL23*, we inoculated *P. fluorescens* EtHAN expressing *PsAvh73* onto the same 48 *Arabidopsis* ecotypes tested with *HaRxL23*. The *HaRxL23*-responsive ecotypes Ei-4 and Ob-0 also responded to *PsAvh73*, but the HR was much weaker. Pla-1 did not respond to *PsAvh73*, nor did any of the other tested ecotypes. Thus, *PsAvh73* provoked a similar but weaker spectrum of resistance responses in our collection of *Arabidopsis* ecotypes.

HaRxL23 and *PsAvh73* suppress programmed cell death (PCD) in *N. benthamiana*.

As a first test of whether *HaRxL23* and *PsAvh73* contribute to pathogen virulence, we assayed whether these effectors could suppress PCD, when transiently expressed in *N. benthamiana* via *Agrobacterium*-mediated delivery. Neither of the effectors could induce cell death when expressed by themselves in *N. benthamiana* (Fig. 4). We induced PCD by the *P. infestans* elicitor, Infestin 1 (INF1), which is a PAMP, or by the *P. sojae* RxLR effector *PsAvh163*. Both of these proteins trigger a strong HR in leaves of *N. benthamiana* (Anderson et al. 2012; Bos et al. 2006; Wang et al. 2011). *Agrobacterium tumefaciens* GV3101 containing binary T-DNA vectors with either *CaMV35-HaRxL23* or *CaMV35-PsAvh73* were infiltrated into the leaves of *N. benthamiana*. These leaves were challenged, 24 or 48 h later, with *CaMV35-INF1* or *CaMV35-PsAvh163* via *Agrobacterium*-mediated delivery. Five to 7 days later, we visually scored each infiltration site for cell death. We observed that INF1-induced cell death is suppressed by *PsAvh73* (Fig. 4A) but not by *HaRxL23*. Both *HaRxL23* and *PsAvh73* suppressed *PsAvh163*-induced cell death (Fig. 4B). An *Agrobacterium* strain with *CaMV35-YFP* (yellow fluorescent protein) served as the control for this experiment and did not suppress cell death triggered by INF1 or *PsAvh163*. These results suggested that these two effectors were also capable of suppressing PCD in *N. benthamiana*, similar to other bacterial and oomycete effectors (Alfano 2009; Cabral et al. 2011; Wang et al. 2011). The cell death-suppressive activity of *PsAvh73* was further supported by Wang et al. (2011), who, based on coexpression in *Nicotiana benthamiana*, demonstrated that *PsAvh73* is a strong suppressor of cell death triggered by mammalian BAX.

HaRxL23 and *PsAvh73* suppress PCD in soybean.

To assess whether *HaRxL23* and *PsAvh73* could contribute to virulence in soybean, we used a biolistic assay for suppression of ETI (Kale and Tyler 2011). *Avr4/6* is an RxLR effector from *P. sojae* that triggers an HR in soybean cultivars with the *RPS4* or

RPS6 resistance genes (Kale and Tyler 2011). When *Avr4/6* is co-bombarded with a β -glucuronidase (*GUS*) into leaves with *RPS4* or *RPS6*, PCD from a HR elicited by *Avr4/6* reduces the number of blue spots visualized, compared with control leaves bombarded only with the *GUS* gene plus an empty vector (Supplementary Fig. S3). Co-bombardment with a third gene that suppresses PCD will restore the viability of bombarded cells, which manifests itself as a larger number of *GUS*-positive spots in the leaves bombarded with the effector (Dou et al. 2008). This assay allows for quantitative assessments of the ability of a candidate effector to suppress PCD. A second elicitor from *P. infestans*, CRN2, was also used as a cell-death inducer. CRN2 (crinkling and necrosis 2) is a necrosis-inducing effector protein and its ectopic expression results in cell death response in *N. benthamiana*, tomato, and soybean (Torto et al. 2003).

As expected, co-bombardment of *GUS* and *Avr4/6* in *RPS4* soybean significantly reduced the number of *GUS*-expressing cells up to 60%, compared with leaves bombarded with the empty vector control instead of *Avr4/6* (Dou et al. 2010). However, a combination of *GUS* and *Avr4/6* with *HaRxL23* or *PsAvh73* restored the numbers of *GUS*-expressing viable cells (Fig. 5A). Similarly, we observed a 65% decrease of *GUS*-expressing viable cells in tissue bombarded with *CRN2*, relative to control samples. *CRN2*-induced cell death was reduced when *HaRxL23* or *PsAvh73* was included in the bombardment (Fig. 5B). Thus, both *HaRxL23* and *PsAvh73* can suppress cell death induced by either *Avr4/6* or *CRN2* in soybean.

***HaRxL23* and *PsAvh73* enhance susceptibility to virulent *H. arabidopsidis*.**

To test whether *HaRxL23* and *PsAvh73* could contribute to virulence in *Arabidopsis*, we expressed both effectors as stable transgenes and evaluated PTI and ETI compared with non-transgenic control plants (Munkvold and Martin 2009). This method has been previously used to characterize several bacterial and oomycete effectors (Fabro et al. 2011; Hauck et al. 2003;

Munkvold and Martin 2009; Nomura et al. 2006). We generated *Arabidopsis* Col-0 lines expressing either *HaRxL23* or *PsAvh73* under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. We obtained several independent, single insertion-locus lines. Transgene mRNA abundance was verified by qRT-PCR in each experimental line (Supplementary Fig. S4). Homozygous lines with different transcript abundance were selected for all subsequent experiments, to evaluate potential quantitative phenotypic effects. We observed little phenotypic variation between independent transformants suggesting that transgene expression levels were not a significant variable affecting the phenotypes arising from transgene expression.

We first tested whether in planta overexpression of the two effectors suppressed basal resistance against virulent *H. arabidopsidis*. Wild-type and transgenic seedlings were inoculated with spores from virulent *H. arabidopsidis* Emco5, and pathogen growth was quantified by counting sporangio-phores at 7 days after inoculation. Representative *Arabidopsis* lines constitutively expressing either *HaRxL23* or *PsAvh73* showed enhanced susceptibility to virulent *H. arabidopsidis* isolate Emco5, compared with wild-type Col-0 plants (Fig. 6). This result suggests that both effectors are capable of suppressing basal resistance to virulent *H. arabidopsidis* in *Arabidopsis*.

Next, we tested whether the Col:HaRxL23 lines were more susceptible to the avirulent *H. arabidopsidis* isolate Emoy2, which is recognized in Col-0 by the resistance gene *RPP4* (van der Biezen et al. 2002). We observed no differences between control Col-0 and transgenic HaRxL23 lines in Emoy2 sporulation or in HR cell death. This result suggests that *HaRxL23* does not interfere with ETI-mediated by *RPP4*.

***HaRxL23* and *PsAvh73* suppress callose formation in stably transformed *Arabidopsis* in response to *Pseudomonas syringae* DC3000(Δ CEL) mutant.**

Because *H. arabidopsidis* makes intimate contact with host cell walls during pathogenesis, we hypothesized that *H. arabidopsidis*

Fig. 3. *HaRxL23* is recognized in the host in an ecotype-specific manner when delivered by bacteria. **A**, Leaves of *Arabidopsis* Ei-4 or Col-0, infiltrated with a 1×10^8 CFU/ml *Pseudomonas fluorescens* (EthAN) suspension carrying an empty vector (EV), *HaRxL23*, *Pseudomonas syringae* effector gene *AvrRpt2*, or *Hyaloperonospora arabidopsidis* effector gene *Atr13*. Hypersensitive response (HR) symptoms were visually monitored over a period of 24 h and images were taken 24 h after inoculation. **B**, HR score: 4, complete leaf collapse, 3, partial leaf collapse, 2, leaf curling, 1, partial leaf curling, and 0, no change compared with the EV control. The experiment was repeated four times with similar results. **C**, *Pseudomonas syringae* pv. *tomato* DC3000 (*HaRxL23*) multiplication in leaves of *Arabidopsis* ecotype Ei-4 plants syringe-infiltrated with a suspension of 5×10^5 CFU/ml. Bacterial populations were determined at day 0 and day 3 after inoculation. An asterisk (*) indicates $P < 0.1$; t test comparisons representing significant differences with *P. syringae* pv. *tomato* DC3000. Error bars indicate standard error of six independent leaf samples tested at the same time. This experiment was repeated three times with similar results.

effectors may function to suppress cell wall-based defenses, such as callose deposition. Callose is a β -1,3 glucan that is deposited between the cell wall and cell membrane near the invading pathogen and is a quantitative proxy for measuring the *Arabidopsis* PTI response (Hauck et al. 2003). In these experiments, we quantified callose depositions induced in response to infiltration with the *P. syringae* mutant DC3000(Δ CEL). This strain contains deletions of at least four effector genes that are conserved among *P. syringae* strains resulting in dramatically reduced virulence in tomato and *Arabidopsis* and extensive callose deposits in the host plant (DebRoy et al. 2004; Sohn et al. 2007).

Accordingly, we observed extensive callose deposition in wild-type *Arabidopsis* Col-0 plants when syringe-infiltrated with the Δ CEL mutant (Fig. 7A). Contrastingly, a reduction of 30 to 50% in callose deposits was observed in multiple lines of *Arabidopsis* plants constitutively expressing either *HaRxL23* or *PsAvh73* (Fig. 7). This degree of suppression is similar to other *H. arabidopsidis* effectors assayed elsewhere (Anderson et al. 2012; Fabro et al. 2011; Sohn et al. 2007).

Next, we tested whether the *HaRxL23* and *PsAvh73* transgenic plants were capable of enhancing the growth of the nonpathogen *P. syringae* pv. *tomato* DC3000(Δ CEL) mutant. Wild-type and transgenic plants were syringe-infiltrated

with *P. syringae* pv. *tomato* DC3000(Δ CEL) and growth was assayed at 0 and 3 days postinfiltration. Transgenic lines expressing either *HaRxL23* or *PsAvh73* showed enhanced susceptibility to *P. syringae* pv. *tomato* DC3000(Δ CEL) (Supplementary Fig. S7) 3 days postinoculation (dpi), further demonstrating that both *HaRxL23* and *PsAvh73* disable PTI in *Arabidopsis*.

To confirm these results, we used the EDV system to deliver *HaRxL23* and *PsAvh73* from *P. syringae* pv. *tomato* DC3000 (Δ CEL). These strains were infiltrated into *Arabidopsis* Col-0 and callose deposits were quantified. We observed that both effectors suppressed callose when delivered via the T3S, similar to their effects when expressed as plant transgenes (Supplementary Fig. S8). Since the concentration of effectors in the host cytoplasm is presumably low, this result indicates that the phenotypes observed in stably transformed plants are unlikely to be neomorphic artifacts of transgene overexpression.

Stably transformed *HaRxL23* suppress defense gene induction.

We next tested whether *HaRxL23* was capable of suppressing induction of defense genes in response to *H. arabidopsidis*. We used qRT-PCR to measure the transcript abundance of four defense marker genes in Col:35S-*HaRxL23* seedlings infected

Fig. 4. *HaRxL23* and *PsAvh73* suppress programmed cell death in *Nicotiana benthamiana*. *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* GV3101 containing *HaRxL23*, *PsAvh73*, or yellow fluorescent protein (YFP) and were challenged 2 days postinfiltration with *A. tumefaciens* GV3101 carrying either **A**, *INF1* or **B**, *PsAvh163*. Cell-death symptoms were visually monitored over a period of 5 to 7 days. Graphs show the percentage of infiltration sites with macroscopic cell death. An asterisk (*) indicates $P < 0.05$, t test comparisons representing statistically significant differences with YFP. Error bars indicate standard error from four independent biological replicates.

with avirulent *H. arabidopsidis* Emoy2. We assayed mRNA from four marker genes that were selected because they are transiently suppressed during early *H. arabidopsidis* infection: *ACD6* (*Accelerated cell death 6*), *PR-1* (*Pathogenesis-related 1*), *AtMPK3* (*Arabidopsis thaliana mitogen activated protein kinase 3*), and *WAK1* (*Wall-associated kinase 1*) (Anderson et al. 2012; Eulgem et al. 2007). HaRxL23 suppressed induction of PR-1, MPK3, and ACD6 (Fig. 8). In contrast, induction of *WAK1* was not suppressed by either effector, indicating defense gene-specific suppression. The induction profiles of individual genes varied to some degree between replicates, but we consistently observed decreased induction of defense marker genes (except for *WAK1*) in transgenic lines compared with the control plants. These results suggest that HaRxL23 intervenes early in the activation of defense.

DISCUSSION

Functional and mechanistic characterization of RxLR effectors is essential to understanding the molecular mechanisms of oomycete pathogenesis and host adaptation. In this study, we characterized a candidate RxLR effector from *H. arabidopsidis*, HaRxL23, and we included functional comparisons with its ortholog in *P. sojae*, PsAvh73. HaRxL23 is one of only four RxLR genes for which an ortholog is present at syntenic locations in sequenced genomes of *Phytophthora* pathogens (Baxter et al. 2010). Conserved effector genes represent potential opportunities for new insights into important mechanisms of virulence. Moreover, identification and breeding of resistance genes against conserved effector proteins can be utilized as an effective strategy for long-term durable resistance (Vleeshouwers and Oliver 2014).

Our first set of experiments was directed at testing the functions of the bioinformatically predicted effector genes. First, we demonstrated that *HaRxL23* is induced early during infection in planta, consistent with a function as an effector. Data from RNAseq experiments (B. M. Tyler *unpublished*) indicate that *PsAvh73* is down-regulated 10- to 20-fold at 12 hpi, compared with in vitro-grown mycelia or to germinating cysts. It is possible that *PsAvh73* expression peaks before

12 hpi, concomitant with the short duration of the biotrophic state of *P. sojae* infection (Ma et al. 2015).

Additional evidence of effector function was provided by a screen for *R* genes against HaRxL23 in *Arabidopsis* ecotypes. We used the EDV-based system to deliver HaRxL23 via T3S

Fig. 6. HaRxL23 and PsAvh73 enhance susceptibility to virulent *Hyaloperonospora arabidopsidis* (Emco5). Ten- to 12-day-old transgenic *Arabidopsis* Columbia seedlings stably transformed with *CaMV35S-HaRxL23* or *CaMV35S-PsAvh73* were challenged with 5×10^4 spores of the virulent *H. arabidopsidis* isolate Emco5. Infection was quantified 7 days post-inoculation by counting sporangia per cotyledon. *Arabidopsis* Col-0 and Oy-1 are controls for susceptibility and resistance to *H. arabidopsidis* Emco5, respectively. An asterisk (*) indicates $P < 0.01$; *t* test comparisons representing significant differences with Col-0. Error bars indicate standard error. This experiment was repeated three times with similar results.

Fig. 5. HaRxL23 and PsAvh73 suppress programmed cell death in soybean. A plant-expression plasmid expressing **A**, *CaMV35S-Avr4/6* or **B**, *CaMV35S-CRN2* was co-bombarded, with plasmids containing the effectors *CaMV35S-HaRxL23* or *CaMV35S-PsAvh73* or the empty vector (EV), along with a vector expressing *CaMV35S-GUS* reporter gene, into soybean leaves. Leaves were stained for β -glucuronidase (GUS) activity, and cell viability was then estimated by counting blue spots under a dissecting microscope. The percentage of surviving cells was quantified relative to control leaves co-bombarded with a GUS plasmid and a second EV plasmid. An asterisk (*) indicates $P < 0.05$, Wilcoxon rank sum test comparisons representing significant differences with the EV. Error bars indicate standard error from technical replicates. These experiments were repeated at least three times with similar results.

from the nonpathogen *P. fluorescens* EtHAN. This general approach of delivering oomycete effectors from bacteria has been used previously to characterize known avirulence effectors from *H. arabidopsidis* and to rapidly screen for *R* genes against novel *H. arabidopsidis* effectors (Fabro et al. 2011; Goritschnig et al. 2012; Sohn et al. 2007). This approach enabled us to identify an ecotype-specific HR phenotype that correlated with resistance to *H. arabidopsidis*, suggestive of gene-for-gene resistance triggered by HaRxL23. We believe that the ecotype-specificity of the resistance is due to an *R* gene that is functional specifically in the Ei-4 ecotype and, perhaps, also Ob-0 and Pla-1. We note that recognition of HaRxL23 appears to be relatively rare, occurring in only three of the 48 ecotypes that we tested. This rarity is reminiscent of the *RPP39* gene, which confers an HR-based response to the ATR39-1 RXLR effector from *H. arabidopsidis* when delivered by *P. fluorescens*. Only one of 83 tested ecotypes responded to ATR39-1 delivery (Goritschnig et al. 2012). *RPP39* encodes a coiled coil-nucleotide binding-leucine-rich repeat protein.

The identification of a putative *R* gene that recognizes HaRxL23 lays the groundwork for identifying the gene through map-based cloning, as reported by Goritschnig et al. (2012). An *R* gene against HaRxL23 could be valuable for two reasons. First, molecular cloning of the *R* gene might aid in identifying the virulence target if the *R* protein guards that target. Second, the *R* gene could potentially be used as a source of resistance against oomycete pathogens of crop brassicas or, perhaps, even in more distantly related species. In this regard, it is interesting that PsAvh73 triggered a response, albeit weakly, in two of the same *Arabidopsis* ecotypes that recognized HaRxL23. Assuming

that the same *R* gene recognizes both effectors, its specificity for PsAvh73 or homologous effectors from other oomycete species could potentially be improved by mutagenesis (Harris et al. 2013; Segretin et al. 2014).

We next hypothesized that these two effector proteins might act as suppressors of defense pathways in divergent hosts, similar to our previous characterization of HaRxL96 and PsAvh163 (Anderson et al. 2012). We tested this hypothesis with studies involving transient assays in *N. benthamiana*, soybean, and *Arabidopsis* and with detailed characterization of stably transformed *Arabidopsis*. In *N. benthamiana* and soybean, both effectors could suppress cell death triggered by diverse oomycete elicitors. The functional capabilities of both proteins were similar, with the exception that cell death triggered by the *Phytophthora* PAMP INF1 could be suppressed by PsAvh73 but not by HaRxL23. *H. arabidopsidis* does not produce INF1-like elicitor proteins. In *Arabidopsis*, both effectors suppressed *P. syringae* pv. *tomato* (Δ CEL)-induced callose deposition and promoted enhanced virulence of *P. syringae*. Moreover, both effectors conferred increased susceptibility to virulent *H. arabidopsidis* isolate Emco5, similar to other *H. arabidopsidis* effectors (Cabral et al. 2011; Fabro et al. 2011; Sohn et al. 2007). Interestingly, the effectors did not impair *RPP4*-dependent resistance to *H. arabidopsidis* Emoy2, suggesting that one or more of the targets of these effectors does not play an important role in the *RPP4* pathway. However, the broad functionality of these effectors in divergent plant species, in response to diverse elicitors of PTI and ETI, indicates that one or more of the targets are conserved and play a central role in plant immunity.

Fig. 7. HaRxL23 and PsAvh73 suppress callose formation in stably transformed *Arabidopsis* in response to *Pseudomonas syringae* DC3000(Δ CEL) mutant. **A**, Four-week old transgenic *Arabidopsis* Columbia plants stably transformed with **B**, *CaMV35S*-HaRxL23 or **C**, *CaMV35S*-PsAvh73 were infiltrated with a 5×10^7 CFU/ml solution of *P. syringae* Δ CEL mutant. Callose deposits were visualized by staining with aniline blue, and callose was quantified using the Autospot program (Cumbie et al. 2011). Four pictures per leaf from six leaves were analyzed from each transgenic and control line. An asterisk (*) indicates $P < 0.01$; t test comparisons representing significant differences with Col-0. Error bars represent standard error of six independent leaf samples tested at the same time. This experiment was repeated three times with similar results.

The identification of effector molecules from various eukaryotic pathogens enables us to draw parallels between prokaryotic and eukaryotic pathogens and helps to illuminate the extent to which these diverse pathogens share virulence strategies and target similar pathways of plant immunity. The next step in characterization of *HaRxL23* and *PsAvh73* is to identify their targets, thereby addressing the hypothesis that these effectors are targeting common proteins or processes in diverse hosts. The utility of studying conserved effectors is illustrated by previous studies of effectors from bacteria, e.g., the conserved effector locus (Collmer et al. 2000), and fungi, e.g., the SIX effectors from *Fusarium* spp. (Thatcher et al. 2012) and the Ecp6 effector from *Cladosporium* spp. (de Jonge et al. 2010). Mechanistic investigation of *HaRxL23* and *PsAvh73* is expected to yield similarly useful insights into the molecular basis of oomycete pathogenicity.

MATERIALS AND METHODS

Construction of expression plasmids.

HaRxL23 was amplified from genomic DNA extracted from *Arabidopsis* Oy-1 plants infected with *H. arabidopsidis* isolate Emoy2, using proofreading polymerase (Pfu; Invitrogen). Forward and reverse primers were designed to amplify from the SP cleavage site (*HaRxL23* NOSP) with (*HaRxL23* S) or without the stop codon (*HaRxL23* NS), depending on the type of fusion. Similarly, *PsAvh73* was amplified from *P. sojae* (isolate P6497)

genomic DNA and forward (*PsAvh73* NOSP) and reverse (*PsAvh73* S or *PsAvh73* NS) primers were designed.

For cloning into Gateway destination vectors, the sequence CACC was added at the 5' end of the forward primer and PCR was performed using the genomic DNA as template. PCR products were gel-purified (Qiagen) and, finally, were recombined into pENTR-D-TOPO gateway entry vector following the manufacture's protocol (Invitrogen). This step was followed by transformation into *Escherichia coli* DH5 α competent cells. Kanamycin-resistant colonies were selected on agarose plates, followed by colony PCR with plasmid-specific M13 forward and M13 reverse primers. Colonies having the correct size insert were selected for plasmid purification and were confirmed by sequencing. The pENTR clone generated was then used to create Gateway expression plasmids using LR recombination (Invitrogen). For *Agrobacterium*- and *Pseudomonas*-mediated transient expression experiments, the pENTR clones of *HaRxL23* and *PsAvh73* were shuttled into pB2GW7 and pEDV6 by LR recombination (Invitrogen).

Plant materials and growth conditions.

Arabidopsis, soybean, and *Nicotiana benthamiana* were grown in Sunshine ProMix 1. For experiments involving *H. arabidopsidis* and *Pseudomonas* spp., *Arabidopsis* plants were grown in controlled growth chambers under short-day cycles (8 h of light and 16 h of dark and 150 to 200 $\mu\text{E}/\text{m}^2\text{s}$) at 22°C and 60% relative humidity. For all other experiments,

Fig. 8. Stably transformed *HaRxL23* suppress defense gene induction. Ten- to 12-day-old *Arabidopsis* Col-0 and transgenic plants constitutively expressing *HaRxL23* were inoculated with a 5×10^4 spores/ml suspension of the avirulent *Hyaloperonospora arabidopsidis* isolate Emoy2. Transcript abundance was measured using quantitative, real-time polymerase chain reaction at 0, 12, and 24 h postinoculation. Transcript abundance was normalized to *AtActin2*. An asterisk (*) indicates ddCt (cycle threshold) values representing statistically significant ($P < 0.05$) differences with Col-0. This experiment was repeated four times with similar results.

Arabidopsis, soybean, and *N. benthamiana* plants were grown under long-day cycles (16 h of light and 8 h of dark, 90 to 100 $\mu\text{E}/\text{m}^2\text{s}$) at 22°C and 60% relative humidity.

Generation of transgenic *Arabidopsis* plants.

Plants expressing effectors were generated by recombining open reading frames in pENTR with the destination binary vector pB2GW7 (Karimi et al. 2002), under the control of the CaMV 35S promoter. The constructs were transferred to the *A. tumefaciens* GV3101 strain by electroporation and were transformed into *Arabidopsis* Col-0 by the floral dip method (Clough and Bent 1998). T_1 generation transformants were selected using BASTA. For the T_2 generation, 3:1 (BASTA-resistant/BASTA-susceptible) segregating lines were tested for homozygosity in the T_3 and T_4 generation. Presence of the transgene was confirmed by PCR from genomic DNA and transcript abundance was quantified by reverse-transcriptase PCR from RNA isolated from transgenic plants. Three to five independent nonsegregating transgenic lines (T_3 or T_4) displaying different mRNA abundance were used in the experiments.

H. arabidopsidis inoculations.

The *H. arabidopsidis* isolates Emoy2 and Emco5 were propagated and maintained in compatible *Arabidopsis* ecotypes Oy-1 and Ws-0, respectively (McDowell et al. 2011). Conidial suspensions of 5×10^4 spores/ml were applied with a Preval spray unit and the plants were kept under short-day conditions. *H. arabidopsidis* disease assays and staining with trypan blue were performed as described (McDowell et al. 2011).

Bacterial strains.

The following bacterial strains were used in this study: *E. coli* DH5 α , *Pseudomonas syringae* pv. *tomato* DC3000 wild type and ΔCEL (Alfano et al. 2000; Yuan and He 1996), *Pseudomonas fluorescens* Pf0-1 EtHAn carrying a functional T3S system (Thomas et al. 2009) and *A. tumefaciens* GV3101. *E. coli* and *A. tumefaciens* were grown in Luria-Bertani medium at 37°C and 28°C, respectively, in liquid media or petri dishes with appropriate antibiotic selections. *Pseudomonas* strains were grown in King's B medium at 28°C. Plasmids were introduced from *E. coli* DH5 α to wild-type or mutant *P. syringae* pv. *tomato* DC3000 and Pf0-1 strains by standard triparental matings, using *E. coli* RK600 as a helper strain.

RNA extraction, reverse-transcriptase PCR, and real-time PCR.

Total RNA was extracted from *Arabidopsis* tissue with TriSure reagent (Bioline). DNase-treated RNA (2 μg) was reverse-transcribed using the OmniScript cDNA synthesis kit (Qiagen) and an oligo(dT) primer. For semiquantitative RT-PCR analysis, 1 μl of cDNA was used per reaction and 40 and 35 PCR cycles were used to amplify effector targets and *AtActin*, respectively. PCR products were separated on 1% agarose gel in Tris-acetate-EDTA buffer. For RT-PCR analysis, samples were prepared by mixing cDNA template with SYBR green mastermix (Applied Biosystems) with the appropriate amount of primers and water. RT-PCR reactions were performed on an ABI 7300 device and the fold change was calculated relative to the 0-dpi timepoint.

Bacterial assays for HR, virulence, and callose suppression in *Arabidopsis*.

For HR assays, five-week-old *Arabidopsis* leaves were syringe-infiltrated with a needle-less syringe with 1×10^8 CFU/ml suspensions in 10 mM MgSO_4 . A total of six plants, three leaves each, were infiltrated and visual scoring was performed 16 h later.

For bacterial growth assays, five-week-old *Arabidopsis* leaves were syringe-infiltrated with a needle-less syringe with 1×10^5

CFU/ml suspensions in 10 mM MgSO_4 . Leaf discs were cored at 0 and 3 dpi, were surface-sterilized with 70% ethanol, and were homogenized using a mini-bead beater (Biospec Products). Serial dilutions were performed to count colony forming units. For each sample, three leaf discs were pooled three times per data point and bacterial growth was quantified by serial dilution and plating on selective media.

For callose suppression assays, whole leaves were harvested at 16 hpi, were treated with alcoholic lactophenol, and were stained with 0.01% (wt/vol) aniline blue stain in K_2HPO_4 buffer, as described previously (Sohn et al. 2007). Aniline blue-stained leaves were mounted on glass slides using 50% glycerol and were imaged with a Zeiss Axio Imager M1, using the filter settings for diamidino-2-phenylindole. Quantification of callose spots was performed using Autospots software (Cumbie et al. 2011).

Transient assays in soybean.

For transient assays in soybean, two-week-old leaves were transformed by particle bombardment using a mixture of plasmid DNA, following the modified BioRad PDS1000 gene gun protocol as described (Kale and Tyler 2011). The plasmid DNA mixtures were comprised of the effector, elicitor, and control setups. The effector setup comprised a mixture of 115 μg of the effector DNA, 50 μg of the elicitor DNA, and 50 μg of *GUS* DNA, as described previously (Dou et al. 2008). The elicitor setup comprised a mixture of 70 μg of the empty vector DNA, 50 μg of the *Avr4/6* or *CRN2* DNA, and 50 μg of *GUS*. The control mixture comprised 115 μg of the empty vector DNA and 50 μg of *GUS* plasmid DNA. Tungsten was prepared with the above mixtures as described earlier (Dou et al. 2008). Individual detached soybean leaves were transformed by particle bombardment using the tungsten preparation. After bombardment, the leaves were placed under controlled conditions of high humidity conditions in petri dishes at 8 h of light and 16 h of dark at 22°C for 2 to 3 days. Next, the leaves were stained with X-Gluc solution and were destained with 70% ethanol for 3 to 4 days. The number of living transformed cells was determined by counting *GUS*-expressing blue-colored cells under a dissecting microscope, using the Autospots software program (Cumbie et al. 2011). The percentage of surviving cells was quantified relative to the co-bombarded empty vector control. Statistical analyses were performed using the Wilcoxon rank sum test as described (Kale and Tyler 2011).

Transient assays in *N. benthamiana*.

Recombinant *A. tumefaciens* were grown, as described previously (Van der Hoorn et al. 2000), with the appropriate antibiotics. Overnight-grown *Agrobacterium* liquid cultures were centrifuged, and the pellets were resuspended in MMA induction buffer (10 mM MgCl_2 , 10 mM MES, 200 mM acetosyringone). The bacterial suspensions were incubated at room temperature for 1 to 3 h, and agroinfiltration using a needle-less syringe was performed on the abaxial side of 3- to 5-week-old *N. benthamiana* leaves at a final optical density at 600 nm of 0.3 to 0.5. Cell death or suppression of cell death were monitored for 4 to 5 days and were visually quantified after 5 days.

ACKNOWLEDGMENTS

Support was provided by the National Science Foundation grant numbers IOS-0744875 and IOS-1353366 and by Agriculture and Food Research Initiative Competitive grant number 224426 from the United States Department of Agriculture National Institute of Food and Agriculture.

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