The *Erwinia amylovora* *avrRpt2*_{EA} Gene Contributes to Virulence on Pear and *AvrRpt2*_{EA} Is Recognized by *Arabidopsis* RPS2 When Expressed in *Pseudomonas syringae*

Youfu Zhao,^{1,2} Sheng-Yang He,^{2,3,4} and George W. Sundin^{1,2}

¹Department of Plant Pathology and ²Center for Microbial Pathogenesis, 103 Center for Integrated Plant Systems, Michigan State University, East Lansing, MI 48824, U.S.A.; ³Department of Plant Biology and ⁴Department of Energy-Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, U.S.A.

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The enterobacterium *Erwinia amylovora* is a devastating plant pathogen causing necrotrophic fire blight disease of apple, pear, and other rosaceous plants. In an attempt to identify genes induced during infection of host plants, we identified and cloned a putative effector gene, *avrRpt2*_{EA}. The deduced amino-acid sequence of the translated *AvrRpt2*_{EA} protein is homologous to the effector protein *AvrRpt2* previously reported in *Pseudomonas syringae* pv. *tomato*. These two proteins share 58% identity (70% similarity) in the functional domain; however, the secretion and translocation signal domain varied. The *avrRpt2*_{EA} promoter region contains a typical ‘*hrp* box,’ which suggests that *avrRpt2*_{EA} is regulated by the alternative sigma factor, HrpL. *avrRpt2*_{EA} was detected in all *E. amylovora* strains tested but not in other closely related *Erwinia* species. An *avrRpt2*_{EA} deletion mutant was reduced in its ability to cause systemic infection on immature pear fruits as compared with the wild-type strain, indicating that *avrRpt2*_{EA} acts as a virulence factor on its native host. Growth of *P. syringae* pv. *tomato* DC3000 expressing *avrRpt2*_{EA} was 10-fold higher than that of *P. syringae* pv. *tomato* DC3000 in an *Arabidopsis* *rps2* mutant, indicating that *avrRpt2*_{EA} promotes virulence of *P. syringae* pv. *tomato* DC3000 on *Arabidopsis* similar to *P. syringae* pv. *tomato* *avrRpt2*. When *avrRpt2*_{EA} was expressed in *P. syringae* pv. *tomato* DC3000 in its native form, a weak hypersensitive response (HR) was induced in *Arabidopsis*; however, a hybrid protein containing the *P. syringae* pv. *tomato* *avrRpt2* signal sequence, when expressed from the *P. syringae* pv. *tomato* *avrRpt2* promoter, caused a strong HR. Thus, the signal sequence and promoter of *avrRpt2*_{EA} may affect its expression, secretion, or translocation, singly or in combination, in *P. syringae* pv. *tomato* DC3000. These results indicated that *avrRpt2*_{EA} is genetically recognized by the RPS2 disease resistance gene in *Arabidopsis* when expressed in *P. syringae* pv. *tomato* DC3000. The results also suggested that although distinct pathogens such as *E. amylovora* and *P. syringae* may contain similar effector genes, expression and secretion

of these effectors can be under specific regulation by the native pathogen.

Additional keywords: λ Red system, type III secretion.

Erwinia amylovora is the causal agent of fire blight, a devastating plant disease affecting apple, pear, and other rosaceous plants. This highly virulent necrotrophic pathogen attacks a variety of host tissues, including blossoms, actively growing shoots, and fruits. The pathogen is also capable of rapid systemic movement downward through stem tissue, resulting in infection and killing of rootstock crowns (Vanneste and Eden-Green 2000). Fire blight is a difficult disease to manage, because most varieties of apple and pear are highly susceptible to infection. Although apple varieties with tolerance to fire blight and crab-apple varieties and apple rootstocks with fire blight resistance are known, gene-for-gene interactions in these pathosystems have not been documented.

Like many other gram-negative plant pathogenic bacteria, *E. amylovora* synthesizes a hypersensitive response and pathogenicity (Hrp) type III secretion apparatus that delivers effector proteins into host plants (He et al. 2004; Kim and Beer 2000; Oh et al. 2005). Most *hrp* genes, which are clustered into a 40-kb genomic region termed the Hrp pathogenicity island (PAI), have been found to encode proteins involved in gene regulation or in assembly of the type III secretion system (T3SS) (Alfano and Collmer 2004; He et al. 2004; Kim and Beer 2000; Oh et al. 2005). The T3SS in *E. amylovora* enables the pathogen to cause disease in susceptible host plants and to elicit the hypersensitive response (HR) in nonhost plants. The T3SS of *E. amylovora* is phylogenetically related to the T3SS of *Pseudomonas syringae* and belongs to the group 2 Hrp1 T3SS (He et al. 2004). In addition to the T3SS, other major *E. amylovora* virulence factors that contribute to pathogenesis and plant colonization include the exopolysaccharides amylovanan and levan and the iron-scavenging siderophore desferrioxamine (Expert et al. 2000; Geier and Geider 1993; Venisse et al. 2001, 2003; Zhang and Geider 1999).

The T3SS of *E. amylovora* secretes several virulence proteins, including HrpA, HrpN, HrpW, and disease-specific protein DspA/E (hereafter referred to as DspE) (Bogdanove et al. 1998a and b; Gaudriault et al. 1997; Kim and Beer 1998, 2000; Wei et al. 1992, 2000). The HrpA protein is the major structural protein of the Hrp pilus, which is the extracellular

Corresponding author: G. W. Sundin; Telephone: 517-355-4573; Fax: 517-353-5598; E-mail: sundin@msu.edu

Nucleotide sequence data for *P. syringae* pv. *tomato* *AvrRpt2*_{EA} are available in the GenBank database under accession numbers DQ344487 (Ea110), and DQ344488 (Ea1189).

part of the T3SS (Jin et al. 2001). DspE, HrpN, and HrpW are known effector proteins of the T3SS (Bogdanove et al. 1998a and b; Kim and Beer 1998; Wei et al. 1992; Zhao et al. 2005). The large multidomain protein DspE is essential for *E. amylovora* pathogenesis, and possesses one or more additional functions in inhibiting salicylic acid-mediated innate immunity (Bogdanove et al. 1998a and b; DebRoy et al. 2004). HrpN and HrpW are harpins, glycine-rich, heat-stable proteins with HrpN functioning as a major HR elicitor and virulence factor (Barny 1995; Wei et al. 1992). HrpW is partly homologous to class III pectate lyases but has not been shown to have a role in virulence (Kim and Beer 1998). A new putative effector, *hopPtoC_{EA}*, was recently shown to be induced in *E. amylovora* during infection of immature pear fruit; however, analysis of a *hopPtoC_{EA}* insertional mutant revealed no effect on virulence (Zhao et al. 2005). Recently, three *hrp*-associated systemic virulence (*hsv*) genes were reported to be required for full virulence of *E. amylovora* in apple (Oh et al. 2005). All of the *hsv* genes, *hrp* operons that encode structural components of the T3SS, and most effector genes have a conserved promoter sequence called the '*hrp box*' in *E. amylovora* and are regulated by HrpL, an alternative sigma factor (Chang et al. 2004; Collmer et al. 2000, 2002; Fouts et al. 2002; Kim and Beer 2000).

To date, all *E. amylovora* effector genes except for *hopPtoC_{EA}* are located within the *hrp* PAI. Since so few effectors are currently known, it is tempting to speculate that additional genes encoding effector proteins may exist in the *E. amylovora* genome. In the present study, we cloned and characterized *avrRpt2_{EA}*, a homolog of *avrRpt2* from *P. syringae* pv. *tomato* (*P. syringae* pv. *tomato* *avrRpt2*) and a gene that is located outside of the *E. amylovora* *hrp* PAI. We show that *avrRpt2_{EA}* contributes to virulence by enhancing the ability of *E. amylovora* to infect and grow within immature pear fruit. We also present evidence demonstrating that *avrRpt2_{EA}* acts both as an avirulence and as a virulence gene, and AvrRpt2_{EA} is recognized by the RPS2 disease resistance gene in *Arabidopsis thaliana* when expressed in *P. syringae* pv. *tomato* DC3000. Finally, we show that expression, secretion, or translocation, singly or in combination, of *avrRpt2_{EA}* in *P. syringae* pv. *tomato* DC3000 was enhanced in chimeric constructs containing

the signal sequence and promoter of *P. syringae* pv. *tomato* *avrRpt2*, suggesting that either expression, secretion, or both of *avrRpt2_{EA}* may be under specific regulation by the native pathogen.

RESULTS

Cloning and analysis

of *avrRpt2_{EA}* from *Erwinia amylovora*.

We recently utilized an in vivo expression technology system to identify genes from *E. amylovora* Ea110 that are induced during infection of immature pear fruit (Zhao et al. 2005). As part of this study, a genomic sequence was identified exhibiting homology to *P. syringae* pv. *tomato* *avrRpt2*, and we used thermal asymmetric interlaced-polymerase chain reaction (TAIL-PCR) to obtain the full sequence of this gene. An alignment of the translated amino acid sequence of AvrRpt2_{EA} with *P. syringae* pv. *tomato* AvrRpt2 indicated that the deduced amino acid sequences were homologous (Fig. 1), leading us to designate this new gene as *avrRpt2_{EA}*. A typical *hrp box* (GGAAGT-N₁₅-TCACCTCA) was found in the promoter region of *avrRpt2_{EA}*, suggesting that this gene may be regulated by the alternate sigma factor HrpL. The 255-amino acid (aa) sequence of *P. syringae* pv. *tomato* AvrRpt2 protein contains two domains, a secretion and translocation signal domain (about the first 71 aa) and a functional effector domain (Mudgett and Staskawicz 1999). Based on amino acid alignment and secondary structure analysis, the *P. syringae* pv. *tomato* AvrRpt2 protein is predicted as a cysteine protease capable of self-cleavage at aa 71 and 72 (aa 67 to 75, VPAFGGWFK) and the predicted catalytic triad of *P. syringae* pv. *tomato* AvrRpt2 is cysteine (aa 122), histidine (aa 208), and aspartic acid (aa 226) (Fig. 1) (Mudgett and Staskawicz 1999). The AvrRpt2_{EA} amino acid sequence was smaller than *P. syringae* pv. *tomato* AvrRpt2 in the N-terminal secretion and translocation signal sequence domain, which was also poorly conserved with *P. syringae* pv. *tomato* AvrRpt2 (Fig. 1). In contrast, the effector domain of the two proteins was conserved sharing 58% aa identity and 70% aa similarity (Fig. 1). In addition, the self-cleavage site (aa 41 to 49, APSFGGCFG) and the predicted catalytic triad between these two proteins were both conserved

Fig. 1. Alignment of deduced amino-acid sequences of the putative effector protein AvrRpt2_{EA} from *Erwinia amylovora* with effector *Pseudomonas syringae* pv. *tomato* AvrRpt2. Sequence alignment was performed using the Clustal W 1.8 program. Black boxes in both proteins indicate amino-acid residues that are conserved; gray boxes indicate amino acid residues that are similar; an asterisk (*) indicates consensus sequences; the line with arrowheads at each end indicates the serine-rich stretch (amino acids 51 to 66); $\rightleftharpoons$ indicates the predicted self-cleavage site; and | indicates the predicted catalytic triad. The start codon for *avrRpt2_{EA}* is GTG. The GenBank accession numbers for *P. syringae* pv. *tomato* AvrRpt2 and AvrRpt2_{EA} are CAA79815, DQ344487 (Ea110), and DQ344488 (Ea1189), respectively.

(Fig. 1). A similar serine-rich stretch was also found in both proteins (Fig. 1).

Distribution of the *avrRpt2_{EA}* gene in closely-related *Erwinia* spp.

We utilized the oligonucleotide primer pair (*avrRpt2_IF1* and *IR1*) (Table 1) targeting a 446-bp sequence within *avrRpt2_{EA}* to check whether this gene was also present in other *E. amylovora* strains. As presented in Table 2, we found that the *avrRpt2_{EA}* PCR product was amplified in all *E. amylovora* strains tested, but no PCR product was amplified from *E. pyrifoliae* Ep1/96 or *Erwinia* sp. strain Ejp557 DNA (Table 2). In addition, Southern hybridization analyses showed that the *avrRpt2_{EA}* probe only weakly hybridized to DNA from these two strains (data not shown). We also cloned and sequenced *avrRpt2_{EA}* from *E. amylovora* Ea1189 using the primer pair *avrRpt2-3* and *avrRpt2-4* (Table 1). The *avrRpt2_{EA}* sequence from Ea1189 was identical to that from Ea110. As we were

performing this work, a database of sequences from a genome-sequencing project of *E. amylovora* Ea273 became available. A BLAST search of the Ea110/Ea1189 sequence against the Ea273 database revealed the presence of the *avrRpt2_{EA}* gene and that there was only a single base pair difference between the sequences. This difference would result in an amino acid change at position 156, with either a serine or cysteine present at this position in Ea273 and Ea110/Ea1189 *AvrRpt2_{EA}*, respectively.

In silico analysis showed that sequences similar to *avrRpt2_{EA}* were not present in the genome of *E. (Pectobacterium) carotovora* pv. *atroseptica* SCRI1043 or *E. chrysanthemi* 3937 (Table 2; Bell et al. 2004; Yang et al. 2004). Interestingly, the gene organization surrounding *avrRpt2_{EA}* in Ea273 was similar to that in *E. carotovora* pv. *atroseptica*, with genes encoding elongation factor P and an outer membrane lipoprotein flanking *avrRpt2_{EA}* and sharing extensive similarity with the corresponding genes in *E. carotovora* pv. *atroseptica* (Fig. 2). However,

Table 1. Bacterial strains, plasmids, and primers used in this study

Strains, plasmids, and primers	Relevant characters or sequences (5' to 3') ^a	Reference or source
<i>Erwinia amylovora</i>		
Ea110	Wild type, isolated from apple	Maxson-Stein et al. 2003
Ea1189	Wild type, isolated from apple	Burse et al. 2004
Ea273	Wild type, isolated from apple	Bogdanove et al. 1998a
CFBP1430	Wild type, isolated from <i>Crataegus</i>	Gaudriault et al. 1997
MR1	Wild type, isolated from <i>Rubus</i> sp.	Maxson-Stein et al. 2003
ZYRKD3-1	<i>avrRpt2_{EA}::Cm</i> ; Cm ^r insertional mutant of <i>avrRpt2_{EA}</i> of Ea1189, Cm ^r	This study
<i>E. pyrifoliae</i> Ep1/96	Wild type, isolated from Asian pear, Korea	Maxson-Stein et al. 2003
<i>Erwinia</i> sp. Ejp557	Wild type, isolated from Asian pear, Japan	Maxson-Stein et al. 2003
<i>Escherichia coli</i> DH10B	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) Φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>ara</i> Δ139 Δ(<i>ara, leu</i>)7697 <i>galU</i> <i>galK</i> λ- <i>rpsL</i> (Sm ^r) <i>nupG</i>	Invitrogen, Carlsbad, CA, U.S.A.
Plasmids		
pBluescript II SK(+)	Ap ^r , cloning vector	Stratagene, La Jolla, CA, U.S.A.
pGEMT-Easy	Ap ^r , PCR cloning vector	Promega, Madison, WI, U.S.A.
pKD46	Ap ^r , P _{BAD} gam bet exo pSC101 oriTS	Datsenko and Wanner 2000
pKD3	Ap ^r , FRT cat FRT PS1 PS2 oriR6K <i>rgbN</i>	Datsenko and Wanner 2000
pUCP19	Ap ^r , shuttle vector	Schweizer 1991
pRK415	Tc ^r , broad host vector	Keen et al., 1988
pDSK600	Sp ^r , Sm ^r , IncQ replicon, 3× <i>lacU</i> V5 promoter	Murillo et al. 1994
pDSK600 (<i>avrRpt2_{PST}</i>)	1.8-kb <i>HindIII</i> fragment containing <i>Pseudomonas syringae</i> pv. <i>tomato</i> <i>avrRpt2</i> promoter and open reading frame in pDSK600	Mudgett and Staskawicz 1999
pZYR2-19/-415	950-bp <i>EcoRI</i> fragment containing <i>avrRpt2_{EA}</i> in pUCP19, in pRK415, or in both	This study
pChimeric1	949-bp PCR products containing promoter of <i>avrRpt2_{PST}</i> and the coding region of <i>avrRpt2_{EA}</i> in pUCP19	This study
pChimeric2	1,043-bp PCR products containing promoter and signal sequence of <i>avrRpt2_{PST}</i> and the function domain of <i>avrRpt2_{EA}</i> in pUCP19	This study
Primers ^b		
Rpt2-N1	GTGAAAGTCAGTCATCTCACATCCCCGCCCTGTCGTAATCGAGCA CCA GCGATTGTGTAGGCTGGAGCT	
Rpt2_N2	CTAATTTTCACTGTATAACATGGCGTGTGGCACCCGCCATGCCAGAC GCT CCATGGTCCATATGAATATCCTCC	
<i>avrRpt2-3</i>	CGTGCAGATTGGCGAAGTGATTA	
<i>avrRpt2-4</i>	GGTGCTTATCCATGCGGTCGTTT	
<i>avrRpt2_PST_F1</i>	CCCAGCTTCAGTGCGAGTAGTCCATTTGAGAGC (<i>HindIII</i>)	
<i>avrRpt2_PST_R1</i>	CTAGTCTAGAGGCAACTGGAGCAATTTTCATCAT (<i>XbaI</i>)	
<i>avrRpt2_PST_R2</i>	CTAGTCTAGAGGCGGAACCTCTATCTTGTGTCTC (<i>XbaI</i>)	
<i>avrRpt2_EA_F1</i>	CTAGTCTAGAACATCCCCGCCCTGTCGTA (<i>XbaI</i>)	
<i>avrRpt2_EA_F2</i>	CTAGTCTAGACTGGTCCCAGCTACCGGCCGC (<i>XbaI</i>)	
<i>avrRpt2_EA_R1</i>	CCGGAATTCAGGTGCTTATCCATGCGGTGCTTCC (<i>EcoRI</i>)	
<i>avrRpt2_IF1</i>	GCCGACGCGCCATCTTTTG	
<i>avrRpt2_IR1</i>	ATCGCATCGTTGGGCTTGTCTATC	
Cm1	TTATACGCAAGGCGACAAGG	
Cm2	GATCTTCCGTCACAGGTAGG	
RT1	GGGCGCTGCTGATGCTATGG	
RT2	GCCCATTCGCTCATTCTGTTG	
RT3	AAAGCCGGCGTTACTGCGAGAGC	
AD1	NTCGASTWTSWGGTT	
AD2	NGTCGASWGANAWGAA	
AD3	WGTGNAGWANCANAGA	

^a Km^r, Ap^r, Cm^r, Sm^r, and Sp^r = kanamycin, ampicillin, chloramphenicol, streptomycin, spectinomycin resistance, respectively.

^b Underlined nucleotides are restriction sites added and the restriction enzymes are indicated at the end of primers. Mixed nucleotides: S = C+G; W = A+T; N = A+T+C+G.

the *avrRpt2_{EA}* gene, present in *E. amylovora* Ea273, was absent from *E. carotovora* pv. *atroseptica* and was replaced with genes encoding entericidin B, a bacteriolytic lipoprotein (Bishop et al. 1998), and a putative membrane protein (Fig. 2).

Mutation of *avrRpt2_{EA}* reduces the virulence of *E. amylovora*.

In order to test whether *avrRpt2_{EA}* contributes to the virulence of *E. amylovora* on its native host, we generated an *avrRpt2_{EA}* mutant in which the endogenous copy of *avrRpt2_{EA}* was disrupted and replaced with an antibiotic resistance cassette. We utilized the λ Red system (Datsenko and Wanner 2000) to construct a nonpolar insertional mutation within *avrRpt2_{EA}* of *E. amylovora* Ea1189 as described below.

We next examined the virulence of mutant strain ZYRKD3-1 on immature pear fruits in comparison with the (wild-type) WT strain. Immature pear fruits have been routinely used to test the pathogenicity of *E. amylovora* field isolates or mutants, and infection of immature pears requires major pathogenicity factors such as HrpA and DspE (Zhao et al. 2005). The WT

strain Ea1189 caused necrotic lesions and produced bacterial ooze on the immature pear fruits at four days after inoculation (Fig. 3A). Although the mutant ZYRKD3-1 also caused symptoms, symptom progression was greatly reduced as compared with the WT strain (Fig. 3A). In order to ensure that the reduction in disease progress of the *avrRpt2_{EA}* mutant ZYRKD3-1 was specifically related to the mutation in the *avrRpt2_{EA}* gene, we tested the virulence of the *avrRpt2_{EA}* mutant ZYRKD3-1 complemented with the original *avrRpt2_{EA}* gene. The complemented strain produced similar symptoms and comparable disease progression on immature pear fruits as the WT strain (Fig. 3A).

Quantitation of bacterial populations recovered from inoculated pears revealed an effect of *avrRpt2_{EA}* on pathogen growth that was largest at one day following inoculation, as growth of the *avrRpt2_{EA}* mutant ZYRKD3-1 was reduced to approximately tenfold less than that of the WT strain (Fig. 3B). Within two days after inoculation, the WT strain Ea1189 increased in population approximately six orders of magnitude on immature pear fruits, and we observed three- to fivefold less growth of ZYRKD3-1 than that of the WT strain at two to four days postinoculation (Fig. 3B). Ea1189 and ZYRKD3-1 data means were compared using a *t* test that showed significant differences (*P* ≤ 0.10) on days 1, 2, and 3 following inoculation (day 1, *P* = 0.017; day 2, *P* = 0.076; day 3, *P* = 0.10; and day 4, *P* = 0.12).

In order to ensure that the reduction of bacterial growth in pear was resulting specifically from the disruption of the *avrRpt2_{EA}* gene, growth of the complemented strain of the *avrRpt2_{EA}* mutant ZYRKD3-1 was also monitored and this strain was partially restored in growth as compared with that of the mutant strain (Fig. 3B). Comparison of data means using a *t* test that showed significant differences only on day 1 following inoculation (day 1, *P* = 0.047; day 2, *P* = 0.12; day 3, *P* = 0.26; and day 4, *P* = 0.16). In summary, these experiments suggest that the *avrRpt2_{EA}* gene contributes to the virulence of *E. amylovora* on pears.

Table 2. Detection of *avrRpt2_{EA}* in *Erwinia amylovora* and related bacteria

Bacteria	Strains	Host	Presence of <i>avrRpt2_{EA}</i> ^a
<i>E. amylovora</i>	Ea1189	Apple, pear	+
	Ea110	Apple, pear	+
	Ea273	Apple, pear	+
	CFBP1430	Apple, pear	+
	MR1	<i>Rubus</i> sp.	+
<i>E. pyrifoliae</i>	Ep1/96	Asian pear	±
<i>Erwinia</i> sp.	Ejp557	Asian pear	±
<i>E. carotovora</i> pv. <i>atroseptica</i>	SCRI1043	Potato	...
<i>E. chrysanthemi</i>	3937	Various crops	...

^a + = PCR products were amplified; ± = No PCR products were amplified, but Southern hybridization showed weak signal; and ... = in silico analysis of the genome sequence, no gene present.

Fig. 2. Schematic map illustrating the genomic location of *avrRpt2_{EA}* in *Erwinia amylovora* Ea273 and comparison with similar genomic region of *E. carotovora* pv. *atroseptica* (ECA) SCR1043. Genes from *E. carotovora* pv. *atroseptica* are numbered according to the annotated genome of strain SCR1043, and putative functions are shown (Bell et al. 2004). GenBank accession numbers for *E. carotovora* pv. *atroseptica* genes 3968 to 3977 are YP_052055 to YP_052064. Solid lines linking genes from *E. amylovora* and *E. carotovora* pv. *atroseptica* indicate homologous genes; numbers indicate the percentage of amino acid similarity of the corresponding genes.

AvrRpt2_{EA} is genetically recognized by the *Arabidopsis* RPS2 disease resistance gene when expressed in *P. syringae* pv. *tomato* DC3000 and enhances bacterial growth in *Arabidopsis* lacking RPS2.

P. syringae pv. *tomato* *avrRpt2* was originally cloned from *P. syringae* pv. *tomato* JL1065, a strain that infects tomato but not *Arabidopsis* (Whalen et al. 1991). However, most studies

of the virulence and avirulence activity of *P. syringae* pv. *tomato* *AvrRpt2* have been performed by heterologously expressing the gene in *P. syringae* pv. *tomato* DC3000, which does not carry an endogenous copy of *P. syringae* pv. *tomato* *avrRpt2* and can infect both tomato and *Arabidopsis* (Buell et al. 2003). *P. syringae* pv. *tomato* *avrRpt2*, when expressed in *P. syringae* pv. *tomato* DC3000 cells inoculated into *Arabidopsis* plants, genetically recognizes the disease resistance gene RPS2 in a gene-for-gene basis eliciting the HR (Kunkel et al. 1993). We asked whether *avrRpt2_{EA}* could likewise induce the HR in *Arabidopsis* carrying the RPS2 gene. Since *E. amylovora* is not a pathogen of *Arabidopsis*, we cloned the *avrRpt2_{EA}* gene into the shuttle vector pUCP19 (Schweizer 1991), creating the plasmid pZYZR2, which was introduced into *P. syringae* pv. *tomato* DC3000. As shown in Figure 4A, *P. syringae* pv. *tomato* DC3000 harboring *P. syringae* pv. *tomato* *avrRpt2* elicited a severe HR in *Arabidopsis* with the RPS2 gene, as previously reported (Bent et al. 1994; Kunkel et al. 1993). In contrast, a weak HR was observed 24 h after inoculation with *P. syringae* pv. *tomato* DC3000 expressing *avrRpt2_{EA}* (Fig. 4B), and no HR was seen for *P. syringae* pv. *tomato* DC3000 harboring the vector control (Fig. 4C). Upon infiltration into *Arabidopsis* plants carrying a mutation at the RPS2 locus (*rps2* mutant), no HR was observed for all three *P. syringae* pv. *tomato* DC3000 strains expressing *avrRpt2_{EA}*, *P. syringae* pv. *tomato* *avrRpt2* or the vector control (data not shown). This result suggested that *AvrRpt2_{EA}* is also genetically recognized by the RPS2 disease resistance gene in *Arabidopsis*, as is *P. syringae* pv. *tomato* *AvrRpt2*, and indicated that *AvrRpt2_{EA}* is functional when expressed in *P. syringae* pv. *tomato* DC3000 in its native form and recognized by plant cells expressing the RPS2 activity. This recognition resulted in disease resistance-mediated localized cell death in planta, although the reaction was weak compared with that with *P. syringae* pv. *tomato* *AvrRpt2*.

The growth of *P. syringae* pv. *tomato* DC3000/pZYZR2 (*avrRpt2_{EA}*), *P. syringae* pv. *tomato* DC3000 (*P. syringae* pv. *tomato* *avrRpt2*), and *P. syringae* pv. *tomato* DC3000(pUCP19) was assessed in *Arabidopsis* Col-0 plants with an intact RPS2 gene or *rps2* mutant plants. Although we observed a weak HR in *Arabidopsis* WT plants when inoculated with high inoculum levels (10^8 CFU/ml) of *P. syringae* pv. *tomato* DC3000/pZYZR2 (*avrRpt2_{EA}*), there were no differences in growth in *Arabidopsis*

Fig. 3. Symptoms and growth of *Erwinia amylovora* wild type (WT) and various mutant strains on immature pear fruits. **A**, Symptoms caused by *E. amylovora* WT Ea1189, *avrRpt2_{EA}* mutant, complemented strain of *avrRpt2_{EA}* mutant in immature pear fruits. DPI = days postinoculation. **B**, Growth of *E. amylovora* WT Ea1189, *avrRpt2_{EA}* mutant ZYRKD3-1, and the complemented strain of *avrRpt2_{EA}* mutant during infection of immature pears. The growth of bacterial strains was monitored at 0, 1, 2, 3, and 4 days after inoculation. Datapoints represent the means of three replicates $\pm$ standard error. Significant differences between data means of Ea1189 and ZYRKD3-1 were observed on days 1, 2, and 3 postinoculation, and significant differences between data means of ZYRKD3-1 and the complemented strain ZYRKD3-1 (pZYZR2) were observed on day 1 postinoculation. Similar results were obtained in three repeated independent experiments.

Fig. 4. Avirulence function of *AvrRpt2_{EA}* when expressed in *Pseudomonas syringae* pv. *tomato* DC3000. Localized cell death in *Arabidopsis thaliana* containing the RPS2 gene induced by *Pseudomonas syringae* pv. *tomato* DC3000 strains expressing **A**, *P. syringae* pv. *tomato* *avrRpt2*, **B**, *avrRpt2_{EA}*, and **C**, vector control. *A. thaliana* leaves were infiltrated with a 1×10^8 CFU/ml suspension of bacteria. Symptoms were photographed 24 h after inoculation.

Fig. 5. Virulence function of AvrRpt2_{EA} when expressed in *Pseudomonas syringae* pv. *tomato* DC3000. **A**, Comparison of growth of *P. syringae* pv. *tomato* DC3000 harboring the vector pUCP19 with *P. syringae* pv. *tomato* DC3000 expressing *P. syringae* pv. *tomato* avrRpt2 or avrRpt2_{EA} in *Arabidopsis thaliana* plants containing the RPS2 gene. **B**, Comparison of growth of *P. syringae* pv. *tomato* DC3000 harboring the vector pUCP19 with *P. syringae* pv. *tomato* DC3000 expressing *P. syringae* pv. *tomato* avrRpt2 or avrRpt2_{EA} in *A. thaliana* plants lacking the RPS2 gene (*rps2* mutant). Plants were inoculated by vacuum infiltration with approximately 5×10^4 CFU/ml bacterial suspensions. The growth of bacterial strains was monitored at 0, 1, 2, and 3 days after inoculation. Data points represent the means of three replicates $\pm$ standard error. Similar results were obtained in repeated independent experiments.

with RPS2 gene for *P. syringae* pv. *tomato* DC3000 (pUCP19), *P. syringae* pv. *tomato* DC3000/pZYZR2 (avrRpt2_{EA}), *P. syringae* pv. *tomato* DC3000 (*P. syringae* pv. *tomato* avrRpt2) when inoculated with low inoculum levels (10^6 CFU/ml) (Fig. 5A). We reasoned that growth of *P. syringae* pv. *tomato* DC3000 containing avrRpt2 was not affected in *Arabidopsis* WT plants because the initial inoculum (about 10^6 CFU/ml) was so low that a large-scale HR response could not be initiated. However, at one day after inoculation, DC3000/pZYZR2 (avrRpt2_{EA}) and *P. syringae* pv. *tomato* DC3000 (*P. syringae* pv. *tomato* avrRpt2) had grown to approximately tenfold greater cell numbers compared with that of *P. syringae* pv. *tomato* DC3000 (pUCP19) in the *Arabidopsis* *rps2* mutant (Fig. 5B). This tenfold difference in recoverable populations remained relatively constant as bacterial cell numbers increased on day 2 and slightly decreased on day 3 after inoculation (Fig. 5B). In terms of growth comparison, the growth of *P. syringae* pv. *tomato* DC3000 expressing either *P. syringae* pv. *tomato* avrRpt2 or avrRpt2_{EA} was higher in the *rps2*

Fig. 6. The promoter and secretion and translocation signal sequence of avrRpt2_{EA} affects its expression, its secretion and translocation, or both in *Pseudomonas syringae* pv. *tomato* DC3000. **A**, Schematic diagram depicting the construction of two hybrid proteins. Color-coded area indicates the origin of the sequences. P = promoter, ST = secretion and translocation signal domain, and FD = functional domain. **B**, Localized cell death in *Arabidopsis thaliana* containing the RPS2 gene induced by *P. syringae* pv. *tomato* DC3000 strains expressing the vector control (1), avrRpt2_{EA} (2), Chimeric1 (3), Chimeric2 (4), and *P. syringae* pv. *tomato* avrRpt2 (5). *A. thaliana* leaves were infiltrated with a 1×10^8 CFU/ml suspension of bacteria. At least ten leaves were scored for each strain tested, and 90 to 100% leaves showed the indicated hypersensitive response (HR) symptoms. + symbols reflect a qualitative assessment of the HR, with increased numbers of + symbols indicating a stronger HR. Symptoms were photographed 24 h after inoculation.

mutant than in WT *Arabidopsis* plants (Fig. 5A and B). These results differ from those previously reported, in which no difference was found for bacterial growth of *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *tomato* DC3000 expressing *P. syringae* pv. *tomato* *avrRpt2* in *rps2* mutants (Chen et al. 2000). Our results showed that, like *P. syringae* pv. *tomato* *avrRpt2*, *avrRpt2_{EA}* enhanced *P. syringae* pv. *tomato* DC3000 growth in *Arabidopsis* plants lacking the RPS2 disease resistance gene.

The promoter and the secretion and translocation signal sequence of *avrRpt2_{EA}* affects either its expression, its secretion and translocation, or both in *P. syringae* pv. *tomato* DC3000.

We have demonstrated that *P. syringae* pv. *tomato* DC3000 expressing *avrRpt2_{EA}* only induced a weak HR in *Arabidopsis* carrying the RPS2 gene (Fig. 4). This indicated that *AvrRpt2_{EA}* was recognized by plant cells expressing the RPS2 activity, but we did not know whether the weak response was due to decreased expression of *AvrRpt2_{EA}* in *P. syringae* pv. *tomato* DC3000, defects in the secretion and translocation of *AvrRpt2_{EA}* into the host cells, or both. *P. syringae* pv. *tomato* *AvrRpt2* is expressed in and secreted from *P. syringae* pv. *tomato* DC3000 and is delivered to plant cells in an Hrp-dependent manner (Chen et al. 2000; Leister et al. 1996; Mudgett 2005). In addition, the N-terminus (first 79 aa) of *P. syringae* pv. *tomato* *AvrRpt2* is not required for RPS2 recognition in planta (Mudgett 2005) but is essential for *P. syringae* pv. *tomato* DC3000-mediated elicitation of RPS2-dependent cell death in *A. thaliana* and acts as a secretion and translocation signal sequence (Guttman et al. 2002; Mudgett and Staskawicz 1999; Vinatzer et al. 2005).

By comparing the deduced aa sequences of these two putative proteins (Fig. 1), we found that the N-terminal region of *AvrRpt2_{EA}* was much shorter and diverged from *P. syringae* pv. *tomato* *AvrRpt2*. To further examine the *AvrRpt2_{EA}*-mediated HR response in *Arabidopsis*, we constructed two chimeric proteins, with the first expressed under the control of the *P. syringae* pv. *tomato* *avrRpt2* promoter and containing the first eight aa of *P. syringae* pv. *tomato* *AvrRpt2* fused in frame to the *AvrRpt2_{EA}* protein lacking five aa of the N-terminus (Fig. 6A). The second chimeric protein was also expressed under the control of *P. syringae* pv. *tomato* *avrRpt2* promoter and contained the first 70 aa of *P. syringae* pv. *tomato* *AvrRpt2* fused in-frame to the predicted functional domain of *avrRpt2_{EA}* gene (the N-terminal 35 aa were deleted) (Fig. 6A). These constructs were then introduced into *P. syringae* pv. *tomato* DC3000 and the transformants were tested for the HR response in *Arabidopsis* containing the RPS2 gene and were compared for the severity of HR with *P. syringae* pv. *tomato* DC3000 expressing the native *P. syringae* pv. *tomato* *avrRpt2* and *avrRpt2_{EA}*. As expected, *P. syringae* pv. *tomato* DC3000 expressing the vector itself did not induce any HR, *P. syringae* pv. *tomato* DC3000 expressing *avrRpt2_{EA}* induced a weak HR, whereas a very strong HR response was produced for *P. syringae* pv. *tomato* DC3000 expressing *P. syringae* pv. *tomato* *avrRpt2* (Fig. 6B). The severity of the HR observed for the two chimeric proteins was greatly increased when compared with DC3000 expressing *avrRpt2_{EA}* (Fig. 6B). Thus, the signal sequence and promoter of *avrRpt2_{EA}* may affect its expression and its secretion, translocation, or both in *P. syringae* pv. *tomato* DC3000 and into the host plants.

DISCUSSION

We report the characterization of the effector protein *AvrRpt2_{EA}* from *E. amylovora*, the first homolog of *P. syringae* pv. *tomato* *AvrRpt2* found in bacterial plant pathogens other

than *P. syringae*. The *AvrRpt2* protein is a unique effector protein only found previously in *P. syringae* pv. *tomato* JL1065 (Whalen et al. 1991). These two putative proteins share high amino-acid similarity in the functional effector domain, whereas the secretion domains were diverged. Axtell and associates (2003) showed that the predicted secondary structure of *P. syringae* pv. *tomato* *AvrRpt2*⁸⁰⁻²⁵⁵ matches the catalytic core of the staphopain cysteine protease, with a putative catalytic triad composed of Cys 122, His 208, and Asp 226. By amino-acid alignment, we found that *AvrRpt2_{EA}*⁵¹⁻²²² may have a secondary sequence structure similar to that of *P. syringae* pv. *tomato* *AvrRpt2*⁸⁰⁻²⁵⁵ and shared a similar putative catalytic triad, with Cys 88, His 178, and Asp 191 (Fig. 1). Recently, Vinatzer and associates (2005) showed that aa 81 to 100, a serine-rich stretch of *P. syringae* pv. *tomato* *AvrRpt2* (contains 7 serine aa), does not contribute to the secretion or translocation of *P. syringae* pv. *tomato* *AvrRpt2* but is important for protein accumulation in bacteria. We found that aa 51 to 66 of *AvrRpt2_{EA}*, corresponding to aa 85 to 100 of *P. syringae* pv. *tomato* *AvrRpt2*, are also serine-rich (7 serine aa), indicating that this region in *AvrRpt2_{EA}* may have similar function.

P. syringae pv. *tomato* *AvrRpt2* is a type III effector secreted by *P. syringae* that is genetically recognized by the disease resistance gene RPS2 in *Arabidopsis* (Chen et al. 2000; Kunkel et al. 1993; Mudgett and Staskawicz 1999). *P. syringae* pv. *tomato* DC3000 expressing *P. syringae* pv. *tomato* *avrRpt2* causes more severe disease on susceptible lines of *Arabidopsis* as compared with *P. syringae* pv. *tomato* DC3000 (Chen et al. 2000, 2004; Guttman and Greenberg 2001; Lim and Kunkel 2004). *P. syringae* pv. *tomato* *avrRpt2* also has virulence function in its native strain *P. syringae* pv. *tomato* JL1065 on a natural host, and disruption of *P. syringae* pv. *tomato* *avrRpt2* reduces the virulence of *P. syringae* pv. *tomato* JL1065 on tomato (Lim and Kunkel 2005). In this study, we showed that disruption of *avrRpt2_{EA}* in *E. amylovora* Ea1189 impaired its ability to cause systemic infection in pears, indicating that *avrRpt2_{EA}* also contributes to virulence in *E. amylovora* on its native host. Furthermore, when expressed in *P. syringae* pv. *tomato* DC3000, *avrRpt2_{EA}* increased the ability of *P. syringae* pv. *tomato* DC3000 to grow in susceptible *Arabidopsis* lines lacking the RPS2 resistance gene and induced an HR response in *Arabidopsis* lines containing the RPS2 gene. Our results indicated that *avrRpt2_{EA}*, like *P. syringae* pv. *tomato* *avrRpt2*, is a virulence factor in its native host, confers avirulence in *Arabidopsis* lines containing the RPS2 gene, and has virulence function in susceptible lines of *Arabidopsis* when expressed in *P. syringae* pv. *tomato* DC3000.

When expressed in *P. syringae* pv. *tomato* DC3000, the activity of *P. syringae* pv. *tomato* *AvrRpt2* is completely dependent upon the function of the T3SS (Mudgett and Staskawicz 1999), an apparatus used to secrete and translocate effector proteins directly into the host cell. Transgenic *Arabidopsis* lines lacking the RPS2 gene that constitutively express *P. syringae* pv. *tomato* *AvrRpt2* exhibit enhanced susceptibility to *P. syringae* pv. *tomato* DC3000 (Chen et al. 2000; Leister et al. 1996). These studies indicated that *P. syringae* pv. *tomato* *AvrRpt2* exerts its virulence activity from inside the plant cell. *E. amylovora* also contains a T3SS that is phylogenetically related to the T3SS in *P. syringae*. Phylogenetic analysis suggests that T3SS in *Erwinia* and *Pseudomonas* spp. belong to the same phylogenetic group of T3SS, i.e., the hrp1 group (He et al. 2004). This indicated that T3SS in these two quite-different plant pathogens may have evolved from the same ancestor. When *avrRpt2_{EA}* was expressed in *P. syringae* pv. *tomato* DC3000 in its native form, that is, under its own promoter and secretion signal sequence, only a weak HR response was observed in *Arabidopsis* containing the RPS2 gene. When

avrRpt2_{EA} was expressed in *P. syringae* pv. *tomato* DC3000 from the promoter of *P. syringae* pv. *tomato* *avrRpt2*, a much stronger HR was observed (Fig. 6B). This result suggests that expression of *avrRpt2_{EA}* in *P. syringae* pv. *tomato* DC3000 is not as efficient as *P. syringae* pv. *tomato* *avrRpt2* in *P. syringae* pv. *tomato* DC3000. An *hrp*-box sequence exists in the promoter region of both *avrRpt2_{EA}* and *P. syringae* pv. *tomato* *avrRpt2* (Innes et al. 1993), suggesting that these genes may be regulated by the alternative sigma factor HrpL. However, it is possible that recognition of the *hrp*-box sequence by the HrpL and regulation of HrpL in *P. syringae* may differ from that of *E. amylovora* HrpL.

A hybrid AvrRpt2_{EA} protein (Chimeric2) containing the secretion signal sequence (first 70 aa) of *P. syringae* pv. *tomato* AvrRpt2, when expressed from the promoter of *P. syringae* pv. *tomato* *avrRpt2*, caused a strong HR in *Arabidopsis* similar to that elicited by *P. syringae* pv. *tomato* AvrRpt2 (Fig. 6B). This result suggests that either the secretion, translocation, or both of *avrRpt2_{EA}* in *P. syringae* pv. *tomato* DC3000 is not as efficient as *P. syringae* pv. *tomato* *avrRpt2* in *P. syringae* pv. *tomato* DC3000. In other words, both the promoter and the secretion signal domain from *P. syringae* pv. *tomato* AvrRpt2 may increase the expression and either the secretion, translocation, or both of AvrRpt2_{EA} in *P. syringae* pv. *tomato* DC3000. This result further confirms our initial hypothesis that the AvrRpt2_{EA} effector domain functions similarly to that of *P. syringae* pv. *tomato* AvrRpt2 and is genetically recognized by the RPS2 disease-resistance gene in *Arabidopsis*. These results suggest that although different pathogens may contain similar effector genes, the expression and secretion of these effectors can be under specific regulation by the native pathogen. Since we do not yet know how efficiently AvrRpt2_{EA} is expressed and secreted in *E. amylovora*, further study is needed to address this question of how different bacterial pathogens control the expression and secretion of similar effectors.

The effector protein *P. syringae* pv. *tomato* AvrRpt2 and disease resistance gene RPS2 in *Arabidopsis* is one well-studied example of gene-for-gene interactions (Bent et al. 1994; Mudgett 2005). The disease resistance RPS2 protein, found in many *Arabidopsis* ecotypes, is a plasma membrane-associated intracellular nucleotide-binding site-leucine rich repeat protein (Bent et al. 1994). This protein specifically detects and mounts an immune response to *P. syringae* that produce the AvrRpt2 protein, triggering HR response and disease resistance. Several recent elegant studies have provided experimental support for the so-called the “guard” hypothesis to explain interaction of *P. syringae* pv. *tomato* AvrRpt2 and RPS2 proteins (Axtell and Staskawicz 2003; Axtell et al. 2003; Coaker et al. 2005; Dangl and Jones 2001; Jones and Takemoto 2004; Kim et al. 2005; Mackey et al. 2003; Schulze-Lefert and Bieri 2005). This hypothesis postulates that bacterial T3SS effectors function to suppress or inactivate cellular virulence targets that are guarded by R-protein complex. The virulence activity of an effector or the virulence target triggers the activation of RPS2, resulting in the activation of signal transduction pathways and disease resistance (Belkadir et al. 2004; Day et al. 2005; Shirasu and Schulze-Lefert 2003). In this case, the RIN4 protein (RPM1-interacting protein 4), a novel plasma membrane-localized protein, is one of the virulence targets of *P. syringae* pv. *tomato* AvrRpt2 in *Arabidopsis* (Belkadir et al. 2004; Chisholm et al. 2005; Mackey et al. 2002, 2003). In healthy *Arabidopsis* plants, RIN4, as a negative regulator, binds and suppresses the activity of RPS2 (Belkadir et al. 2004). After secretion into plant cells by the T3SS, the *P. syringae* pv. *tomato* AvrRpt2 interacts with host proteins such as ROC1 (cyclophilins), which, in turn, activates the self-cleavage function of *P. syringae* pv. *tomato* AvrRpt2 (Coaker et al. 2005; Jin et al.

2003). The activated *P. syringae* pv. *tomato* AvrRpt2 protease not only cleaves its own secretion signal peptide but also RIN4 and at least three other *Arabidopsis* proteins (Chisholm et al. 2005; Coaker et al. 2005; Day et al. 2005; Schulze-Lefert and Bieri 2005). RIN4 cleavage products no longer suppress RPS2 activity and serve as a signal to activate RPS2-mediated resistance, leading to the HR and other defense responses (Coaker et al. 2005). It will be interesting to see what host proteins interact with AvrRpt2_{EA} in apples and pears, and we are now working towards this goal.

In summary, we identified a putative effector protein AvrRpt2_{EA}, a homolog of *P. syringae* pv. *tomato* AvrRpt2, from the fire blight pathogen *E. amylovora*. We also showed that the *avrRpt2_{EA}* gene was detected in all *E. amylovora* strains tested. We provided evidence that AvrRpt2_{EA} is genetically recognized by the RPS2 disease-resistance protein in *Arabidopsis* when expressed in *P. syringae* pv. *tomato* DC3000 and has virulence and avirulence functions during interaction with its native host or *Arabidopsis*. Although gene-for-gene interactions are currently unknown in the *Erwinia*-apple/pear system, our results clearly indicate that we could explore the potential of RPS2 as a disease-resistance gene for apple and pear.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The bacterial strains and plasmids utilized in this study are listed in Table 1. *Erwinia amylovora* WT and mutant strains and *Escherichia coli* strains were grown in Luria Bertani (LB) medium at 28 and 37°C, respectively. Antibiotics were added to the culture medium at the following concentrations: kanamycin, 20 µg/ml; chloramphenicol [Cm], 20 µg/ml, tetracycline, 15 µg/ml, and ampicillin, 100 µg/ml. Oligonucleotide primers used for PCR and mutant construction in this study are also listed in Table 1.

DNA manipulation and sequence analysis.

Plasmid DNA purification, PCR amplification of genes, isolation of fragments from agarose gels, cloning and restriction enzyme digestion, T4 DNA ligation, and Southern hybridization were performed using standard molecular procedures (Sambrook et al. 1989). Chromosomal DNA was isolated using a genomic DNA purification kit (Qiagen, Valencia, CA, U.S.A.). DNA sequencing was performed at the Genomic Technology Support Facility at Michigan State University. Sequence management and contig assembly were conducted using DNASTar software (DNASTar Inc., Madison, WI, U.S.A.). Database searches were conducted using the BLAST programs at the National Center for Biotechnology Information (Altschul et al. 1997). Amino acid alignments were done with ClustalW, v. 1.83 (European Bioinformatics Institute, Cambridge, U.K.).

TAIL-PCR.

The Ea110 genome was used as template for TAIL-PCR as described previously (Liu and Whittier 1995). Primers RT1, RT2, and RT3, designed from the sequence of *avrRpt2_{EA}*, were used for the primary, secondary, and tertiary nested PCR reactions, respectively. The degenerate primers AD1, AD2, and AD3 as described by Liu and Whittier (1995) were used in the PCR reactions. PCR products from secondary and tertiary nested PCR were used for sequencing.

Immature-pear infection assays.

For *E. amylovora* WT and mutant strains, bacterial suspensions were grown overnight in LB broth, were harvested by centrifugation, and were resuspended in 0.5× sterile phosphate-

buffered saline (PBS) with the cells adjusted to approximately 1×10^3 to 1×10^4 CFU/ μ l (optical density at 600 nm [OD₆₀₀] = 0.1 and then diluted 100 times) in PBS. Immature pears (*Pyrus communis* L. cv. Bartlett) were surface-sterilized and pricked with a sterile needle as described previously (McGhee and Jones 2000). Wounded pears were inoculated with 2 μ l of cell suspensions and were incubated in a humidified chamber at 28°C. Symptoms were recorded at 2, 4, 6, and 8 days postinoculation. For bacterial population studies, the pear tissue surrounding the inoculation site was excised using a #4 cork borer as described previously (Bogdanove et al. 1998b; Zhao et al. 2005) and was homogenized in 0.5 ml of 0.5 $\times$ PBS. Bacterial growth within the pear tissue was monitored by dilution-plating of the ground material on LB medium amended with the appropriate antibiotics. For each strain tested, fruits were assayed in triplicate and each experiment was repeated at least twice.

Arabidopsis infection and HR test.

Arabidopsis thaliana WT Col-0 and its corresponding *rps2* mutant plants were grown in soil in growth chambers with a 12-h day and 12-h night cycle, a light intensity of 100 μ E, and a constant temperature of 20°C. The experiments used 4- to 5-week-old plants. Bacteria were grown in low-salt LB broth to the mid- to late-logarithmic phase at 28°C. Bacterial cultures were centrifuged to recover bacteria, which were resuspended in sterile water to a final OD₆₀₀ of 0.2 and, then, were diluted 100 times (equivalent to 1×10^6 CFU/ml). Fully expanded leaves were infiltrated with bacterial suspensions, and bacteria were enumerated as described (Hauck et al. 2003). The experiments were repeated three times. For the HR test, the bacterial suspension was adjusted to a final of OD₆₀₀ of 0.2 (equivalent to 1×10^8 CFU/ml). Symptoms were recorded 24 h after infiltration, and at least 15 to 20 leaves per strain were recorded.

Construction of *E. amylovora* mutants by Red cloning.

We generated *E. amylovora* stable mutants by using the λ phage recombinases as previously described for *Escherichia coli* (Datsenko and Wanner 2000). Briefly, we transformed *E. amylovora* Ea1189 with plasmid pKD46 harboring recombinases *red α* , β , and γ . The transformant Ea1189(pKD46) was grown overnight at 28°C and reinoculated in LB broth containing 0.1% arabinose and was grown to exponential phase. The cells were then made electrocompetent and were stored at -80°C. Recombination fragments consisting of a Cm^r gene with its own promoter, flanked by a 50-nucleotide (nt) homology arm, were generated by PCR using plasmid pKD3 as a template. For generating the *avrRpt2*_{EA} mutant, the primer pair Rpt2-N1 and Rpt2-N2 was used to generate the recombination fragments. The primer pair *avrRpt2*-3/2-4 and an internal primer pair Cm1 and Cm2 of the Cm^r gene were used to confirm the *avrRpt2*_{EA} mutants by PCR. For *avrRpt2*-N1/2 primers, the 5' end of each primer contained the 50-nt homolog arms corresponding to the first and last 50 bases of the corresponding gene, whereas the remaining 21 or 24 nt of each primer at the 3' end were designed to amplify the Cm^r cassette from pKD3. The PCR products were gel-purified using a gel purification kit from Qiagen (Qiagen). Electroporation was done as normal, and transformants were plated on LB with ampicillin and Cm. In the resulting mutants, the majority of the coding region of each gene would be replaced by the Cm^r marker, except for the first and last 50 nt. The *avrRpt2*_{EA} mutant was designated as ZYRKD3-1.

Construction of *avrRpt2* and chimeric expression clones.

The 950-bp DNA fragment containing the promoter and open reading frame of the *avrRpt2*_{EA} gene was PCR amplified using the primer pair *avrRpt2*-3 and *avrRpt2*-4 and was then

cloned into the pGEM-T easy vector (Promega, Madison, WI, U.S.A.). The insert was confirmed by sequencing and excised using *Eco*RI. The *Eco*RI fragment was then cloned into the shuttle vectors pUCP19 and pRK415, and the resulting plasmids were designated as pZYR2-19 and pZYR2-415, respectively. These plasmids (pZYR2-19 and pZYR2-415) were used to transform *P. syringae* pv. *tomato* DC3000 and ZYRKD3-1 (*avrRpt2*_{EA} mutant), respectively. The fragments for making the chimeric expression clones were PCR-amplified and cloned into shuttle vector pUCP19, using appropriate restriction enzymes as shown in Table 1. The primer pairs *avrRpt2*-PST-F1/R1 and *avrRpt2*-PST-F1/R2 amplified 174- and 360-bp DNA fragments, respectively, using pDSK600 (*avrRpt2*) as DNA template. These fragments containing 24- and 210-bp 5' coding region of *P. syringae* pv. *tomato avrRpt2* were digested using *Hind*III and *Xba*I and were cloned into pUCP19 digested with the same enzymes. The resulting plasmids were designated as pChimeric1-1 and pChimeric2-1. The 749- and 659-bp fragments containing 654- and 564-bp 3' coding region of *avrRpt2*_{EA} were amplified using primer pairs *avrRpt2*-EA-F1/R1 and *avrRpt2*-EA-F2/R1 and were digested with *Xba*I and *Eco*RI. These two fragments were then cloned into pChimeric1-1 and pChimeric2-1, respectively. The final plasmids were designated as pChimeric1 and pChimeric2 and were confirmed by sequencing and restriction digestion. The plasmids pChimeric1 and pChimeric2 were then introduced into *P. syringae* pv. *tomato* DC3000 by transformation and were confirmed by reisolating the plasmids and restriction enzyme digestions.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Wellcome Trust Sanger Institute's *E. amylovora* webpage:
www.sanger.ac.uk/Projects/E_amylovora
- National Center for Biotechnology Information Blast webpage:
www.ncbi.nlm.nih.gov/BLAST