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Computational and Biochemical Analysis of the Xanthomonas Effector AvrBs2 and Its Role in the Modulation of Xanthomonas Type Three Effector Delivery

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Abstract

Effectors of the bacterial type III secretion system provide invaluable molecular probes to elucidate the molecular mechanisms of plant immunity and pathogen virulence. In this report, we focus on the AvrBs2 effector protein from the bacterial pathogen Xanthomonas euvesicatoria (Xe), the causal agent of bacterial spot disease of tomato and pepper. Employing homology-based structural analysis, we generate a three-dimensional structural model for the AvrBs2 protein and identify catalytic sites in its putative glycerolphosphodiesterase domain (GDE). We demonstrate that the identified catalytic region of AvrBs2 was able to functionally replace the GDE catalytic site of the bacterial glycerolphosphodiesterase BhGlpQ cloned from Borrelia hermsii and is required for AvrBs2 virulence. Mutations in the GDE catalytic domain did not disrupt the recognition of AvrBs2 by the cognate plant resistance gene Bs2. In addition, AvrBs2 activation of Bs2 suppressed subsequent delivery of other Xanthomonas type III effectors into the host plant cells. Investigation of the mechanism underlying this modulation of the type III secretion system may offer new strategies to generate broad-spectrum resistance to bacterial pathogens.

Introduction

Plants have evolved sophisticated innate immune systems to counter the attack of various microbial pathogens through a combination of diverse molecular mechanisms [1]. Plant innate immunity is controlled by two overlapping signaling pathways. The first pathway, PAMP-Triggered Immunity (PTI), is a basal defense response that is triggered by the recognition of pathogen-associated molecular patterns (PAMPs) through a set of specialized plant extracellular receptor kinase proteins [2–5]. Plants use PTI to suppress the growth of non-pathogens. However, successful bacterial pathogens can interfere with PTI via effector proteins that are delivered into plant cells through the type three secretion and translocation system (TTSS). Many bacterial TTSS effectors have identified virulence functions that modulate the pathways involved in PTI, making the plants more susceptible to the proliferation of microbial pathogens [1]. Most of these TTSS effector proteins are not homologous, and the majority have no obvious biochemical function, although a few have been shown to have enzymatic activity [6–9]. Characterizing the biochemical functions of pathogen effectors and identifying the plant targets of each effector will shed light on bacterial pathogenesis and plant immunity. In response to effector proteins, plants have evolved a second layer of defense signaling pathways controlled by resistance genes (R genes). The plant R proteins directly or indirectly recognize the bacterial TTSS effectors and initiate effector-triggered immunity (ETI) [10]. This response is often a localized, programmed cell death-related defense response, also known as the hypersensitive reaction (HR) [11]. Despite intensive study of the molecular mechanisms of PTI and ETI, the interplay between these two primary defense mechanisms remains elusive [12,13].

The TTSS machinery of phytopathogenic bacteria encoded by the clustered hpa (hypersensitive reaction and pathogenicity) genes is essential for the delivery of effectors to the interior of the plant cell [14]. Mutations in the pathogen that block the TTSS will subsequently prevent the translocation of the type III effectors and impair the virulence of the pathogen on host plants [14–16]. Therefore, the TTSS plays a critical role in bacterial pathogenesis. The translocation of TTSS effectors can be quantitatively measured by monitoring adenylate cyclase enzyme activity in plant cells by fusing the effector protein with the calmodulin-dependent adenylate cyclase domain (Cya) of Bordetella pertussis cyclolysin [17,18]. Despite intensive characterization of the TTSS in model bacterial pathogens, including several Pseudomonas and Xanthomonas species, detailed information describing the establishment and regulation of the TTSS is still missing. It is also not clear if plants have evolved defense mechanisms that can recognize the establishment of bacterial TTSS. However, a recent report

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demonstrated that PTI of the host plant can inhibit the injection of bacterial type III effectors [19], suggesting that the suppression of TTSS may contribute to the plant immunity.

Xanthomonas euvesicatoria (Xe) is the causal agent of bacterial leaf spot disease of pepper and tomato, which can deliver more than 28 TTSS effectors into plant cells [20,21]. One type III effector AvrBs2 is highly conserved not only in Xe strains but also in many other Xanthomonas pathogens that cause disease in a wide range of crops [22,23]. The presence of avrBs2 in many of these pathogens makes a significant contribution toward their virulence [22]. Previous analyses have determined that the avrBs2 gene encodes a protein containing a domain homologous to the E. coli glycerol-phosphodiesterase (GDE) and the agrocinopine synthase (ACS) of Agrobacterium tumefaciens. However, it has not been shown whether AvrBs2 possesses GDE or ACS enzyme activity and whether such activity is relevant to AvrBs2 function [23,24].

Pepper plants (Capsicum annuum) carrying the bacterial leaf spot disease resistance gene ( Bs ) are resistant to strains of Xe that contain AvrBs2. This host-pathogen interaction results in a resistance response that inhibits the growth of Xe [22-25]. The Bs gene has been isolated by map-based cloning and encodes a protein that belongs to the largest class of plant disease resistance proteins. The protein contains a central putative nucleotide-binding site (NBS) and a carboxyl-terminal leucine-rich repeat (LRR) region [25]. Bs2 has been shown to associate with the molecular chaperone SGT1 through its LRR domain to specifically recognize AvrBs2 and trigger the HR in plants [26]. However, it is still not clear whether Bs2 recognizes AvrBs2 directly or indirectly in planta.

In addition to the Bs2 gene, two other pepper resistance genes, Bst1 and Bst3, have been identified that confer resistance to Xe strains carrying the avrBs1 and avrBs3 effector genes, respectively [27]. Near-isogenic lines carrying the Bst1, Bst2, and Bst3 genes have been generated by introgression of individual or combinations of Bs genes into the susceptible pepper cultivar Early Cal Wonder (ECW) [28,29]. The avrBs1 and avrBs3 genes have also been identified and cloned [23,30-32]. The Bst1 gene has not been cloned [32], but Bst3, which encodes a flavin monoxygenase enzyme, has recently been isolated from the pepper genome [33].

In this study, the pepper and Xe pathosystem is used to study the interaction between Bs2 and AvrBs2. We demonstrate that the catalytic sites of the putative GDE domain of AvrBs2 are under purifying selection, and that the GDE catalytic sites are required for AvrBs2 virulence function but not the activation of Bs2. Although we were unable to demonstrate the GDE enzymatic activity using purified, full-length AvrBs2, we determine that the AvrBs2 GDE catalytic site could functionally replace the GDE catalytic site of BhGdpQ (Borrelia hermsii) [34]. We also identify a minimum domain of AvrBs2 that included the GDE homologous region and a carboxyl Bs2 activation domain. Therefore, we are able to genetically separate the virulence function of AvrBs2, which is dependent on its GDE catalytic site, from the Bs2 activation, which is independent of the GDE catalytic site.

Finally, we describe a novel plant disease resistance phenotype related to the AvrBs2/Bs2 host-pathogen interaction. When AvrBs2 activates the B2 R gene function, the TTSS is reduced in the delivery of effectors to the plant host. Investigation of the mechanism of the AvrBs2 virulence function and TTSS suppression during its recognition by Bs2 could offer new strategies to generate broad-spectrum resistance to the Xe bacterial pathogen.

Results

Computational and biochemical evidence that AvrBs2 contains an active GDE catalytic domain

Previous characterization of AvrBs2 (YP 361783) from Xe revealed a domain [amino acids (aa) 280 to 340] with homology to a bacterial GDE [23]. To further characterize this Xe AvrBs2 domain, we searched the current GenBank database with the BLASTP program using the full-length AvrBs2 protein as a query. This search allowed us to compile remote homologs from plants, animals, fungi, and bacteria that contain GDE domains homologous to AvrBs2. In Figure 1A, selected GDE proteins from plants [4GDE (NP_177561) and OsGDE [(AP003274)], human [HhMIR16 (NP_057725)], fungi [XGDE1 (NP_015215)], and bacteria [TmGdpd (TM1621) of Thermotoga maritima, BhGlpQ (ADD63790) from Borrelia hermsii, and AgACS (AAO15364) from Agrobacterium tumefaciens] aligned with the GDE domain of AvrBs2 (aa 274 to 328) are shown. Several AvrBs2 homologs from Xanthomonas pathogens of tomato, euvesicatoria (Xe) (YP_361783; alfalfa, campestris pv. alfafla (Xac) citrus, axonopodis pv. citri (Xac) (NP_640432); cabbage, campestris pv. campestris (Xe) (NP_635447); and rice, oryzae pv. oryzae (Xoo) (YP_449177) or oryzae pv. oryzae (Xoo) (ZP_02421238) were included in the alignment. The overall sequence identity between AvrBs2 and the different GDEs in this region was approximately 33% (with >37% sequence similarity) (Figure 1A) [35]. The putative GDE domain in AvrBs2 aligned well with the glycerophosphodiester phosphodiesterase (GdPd) protein from Thermotoga maritima, for which the three-dimensional crystal structure had been previously determined (PDB ID: 1O1Z) [36]. The GDE domains of AvrBs2 and TmGdpd share 60% amino acid sequence similarity and 47% identity. The high amino acid sequence similarity between the GDE domains of AvrBs2 and TmGdpd predicts that these two proteins will have similar three-dimensional structures.

A homology-based modeling method was employed to generate a three-dimensional structural model for AvrBs2 (aa 274 to 328) using the solved crystal structure of TmGdpd as a template [36,37]. The resulting three-dimensional structural model of AvrBs2 closely matched the solved crystal structure of Tm 101z A (Figure 1B). Both structures consist of two antiparallel beta-sheets capped by nine putative alpha-helices. Recently, GDE enzyme activity and the putative catalytic sites of the human GDE (HhMIR16) have been characterized [38,39]. Point mutations in the GDE catalytic sites (E97A, D99A, and H112A) in HhMIR16 eliminated GDE enzyme activity [38,39]. The putative catalytic sites of HhMIR16 are conserved in all of the GDE homologs, including the six AvrBs2 homologs (Figure 1A). In the three-dimensional structural model of AvrBs2, the catalytic sites are present in regions of high structural homology between the two proteins (TmGdpd in blue and AvrBs2 in red), which suggests that AvrBs2 utilizes the same residues for enzymatic function (Figure 1B).
To investigate whether the AvrBs2 protein possesses GDE enzyme activity, both the wild type and the catalytic mutants of avrBs2 were expressed in E. coli as GST-AvrBs2 fusion proteins. The fusion proteins were assayed for GDE enzyme activity using a method that was originally adapted for E. coli and Borrelia GDEs, with glycerophosphocholine as a substrate [40,41]. However, we were unable to detect GDE enzyme activity of AvrBs2 with this substrate. Because the GDE catalytic sites of the BhGlpQ enzyme were conserved with predicted catalytic sites in AvrBs2 (Figure 1A), we hypothesized that if we replaced the core GDE catalytic site of the active BhGlpQ enzyme [41] (24 amino acids) with the putative GDE catalytic site of AvrBs2, we might be able to detect enzyme activity with glycerophosphocholine substrate in vitro. To test this possibility, the GDE catalytic site of BhGlpQ was replaced with either the wild-type AvrBs2 catalytic site or a GDE catalytic site mutant (E304A/D306A) (Figure 1C). The GDE enzyme activities of purified GST:BhGlpQ (positive control), GST:BhGlpQ-AvrBs2-WT, and GST:BhGlpQ-AvrBs2-E304A/D306A were analyzed using an indirect coupled enzyme assay [41]. The higher light adsorptions at 340 nm for GST:BhGlpQ (positive control) and GST:BhGlpQ-AvrBs2-WT compared to the inactive GST:BhGlpQ-AvrBs2-E304A/D306A indicated that AvrBs2 had a functional GDE catalytic site (Figure 1C and 1D).

The GDE catalytic sites of AvrBs2 are required for virulence but not for activation of the B2-specified disease resistance signaling pathway

To test whether the GDE catalytic site of AvrBs2 is important for Xe virulence in susceptible bs2 plants or for B2 disease resistance activation, we mutated the GDE catalytic sites E304A, D306A and H319A by site-directed mutagenesis of the wild-type avrBs2 gene (Figure 2A). We replaced the chromosomal copy of avrBs2 in strain Xe GM98-38-1 with various avrBs2 mutants by homologous recombination. The effects of these mutations on AvrBs2 virulence function and/or Bs2-activation were evaluated by in planta bacterial growth assays in near-isogenic pepper and tomato lines with and without the R gene Bs2 (Figure 2B). In pepper and tomato lines without Bs2, the Xe strain with wild-type avrBs2 was more virulent and grew approximately five-fold higher than the null strain Xe without avrBs2 (Figure 2B). The Xe strains with mutations in GDE domain (E304A/D306A and H319A) lost AvrBs2 virulence function and were similar to the null strain Xe without avrBs2 (Figure 2B). However, on near-isogenic pepper and transgenic tomato lines with Bs2 (25), Xe strains carrying the AvrBs2 GDE mutants were still able to activate Bs2-based resistance, similar to the Xe strain carrying wild-type avrBs2 (Figure 2B). These results demonstrate that the putative GDE catalytic sites of avrBs2 are required for its virulence function but not for recognition by Bs2.

Additionally, we tested two control Xe strains that contain point mutations (R403P and A410E) [24] that evade Bs2 activation while maintaining most of the virulence functions of AvrBs2 (Figure 2A). Similar to previously reported results in pepper plants without Bs2 [24], these mutants were intermediate in virulence between Xe carrying wild-type avrBs2 and Xe without avrBs2. However, the mutants were unable to activate Bs2 resistance in pepper plants containing Bs2 (Figure 2B).

Another method for assaying the induction of plant immunity is to challenge a plant with a high-density bacterial dose that triggers a macroscopic hypersensitive cell death reaction, or HR response. High-density inoculations (2 x 10^8 CFU/ml) of pepper with Bs2 caused a similar, strong brown necrosis with the Xe strain without wild-type avrBs2 and the Xe strains with avrBs2 GDE mutations (E304A/D306A and H319A) [Supplemental Figure S1]. However, high-density inoculations of pepper plants containing Bs2 with the Xe avrBs2 mutant strain (A410E) caused a light brown necrosis,
To test whether the GDE mutations had a negative effect on AvrBs2 delivery by Xe TTSS, the TTSS effector delivery reporter Cya [18] was utilized to quantitatively measure the translocation of two different AvrBs2 GDE mutant Xe effectors. The AvrBs2 GDE mutations caused no reduction of detectable effector delivery (Supplemental Figure S2A). Additionally, the Xe (avrBs2-Cya) wild type and catalytic site mutant strains were not altered from the non-Cya strains in the activation Bs2 HR (Supplemental Figure S2B).

The AvrBs2 GDE virulence domain is under strong purifying selection

Demonstrating that the GDE domain of AvrBs2 is required for virulence prompted us to evaluate the natural variations in various avrBs2 alleles with respect to the evolutionary selection. In addition to the previously published avrBs2 homologs (Xp in pepper [YP_361783], Xao in alfalfa and Xcc in cabbage [NP_653447] [23], three additional uncharacterized homologs of avrBs2 (Xanthomonas axonopodis pv. citri [Xac] [NP_640432], Xanthomonas oryzae pv. oryzae [Xo] [YP_449177], and Xanthomonas oryzae pv. oryzicola [Xo] [ZP_02241238]) from newly released genome sequences were aligned using the CLUSTALW program [35]. The overall sequence identity of the different avrBs2 homologs in Xanthomonas was high (>70%). Phylogenetic analysis by maximum likelihood (PAML) software was used to determine which evolutionary model acts on these six homologs of avrBs2 from different Xanthomonas pathovars that have adapted to cause disease in different host plant species [42]. This statistical analysis of nucleotide changes with respect to amino acid changes calculated an average rate of non-synonymous (Ka) and synonymous (Ks) substitutions per site for all six avrBs2 homologs. The ratio (ω) = Ka/Ks measures the difference between the two rates. For neutral amino acid changes or neutral selection, the ω ratio is 1.0. For advantageous amino acid changes or adaptive selection, the ω ratio is >1.0, and for deleterious amino acid changes or purifying selection, the ω ratio is <1.0 [42,43]. The average ω ratio over all six homologs was estimated to be 0.1534, indicating a strong purifying selection on the Xanthomonas pathovars to maintain avrBs2 for its contribution to pathogenic virulence in a range of different host plant species. In addition, PAML analysis revealed a significant variation in the ω ratio over the length of the avrBs2 sequence. Sliding window analysis using the SWAKK program [43] was used to determine the distribution of variation in the ω ratio across avrBs2 from Xe and Xcc. The low ω over the GDE-virulence region is consistent with purifying selection to maintain the virulence function of avrBs2 (Figure 2C). Although the ω for the TTSS signal peptide remained below one, there was an increase in ω in this region, possibly associated with differences in TTSS effector delivery for specific Xanthomonas pathovars as they infect different host plants (Figure 2C).

The minimum AvrBs2 domain required for Bs2-activation includes the entire GDE homologous region and an additional C-terminal region

Having established that the GDE catalytic sites are required for AvrBs2 virulence function but not Bs2-activation, we generated additional deletions of the N-terminus of AvrBs2 to define a minimal region required for Bs2 activation. The deletions were cloned into a binary vector and screened for HR in stable transgenic Bs2 Nicotiana benthamiana using Agrobacterium-mediated transient expression (Figure 3A). The previously reported [44] avrBs2 deletion construct (aa 97 to 520) was still able to trigger a Bs2 HR; the N-terminal deletion (aa 271 to 520) produced a similar result (Figure 3A and 3B). Further deletions at either the
Activation. We confirmed the Agrobacterium-minimal Bs2 recognition region included the GDE domain, to elicit a Bs2-dependent HR. Thus, the fragment (aa 271 to 520) was amino or the carboxyl terminus of the minimal domain failed to activate a rapid Bs1-dependent HR visible at 18 hpi [30]. When we inoculated the Xe (avrBs2, avrBs1) strain delivering both AvrBs1 and AvrBs2 into a pepper line containing both Bs1 and Bs2 R genes, we observed that AvrBs2 activation of a slower Bs2-HR was epistatic to the AvrBs1 activation of a more rapid Bs1-HR (Figure 4). Control strains Xe (avrBs1) and Xe (avrBs2) along with control pepper (B1) and pepper (B2) were included for comparison to detect the epistatic, slow Bs2-HR at 48 hpi instead of the expected faster Bs1-HR at 18 hpi (Figure 4). The epistasis of the Xe activated slower Bs2 HR over the Xe activated faster Bs1 HR was also confirmed by measuring electrolyte leakage (Supplemental Figure S3A and S3B).

To test whether the Bs2 activation dependent suppression of the AvrBs1/Bs1 fast HR phenotype could be activated in trans, we co-inoculated a mixed inoculum of two strains of Xe containing either avrBs1, avrBs2 or no effector onto pepper (B1, B2). Again we observed the Bs2 activation dependent suppression of the AvrBs1/Bs1 fast HR phenotype (Supplemental Figure S6A). Control
inoculations with single Xe effectors, either by individual or mixtures, gave the expected responses on pepper plants with and without the corresponding R gene (Supplemental Figure S6). Additionally, the epistasis of the Xe activated slower Bs2 HR over the Xe activated faster Bs1 HR was again confirmed by measuring electrolyte leakage (Supplemental Figure S7A).

We hypothesized that this suppression might be accounted for by one of the following: (i) Bs2 activation disrupts Bs1 activation or (ii) Bs2 activation disrupts TTSS-mediated translocation of AvrBs1 or (iii) Bs2 activation causes a reduction or loss of induction of AvrBs1. To test the first hypothesis, three Agrobacterium strains containing either 35S-avrBs1, 35S-avrBs2 alone or a 35S-avrBs1/35S-avrBs2 tandem construct were inoculated on pepper containing both the Bs1 and Bs2 R genes. If Bs2 activation disrupts Bs1 activation, then suppression of AvrBs1/Bs1-dependent HR should occur. However, we did not observe alteration of the fast Bs1 HR by the slow Bs2 HR activation when both effectors were transiently expressed (Supplemental Figure S8A). The fast Bs1 HR for the co-expressed AvrBs2 and AvrBs1 on pepper (Bs2, Bs1) was confirmed by measuring electrolyte leakage (Supplemental Figure S8B). In addition, immunoblot analysis detected similar levels of expression for both HA epitope tagged effectors after 24 hours (Supplemental Figure S8C). Therefore, when AvrBs1 and AvrBs2 were simultaneously expressed in plant cells, the Bs2/AvrBs2-
dependent HR no longer suppressed the Bs1/AvrBs1-dependent HR. This finding is not consistent with the first hypothesis.

To test our second hypothesis, whether B2 activation modulates subsequent Xe TTSS effector delivery, the TTSS effector delivery reporter Cya [18] was utilized to quantitatively measure the translocation of two different Xe effector-reporters for avrBs1 and xopX. In this assay, the type three secretion and translocation signal peptides for each effector were translationally fused to the reporter Cya. Using homologous recombination, the reporters were marker-exchanged in tandem with the corresponding chromosomal allele of different Xe strains so that the wild-type copy of the particular effector was also maintained [18]. Pairs of effector-Cya reporter strains with and without avrBs2 included the pair of strains Xe (avrBs3) and Xe (avrBs1, avrBs2) with either AvrBs1,212-Cya reporter (Figure 5A) or XopX1,183-Cya reporter (Figure 5B).

Pairs of Xe Cya reporter strains, with and without avrBs2, were inoculated on pepper (no R genes), pepper (B2) and pepper (B1). Plants were sampled eight hours post-inoculation to avoid in planta multiplication of the reporter strains [18]. Eight hours post-inoculation is also before visible R gene-mediated HR. Because each effector-Cya reporter construct has a unique rate of translocation, each reporter construct was evaluated separately.

When the translocation of AvrBs1 and XopX Cya reporters was assessed in the presence of Bs2/avrBs2, the detectable levels of cyclic AMP for both effector-Cya reporters were significantly reduced in comparison to all other combinations where Bs2 was not activated including the Bs1/AvrBs1 interaction (Figure 5A, 5B).

Additionally, we tested three other pairs of effector-Cya reporter strains with and without avrBs2 that included the pair of strains Xe (avrBs3) and Xe (avrBs3, avrBs2) with either AvrBs2,212-Cya reporter, AvrBs3,212-Cya reporter or XopX1,183-Cya reporter (Supplemental Figure S9). Again only Bs2 activation was associated with reduced levels of effector-Cya reporter delivery to the host. This is consistent with the hypothesis that the Bs2 activation disrupts general TTSS-mediated translocation of effectors.

To preclude the possibility that Bs2 activation might block calmodulin dependent Cya elevation of in planta cyclic AMP levels, we tested Agrobacterium transient expression of 35S-AvrBs2:Cya in the presence and absence of Bs2 at 15 hpi in N. benthamiana. Similar elevated levels of cyclic AMP were observed in the presence and absence of Bs2 activation (Supplemental Figure S10A).

Additionally, we evaluated the effect of the GDE catalytic site mutations in AvrBs2 on the TTSS disruption by Bs2 activation with the AvrBs3-Cya reporter Xe strain. The set of four effector-Cya reporter Xe strains (avrBs2, avrBs2-E304A/D306A, avrBs2-H319A and without avrBs2) with the AvrBs3,212-Cya reporter were tested on pepper with or without Bs2. The loss of the GDE catalytic sites in AvrBs2 did not alter the TTSS repression effect of the Bs2/avrBs2 interaction (Supplemental Figure S10B).

To preclude the possibility that Bs2 activation causes a reduction or loss of induction of TTSS effectors in Xe, AvrBs2-Cya, an effector that is also disrupted in delivery to the host by Bs2 activation (Supplemental Figure S9A), was tested for reduction in protein level. Immunoblot assays of high titers inoculation of pepper (w/o B2) and pepper (B2) with Xe (avrBs2), Xe (avrBs2:Cya), Xe (avrBs2-E304A/D306A:Cya) and Xe (avrBs2-H319A:Cya) detected no reductions of protein levels associated with Bs2 activation (Supplemental Figure S10C). Although these results do not support hypothesis (iii) as a broad mechanism targeting all TTSS effectors it does not preclude an AvrBs1 specific targeting for degradation or loss of induction by Bs2 activation. While both 35SAvrBs2:HA and 35S-AvrBs1:HA transiently expressed in pepper were detected in immunoblot analysis we were only able to detect Xe expressed AvrBs2:HA but not AvrBs1:HA (data not shown). Low Xe expression of AvrBs1 may contribute to the overall low levels of TTSS delivered AvrBs1-Cya reporter compared to all other effector-Cya reporters evaluated. There is also a Bs2 activation specific reduction in the detectable Xe delivered AvrBs1-Cya reporter that should correlate with a Bs2 activation specific reduction in the Xe delivered AvrBs1. This indirect evidence is all consistent with a Bs2 activation dependent reduction in TTSS delivery of an already lowly expressed AvrBs1 resulting in a lack of the minimal amount of AvrBs1 required to activate a confluent Bs1 HR.

Figure 5. Bs2 activation by AvrBs2 blocks TTSS delivery of two independent effector reporters to host cells. The TTSS effector delivery reporter constructs consisted of the effector promoter and the secretion and translocation signal peptide translationally fused to adenylate cyclase (Cya). These constructs were introduced into Xe in tandem with the native effector by single homologous recombination. Pepper (no R genes), pepper (B1) and pepper (B2) were sampled 8 hours post-inoculation to avoid in planta multiplication of the reporter strain pairs (with and without AvrBs2) and assayed for cyclic AMP (cAMP). TTSS delivered effector-Cya translational fusions into the plant cell and calmodulin from the plant cell leads to elevated levels of cAMP. A. AvrBs1,212-Cya reporter in strains Xe (avrBs2 and avrBs1) and Xe (avrBs1) were inoculated into pepper plants (no R genes, B1 or B2). In planta cAMP levels were assayed. Student t-test was used to compare TTSS delivery of effector reporter in an Xe strain with and without avrBs2 on Pepper (+) Bs2, p-values were <0.05. B. XopX1,183-Cya reporter in strains Xe (avrBs2 and avrBs1) and Xe (avrBs1) were inoculated into pepper plants (no R genes, B1 or B2). In planta cAMP levels were assayed. Student t-test was used to compare TTSS delivery of effector reporter in an Xe strain with and without avrBs2 on Pepper (+) B2, p-values were <0.01.
These results led us to conclude that plant cells undergoing a Bs2/AvrBs2 incompatible reaction were able to modulate subsequent effector delivery by the Xe TTSS.

Discussion

Bacterial GDEs contribute to Xe virulence

Several classes of bacterial TTSS effectors have been characterized based on their enzymatic activities targeting host proteins [6–9]. In this study, we identified a GDE domain present in AvrBs2 that is highly conserved in homologs from several species of Xanthomonas. In addition to generating a three-dimensional structural model of the GDE domain of AvrBs2 using the crystal structure of a bacterial GDE, we demonstrated that the putative GDE catalytic site of AvrBs2 could functionally replace the catalytic site of the bacterial GDE from Borrelia hermsii (BhGlpQ). We further demonstrated that Xe strains with mutations in the putative GDE catalytic site of AvrBs2 had reduced bacterial growth in susceptible bs2 plants, suggesting that glycerophosphodiesterase activity has an important virulence function in this pathogen. An evolutionary analysis supports this conclusion and demonstrates that the GDE domain in AvrBs2 is under strong purifying selection. Interestingly, the catalytic mutations in GDE did not interfere with the ability of the plant to recognize AvrBs2 through the cognate R protein Bs2 and trigger disease resistance. This finding suggests that recognition of AvrBs2 is independent of its GDE enzyme activity.

Genes with GDE domains have been identified in species across the animal, plant, fungal and bacterial kingdoms [45–47]. Although the exact biological functions of most GDE genes are unknown, it has been documented that GDE enzyme activity is directly linked to bacterial pathogenesis in other systems [45–47]. For example, in Borrelia species, some but not all spirochetes carry GDE genes. It has been demonstrated that spirochetes carrying GDE genes were able to achieve high cell densities (>10^9/ml) in the blood, whereas spirochetes lacking GDE genes grew too much lower densities (<10^7/ml) [41,48]. These results clearly suggest that the GDE gene product could contribute to bacterial virulence, although the exact mechanism is still unclear [40]. Genes similar to GDE have been identified in plants; their products may contribute to plant cell wall biogenesis [49–51]. It is possible that bacterial pathogens interfere with the functions of endogenous plant GDEs by either blocking or competing for the same substrates. This hypothesis could be tested in future studies as more information is revealed about plant GDEs and their endogenous substrates.

The AvrBs2 protein may require a plant cofactor to activate its GDE enzyme activity

In this study, we purified the GST-AvrBs2 fusion protein from E. coli and subjected it to a common procedure used to test bacterial proteins for GDE enzyme activity [41]. However, GDE enzyme activity was not detectable using the recombinant GST-AvrBs2. This result could be due to the buffer conditions or the substrates employed, which may not be optimal for AvrBs2 enzyme activity in vitro. Interestingly, the in vitro GDE enzyme activity of the Arabidopsis putative GDE (AT4G26690) was not confirmed by using a similar testing condition as described in this report [51]. It may suggest that certain plant GDEs prefer different substrates compared to E. coli GDE. Our results (Figure 1C and 1D) confirmed that AvrBs2 has a functional GDE catalytic site. However, the amino acid sequences flanking the GDE catalytic site may be important for substrate binding. Since the flanking sequences in AvrBs2 are different from BhGlpQ, AvrBs2 could have a different substrate specificity and not use glycerophospholipol as substrate.

It is also possible that AvrBs2 requires other plant co-factors to activate its proper folding or its GDE enzyme activity. It is not unusual for a bacterial TTSS effector protein to require plant co-factors for full enzyme activity [1,6]. For example, the bacterial TTSS effector AvrRpt2 requires plant cyclophilin to activate its protease activity [1,6]. In this study, however, it was not possible to test whether AvrBs2 required plant cofactors for its GDE enzyme activity by mixing plant total protein extracts because of the high background of endogenous plant GDE activity. By using chimeric proteins, we confirmed that AvrBs2 did possess the functional GDE catalytic site that is essential for GDE enzyme activity. Because the GDE domain is required for the virulence function of AvrBs2, it is possible that AvrBs2 fulfills its virulence function through the GDE-activated hydrolysis of substrates in plant cells. Further investigation to identify the substrates for AvrBs2 enzyme function may help to elucidate the mechanism of the AvrBs2 virulence function and the modulation of Xe TTSS.

The virulence domain of AvrBs2 can be genetically separated from the region that triggers the Bs2-dependent plant immune response

We demonstrated that AvrBs2 carries a GDE domain with catalytic sites required for promoting bacterial virulence. However, GDE activity is not required for the activation of Bs2-dependent disease resistance. Through further genetic analyses, two overlapping AvrBs2 domains were identified: one corresponding to the GDE homologous region and one to a minimal Bs2-activating domain that includes the GDE domain and a C-terminal region. We confirmed that the previously identified mutations in this C-terminal region of AvrBs2 no longer activated Bs2-dependent resistance [24] and several novel mutations were identified that compromised Bs2 activation while having little effect on bacterial virulence. These results show that Xanthomonas can overcome Bs2 resistance without losing the virulence function of AvrBs2. These findings are significant for optimizing the deployment of Bs2 resistance in field studies because it is important to understand how Xe strains can overcome Bs2 activation but retain the AvrBs2 virulence function. For example, anticipatory breeding could be used to identify new Bs2 alleles that recognize the AvrBs2 loss-of-recognition mutants (R403P, A410E and Y419A). This scheme would allow us to use molecular breeding to stay ahead of evolving pathogens.

AvrBs2 activation of Bs2 leads to suppression of subsequent Xe TTSS effector delivery to host cells

In this study, we used the AvrBs2/Bs2 system to identify a potentially novel mechanism in plant disease resistance. AvrBs2-dependent activation of Bs2 triggers an unknown plant immunity mechanism, resulting in the suppression or modulation of the TTSS of the bacterial pathogen. In host plants containing the two R genes Bs1 and Bs2, we observed epistasis of the Bs2 activity with a slow, 48-hour HR over the Bs1 activity with a rapid, 18-hour HR when avrBs1 and avrBs2 were present in either a single Xe strain or during co-infection into the appropriate pepper plants. A Cya reporter assay demonstrated that this interference was most likely due to the inhibition of the bacterial TTSS following the AvrBs2/Bs2 interaction. This general inhibition of the subsequent Xe TTSS effector-reporter delivery could be detected as early as one hour after inoculation of Xe delivering wild-type AvrBs2 to Bs2 pepper plants.
Recently, it has been reported that the pre-inoculation of non-pathogenic *Pseudomonas fluorescens* or Flg21 (a 21-amino-acid peptide from bacterial flagellin) induces PAMP-triggered immunity (PTI) in *Nicotiana tabacum* (tobacco) plants [19]. The PTI subsequently induced the HR triggered by the secondary inoculation with *Pseudomonas* carrying TTSS effector genes [19]. Effector-Cya assays confirmed that HR suppression was caused by the restriction of injection of the TTSS effectors into plant cells. From this result, the authors concluded that PTI could directly or indirectly inhibit the injection of TTSS effectors into plant cells [19]. In this report, we demonstrated that the effector-triggered immunity, which was triggered by the interaction of Bs2 and AvrBs2, led to the suppression of the delivery of TTSS effectors into plant cells. It would be interesting to test whether the mechanism of the PTI-based suppression of TTSS is similar to that of the AvrBs2/Bs2 interaction.

Because almost all Gram-negative pathogens, some symbiotic bacteria and several phytopathogenic bacteria have similar TTSS machineries [52–54], it is possible that the conserved components of the TTSS machinery also serve as PAMPs that are specifically recognized by plant extra- or intracellular receptors, triggering plant immunity [55]. It would be intriguing to test the hypothesis that the interaction of AvrBs2 with Bs2 directly or indirectly modifies the plant cell walls, subsequently blocking the penetration of the TTSS pilus across the plant cell walls. It would also be interesting to explore whether the TTSS suppression triggered by AvrBs2/Bs2 is common in other R protein/effector interactions in other plant species. Answering these questions may reveal whether plants employ TTSS suppression as a general immune response to help inhibit the growth of invasive bacterial pathogens.

**Materials and Methods**

**Strains and growth**

*Escherichia coli* strains DH5α, Top10, BL21(DE3) and DB3.1 as well as *Agrobacterium tumefaciens* strain C58C1 were grown on Luria-Bertani agar containing the appropriate antibiotics at 37°C (for *E. coli*) and 28°C (for *A. tumefaciens*). *Xanthomonas* strains were grown on nutrient yeast glucose agar [56] containing the appropriate antibiotics at 28°C. The *Xanthomonas* strains used were GM98-38 *Xe* (avrBs2), GM98-38-1 *Xe* (avrBs2, avrBs2) [24], 85–10 *Xe* (avrBs2, avrBs1) [31] and 69–1 *Xe* (avrBs2) [25]. Various constructs in *E. coli* were transferred to *Xanthomonas* and *A. tumefaciens* C58C1 by tri-parental mating with DH5α (RK600) acting as helper strain [57].

Electrolyte leakage of 1.5 cm² pepper leaf disc post inoculation with *Xe* strains at 2 × 10⁶ CFU/ml and rocked gently in 4 ml water for 1 hour. Conductance was measured with an Thermo Orion conductance meter (model 105A+) in microSiemens/cm (µS).

*Nicotiana benthamiana*, tomato cv. VF36, Bs2 transgenic *Nicotiana benthamiana* and VF36 and pepper lines ECW-0 (no R gene control), ECW-20R (Bs2), ECW-10R (Bs1) and ECW-123R (Bs1, Bs2 and B3) were grown in the greenhouse before and after inoculation at 24°C under 16 hours light/8 hours dark cycles.

**Homology-based modeling**

The MODELLER software package [37] was used to create a comparative protein structural model for AvrBs2 using the solved crystal structure of 1o1z A as a template. The Chimera package was used to perform structural alignments and generate molecular graphics images [58].

**AvrBs2 GDE catalytic site subcloning and protein purification**

The full-length *avrBs2* gene was amplified as a BamHI-StuI fragment by using the following primer set: 5’-caccGGATCC-CTGATGTCGTTTAGCTGACCTTCTGCAACCTTC-3’ and 5’-GTCGACATCCGGTCTTCCGTGCGTGGCCGCT-3’. The resulting PCR fragment was cloned into the same sites of the protein expression vector pGEX4T-1 (GE Healthcare, NJ). The GDE positive control gene *Borelia hermsii* BhGlpQ was amplified from a plasmid provided by Dr. Tom Schwan (University of Montana, Missoula, MT, USA) by using the following primer set: 5’-caccGGATTCCTGAGCTACGAGGCAAATGAGTCA-3’ and 5’-GTCGACGTTGTTTATTTTTTGATGGA3’. The PCR product was cloned into the BamHI/StuI sites of pGEX4T-1 (GE Healthcare, Piscataway, NJ). An overlap extension PCR method was applied to generate the chimeric genes BhGlpQ-avrBs2-wt and BhGlpQ-avrBs2-D304A/D306A. The catalytic domain of wild-type avrBs2 was first amplified with the following primer set: 5’-caccGGATTCCTGAGCTACGAGGCAAATGAGTCA-3’ and 5’-GACCGACATCCGGTCTTCCGTGCGTGGCCGCT-3’. The catalytic domain of avrBs2-E304A/D306A was amplified with the following primer set: 5’-caccGGATTCCTGAGCTACGAGGCAAATGAGTCA-3’ and 5’-GACCGACATCCGGTCTTCCGTGCGTGGCCGCT-3’. The derived PCR products were used as templates for another round of amplification with the following primer set: 5’-caccGGATTCCTGAGCTACGAGGCAAATGAGTCA-3’ and 5’-GTCGACGTTGTTTATTTTTTGATGGA3’. The PCR products were purified by using a gel-purification kit (Bioneer, CA). An overlap extension PCR method was applied to generate the chimeric gene BhGlpQ-avrBs2-wt and BhGlpQ-avrBs2-D304A/D306A. The catalytic domain of wild-type avrBs2 was first amplified with the following primer set: 5’-caccGGATTCCTGAGCTACGAGGCAAATGAGTCA-3’ and 5’-GACCGACATCCGGTCTTCCGTGCGTGGCCGCT-3’. The catalytic domain of avrBs2-E304A/D306A was amplified with the following primer set: 5’-caccGGATTCCTGAGCTACGAGGCAAATGAGTCA-3’ and 5’-GTCGACGTTGTTTATTTTTTGATGGA3’. The PCR products were purified by using a gel-purification kit (Bioneer, CA) and cloned into the BamHI/StuI sites of pGEX4T-1 (GE Healthcare, NJ). The DNA sequences of all clones were confirmed by sequencing.

The protein expression constructs were transformed into *E. coli* strain BL21(DE3) by electroporation and were grown in liquid LB medium supplemented with 50 µg/ml ampicillin at 28°C/220 rpm to OD₆₀₀ = 0.4; 0.5 mM IPTG was added to the culture for 6 hours to induce protein expression. The cells were harvested and disrupted by sonication in cold PBS buffer (147 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH = 7.4) supplemented with 1% Triton X-100. The cell debris was cleared by centrifugation at 12,000 g for 20 min. The soluble GST fusion proteins were purified using Glutathione Sepharose following the manufacturer's protocol provided by the manufacturer (GenScript USA Inc., NJ, USA). The fusion proteins were eluted in 50 mM Tris-Cl, pH = 8.0, supplemented with 10 mM reduced glutathione. All protein samples were stored on ice before the enzyme assays.

**GDE enzyme assays**

The enzyme activity of the purified GST-fusion proteins was measured using an enzyme-coupled spectrophotometric assay to measure the amount of G3P that was released by the glycerophosphodiester phosphodiesterase reaction. The reaction
mixture contained 0.2 M hydrazine-glycine buffer, pH = 9.0, 0.5 mM NAD, 10 U/ml G3P dehydrogenase (Sigma G6880), 10 mM CaCl₂, 0.5 mM Sn-glycerol-3-phosphocholine (G5291), and the GST-fusion proteins at several pre-set concentrations. The reaction mixture was incubated at 30 °C in a 96-well plate for 1 h until the oxidation of G3P by G3P dehydrogenase was complete. The G3P concentration was determined from the absorbance change at 340 nm by using the BioTek plate reader (BioTek Instruments, Inc., VT, USA).

Site-directed mutagenesis of avrBs2 in the Xe genome and construction of Cya fusions for TTSS effector delivery reporters

Mutants formed by homologous recombination of the genomic copy of avrBs2 in Xe were constructed as previously described [18,59]. The avrBs2 open reading frame was first PCR amplified with a SacI site at the 5'-end and a BamHI site at the 3'-end and cloned directionally into pBluescript KS+. This intermediate construct was mutagenized using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, CA) to incorporate the two GDE catalytic site mutations (E304A/D306A, H319A and Y419A). The three resultant mutant constructs were digested with NotI and BamH1, and T3 DNA polymerase was used to create blunt ends. The blunt-ended fragments were then cloned into the suicide vector pLVCI8L, which has a Col E1 replicon and contains the highly efficient mob region from pRSF1010 [18], cut with XhoI and SmalI, and filled using T4 DNA polymerase to make pLVCI8AvrBs2 (E304A/D306A, H319A and Y419A). The three constructs were then mobilized into Xe (avrBs2, avrBs3) and rescued by tetracycline selection of a single recombination event into the genomic copy of avrBs2. Second-site resolution crossover events were identified as tetracycline-sensitive single colonies from cultures grown in the absence of tetracycline. PCR amplification and sequencing were used to confirm a double homologous recombination event for either the E304A/D306A, H319A or Y419A. All bacterial growth assays in pepper and tomato were performed as previously described [25].

Two mutant strains Xe (E304A/D306A and H319A) were further modified by homologous recombination to add Cya as a C-terminal translational fusion as previously reported [18]. Double homologous genomic recombination was used to delete the avrBs2 locus in strains 85–10 Xe (avrBs2, avrBs1) and 69–1 Xe (avrBs2) to make Xe (avrBs1) and Xe (no effector) respectively using p815:avrBs2:GM as previously described [23].

Epitope-tagged AvrBs2 deletions and mutations and Agrobacterium-mediated transient expression

All avrBs2 deletions and mutations were first cloned into pENTR/D-TOPO (Invitrogen) as previously described [59]. Each construct began with a start codon and ended without a stop codon so that the HA epitope and stop codon of the destination vector would be maintained after transfer. For Agrobacterium-mediated transient expression from the 35S promoter and C-terminal HA epitope tagging, pMD1 was first digested with XhoI. The HA epitope and the stop codon linker (5’- GCGAGTGTTGGATGGCCGATTTCAG-3’) were cloned in and then re-opened at the Smal site, and the ccdB cassette A (Invitrogen) was cloned in to create the destination vector pMD1-Des-HA. All pENTR-avrBs2 constructs were then transferred to pMD1-Des-HA using LR clonase (Invitrogen).

For AvrBs1:HA and AvrBs2:HA Agrobacterium-mediated transient expression constructs both full length effectors were cloned into pENTR/D-TOPO with N-terminal Xbod site and a C-terminal HA epitope tag (5’- GGAGTCTCACCGATCATGATGTTCGCACTATGCCTAATGACGTCCGGGACTATCGAGTAGGSGCTGCTG-3’) followed by a SacI site. These were then subcloned into pMD1. The pMD1-AvrBs2:HA construct was further modified by re-opening at the single Bsd site and the ccdB cassette B (Invitrogen) cloned in to create a destination vector. The HindIII-EcoRI 33S-nos Terminator fragment was cloned into pENTR/D-TOPO and then the AvrBs1:HA Xbod-SacI fragment was subcloned in. This pENTR-33S-AvrBs1:HA was transferred into the pMD1-AvrBs2:HA destination vector using LR clonase (Invitrogen) to create a double effector binary vector for Agrobacterium transient expression.

The binary deletion and mutation constructs were transferred to Agrobacterium (C58C1) for transient expression in Nicotiana benthamiana and pepper, as previously described [25].

Immunoblot analysis protocol was previously described [26].

Generating the Cya reporter fusion with the Xe effectors avrBs1, avrBs2, avrBs3 and XopP

Two effector-Cya reporters from avrBs1 and avrBs3 were made by directional cloning PCR products into Gateway-compatible pENTR/D-TOPO (Invitrogen) and then translationally fused to Cya by LR clonase (Invitrogen) into the suicide destination vector pDestCya [59]. The effector PCR products of 1352 base pair for avrBs1 and 950 bp for avrBs3 included the promoter region and the first 212 codons of AvrBs1 and the first 107 codons of AvrBs3 were used to create AvrBs11-212-Cya and AvrBs31-107-Cya, respectively. The two previously constructed pDestCya effector-Cya reporters for AvrBs21-98-Cya and XopX1159-Cya, along with AvrBs31-107-Cya and AvrBs11-212-Cya, were introduced into Xe by genomic single recombination rescues of these constructs. This recombination still maintained the wild-type genomic copy of the particular effector [18]. The pairs of effector-Cya reporter strains with and without avrBs2 included the three-strain pairs of Xe (avrBs3), Xe (avrBs3, avrBs2) with either reporter AvrBs21-212-Cya, AvrBs31-107-Cya or XopX1159-Cya. Also included were the two-strain pairs of Xe (avrBs1) and Xe (avrBs1, avrBs2) with either XopX1159-Cya or AvrBs11-212-Cya. Additionally the pDestCya with AvrBs31-107-Cya was introduced into strains Xe (avrBs2-E304A/D306A and H319A) by genomic single recombination rescues of these constructs. The Cya was added to the C-terminus of Xe catalytic mutants of AvrBs2 as previously described [18]. The 35S-avrBs2:Cya construct was made by replacing the BamHI-Sacl GFP fragment from pMD1-avrBs2::GFP [18], with a BamHI-Sacl Cya fragment. This construct was introduced into Agrobacterium for transient expression as previously described [26]. Plant cyclic AMP (cAMP) levels were measured as previously described [18]. Sampling at eight hours post-inoculation will avoid in planta multiplication of the reporter strains. Eight hours post-inoculation is also long before the development of any R gene-mediated HR.

Supporting Information

Supplemental Figure S1 Inoculation of near-isogenic pepper (Bs2) and pepper (w/o Bs2) with high-density suspensions of Xe (2 × 10⁶ CFU/ml). Bs2-dependent brown necrotic HR detected at 96 hours post-inoculation for Xe (avrBs2) strain GM98-38-1 and double homologous recombination mutants for the putative GDE catalytic site Xe (avrBs2-E304A/
D306A) and Xc (avrBs3-H319A). Also the AvrBs2 mutations identified in Xc strains isolated from bacterial spot diseased pepper (B2) were recombined into GM98-38-1 for Xe (avrBs2-R403P) produced no HR, similar to the control Xc (w/o avrBs2). The other mutant Xe (avrBs2-V410E) produced intermediate HR with light brown necrosis detected. In pepper (w/o B2), only the strains w/o avrBs2 or with putative catalytic site mutations gave an altered high-density virulent phenotype.

**Supplemental Figure S2** Confirm Xe strains with GDE catalytic site mutations are not altered in TTSS delivery to host and are not altered in Bs2 activated HR. A. The TTSS effector reporter adenylate cyclase (Cya) was translationally fused on the C terminus of the gemonic copy of AvrBs2 and the AvrBs2 catalytic site mutants. These Xe TTSS reporter inoculations on pepper were sampled 8 hours post-inoculation to avoid *in planta* multiplication and in planta cyclic AMP (cAMP) levels were assayed. No alteration of AvrBs2 delivery for catalytic site mutations. B. Inoculation of pepper (B2) and pepper (w/o B2) with high-density suspensions of Xe (2×10^8 CFU/ml) at 48 hpi. No alteration in HR phenotype for Xe strains with Cya translational reporters.

**Supplemental Figure S3** Confirmation of protein expression for *Agrobacterium* transient constructs for minimum domain and key amino acids mutations of AvrBs2 required for Bs2 activation. A. Immunoblot analysis with anti-HA showing *Agrobacterium*-mediated transient protein expression of HA epitope-tagged constructs of the various avrBs2 deletions and mutations of the minimal Bs2-HR activation domain (~31 kDa). Ponceto S staining of immunoblot as loading control. B. Near-isogenic pepper with and without Bs2 inoculated for *Agrobacterium* transient expression (48 hpi at 2×10^6 CFU/ml) with the 35S-HA epitope tagged constructs of the various AvrBs2 deletions and mutations of the minimal Bs2-HR activation domain.

**Supplemental Figure S4** Evaluation of loss of Bs2 activation mutant AvrBs2 (Y419A) in Xe. A. In planta pathogen growth assay for *Xanthomonas* strains GM98-38 Xe (w/o avrBs2), GM 98-38-1 Xe (avrBs2), and GM98-38-1 Xe (Y419A) exchange mutant. Host plants pepper (w/o B2) and pepper (B2). Exchange mutant Xe (Y419A) was unable to completely overcome Bs2 resistance. B. Inoculation of near-isogenic pepper (B2) and pepper (w/o B2) with high-density suspensions of Xe (2×10^8 CFU/ml). Exchange mutant Xe (Y419A) gave a light brown necrotic HR on pepper (B2).

**Supplemental Figure S5** Electrolyte leakage to confirm slower Bs2-HR (48 hpi) from mixed high-density (1.5×10^8 CFU/ml) inoculation of independent Xe strains one with AvrBs1 and one with AvrBs2 was epistatic to the faster Bs1-HR (18 hpi) for pepper (Bs1, Bs2). A. Near-isogenic pepper lines with bacterial spot resistance genes (Bs1, Bs2) and the combination of Bs1 and Bs2, at 10 and 48 h hours post-inoculation (hpi) with the mixed strains Xe (avrBs1) and Xe (avrBs2). When the mixed strains Xe (avrBs1) and Xe (avrBs2) were co inoculated on pepper (B1, B2), the fast Bs1/AvrBs1 HR was again not detected at 18 hpi. B. All pepper (no R-gene) control inoculations with single Xe effector, either by individual or mixtures, gave the expected responses.

**Supplemental Figure S6** Electrolyte leakage to confirm slower Bs2-HR (48 hpi) from mixed high-density (1.5×10^8 CFU/ml) inoculation of independent Xe strains one with AvrBs1 and one with AvrBs2 was epistatic to the faster Bs1-HR (18 hpi) for pepper (Bs1, Bs2). A. Near-isogenic pepper lines with bacterial spot resistance genes (Bs1, Bs2) and the combination of Bs1 and Bs2, at 10 and 48 h hours post-inoculation (hpi) with the mixed strains Xe (avrBs1) and Xe (avrBs2). When the mixed strains Xe (avrBs1) and Xe (avrBs2) were co inoculated on pepper (B1, B2), the fast Bs1/AvrBs1 HR was again not detected at 18 hpi. B. All pepper (no R-gene) control inoculations with single Xe effector, either by individual or mixtures, gave the expected responses.

**Supplemental Figure S7** Electrolyte leakage to confirm slower Bs2-HR (48 hpi) from mixed high-density (1.5×10^8 CFU/ml) inoculation of independent Xe strains one with AvrBs1 and one with AvrBs2 was epistatic to the faster Bs1-HR (18 hpi) for pepper (Bs1, Bs2). At 18 hpi electrolyte leakage of inoculated leaf disc in water were measured with a conductance meter in microSiemens/cm (uS). High levels of electrolytes correlated with the corresponding HR phenotypes reported in Supplemental figure 6A.

**Supplemental Figure S8** Bs2 activation dependent suppression of the AvrBs1/Bs1 fast HR phenotype not observed when expressed inside plant cells via *Agrobacterium* transient expression. A. *Agrobacterium* transient expression strains containing 1. Agro 35S-avrBs1, 2. Agro 35S-avrBs1 + 35S-avrBs2 and 3. Agro 35S-AvrBs2 were inoculated on pepper plants containing the Bs1, (B1 and B2) and no resistance genes. During the co-expression of AvrBs2 and AvrBs1 no epistasis was observed as the phenotype of the Bs1 HR was not altered by the co-expression of AvrBs2. B. Electrolyte leakage was observed for the same combinations as shown in panel A. The electrolyte leakage phenotype of the Bs1 HR was observed when both AvrBs2 and AvrBs1 were co-expressed in Agro confirming that there was no epistasis when the genes are co-expressed in planta. C. Immunoblot detection of AvrBs2-HA and AvrBs1-HA expressed in pepper plants via *Agrobacterium* transient expression at 0 and 24 hpi. This result showed that the activation of Bs2-specified resistance did not interfere with the detection of the Bs1 protein.

**Supplemental Figure S9** Bs2 activation blocked subsequent TTSS delivery of multiple effectors to host cells. TTSS effector delivery reporter constructs consisted of the effector promoter and the secretion and translocation signal peptides translationally fused to adenylate cyclase (Cya). Pepper plants (no R genes, Bs2 or Bs3) were sampled 8 hours post-inoculation to avoid in planta multiplication of the reporter strain pairs (with and without avrBs2) and assayed for cyclic AMP (cAMP). Eöter-Cya translational fusion and calmodulin from the plant cell led to elevated levels of cAMP. A. AvrBs2,1-212-Cya reporter in *Xanthomonas* strains GM98-38-1 Xe (avrBs2) and Xe (avrBs3) and Xe (avrBs2) inoculated onto pepper plants (no R genes, Bs3 or Bs2). In planta cAMP levels were assayed. Student t-test was used to compare TTSS delivery of effector reporter in an Xe strain with and without avrBs2 on B2 pepper plants; p-values were <0.01. B. AvrBs3,1-212-Cya reporter in *Xanthomonas* strains GM98-38-1 Xe (avrBs2 and avrBs3) and Xe (avrBs3) inoculated onto pepper plants (no R genes, Bs3 or Bs2). In planta cAMP levels were assayed. Student t-test was used to compare TTSS delivery of effector reporter in an Xe strain with and without avrBs2 on B2 pepper plants; p-values were <0.01. C. XopX1,1-117-Cya reporter in *Xanthomonas* strains GM98-38-1 Xe (avrBs2 and avrBs3) and Xe (avrBs3) inoculated onto pepper plants.
Supplemental Figure S10 Elevated levels of in planta cyclic AMP resulting from Agrobacterium transient expression of 35S-avrBs2:Cya was not blocked by Bs2 activation. Also avrBs2 GDE catalytic mutations were still able to block subsequent TTSS effector delivery to pepper host. Also Bs2 activation does not change effector protein levels in Xe. A. Agrobacterium transient expression of 35S-avrBs2:Cya in Nicotiana benthamiana with and without Bs2 sampled at 15 hpi were similar for elevated levels of cyclic AMP. B. AvrBs3 (signal peptide):Cya TTSS effector recombine into Xanthomonas strains GM961-38-1 Xe (avrBs2, avrBs2-E304A/D306A or avrBs2-H319A) had similar reduced levels of cyclic AMP in the presence of Bs2 compared to pepper host without Bs2. C. Immoblot assays of high titer inoculation (5 \times 10^9 CFU/ml) of pepper (w/o Bs2) and pepper (Bs2) at 6 hpi with Xe (avrBs2), Xe (avrBs2-Cya), Xe (avrBs2-E304A/D306A-Cya) and Xe (avrBs2-H319A-Cya) detected no reductions of protein levels associated with Bs2 activation.

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Author Contributions

Conceived and designed the experiments: BJS BD DKV. Performed the experiments: BJS BD KKV RWF. Analyzed the data: BJS BD DKV RWF. Contributed reagents/materials/analysis tools: BJS BD KKV. Wrote the paper: BJS BD DKV.

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