ABSTRACT

KATAWCZIK, MELANIE L. Characterization of *Xanthomonas euvesicatoria,* Adaptation of Bacterial Strains Carrying Alleles of *avrBs2* and Association with Pathogen Aggressiveness Under Field Conditions. (Under the direction of Dr. D.F. Ritchie.)

Bacterial spot of pepper (*Capsicum annum*), caused by *Xanthomonas euvesicatoria,* (syn. *Xanthomonas axonopodis* pv. vesicatoria and *Xanthomonas campestris* pv. vesicatoria) is a serious disease controlled by a combination of pathogen-free seed and transplants, bactericides containing copper, and host resistance. The predominantly used resistance gene (R gene), *Bs2,* has been deployed in commercially grown peppers since the mid 1990s. The *Bs2* gene was hypothesized to provide durable resistance because the corresponding pathogen effector gene (*avrBs2*) is chromosomally conserved across many species of *Xanthomonas* and has been shown to be necessary for full fitness and virulence on hosts lacking the cognate R gene. To date, many of the bacteria causing bacterial spot of peppers overcome the *Bs2* gene through mutations in the *avrBs2* locus, but are presumed to be less aggressive than races that have a functional *avrBs2* gene.

Using a collection of strains from the southeastern United States including strains isolated before the commercial introduction of the *Bs2* R gene, phenotypic and genotypic characteristics, including frequency of mutation types in *avrBs2* and aggressiveness under field conditions were evaluated. Ten types of mutations have been detected in *avrBs2*. These are the complete deletion of the *avrBs2* locus, a 5-bp insertion or deletion, a 12-bp repeat, CC to AA transversion, IS-element insertion, and four point mutations. To determine the effect of the mutation type in the *avrBs2* locus on aggressiveness, strains comprising the 10 different mutations in the *avrBs2* locus were inoculated on Early Calwonder (ECW, carries no major resistance genes) pepper plants at the Sandhills Research Station, Jackson Springs, North Carolina. Fifteen strains each of the four races 1, 3, 4 and 6 were inoculated on ECW pepper plants in field plots and repeated over three locations. Disease severity ratings were recorded weekly and used to evaluate strain aggressiveness in the field. Strains with the mutated *avrBs2* alleles belong to races 4 (nonfunctional *avrBs2*, functional *avrBs3*) and 6 (nonfunctional *avrBs2* and *avrBs3*). Races 1 (functional *avrBs2* and *avrBs3*) and 3 (functional *avrBs2* and nonfunctional *avrBs3*) were used for comparison with races 4 and 6, respectively. Race 1 was always more aggressive as a population than race 4 and similarly

race 3 was more aggressive than race 6 as a population. While the races with the functional *avrBs2* gene were more aggressive than those with nonfunctional *avrBs2* genes, strains of each race caused severe disease in the field although variation in aggressiveness among strains within each race occurred. The type of mutation in the *avrBs2* gene also was correlated with pathogen aggressiveness. Strains with a 5-bp insertion and a 5-bp deletion were significantly less aggressive than strains with point mutations. Mutations in *avrBs2*, within strains of races 4 and 6, preserving aggressiveness were more prevalent in the sample collection than those that did not.

The majority of mutations occurred in AvrBs2 coding regions required for recognition by *Bs2* and not in the 5' N-terminal region of *avrBs2* where coding for the type III secretion system exists. This suggests that the effector protein, AvrBs2, may be translocated into the plant cellular region, while able to bypass the host defense system. These results show that in the arms race between *X. euvesicatoria* and cultivated pepper, that the pathogen is able to accumulate mutations that preserve aggressiveness while evading host recognition.

Characterization of *Xanthomonas euvesicatoria,* Adaptation of Bacterial Strains Carrying Alleles of *avrBs2* and Association with Pathogen Aggressiveness Under Field Conditions

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BIOGRAPHY

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LITERATURE REVIEW

Single-gene resistance is determined by specific allele interactions of a dominant resistance gene (R gene) in the host that corresponds to a dominant avirulence (Avr) gene in the pathogen (Flor 1971). When such complementary genes are present, an incompatible reaction occurs usually expressed as a hypersensitive response (HR), defined by localized self-induced cell death and collapse of tissue, at the site of infection. The HR is accompanied with calcium accumulation, ion movement through the cell membrane, oxidative bursts and local transcriptional modification around the infection site (Nimchuk et al. 2003; Grant and Loake 2000; Greenberg 1997). The HR effectively walls off the pathogen in the dead intercellular space so the pathogen can no longer multiply and spread (Greenberg 1997). Disease ensues if either the host resistance gene or the corresponding pathogen avirulence gene is not present.

The recognition between host R products and pathogen Avr products can limit the host range of individual strains. A pathogen's host range may increase through mutations in or loss of the corresponding Avr genes (Kearney and Staskawicz 1990a). The mutated Avr allele, if no longer functional, is not recognized by the corresponding R gene product and infection ensues (Kearney and Staskawicz 1990b). It appears counter intuitive for a pathogen to retain a gene that allows host recognition and prevention of further infection. One possible explanation is that in addition to being a target for host detection, Avr gene products perform an essential function for the pathogen (Alfano and Collmer 2004; Kjemtrup et al. 2000; Kearney et al. 1988). Some genes exhibit a dual role for the pathogen when infecting hosts without the corresponding R gene. These Avr genes contributed to virulence and fitness of the pathogen through suppression of plant basal defenses in the absence of the corresponding R gene (Alfano and Collmer 2004; Jakobek et al. 1993). The term of effector gene, instead of Avr gene, is used here to refer to type III secretion system proteins that function inside host cells (Alfano and Collmer 2004). Thus, depending on the host genotype, the effector gene of a pathogen can display virulence or avirulence functions.

The type III secretion system is an essential component for pathogenicity in many gram-negative bacterial pathogens including plant pathogens (Alfano and Collmer 2004;

Abramovitch and Martin 2004; Cornelis and van Gijsegem 2000). This secretion system is responsible for trafficking effector proteins into the plant cell through use of a molecular syringe (Alfano and Collmer 2004; Szurek et al. 2002). The type III secretion system is crucial in *Xanthomonas euvesicatoria* pathogenicity (Büttner and Bonas 2002a; Büttner and Bonas 2002b; Büttner et al. 2003) since the *avrBs2* effector product is secreted into the host by this system (Casper-Lindley et al. 2002; Mudget et al. 2000). The structure of the type III secretion system is highly conserved among plant and animal bacterial pathogens, whereas the secreted effectors are more diverse, lacking sequence similarity (Tampakaki et al. 2004; Cornelis and Van Gijsegem 2000). The structural components of the type III secretion system are encoded by a large gene cluster of *hrp* (hypersensitive response and pathogenicity) and *hrc* (hypersensitive response and conserved) genes, whose products direct type III secretion system molecules across the bacterial envelope and through host cell walls, respectively (Alfano and Collmer 2004; Bogdanove et al. 1996). The only potential type III secretion system chaperone, proteins that can aid effectors in proper folding, found in *X. euvesicatoria* is HpaB, interacting with AvrBs1 and AvrBs3, but not AvrBs2 (Büttner et al. 2006; Büttner et al. 2004). HpaB may not only be a chaperone but may play an essential role in monitoring translocation of effectors versus inhibition of other proteins (Büttner et al. 2006; Büttner et al. 2004). Knowledge of effector protein chaperones is important as chaperones potentially allow for earlier delivery of some effectors versus others into the plant cell (Thomas and Finlay 2003). This may help explain why some effectors seem to have a more dominant effect then others. The discovery of a possible mRNA signal within the first 18 amino acids of *avrBs2* suggests that *avrBs2* is a chaperone-independent effector gene (Mudgett et al. 2000).

In the plant cell, effector proteins either have an avirulence activity by revealing the pathogen to the plant defense system or may interfere with host cellular processes to the benefit of the pathogen. Effector proteins suppress host defense responses, allowing pathogen multiplication within plant tissue (Nomura et al. 2005; Chisholm et al. 2006). Specific examples include the interference of the type III secretion system effectors with R-protein activation, repression of the resistance inducing salicylic acid pathway, regulation of plant transcription by members of the AvrBs3 effector family, prevention of papillae formation

and HR development (Mudgett 2005; Durrant and Dong 2004; Reymond and Farmer 1998; Szurek et al. 2001; Szurek et al. 2002; Hauck et al. 2003; Jamir et al. 2004). The disadvantage of type III secretion system effector products is that they are targets for the host-resistance proteins of the nucleotide-binding site leucine-rich repeats (NBS-LRR) class to recognize and can lead to the stoppage of pathogen spread (Nimchuk et al. 2003). Unlike effector genes, the composition of most R proteins display key similarities of homology with a series of leucine rich repeats (LRRs), a conserved nucleotide binding site (NBS), and a putative amino-terminal signaling domain (Belkhadir et al. 2004; Tameling et al. 2002; Cornelis and Van Gijsegem 2000).

The *Bs2* gene was discovered in *Capsicum chacoense* (Cook and Guevara 1984) and shown to encode a protein of the prevalent NBS-LRR class of plant disease resistance genes (Meyers et al. 2003; Tai et al. 1999). The conserved NBS binds and hydrolyses ATP (Tameling et al. 2002), while the LRRs serve as bacterial protein recognition sites in addition to host protein activation potentially leading to an HR (Belkhadir et al. 2004; Tanabe et al. 2004; Chen et al. 2004). It has been shown that R proteins of the NBS-LRR class directly or indirectly detect effectors of the type III secretion system (Dangl and Jones 2001; van der Biezen and Jones 1998). The simplest explanation of pathogen effector and host R gene interaction is though direct interaction of effector-produced ligands with host-encoded receptors. Few cases have been reported where the "receptor-ligand" model is actually the case (Jia et al. 2000; Deslandes et al. 2003). This may be explained by the difficult task a R gene, with conserved recognition features, would have in maintaining specific recognition of the extreme diversity of potential pathogens and their effectors (Dangl and McDowell 2006; Chisholm et al. 2006; Dangl and Jones 2001). The other interaction model, known as the "guard-hypothesis", hypothesizes an indirect recognition. Plant virulence targets of pathogen effectors are monitored or "guarded" by R proteins (Dangl and Jones 2001; van der Biezen and Jones 1998). Once the type III secretion system effectors modify the virulence target, the host R protein activates signal transduction pathways of defense resulting in limiting bacterial multiplication (Dangl and Jones 2001; van der Biezen and Jones 1998). Indirect detection allows the plant to monitor a limited number of targets and respond when those

targets are altered, thus limiting resistance proteins required for effective resistance (Dangl and Jones 2001; Chisholm et al. 2006).

Transient expression of *Bs2* in pepper, in addition to tomato and *Arabidopsis*, demonstrated that effector *avrBs2* is required for the activation of *Bs2*-dependent disease resistance (HR) *in planta* (Tai et al. 1999; Mudget et al. 2000). The predicted AvrBs2 protein has homology with agrocinopine synthase (ACS) of *Agrobacterium tumefaciens* and the glycerophosphoryl diester phosphodiesterase (UgpQ) of *Escherichia coli* (Swords et al. 1990), suggesting that AvrBs2 may function in pathogenicity as an enzyme to synthesize or hydrolyze phosphodiester linkages between carbohydrates or phospholipids in plant cells (Brzoska and Boos 1988, Ryder et al. 1984). The region with homology to the synthase is where the regions determining avirulence and virulence functions are thought to be localized in AvrBs2 (Mudgett et al. 2000). Like many other plant pathogenic bacteria, *X. euvesicatoria* harbors additional effectors (Thieme et al. 2005; Roden et al. 2004; Guttman et al. 2002; Petnicki-Ocwieja et al. 2002). The AvrBs3 effector family is found in many *Xanthomonas* species and is thought to promote disease by causing hypertrophy in pepper mesophyll tissue, allowing further multiplication of bacteria (Leach et al. 2001; Marois et al. 2002). AvrBs3 is the only known effector to be translocated into the plant nucleus where it binds DNA and induces host gene transcription (Marois et al. 2002). Less is known about the function of the AvrBs1 effector although mutations in *avrBs1* have been shown to reduce bacterial multiplication in plants (Kearney and Staskawicz 1990b). Another study found that only in *avrBs2* deficient backgrounds did the loss of *avrBs1* function cause a decrease in bacterial multiplication *in planta* in addition to a reduction in development of disease symptoms, and even then the effect of loss of *avrBs1* function is small (Wichmann and Bergelson 2004). Unlike *avrBs2*, both *avrBs1* and *avrBs3* are located on self-transmissible plasmids (Stall et al. 1986; Minsavage et al. 1990). Discoveries of seven type III effectors in *X. euvesicatoria* (i.e. *X. campestris* pv. vesicatoria), of which three are *Xanthomonas* species specific, has been accomplished though genetic screens and these are termed Xops (*Xanthomonas* outer proteins) (Roden et al. 2004). Only *X. euvesicatoria xopN* has been found to effect pathogen multiplication in pepper and tomato plants (Roden et al. 2004). Because type III effector function can be redundant, often the effect of nonfunctional

effectors is difficult to deduce due to strains harboring a large number of effectors (Nomura and He 2005; Roden et al. 2004).

The diversity in DNA sequence of pathogen effectors has been a hindrance in determining effector virulence function. Additionally, the biochemical function of most pathogen effectors, including *X. euvesicatoria*, in virulence activity has not been established. Most knowledge of effector function comes from comparisons of sequence homology to eukaryotic proteins (Chang et al. 2004; Mudgett 2005; Petnicki-Ocwieja et al. 2002). Comparisons have revealed possible functions of basal defense suppression and specific interference with defensive pathways by cleaving or degrading specific host substrates (Mackey et al. 2003; Jakobek et al. 1993; Ritter and Dangl 1996).

Effector genes in a pathogen may be lost or altered, which may increase a pathogen's host range by allowing infection of hosts that carried a corresponding R gene. Mutations and physical loss of effector genes have been detected in *X. euvesicatoria* (Gassmann et al. 2000; Wichmann et al. 2005; Swords et al. 1996; Kousik and Ritchie 1998). Genome sequence analysis of *X. euvesicatoria* strain 85-10 has revealed a genome with features such as high number of insertion sequence (IS) elements and tRNA genes with deviations in G+C content indicating gene acquisitions through horizontal transfer (Thieme et al. 2005). This plasticity is advantageous for pathogens under continuous selection pressure from host R genes and allows them to evolve toward evasion or suppression of host defenses. Other potential pathogenicity genes uncovered code for adhesion, cell wall-degrading enzymes, and extracellular polysaccharide production (Thieme et al. 2005). Genome analysis also revealed the possession of genes that code for all known types of protein secretion systems (Thieme et al. 2005). So, in addition to the type III secretion system which is required for bacterial pathogenicity, genes for type I, II, IV, and V secretion systems for gram-negative bacteria plus other transport systems were found. Six new putative type III effectors were revealed through BLAST comparisons, in addition to the 14 previously identified. 10 of the 20 putative effectors had significantly lower G+C content when compared to the entire genome, suggesting they arose via horizontal gene transfer (Thieme et al. 2005; Dobrindt et al. 2004; Gürlebeck et al. 2005). Strain 85-10 did not have homologs to the AvrBs3 family of effectors but this is not surprising as the comparison of the sequenced *X. euvesicatoria* strain

with closely related *Xanthomonads* (*X. axonopodis* pv citri, *X. campestris* pv. campestris, *X. oryzae* pv. oryzae) differed in effectors contained (Thieme et al. 2005). This suggests that a pathogens effector set may determine the pathogen's host range; however, the type III secretion system machinery is excluded from host range determination as it is conserved across species (Fouts et al. 2003; Galan and Collmer 1999). Although it has been suggested that the loss of a single type III effector does not typically effect pathogenicity, certain effectors such as AvrBs2 and *X. euvesicatoria xopN* do show effects on bacterial multiplication and pathogenicity when mutated (Kearney and Staskawicz 1990b; Roden et al. 2004).

Variations or mutations in effector genes retained across pathovars were shown to be important determinants of host specificity (Stevens et al. 1998). Examination of the *avrBs2* gene showed that a single-open-reading-frame (ORF1) is required for both the production of the effector product that is recognized by the *Bs2* gene for resistance and for full pathogen fitness (Swords et al. 1996). Genome analysis revealed nine classes of mutations that defeat the *Bs2* gene with all the mutations potentially affecting protein coding (Swords et al. 1996; Gassmann et al. 2000; Wichmann et al. 2004; Wichmann et al. 2005). Most of those mutations lie in the region of the *avrBs2* locus that is crucial for AvrBs2 effector domain recognition by the *Bs2* gene product (i.e. crucial for HR development) but not for protein translocation (Mudgett et al. 2000). In response to continuous *Bs2* selection pressure, the dual function of the effector gene was uncoupled (Gassmann et al. 2000). *X. euvesicatoria* strains were found with mutations in the *avrBs2* gene that prevented recognition by the host carrying the corresponding R gene without an apparent decrease in pathogen fitness (Gassmann et al. 2000; Wichmann et al. 2005; Kousik and Ritchie 1998). This also was found in two other Avr genes, *avrRpt2* and *avrPto*, of *Pseudomonas syringae* pv. tomato (Lim and Kunkel 2004; Shan et al. 2000).

Under an "arms race" model, the pathogen should conserve mutations that retain virulence function while avoiding detection of the host. Of the *avrBs2* mutant alleles examined, while avoiding host detection, most did not retain aggressiveness on susceptible hosts (Wichmann et al. 2005; Gassmann et al. 2000). Results for one of the mutants did

suggest that *X. euvesicatoria* is maintaining virulence under the selection of *Bs2* while avoiding detection (Wichmann et al. 2005, Gassmann et al. 2000).

Bacterial spot on pepper (C*apsicum annuum*) is caused by *Xanthomonas euvesicatoria* (Jones et al. 2004), previously known as *Xanthomonas campestris* pv. vesicatoria (Dye et al. 1980) and more recently as *Xanthomonas axonopodis* pv. vesicatoria, after it was separated from *X. vesicatoria* based on amylolytic activity and other characteristics (Vauterin et al. 1995). This disease effects pepper worldwide (Cook and Stall 1963) and is most damaging during warm, moist conditions that allow the pathogen to multiply intercellularly while eluding host detection (Marco and Stall 1983; Sahin and Miller 1998). The causal agent of bacterial spot was first observed as a canker on tomato in 1914 and officially reported in 1920 (Doidge 1921) and first described as bacterial spot on pepper around 1918 (Sherbakoff 1918). Characteristic symptoms of bacterial spot are angular, water-soaked lesions defined by leaf veinlets (Jones and Pernezny 2003). Under dry conditions, the interior of the lesions may disintegrate, giving the leaf a shot-hole appearance. Fruit lesions, defoliation, and premature blossom and fruit drop have the greatest potential to cause yield loss (Jones and Pernezny 2003). X. *euvesicatoria* is not a vascular pathogen therefore bacterial transmission to new plant parts depends primarily on rain splash and aerosols with bacteria entering the plant tissue through stomata and wounds to multiply intercellularly (Jones and Pernezny 2003).

Bacterial spot control uses copper and other bactericides, with host resistance as a component of disease management. Host resistance in the *X. euvesicatoria*-pepper system follows the gene for gene model (Minsavage et al. 1990). Currently three major R genes, *Bs1*, *Bs2*, and *Bs3*, are deployed in pepper, corresponding to the effector genes *avrBs1*, *avrBs2*, and *avrBs3* found in *X. euvesicatoria* (Minsavage et al. 1990; Bonas et al. 1989; Stall et al. 1986). The *avrBs2* gene is located chromosomally; *avrBs1* and *avrBs3* are located on separate plasmids. *Xanthomonas euvesicatoria* is divided into races, which correspond to the pepper resistance genes they defeat. Since the early 1990s, hybrid pepper cultivars carrying the *Bs2* gene have been used to specifically target the *avrBs2* gene (Minsavage et al. 1990; Kousik and Ritchie 1996). The *Bs2* gene was hypothesized to be an effective and durable gene for bacterial spot control due to the chromosomal conservation of *avrBs2* across

many species of *Xanthomonas*, suggesting it served a crucial function to the bacteria and was consequently shown to be necessary for full pathogen fitness and virulence on susceptible hosts (Swords et al. 1996; Minsavage et al. 1990; Kearney and Staskawicz 1990b). Soon after the deployment of *Bs2*, shifts to races 4, 5, and 6 that carry a nonfunctional *avrBs2* allele were commonly detected (Kousik and Ritchie 1996; Sahin and Miller 1995; Kousik and Ritchie 1995; Jones et al. 1998). A field study examining races that overcome *Bs2* confirmed that strains without functional *avrBs2* genes retained the potential to cause severe disease on peppers carrying the *Bs2* gene (Pernezny and Collins 1999; Kousik and Ritchie 1996).

Previous research examining effects on pathogen aggressiveness in the *Xanthomonas*-pepper system have had a key component missing. First, while *avrBs2* effects were examined in the field, they were done using frame-shift effector genes which were assumed to result in truncated effector proteins that would abolish gene function (Wichmann and Bergelson 2004). These laboratory derived mutations did not produce any *avrBs2* RNA transcript, while some *avrBs2* mutations isolated under field conditions do produce an *avrBs2* transcript (Wangsomboondee 2002). Second, mutations in field-derived strains were examined but were evaluated for aggressiveness in greenhouse conditions (Gassmann et al. 2000). The research presented here was conducted using strains possessing mutations selected for under field conditions from research plots and pepper production fields to further the understanding of the adaptation of effectors under selection of host resistance.

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Characterization of *Xanthomonas euvesicatoria,* Adaptation of Bacterial Strains Carrying Alleles of *avrBs2* and Association with Pathogen Aggressiveness Under Field Conditions

ABSTRACT

Bacterial spot of pepper (*Capsicum annum*), caused by *Xanthomonas euvesicatoria,* (syn. *Xanthomonas axonopodis* pv. vesicatoria and *Xanthomonas campestris* pv. vesicatoria) is a serious disease controlled by using a combination of pathogen-free seed and transplants, bactericides containing copper, and host resistance. The predominantly used resistance gene (R gene), *Bs2,* has been deployed extensively n commercially grown peppers since the mid 1990s. The *Bs2* gene was hypothesized to provide durable resistance because the corresponding pathogen effector gene (*avrBs2*), which is chromosomally conserved across many *Xanthomonas* species, has been shown to be necessary for full fitness and virulence on hosts lacking the cognate R gene. To date, many of the bacteria causing bacterial spot of peppers overcome the *Bs2* gene through mutations in the *avrBs2* locus, but are presumed to be less aggressive than strains that have a functional *avrBs2* gene.

Using a collection of strains from the southeastern United States including strains isolated before the commercial introduction of the *Bs2* R gene, phenotypic and genotypic characteristics, including frequency of mutation types in *avrBs2* and aggressiveness under field conditions were evaluated. Ten types of mutations have been detected in *avrBs2*. These are the complete deletion of the *avrBs2* locus, a 5-bp insertion or deletion, a 12-bp repeat, CC to AA transversion, IS- element insertion, and four point mutations. To determine the effect of the mutation type in the *avrBs2* locus on aggressiveness, strains comprising 10 different mutations in the *avrBs2* locus were inoculated on Early Calwonder (ECW, carries no major resistance genes) pepper plants at the Sandhills Research Station, Jackson Springs, North Carolina. Fifteen strains each of the four races, 1, 3, 4 and 6 were inoculated on ECW pepper plants in field plots and repeated over three locations. Disease severity ratings were recorded weekly and used to evaluate strain aggressiveness in the field.

Strains of races that contained the functional *avrBs2* gene (races 1 and 3) were collectively more aggressive than strains of races containing a nonfunctional *avrBs2* gene (races 4 and 6). However, particular strains of each race caused severe disease in the field and variation in aggressiveness among strains within each race occurred. The type of mutation in the *avrBs2* gene also was correlated with pathogen aggressiveness. Strains with a 5-bp insertion and a 5-bp deletion were significantly less aggressive than strains with point mutations. Mutations in *avrBs2*, within strains of races 4 and 6 that preserve aggressiveness, were more prevalent in the sample collection than those that did not. These results show that in the arms race between the R gene, *Bs2* and *avrBs2* gene, the pathogen is able to accumulate mutations that preserve aggressiveness while evading host recognition.

INTRODUCTION

Bacterial spot on pepper (C*apsicum annuum*) is caused by *Xanthomonas euvesicatoria* (Jones et al. 2004), previously known as *Xanthomonas campestris* pv. vesicatoria (Dye et al. 1980) and more recently as *Xanthomonas axonopodis* pv. vesicatoria, after it was separated from *X. vesicatoria* based on amylolytic activity and other characteristics (Vauterin et al. 1995). Bacterial spot is a serious disease worldwide with host resistance as a favored component of disease management (Cook and Stall 1963). Resistance to *X. euvesicatoria* follows the gene-for-gene system with the three major resistance genes, *Bs1*, *Bs2*, and *Bs3* all having been defeated by their corresponding avirulence genes *avrBs1*, *avrBs2* and *avrBs3* (Table 1). The *Bs2* gene was hypothesized to be an effective and durable R gene for bacterial spot control due to the chromosomal conservation of its cognate *avrBs2* gene across many species of *Xanthomonas* suggesting *avrBs2* serves a crucial function for the bacteria, which was shown to be necessary for full pathogen fitness and virulence on susceptible hosts (Swords et al. 1996; Minsavage et al. 1990; Kearney and Staskawicz 1990b). Deployment of the *Bs2* R gene in commercial bell peppers places a strong selection pressure for nonfunctional alleles of *avrBs2* and can lead to defeat of the *Bs2* gene.

A confounding aspect of the gene-for-gene system is the dual function that avirulence (i.e., effector) genes perform. Effector gene products enable the host plant to

recognize the pathogen, eliciting the defense system normally leading to a hypersensitive response (Goodman and Novacky 1994; Minsavage et al. 1990). In contrast, if the host plant lacks the corresponding R gene, the effector gene may suppress basal plant defenses, increasing bacterial fitness or virulence (Jakobek et al. 1993; Kearney and Staskawicz 1990b). The type III secretion system is an essential component for pathogenicity in many gram-negative bacterial pathogens and is responsible for translocating effector proteins into the plant cytoplasm (Cornelis and van Gijsegem 2000). Effector gene mutations result in failure of recognition by a previously resistant host through either prevention of effector product translocation or modified protein structure, but at a supposed cost to the pathogen (Alfano and Collmer 2004). A lack of fitness was demonstrated in *X. euvesicatoria* strains with a mutated *avrBs2* gene (Swords et al. 1996). Yet races that overcome the *Bs2* gene in commercial field settings have caused severe yield loss (Pernezny and Collins 1999). Field experiments confirmed that races defeating the *Bs2* gene retain the ability to cause severe disease (Kousik and Ritchie 1996b). It was reported that in response to continuous *Bs2* selection pressure, the dual function of the avirulence gene was uncoupled (Gassmann et al. 2000). *X. euvesicatoria* strains were found with a mutation in the *avrBs2* gene that prevented recognition by the host without an apparent decrease in pathogen aggressiveness (Gassman et al. 2000). These mutated avrBs2 alleles theoretically are responsible for the shift in races from those retaining functional avrBs2 genes (races 1, 2, and 3) to those possessing nonfunctional mutated avrBs2 alleles (races 4, 5, and 6).

Previous research done in the Xanthomonas-pepper system to examine affects on pathogen aggressiveness has had a key component missing. First, while effects of *avrBs2* were examined in the field, the mutations used to analyze gene function were laboratory derived and completely knocked out gene function as no transcript was detected (Wichmann and Bergelson 2004). Second, mutations in field-derived strains were examined, but were only evaluated for aggressiveness under greenhouse conditions (Gassmann et al. 2000). Yet previous research indicated environmental conditions have an effect on pathogen aggressiveness (Wangsomboondee 2002; Kousik and Ritchie 1996b). The research presented here was conducted under field conditions using strains possessing mutations

selected under field conditions from research plots and pepper production fields since the late 1980s.

The results presented here will help separate the dual role of effector genes by examining different types of mutations in *avrBs2,* which may enhance or retain pathogen aggressiveness while avoiding host recognition. The dual role of effector genes needs to be better understood if major resistance genes are to be successfully deployed as the targeting of specific effector gene is no longer a dependable solution. Laboratory-derived knockouts are helpful for deducing gene function, but mutations selected for and evaluated under field conditions are more appropriate for gaining a better understanding of the evolution of type III effectors in *X. euvesicatoria* to help reveal the roles of these proteins in pathogenicity.

Variations or mutations in effector genes retained across pathovars have been shown to be important determinants of host specificity (Stevens et al. 1998). Examination of the *avrBs2* gene showed a single-open-reading-frame (ORF1) within the *avrBs2* locus is required both for the production of the effector product that is recognized by the *Bs2* gene for resistance and for full pathogen fitness (Swords et al. 1996). Genome analysis also revealed nine classes of mutations that defeat the *Bs2* gene with all the mutations potentially affecting protein coding (Fig. 1A) (Swords et al. 1996; Gassmann et al. 2000; Wichmann et al. 2004; Wichmann et al. 2005). Most of the mutations lie in the region of the *avrBs2* locus that is crucial for AvrBs2 effector domain recognition by *Bs2* gene product (i.e. crucial for HR development) but not for effector protein translocation (Mudgett et al. 2000). In response to continuous *Bs2* selection pressure, it was shown that the dual function of the effector gene could be uncoupled (Gassmann et al. 2000). *Xanthomonas euvesicatoria* strains with mutations in the *avrBs2* gene were found that prevented recognition by the host carrying the corresponding R gene without an apparent decrease in pathogen fitness (Gassmann, et al. 2000; Wichmann et al. 2005; Kousik and Ritchie 1998). This also was found to be associated with two other Avr genes, *avrRpt2* and *avrPto*, of *Pseudomonas syringae* pv. tomato (Lim and Kunkel 2004; Shan et al. 2000).

Under an "arms race" model, the pathogen would be expected to conserve mutations that retain virulence function while avoiding detection by the host. Most of the mutant alleles of *avrBs2* examined did not support an arms race model, i.e. virulence was not

maintained on susceptible hosts (Wichmann et al. 2005; Gassmann et al. 2000). However, one of the mutations, a G to A transition, did support the maintenance of virulence under the selection of *Bs2* while avoiding detection (Wichmann et al. 2005). Here, we address concerns that *Xanthomonas euvesicatoria* is overcoming host R gene (*Bs2*) recognition and retaining the effector (*avrBs2*) virulence function to determine if there is a cost to the pathogen in terms of aggressiveness.

Using strains of *X. euvesicatoria* collected prior to and spanning the deployment of the *Bs2* gene, this research examined the possibility of race shifts occurring throughout the *X. euvesicatoria* population in response to *Bs2* pressure and if certain effector gene mutations, which result in race shifts, are being conserved in the population. Strains with *avrBs2* mutations that were field derived under selection pressure of *Bs2* were examined for their effect on *X. euvesicatoria* aggressiveness on a susceptible host. Aggressiveness is a component of pathogen virulence that is defined here as the degree of disease severity caused by the pathogen as measured by area under disease progress curve (AUDPC).

MATERIALS AND METHODS

Bacterial strains used and race identification

Bacterial strains of *X. euvesicatoria* were cultured on sucrose peptone agar (SPA) medium (20 g sucrose, 5 g peptone, 0.5 g K_2HPO_4, .25 g $MgSO_4$, and 15 g agar in 1 liter H_2O) at 28ºC for 48 hours. These strains were collected from 1985-2006 mostly in the southeastern United States from commercial pepper production fields and research plots and stored in 20% glycerol at -80ºC. Pathogen race of the isolates was determined through infiltration of the strains into near isogenic lines of Early Calwonder (ECW) peppers, each containing a single resistance gene of *Bs1*, *Bs2* or *Bs3* (as previously described (Ritchie and Dittapongpitch 1991). Individual bacteria strains were suspended to 10^8 CFU/ml and infiltrated into the pepper leaves with a sterile, needless syringe. Plants were monitored during the following 18 to 36 hours to determine if a hypersensitive (incompatible) or susceptible (compatible) reaction occurred. Sensitivity to copper sulfate and streptomycin was tested on SPA media amended with copper (200 µg/ml) and streptomycin (100 µg/ml). Starch utilization was assayed for on nutrient agar amended with potato starch (23 g nutrient

agar and 15 g potato starch in 1.0 liter H_20). Pectolytic activity was assayed for on CVP medium, agar amended with sodium polypectate (9 ml 1 N NaOH, 6 ml, 10% $CaCl_2 \cdot 2H_2O$, 2 g $NaNO_3$, 3 g agar, 20 g sodium polypectate, 1 ml SDS, 2 ml 0.075% crystal violet in 1.0 liter H_20).

Mutation type identification

Four primer sets (Table 2) were used to perform PCR for specific mutation detection (Fig. 1). Three of the primer sets that amplify different regions of the *avrBs2* gene were used to identify a 97-bp deletion, IS-element insertion immediately after the promoter, 12-bp tandem repeat, or a 5-bp insertion or deletion (Fig. 1B). Whole cell PCR, using 1.5 µl of suspended bacterial cells in 10 µl of sterile distilled H_20, was conducted in a 12.5 µl reaction volume with cell transfer size of approximately 10^3 cfu/ml. In addition to the 1.5 µl of suspended cells, the PCR cocktail consisted of 1.25 µl 10X PCR buffer, 7.24 µl sterile distilled H_2O, 0.375 µl 10uM $MgCl_2$, 0.25 µl 2mM dNTPs, 0.25 µl 10uM forward primer (Table 2), 0.25µl 10mM reverse primer (Table 2) 0.5 µl 100% DMSO, 0.825 µl 75% glycerol, and 0.0625 µl of 5.0 units/ul Taq polymerase. A PTC-100 Programmable Thermal Controller (MJ Research, Inc) was used to carry out thermal cycle conditions. Thermal cycling parameters were an initial denaturing at 95ºC for 5 min and 94ºC for 1 min 30 sec. Followed with denaturation at 94ºC for 1 min 10 sec, annealing at 64ºC for 1 min 10 sec, and extension at 72ºC for 2 min, 35 cycles were used. Final extension was at 72ºC for 5 min. Amplicons were run on a 4% agarose gel (Metaphor agarose; MC BioProducts Rockland, ME) for conformation of the 5-bp mutation, otherwise a 1.8% agarose gel was used. Gels were made with 1X TAE (0.04 M Tris-acetate, 0.0001 M EDTA, pH 8.0 in 1 L H_20) and stained afterwards with ethidium bromide.

Primer set 1 amplified a region which identified the 97-bp deletion and IS-element. Primer set 4 amplified a 183-bp product that identified the insertion or deletion of the 5-bp mutation. Primer set 5 identified the tandem 12-bp insertion. The PCR products were run on a gel and the mutation groups were confirmed using a 100-bp ladder as well as size of PCR product from strains with known mutations types. Primer set five (Table 2) was also used to amplify the region where two of the point mutations occurred for identification by use of restriction enzymes (Fig. 1A). The G to C point mutation at base pair 1386 destroys the restriction enzyme *Nae*I cut site in the *avrBs2* allele (Gassmann et al. 2000). The C to A

point mutation at base pair 1407 destroys an *Aci*I cut site. Sequencing identified all other mutation types and two additional were purified using a QIAquick PCR Purification Kit (Qiagen). Sequencing was performed at primers (Table 2) were used to obtain complete coverage of the *avrBs2* gene. PCR products the DNA Facility of the Iowa State University Office of Biotechnology on an Applied Biosystems Inc ABI 3730 DNA Analyzer utilizing Applied Biosystems Big Dye chemistries and were aligned using the program Sequencher (version 4.6, Gene Codes Corp., Ann Abor, MI).

Data organization

The bacterial isolates were randomly collected from 1985 through 2006 with no measures taken to protect against bias from location. As a result some years and locations had more samples than others. For the race frequency data (Fig. 2), each location per year was recognized as one sample. The total sample number was added and divided by the number of each race found for that year. For the mutation data (Fig. 3), each location was analyzed for number of different mutations found and only one of each mutation type was scored for that location. The mutation-type data is for strains after *Bs2* deployment as none prior to deployment were detected.

Field experiment 2005

Experiments to compare aggressiveness between strains were conducted at the Sandhills Research Station, Jackson Springs, North Carolina. The pepper plants were grown on raised beds without plasticulture. Overhead irrigation was applied weekly to maintain optimal moisture for plant growth and pathogen dissemination.

Five strains of *X. euvesicatoria*, Xcv135, Xcv310 and a mixture of cr16, cm 27, Xcv16-22 (Xcv-mix) were utilized. Early Calwonder (ECW) pepper plants which carry no major resistance genes and are susceptible to all known races of the pepper bacterial spot pathogen and ECW-20R pepper plants which carry the *Bs2* gene for resistance served as host plants. The transplants were grown in a greenhouse environment for eight weeks in 3.81 cm cell trays and transplanted April 29.

Two replications were used for this experiment, with each replication split into four blocks, one block for each strain. Each block was separated by 5 m of rye. Within each block there were 15 plants in three rows of five of both ECW and ECW-20R.

Strain selection and inoculation for 2005 field experiment

Xcv135 has a functional *avrBs2* gene, Xcv310 putatively lacks the *avrBs2* locus, and the mixture of cr16, cm27, and Xcv16-22 are field selected mutants that failed to elicit a HR on ECW-20R plants carrying the *Bs2* gene.

The two individual bacterial strains and the mixture of the three mutants were suspended to approximately 10^8 CFU/ml in sterile water. At the field site, May 11, Silwet L-77, an organosilicone surfactant, was added to make a 0.04% concentration to aid in infection. The top leaves of the center plant in each 3 by 5 block of ECW and ECW-20R were bent into 50 mls of the inoculum for 10 seconds. Gloves were changed between strain inoculations.

Weather conditions were hot and dry during most of the experiment. Although rainfall was scarce during the experiment, it occurred almost daily during the first 10 days of June. The relatively dry conditions allowed for the development of low to moderate disease.

Data collection

Weekly, from June 1 to July 6, disease severity data were recorded for each plant on a rating scale ranging from 0 (no disease) to 9 (complete defoliation) in which 0 = no lesions, 1 ≤ 10% leaf area covered with lesions, but no defoliations, 2 = 11 to 20% leaf area covered with lesions, but no defoliation, 3 = 21 to 30% area covered with lesions, one to two leaves defoliated, 4 = 31 to 40% leaf area covered with lesions and defoliation, 7 = 61 to 70% leaf area covered with lesions, few leaves remaining on plant, 9 = 91 to 100% plant covered with lesions, plant dying or dead, complete defoliation as previously described (Kousik and Ritchie 1996b).

Field experiment 2006

Experiments to compare aggressiveness between strains were conducted at the Sandhills Research Station, Jackson Springs, North Carolina. The pepper plants were grown on raised beds without plasticulture. Overhead irrigation was applied weekly to maintain optimal moisture for plant growth and pathogen dissemination. Pepper cultivar Early Calwonder (ECW) was used to evaluate pathogen aggressiveness for strains with functional and nonfunctional *avrBs2* alleles. ECW is fully susceptible to the four races of *X. euvesicatoria* used in this study.

Strain determination and selection for 2006 field experiment

Fifteen strains each of races 1, 3, 4, and 6 were chosen from the collection of *X. euvesicatoria* that spanned from 1987 to 2005, isolated from different locations mostly in the southeastern United States (Tables 3 and 4). The strains were stored in 20% glycerol at -80ºC and grown on sucrose peptone agar (SPA) for 48 hours at 28ºC before use in inoculation. Strains were selected for different mutations in the *avrBs2* gene, in the cases of race 4 and 6 (Table 4), confirmed by PCR, restriction enzymes, and sequencing as previously described. Strains from races 1 and 3 (Table 3) were selected based on growth performance on SPA media amended with copper sulfate at 200 μg/ml or streptomycin at 100 μg/ml and nutrient agar amended with potato starch consisting of 23 g nutrient agar and 15 g potato starch in one liter H_20. These races were selected with the intention of comparing aggressiveness between strains of races 1 and 4, as both have a functional *avrBs3* gene but race 4 has the nonfunctional *avrBs2* allele whereas race 1 has a functional *avrBs2* allele (Table 1). Races 3 and 6 were matched for aggressiveness comparisons, as race 3 has the functional *avrBs2* allele while race 6 lacks the functional allele (Table 1).

Plot design

A randomized, split-plot design with race as the main plot treatment and bacterial strain analyzed as the subplot was used. Each split-plot was replicated over three field sites, A, B, and C, on the research station, each approximately 0.8 km separation. Replication A had the most uniform soil being a sandy loam. Replication B was on a sandy loam soil, but placed above a geologic pan resulting in flooding during periods of rain. Replication C was slightly sloping and progressed from a sandy to gravelly type texture. Each replication was split into four blocks, one for each bacterial race 1, 3, 4, and 6. The four blocks were separated by 4.5 m partitions of rye. Each plot had fifteen subplots of six plants each, in which three alternating plants of each subplot were inoculated with a strain of that race. The other three plants remained for natural spread of the pathogen. Each subplot was 2.5 m from the adjacent. Pepper plants were transplanted on May 4. The exception for plot set-up was replication C, race 3, which lacked five strains because of missing plants. This discrepancy was accounted for during statistical analysis with the use of least square means.

Inoculation

Each of the 60 bacterial inocula was suspended to approximately 10^6 CFU/ml in sterile water. At the field site, June 5, Silwet L-77, an organosilicone surfactant, was added to a final concentration of 0.02% to aid in infection. The top leaves of three alternating ECW plants in each subplot were bent into 50 ml of the inoculum for 10 seconds. Sixteen strains of races 4 and 6 (Table 4) were reinoculated nine days later because of no disease development that could be attributed to the bacterial inocula. Gloves were changed or gloved hands washed in 70% ethanol to maintain sterility between inoculations.

Disease data

Each week (between 2-8 days), for five weeks, disease severity data were recorded for each plant using a rating scale of 0 to 9 in which 0 = no lesions, 1 ≤ 10% leaf area covered with lesions, but no defoliations, 2 = 11 to 20% leaf area covered with lesions, but no defoliation, 3 = 21 to 30% area covered with lesions, one to two leaves defoliated, 4 = 31 to 40% leaf area covered with lesions and defoliation, 7 = 61 to 70% leaf area covered with lesions, few leaves remaining on plant, 9 = 91 to 100% plant covered with lesions, plant dying or dead, complete defoliation as previously described (Kousik and Ritchie 1996b).

At the end of the experiment, bacteria were isolated from leaf lesions to confirm that the original inoculated strain was present. Two leaves were collected from each plant in a subplot with two lesions isolated from each leaf, combined and macerated in 300 µl of water. Ten µl of this suspension were plated twice on SPA media amended with cephalexin (150 µg/ml). Ten *X. euvesicatoria* colonies from the two Petri plates were selected and plated on selective media (copper sulfate, streptomycin, and starch). The colonies were infiltrated into pepper differential lines for race confirmation as previously described (Ritchie and Dittapongpitch 1991).

Data Analysis

Area under the disease progress curve (AUDPC) was calculated (Campbell and Madden 1990) using the disease severity ratings. Aggressiveness for each strain was measured using the AUDPC. All statistical analyses were performed using SAS software (version 9.1; SAS Inc., Cary, NC). An analysis of variance was preformed using PROC GLM to test the effects of replication, race, strain and mutation type. Means were separated

for strain and mutation type with least squares estimates of marginal means using the LSMEANS statement to account for unbalanced data. The Tukey-Kramer least squares means adjustment for multiple comparisons was used for comparison of AUDPC least square means for differences between individual strains within each race and between mutation type within races 4 and 6 (P=0.05).

RESULTS

Race and mutation shifts

Races 1, 2, and 3, with a functional *avrBs2* allele (Table 1) predominated strains isolated from commercial and smaller scale pepper growing operations until the mid 1990s, which coincided with deployment of the *Bs2* gene (Fig. 2). After which, the detection of races 1 and 2 became less frequent. Race 3 was mainly isolated from home gardens and small growers not necessarily growing peppers possessing the *Bs2* resistance gene.

Races 4, 5, and 6, carrying a nonfunctional *avrBs2* allele (Table 1) emerged in the mid 1990s. Races 4 and 6 increased in frequency as isolates with the functional *avrBs2* allele decreased (Fig. 2). Neither of the races containing a functional *avrBs1* allele (races 2 and 5) was predominant in this collection.

Detection of certain mutation types in the *avrBs2* gene has apparently shifted with the loss of the IS insertion and 5-bp insertion or deletions mutations and the development of others point mutations and 12-bp insertions (Fig. 3A). For both races 4 and 6 the 5-bp insertion or deletion was not detected in bacteria collected after 2000. The IS-element insertion and 97-bp deletion were also not detected after 2000. A G to C mutation was most dominant for strains of race 6 followed by the 12-bp insertion (Fig. 3C). The C to A, G to A and CC to AA mutations all were less frequently detected although the C to A, G to A in addition to the G to C were the most recently detected. For race 4, fewer mutation types were detected (Fig. 3B), five verses eight discovered in race 6. The CC to AA and G to A mutations were the most commonly detected but it was the G to A mutation that has dominated race 4 since 1998.

Overlap in mutation types was found between races 4 and 6, with the 5-bp insertion/deletion, CC to AA, and G to A mutations detected in both races (Fig. 3B and 3C). The 5-bp insertion/deletion was more prevalent in race 6 and the G to A and CC to AA was more so in race 4.

2005 Bacterial spot ratings

In 2005, AUDPC values for *X. euvesicatoria* strains Xcv310 (missing *avrBs2* locus) and Xcv135 (functional *avrBs2* locus) were similar on ECW-20R (contains *Bs2*) plants, with both displaying low AUDPC values (Fig. 4). Xcv310, which putatively lacks *avrBs2*, also was shown to be a less aggressive strain on ECW (lacks *Bs2*). Xcv135 and the mixed mutant strains all displayed high AUDPC values on ECW (Fig. 4). Only the strains with nonfunctional *avrBs2* genes (mixed field mutants), displayed high AUDPC values on ECW-20R.

2006 Bacterial spot ratings

A race by replication interaction was found for race 1 strains in replication A. This was explained by two outlying strains with significantly higher AUDPC values. There was a significant difference in AUDPC between inoculated and noninoculated plants but no interaction between inoculated plant and strain, when strain was nested within race. This allowed the data for inoculated and noninoculated plants to be combined for analysis.

Differences between AUDPCs for strains of races 1, 3, 4, and 6 were found for each replication (P=0.05) (Fig. 5). Strains of the two races containing the functional *avrBs2* allele (race 1 and 3) resulted in higher AUDPC levels than strains with a nonfunctional *avrBs2* allele (race 4 and 6). More specifically, strains of race 1 (functional *avrBs2* and *avrBs3*) were always significantly more aggressive (P<0.0001) than strains of race 4 (functional *avrBs3*) and race 3 (functional *avrBs2*) always significantly more aggressive (P<0.0001) than race 6 (nonfunctional *avrBs2* and *avrBs3*). In addition to significant variation between races, AUDPC variation also existed between individual strains within each race (P=0.05) (Fig. 6).

A considerable range of AUDPCs from strains without the functional *avrBs2* gene (race 4 and 6) was associated with the different mutations, with significant difference (P=0.05) among some mutations types (Fig. 7). Most point mutations (missense) and the 12-bp insert had similar AUDPC levels comparable to the highly aggressive race 1 and 3

strains. For race 4, the T to G (P=0.9875) point mutation was as aggressive as race 1 strains. The G to A mutation, while significantly different at the P= 0.05 level (P=0.0289), was more similar in aggressiveness to race 1 strains than to the less aggressiveness race 6 mutations such as the 5-bp insertions/deletions (Fig. 7A). For race 6, strains with C to A (P=1.000) and the 12-bp (P=0.0659) mutations were as aggressive as race 3 strains (Fig. 7B). Strains with the IS element and G to C mutation were not significantly different from each other (P=0.9999) and were considered moderately aggressive strains as both were different in AUDPC values from the strains with the functional *avrBs2* allele (P<0.0001) and more aggressive, although not significantly, than the strain putatively lacking the *avrBs2* gene (Xcv310).

In both races 4 and 6, strains with the 5-bp insertion (AUDPC least-square means 103.0 and 109.9 respectively) were consistently more aggressive than strains with the 5-bp deletion (AUDPC least-square means 87.8 and 103.9 respectively). Strains with the 5-bp insertion and deletion mutations (AUDPC =103.0 and 109.0 for race 4 and 87.8 and 103.9 for race 6 respectively) and the CC to AA mutation (AUDPC=98.4) both had similar AUDPC levels to isolate Xcv310 (AUDPC=104.4), which apparently lacks the *avrBs2* locus and was shown to consistently be a less aggressive strain in the field in both 2005 and 2006 (Fig. 4 and 5).

DISCUSSION

The ineffectiveness of deployed resistance genes as a management strategy for disease control is most likely a consequence of the flexibility of the targeted pathogen to adjust to changes in the host genotype. The durability of the *Bs2* gene for bacterial spot control was based on the conservation of *avrBs2* across *Xanthomonads* and the requirement of *avrBs2* for full pathogen fitness (Kearney and Staskawicz 1990b). Utilizing strains collected from before the widespread commercial deployment of the *Bs2* gene, an analysis of the effector gene, *avrBs2*, showed how R gene pressure on a corresponding effector gene can shift race domination in a bacterial population. Specific analysis of the effector gene, *avrBs2*, demonstrated the plethora of polymorphisms from which selection can occur to overcome

host resistance. From that allele collection, conservation of mutated *avrBs2* alleles that enabled the pathogen to retain an aggressiveness level comparable to wild-type strains were detected. The retainment of pathogen aggressiveness, while at the same time avoiding recognition from the corresponding R gene, effectively uncouples the virulence and avirulence function of the gene.

Analysis of the strains collected from 1985-2006 indicates that a genetic shift has occurred towards strains carrying a nonfunctional *avrBs2* allele. The impact of introducing the *Bs2* gene into commercial pepper cultivars as a control measure against *X. euvesicatoria* can clearly be seen in the increased frequency of races 4 and 6 (both carry a nonfunctional *avrBs2* allele), simultaneously with decreases of races 1, 2 and 3 (carry the functional *avrBs2* allele) over a 15 year period (Fig. 2). The presence of race 5 (carries functional *avrBs1* nonfunctional *avrBs2*) in our collection was only detected in two non-consecutive years. The theoretical counterpart to race 5, race 2 with functional *avrBs1* and *avrBs2*, rapidly declined since deployment of the *Bs2* gene. This suggests that it is the combination of *avrBs1* with *avrBs2* that affects aggressiveness, and that *avrBs1* on its own, may actually incur a fitness cost. Only in *avrBs2* deficient backgrounds has the loss of *avrBs1* function been shown to have an effect on bacterial multiplication *in planta* in addition to development of disease symptoms, and even then the effect of loss of *avrBs1* function is small (Wichmann and Bergelson 2004). That leaves races 1, 3, 4 and 6, which were the primary focus of our study, none of which carry a functional *avrBs1*, although some may retain the *avrBs1* locus, but in a nonfunctional form (Kousik and Ritchie 1996a).

A decline in strains of races with the functional *avrBs2* allele (races 1 and 3) was seen after deployment of *Bs2* (Fig. 2). Although still detected, strains of races 1 and 3 are predominately isolated in smaller scale pepper production (e.g. home gardens) that typically do not use the *Bs2* gene. Races 4 and 6 currently predominate in commercial fields which deploy *Bs2*. Even with a nonfunctional *avrBs2* allele, field reports and research data suggest that such strains remain a threat to pepper culture (Pernezny and Collins 1999; Kousik and Ritchie 1996b). It is interesting to note that from 1997 to 2000 a transition in race dominance was detected in the collection, as no race appeared to dominate during that time (Fig. 2).

That is also the time when the greatest number of mutation types was detected in the collection (Fig. 3).

Although the *avrBs2* allele found in races 4 and 6 is nonfunctional (in the sense that it no longer elicits a HR on plants carrying the *Bs2* gene), the gene still resides in the pathogen genome. Found instead are mutations that were presumed to knock out the virulence function as they knocked out the recognition or avirulence function (Kearney and Staskawicz 1990b). The opposite is observed in the field as bacterial races 4 and 6 have been found in commercial and experimental fields to have significant effects on crop loss (Pernezny and Collins 1999; Kousik and Ritchie 1996b). This would be predicted under an arms race model, as *X. euvesicatoria*, under *Bs2* pressure, would select for conservation of mutations that defeat host recognition while maintaining aggressiveness (Gassmann et al. 2000; Wichmann et al. 2005).

In the absence of *Bs2*, bacteria possessing functional *avrBs2* have a virulence advantage over bacteria lacking the effector gene (Wichmann and Bergelson 2004; Gassmann et al. 2000). Field analysis of race aggressiveness of strains on plants with no R gene confirmed strains of races 1 and 3 (functional *avrBs2*) were significantly more aggressive than strains of races 4 and 6 (nonfunctional *avrBs2*) as a population (Fig. 5). Collectively, strains of race 1 (functional *avrBs2* and *avrBs3*) were always significantly more aggressive than strains of race 4 (nonfunctional avrBs2 but functional *avrBs3*) (Fig. 5). Concurrently, strains of race 3 (functional *avrBs2*, nonfunctional *avrBs3*) were always more aggressive than race 6 (nonfunctional *avrBs2* and nonfunctional *avrBs3*) (Fig. 5); further supporting *avrBs2* as a gene with a dual function of virulence and avirulence. While strains of races 1 and 3 were more aggressive as a population, races 4 and 6 still caused significant disease loss in the field. Additionally, certain strains in races 4 and 6 displayed aggressiveness levels equal to or greater than some strains in races 1 and 3 (Fig. 6). This suggests that other type III effectors or compensating mutations elsewhere in the *X. euvesicatoria* genome may exist to compensate for mutations in *avrBs2* (Nomura and Yang He 2005; Roden et al. 2004). The dominance of race 6 strains over race 4 strains in aggressiveness was shown in this study and has also previously been reported (Wangsomboondee 2002). However, the dominance of strains without functional *avrBs2* or

avrBs3 alleles in aggressiveness is counter to previous findings of an experiment utilizing subsequent knockouts of Avr genes in one strain of *X. euvesicatoria* to deduce the role of effectors in pathogenicity (Wichmann and Bergelson 2004). Our field results show race 6 strains (nonfunctional *avrBs2* and *avrBs3*) are more aggressive then strains of race 4 (nonfunctional *avrBs2* and functional *avrBs3*).The conflict in results underline the need for use of both naturally derived mutations in conjunction with those derived artificially in order to uncover the role of effectors in addition to potential interaction effects between effectors. Our field results suggest that retention of functional *avrBs3* (races 1 and 4) has a negative effect on pathogen aggressiveness, or poses some fitness cost to the pathogen

After determining that strains of races 4 and 6 were of more recent origin compared to strains of races 1 and 3 in the collection, the *avrBs2* gene was analyzed by restriction enzyme analysis, PCR, and sequencing to detect mutations that may allow *avrBs2* to lose host recognition (Fig. 1). The 5-bp insertion/deletion mutations, which result in a frameshift, had been the most prevalent mutations in a study analyzing strains up until 2000 (Wichmann et al. 2005), but were no longer detected in the strains analyzed after 2000, along with the IS insertion and 97-bp deletion (Fig 3). The point mutations and 12-bp insertion dominate *avrBs2* mutants in the collection (Fig. 3). The 5-bp insertion/deletion was similar to other prokaryotic genomic variation related to polymerase slippage during replication of microsatellites (Moxon and Wills 1999; Ellegren 2000) and has been reported to have occurred independently in several different *X. euvesicatoria* strains (Wichmann et al. 2005). The IS element is located directly after the promoter region of *avrBs2* and the 97-bp deletion occurs within the first 28 codons that are required for type III secretion *in vitro*, with the first 58 codons required for *in planta* translocation (Mudgett et al. 2000). The effector domain of AvrBs2 that is required to initiate *Bs2*-specific disease resistance-mediated cell death in plants was mapped between codons 67 and 497 of the mature polypeptide (Mudgett et al. 2000). All strains of *X. euvesicatoria* defeating *Bs2* plant resistance, minus the IS element and 97–bp deletion, possess molecular lesions within codons 67 and 497 of the *avrBs2* gene, which is the region with the highest homology to ACS and UgpQ, proteins that function in synthesis and hydrolysis of phosphodiester linkages (Mudgett et al. 2000; Gassman et al. 2000; Swords et al. 1996). Additionally, all mutations found lead to amino acid changes in

the predicted AvrBs2 protein (Fig. 1A). Under an arms race model, with the dominant presence of races 4 and 6 in areas of *Bs2* deployment, in addition to conservation of specific mutations and subsequent loss of others, it would follow that certain mutations are being selected that retain some fitness component of the Avr gene while performing the essential function of defeating host resistance (Gassmann et al. 2000; Wichmann et al. 2005).

The strains were analyzed for aggressiveness based on disease severity ratings from infected ECW peppers plants that lack major dominant resistance genes. A susceptible host was used to prevent any placement of genetic pressure on the effector genes, allowing for analysis of only pathogen aggressiveness equally over all four races. However, the possibility cannot be ignored that other genetic interactions are occurring between the plant and pathogen genomes that might have some resistance effects. The strains used in this study were not isogenic derivatives of one bacterial clone, but were purposely chosen for the effector gene, *avrBs2*, that was rendered nonfunctional under host selection pressure in the field (Table 3 and 4). In addition, care was taken to select strains over a 10 year period, coming from different locations, and with different phenotypic characteristics. This was done to ensure that we were not selecting from one bacterial population and to confirm that it is the mutation in the *avrBs2* allele that influences pathogen aggressiveness, potentially overriding other genomic variations. Even though the strains used in this experiment were not isogenicly derived, previous work has shown a lack of genetic variation through comparison of chromosomal loci and use of genomic fingerprinting within strains of *X. euvesicatoria* worldwide (Wichmann et al. 2005; Louws et al. 1995). The high homology between genomes is only found within stains of *X. euvesicatoria* and is not characteristic of other *Xanthomonads* (Louws et al. 1995).

An arms race hypothesis was supported with the field study of *avrBs2* mutation effects on pathogen aggressiveness that previously was suggested from other laboratory studies (Gassmann et al. 2000). The data show certain types of mutations in the *avrBs2* locus may produce an effector product that allows the pathogen to retain aggressiveness. When the aggressiveness of mutated strains was compared to strains with functional *avrBs2* alleles, most strains with point mutations (all produce missense mutations) and the 12-bp insertions were not significantly different from their functional *avrBs2* counterparts (Fig. 7).

Additionally, these are the mutations that were most recently found in races 4 and 6 in strains from the field (Fig. 3). The mutations in both races 4 and 6 that associated with least aggressiveness, the 5-bp insertion and deletions, were not found after 2000, when the point mutations became most prevalent. Strain Xcv310, which apparently lacks the *avrBs2* locus, was also one of the least aggressive strains (Fig. 4 and 7B), and was so in previous field studies (Wangsomboondee 2002). Strains with the CC to AA mutation were also less aggressive when compared to strains with functional *avrBs2*, and was only found in a few strains throughout the collection (Fig. 7A). Strains with the 97-bp deletion and IS mutation have not been recovered recently and while the strains with IS elements were significantly different in aggressiveness than strains with functional *avrBs2* gene, it was more aggressive than the 5-bp mutations and the strain lacking the *avrBs2* locus (Fig. 7B). IS476, an insertion element found in *avrBs1*, is not site specific, and depending on the regions of insertion, has variable effects on host phenotype and *in planta* (ECW-10R) multiplication of *X. euvesicatoria* strains (Kearney and Staskawicz 1990a). One of the IS476 inserts located before the coding region of *avrBs1* has been shown to elicit only a partial HR and to have higher level of bacterial multiplication than a strain with functional *avrBs1* in ECW-10R plants (Kearney and Staskawicz 1990a). Both of these IS insertions indicate that it is possible, if the insertion in is the correct region, to retain pathogen aggressiveness and fitness when the Avr function is deleted (Kearney and Staskawicz 1990a; Kearney et al. 1988). Mutations with the 5-bp insertion/deletion, missing locus and CC to AA, while not allowing the pathogen to retain its prior level of aggressiveness still allow it to cause considerable damage in the field (Fig. 7). These results suggest that some specific mutations preserve pathogen aggressiveness more than other mutations.

Although a race is defined by the functional avirulence genes carried in its genome, significant variation in aggressiveness among some strains occurred within each race (Fig. 6), suggesting other factors influence pathogen aggressiveness. For races 4 and 6, this can possibly be attributed to mutation type within the *avrBs2* gene as strains having specific mutation types were found to be more aggressive under field conditions than others. Environmental factors also play an important role, as one strain in particular, Xcv310, while documented as a weak strain in one field study (Wangsomboondee 2002) was observed as an

aggressive strain in a greenhouse study (Kousik and Ritchie unpublished data). In this study, Xcv310 was one of the weakest strains, although it caused enough damage to be a threat in a commercial setting in 2006 when ideal rain and humidity conditions prevailed.

While environmental conditions help explain the variability in aggressiveness between strains, the variation is most likely due to other genetic factors, demonstrated through analysis of mutations that knock out *avrBs2* function leading to attenuation of *X. euvesicatoria* virulence. Additionally, analysis of the complete genome of *X. euvesicatoria* has identified 20 putative type III effector genes (Thieme et al. 2005). Our field studies with Xcv310 (missing *avrBs2* locus) revealed that while it is a less aggressive strain, it still retains a level of virulence activity, suggesting compensation elsewhere in the genome (Wichmann and Bergelson 2004). This is highly likely as this was a mutation derived under field pressures. That combined with signatures of genome flexibility, in addition to other putative virulence factors, make it highly likely that the strain Xcv310 would have evolved to develop or conserve some other effector for virulence activity (Thieme et al. 2005).

avrBs2 gene transcription has been examined for some strains used in this study with mutations of a missing locus, 5-bp deletion and IS element insertion (Wangsomboondee 2002). The 5-bp mutations and the insertion element all produced detectable transcript, yet they all failed to elicit HR on *Bs2* plants (Wangsomboondee 2002), indicating that either the effector is not translated or mutated and no longer able to trigger recognition in plants carrying the corresponding resistance gene. Protein analysis has been completed on a 5-bp addition mutant (Swords et al. 1996), and showed the truncation of the AvrBs2 protein due to the reading of a stop codon, which is a result of a frameshift caused by the 5-bp mutations. All the mutations examined alter the amino acid sequence in the predicted AvrBs2 protein with enough of a protein structure alteration that the strains are not recognized in plants with the *Bs2* gene (Fig. 1A). Some functional product may still be recognized as a study showed a point mutation (C to A) inducing a partial resistant reaction (HR) on *Bs2* plants three days after inoculation although the other point mutation examined (G to C) did not (Gassmann et al. 2000). That study also found strains with the G to C mutation to be more aggressive where our study found the opposite. The likelihood of a complete, although mutated effector product, may help explain why all point mutations examined in this study were more

aggressive than the 5-bp mutations which would produce a truncated protein due to an earlier formed stop codon. In our study, strains with C to A transversions were found to be more aggressive than a strain with the G to C transition, although both evaded host detection demonstrated by lack of HR on the cognate host (Fig. 7). Our studies of aggressiveness were also performed with plants containing no R genes, whereas the study by Gassmann et al. (2000) utilized plants with the *Bs2* gene, suggesting that the G to C transition, while less aggressive on plants without corresponding R genes may avoid more plant defense reactions than the C to A transversion in plants with the cognate R gene.

Based upon the position of the majority of mutations examined, which span approximately 300 bp of the coding region for the AvrBs2 effector, the type III secretion machinery is functional and the proteins are being translocated into the host cytoplasm, but the effector site for *Bs2* recognition is disrupted sufficiently to bypass targeting from the host defense system. The predominance of those mutations recently recovered from fields where the *Bs2* resistance gene was deployed, as opposed to those of the 5-bp mutations, IS element and 97-bp deletion, have led us to conclude that *X. euvesicatoria* is adapting to R gene deployment through conservation of mutations in the *avrBs2* locus that uncouple the virulence function from that of host detection.

Mutations that result in loss of host recognition but not virulence activity have been identified in two other bacterial effector genes, *avrRpt2* and *avrPto*, of *Pseudomonas syringae* pv. tomato (Lim and Kunkel 2004; Shan et al. 2000). This suggests that if use of R gene control is to continue to be an effective disease management strategy, then the focus of deployment will have to be re-evaluated. The results presented here confirm that *X. euvesicatoria* strains are adapting to the deployment of *Bs2* R gene without or limited sacrifice of aggressiveness as specific mutations that defeat R gene resistance and allow the pathogen to maintain aggressiveness are being retained in the bacterial population. It is no longer efficient to target an avirulence gene of reported significance to the pathogen. The pathogen target must be broken down. Protein structure must be analyzed to discern what areas are more or less tolerable of mutation in order to bypass the host surveillance system. Further work should be done to analyze the protein structure of the *avrBs2* mutants that have the highest level of retained aggressiveness, as it is likely that it is in this form that virulence

function is able to be retained, and this is the structure that deployed R genes need to target for in order for R gene mediated resistance to remain durable.

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Table 1. Host-differentiated race designation for strains of *Xanthomonas euvesicatoria* that infect peppers and response elicited in lines of peppers carrying one of four resistance genes

Race	Functional avirulence gene(s)	Differential pepper lines and resistance genes				
		ECW (none)	ECW-10R *(Bs1)*	ECW-20R *(Bs2)*	ECW-30R *(Bs3)*	P1235047 *(Bs4)*
0	*avrBs1, avrBs2, avrBs3, avrBs4*	C	HR	HR	HR	HR
1	*avrBs2, avrBs3, avrBs4*	C	C	HR	HR	HR
2	*avrBs1, avrBs2*	C	HR	HR	C	C
3	*avrBs2 avrBs4*	C	C	HR	C	HR
4	*avrBs3, avrBs4*	C	C	C	HR	RH
5	*avrBs1*	C	HR	C	C	C
6	*avrBs4*	C	C	C	C	HR
7	*avrBs2, avrBs3*	C	C	HR	HR	C
8	*avrBs2*	C	C	HR	C	C
9	*avrBs3*	C	C	C	HR	C
10		C	C	C	C	C

C = compatible, disease HR= hypersensitive reaction, resistance

Table 2. Primer sets used for sequencing the *avrBs2* locus and for detecting different mutation types present in *avrBs2*

Primer	Size (bp)	Sequence 5' - 3'	Annealing site in avrBs2 locus (bp from 5' ends)	Product length (bp)
PS1F	19	GGGCAACGCGTCCAAACAA	27-45	
PS1R	22	ATCCAGATACACCGGCTCCAGT	562-541	536
PS2F	26	GCCCGCCGCGTTTTCGAGGTCATCAT	155-541	
PS2R	29	CAGGAATTGGTCAAAGCCGCCCGTGTAGT	1459-1431	1305
PS3F	25	AGGCTGCGGCGATCAAGGTCTATGC	1401-1425	
PS3R	24	CGCGCCACGGTATTTGTCGGTCAG	2272-2249	872
PS4F	31	CTACACGGGCGGCTTTGACCAATTCCTGTCC	1432-1462	
PS4R	31	AGAAGCCGCGCAAGCGCTCGTCGTTCAACAT	1607-1577	176
PS5F	20	CATCGCCGGTGTGGAGCAGA	1270-1289	
PS5R	24	TTGGTCAAAGCCGCCCGTTAGTA	1453-1430	183
PS-F	19	TCGCATCATCTTCAATCTG	650-668	
PS-R	18	CGCCCTGCTCATTGCTTC	1620-1603	970

Table 3. Characteristics of *Xanthomonas euvesicatoria* strains of races 1 and 3 used in 2006 field experiments

Race	Strain	Selective characteristics			Reaction on plants		Isolation		AUDPC
	(Xcv)	Cu	Sm	Amy	PI 235047	Tomato	Date	State	
1	13	-	+	-	HR	HR	5/1/1987	NC	108
1	33	-	+	-	S	S	4/1/1989	NC	135
1	59	+	+	-	HR	HR	8/2/1989	NC	125
1	70	+	-	-	HR	p	6/7/1990	NC	118
1	79	+	-	-	HR	S	5/30/1991	NC	129
1	105	-	-	-	S	HR	6/11/1992	NC	167
1	121	+	-	-	HR	S	8/26/1992	VA	129
1	146	+	-	-	HR	HR	6/16/1993	IL	115
1	163	+	-	-	HR	S	4/22/1994	FL	132
1	176	-	-	-	S	S	7/21/1994	NC	146
1	282	-	-	-	S	S	5/31/1996	GA	154
1	343	+	-	-	HR	S	9/2/1997	FL	119
1	414	-	-	-	HR	HR	7/8/1998	NC	121
1	443	-	-	-	S	S	10/28/1998	AZ	122
1	561	-	-	-	S	HR	1/19/2001	FL	141
3	61	+	+	-	S	HR	9/15/1989	NC	146
3	65	-	-	-	S	HR	5/17/1990	NC	154
3	96	-	-	-	S	S	5/27/1992	VA	135
3	135	-	-	+	S	HR	4/15/1993	NC	158
3	178	-	-	+	S	S	8/25/1994	OH	178
3	220	+	+	-	S	HR	3/13/1995	FL	133
3	273	-	-	+	S	S	5/1/1996	NC	167
3	325	-	-	+	S	S	5/29/1997	GA	135
3	339	-	-	-	HR	S	7/8/1997	NC	148
3	449	+	+	-	S	HR	11/4/1998	NJ	154
3	468	+	+	-	S	HR	1/7/1999	FL	74
3	499	-	-	-	S	S	8/31/1999	OH	100
3	545	-	-	-	HR	HR	9/15/2000	KY	110
3	567	-	-	-	S	S	6/11/2001	SC	129
3	606	-	-	-	S	S	4/19/2004	NC	131

Cu= copper sulfate, Sm = streptomycin, and Amy = amylolytic activity

+ = resistant or amy activity, - = sensitive or no amy activity

HR = Hypersensitive reaction; incompatible, S = Susceptible; compatible

Table 4. Characteristics of *Xanthomonas euvesicatoria* strains of races 4 and 6 used in 2006 field experiment

Race	Strain	Selective characteristics			Reaction on plants		Isolation		*avrBs2*	Reinoculation	AUDPC
	(Xcv)	Cu	Sm	Amy	PI 235047	Tomato	Date	State			
4	181	+	-	-	HR	HR	9/2/1994	NC	5-bp del	x	93
4	183	+	-	-	HR	HR	9/2/1994	NC	5-bp add	x	89
4	316	-	-	-	S	S	8/1/1996	KY	5-bp del	x	72
4	317	-	-	-	S	S	8/1/1996	KY	5-bp add	x	94
4	319	-	-	-	S	S	8/1/1996	KY	5-bp add		120
4	322	-	-	-	S	S	9/24/1996	KY	5-bp del	x	97
4	380	+	-	-	HR	HR	8/1/1995	NC	T->G		126
4	381	?	-	-	S	S	8/1/1995	FL	5-bp del	x	90
4	395	+	-	-	S	HR	8/1/1995	NC	5-bp add	x	92
4	400	+	-	-	HR	HR	8/1/1995	NC	5-bp add		119
4	451	+	-	-	S	HR	11/19/1998	FL	G->A	x	98
4	519	+	+	-	S	S	12/23/1999	FL	CC->AA	x	100
4	551	+	+	-	S	S	11/6/2000	FL	CC->AA	x	97
4	595	+	+	-	S	HR	6/27/2003	FL	G->A	x	110
4	611	+	-	-	S	S	6/25/2004	NC	G->A		158
6	200	+	-	-	HR	S	12/21/1994	NC	5-bp del	x	96
6	260	-	-	+	S	S	11/1/1995	FL	5-bp ins		139
6	310	-	-	-	HR	S	7/1/1995	OH	missing	x	104
6	329	+	+	-	S	S	6/12/1997	FL	12-bp add		139
6	369	+	+	-	S	S	3/6/1998	FL	G->C		130
6	376	-	-	+	HR	HR	8/1/1995	NC	IS 1646	x	119
6	399	-	-	-	S	S	8/1/1995	NC	5-bp ins	x	97
6	406	-	-	+	HR	S	8/10/1998	NC	IS1646?		118
6	459	+	+	-	S	S	11/11/1998	FL	5-bp ins		94
6	487	+	+	-	S	S	1/20/1999	FL	G->C		127
6	512	+	+	-	S	S	11/17/1999	FL	12-bp add		132
6	546	-	-	-	S	S	9/15/2000	KY	5-bp del		112
6	562	+	+	-	S	S	1/30/2001	FL	G->C		92
6	575	+	+	-	S	S	2/11/2003	FL	12-bp add	x	114
6	612	+	-	-	S	S	7/26/2004	NC	C->A		138

Cu= copper sulfate, Sm = streptomycin, and Amy = amylolytic activity

+ = resistant or amy activity, - = sensitive or no amy activity

HR = Hypersensitive reaction; incompatible, S = Susceptible; compatible

A Classes of Mutations Detected in *avrBs2* Locus

	Mutation	Positions(s)	Effect	Detection Method
*	CGCGC deletion	1522-1526	Frameshift	Gel sizing
*	CGCGC insertion	1522-1526	Frameshift	Gel sizing
**	12-bp tandem insertion	1305	Frameshift	Gel sizing
**	97-bp deletion	218-314		Gel sizing
**	CC->AA transversion	1165-1166	Asp, Pro-> Glu, Thr	Sequencing
**	G->A transition	1100	Glu->Lys	Sequencing
***	G->C transversion	1386	Ary->Pro	Nae I restriction enzyme
***	C->A transversion	1407	Ala->Glu	Aci I restriction enzyme
****	T->G transversion	1335	Leu->Arg	Sequencing
**	IS insertion	113		Gel sizing

* Swords et al. 1996
** Wichmann et al. 2005
*** Gassmann et al. 2000
**** Found from this research
Numbering of nucleotides is according to the work of Swords et al. 1996

B

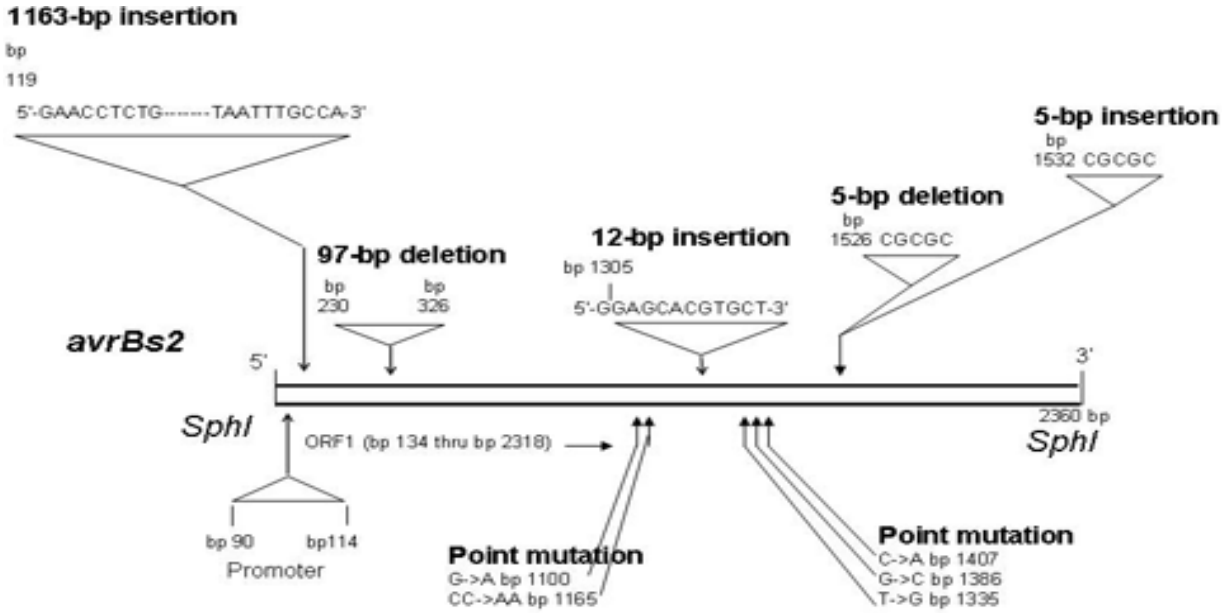

Figure 1. **A:** Changes in the *avrBs2* locus which abolish the hypersensitive reaction in pepper carrying resistance gene *Bs2* and their genomic effects. **B:** Location of mutations in the *avrBs2* locus that abolish the hypersensitive reaction in pepper carrying *Bs2* resistance gene.

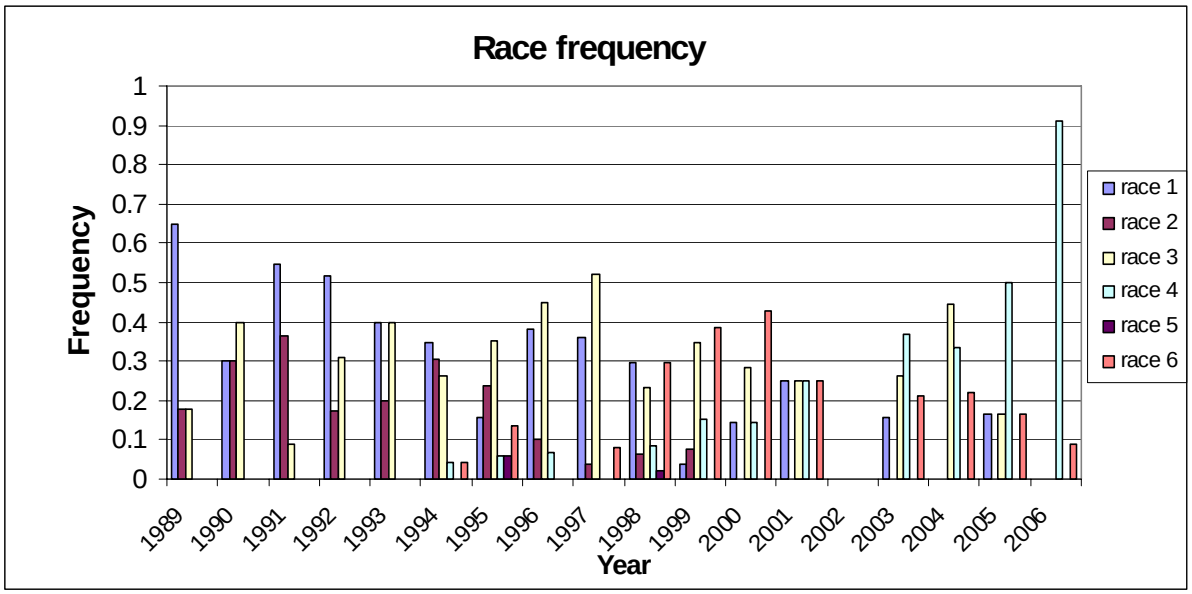

Figure 2. Change in frequency of *X. euvesicatoria* races from 1989 to 2006. Isolates were collected from the southeastern United States from commercial or research fields. *Bs2* was deployed during the mid 1990s. Frequency for each race per year was determined by using only one isolate of a race for each site. All the sample isolates of each year were totaled and divided by the number of isloates for each race.

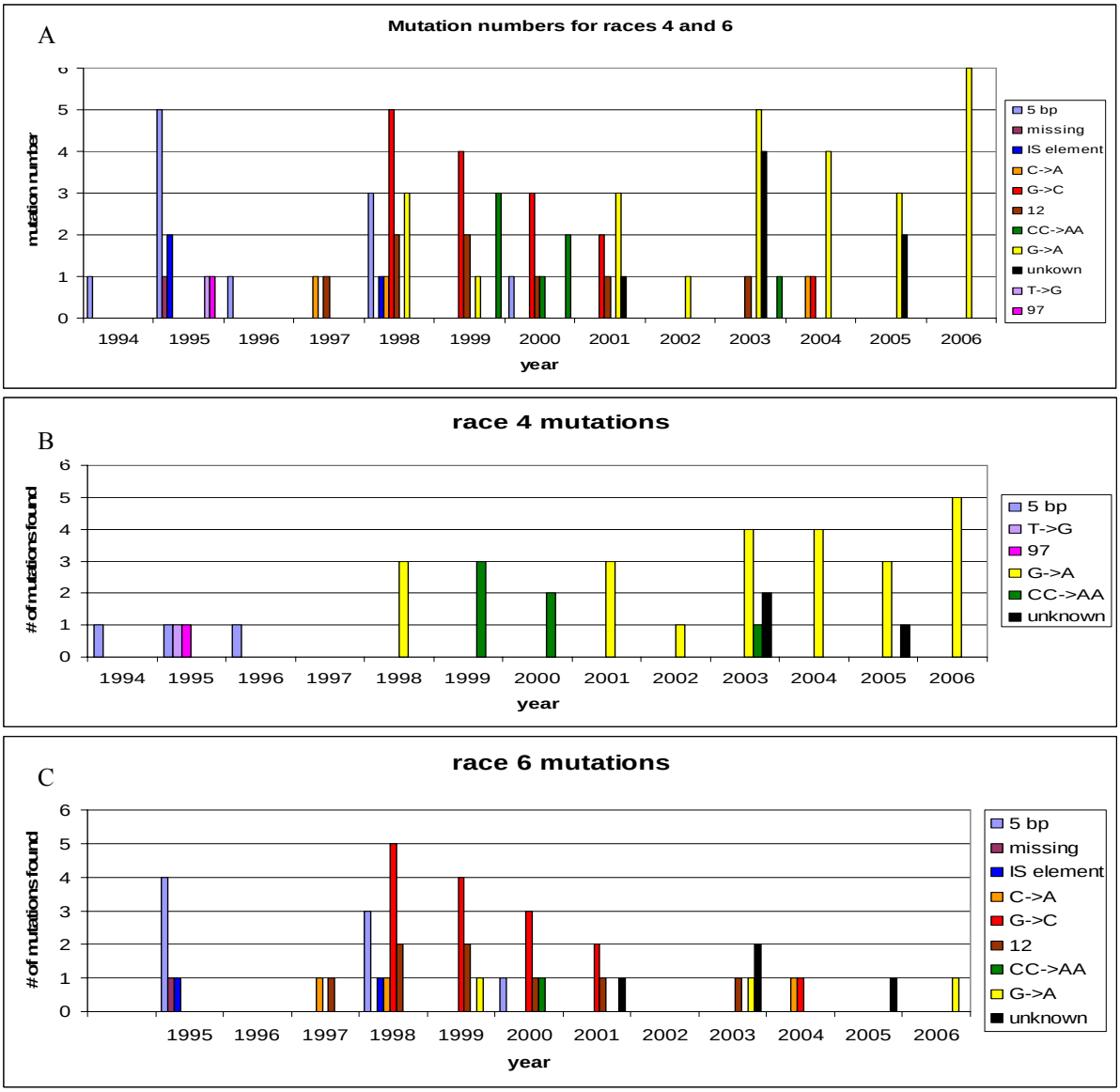

Figure 3. Change in number of *avrBs2* mutation types from 1994 to 2006. Isolates were collected from the southeastern United States from fields of commercial and research fields that deployed *Bs2*. Each bar depicts the number of occurrences of that particular mutation. Only one isolate of an individual mutation type per location was counted for each year. **A:** Total mutation counts. **B:** *avrBs2* mutation counts for race 4. **C:** *avrBs2* mutation counts for race 6.

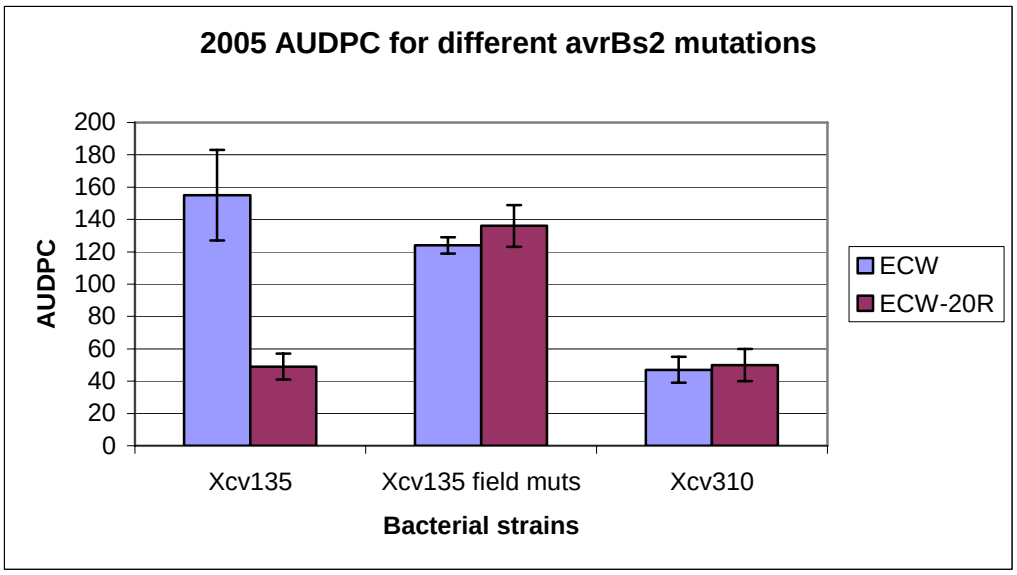

Figure 4. Area under disease progress curve (AUDPC) for bacterial aggressiveness on ECW and ECW-20R peppers from disease severity ratings during 5 weeks in 2005. Peppers were inoculated with strains Xcv135 (functional *avrBs2* gene), a mixture of three Xcv135 field derived mutants, or Xcv310 (apparently lacking *avrBs2* locus).

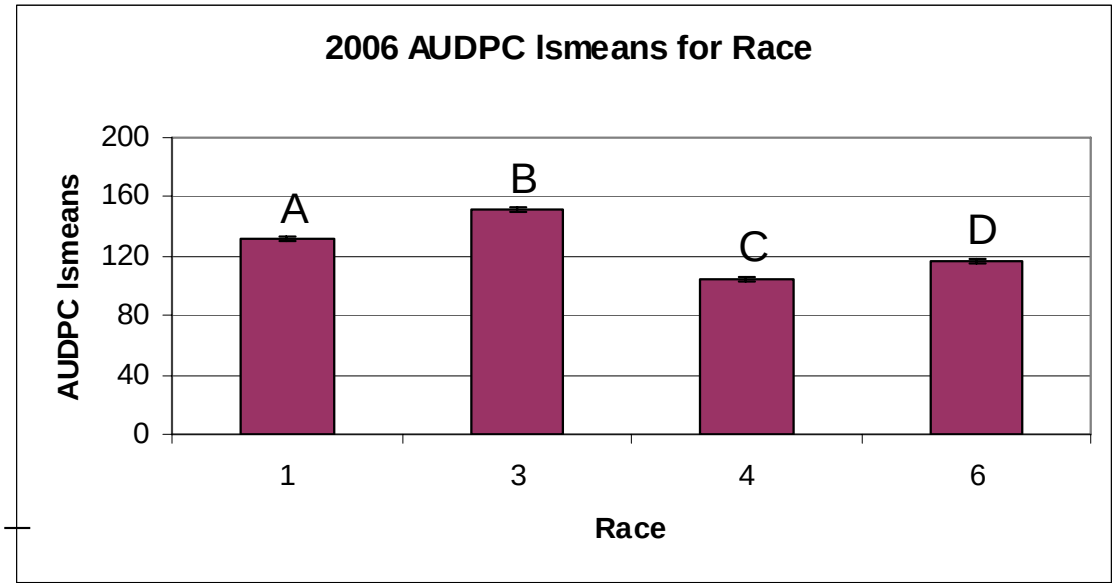

Figure 5. 2006 Area under disease progress curve (AUDPC) for bacterial spot aggressiveness on ECW peppers inoculated with strains of races 1, 3, 4, and 6 in the field. Columns shown with a different letter are significantly different (P=0.05) according to the Tukey-Kramer least-square means adjustment for multiple comparisons. Each bar represents the least-square means of data pooled from strains of a race over three replications.

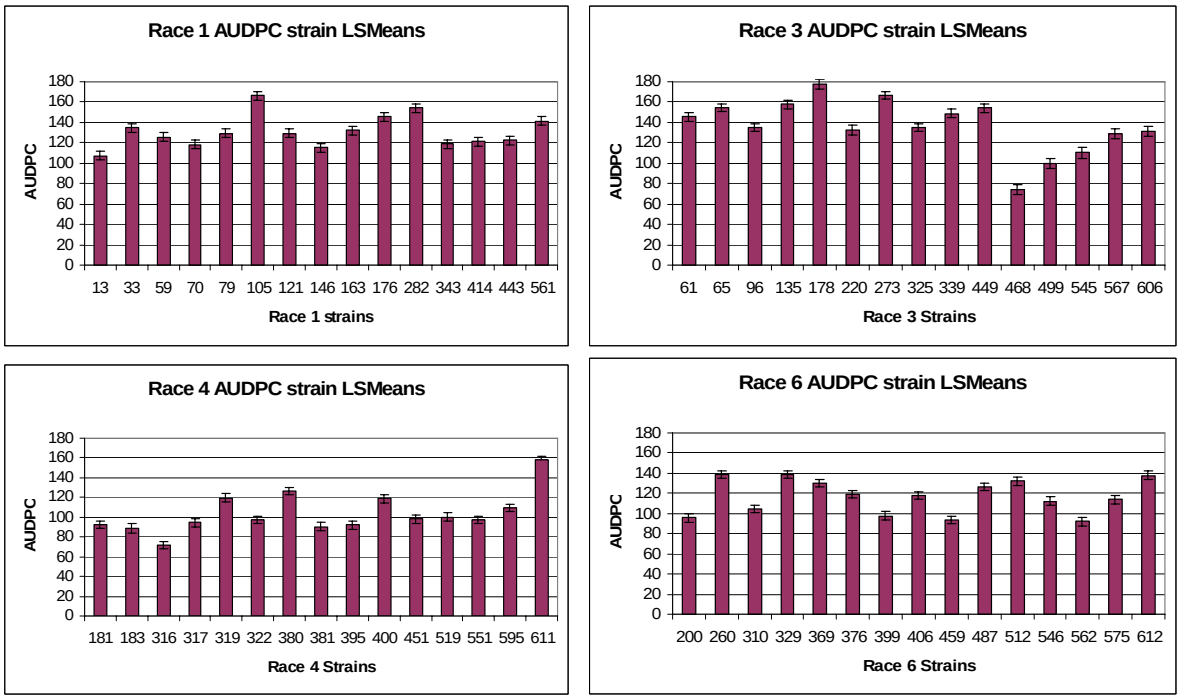

Figure 6. 2006 Area under disease progress curve (AUDPC) for bacterial aggressiveness on ECW peppers determined from biweekly disease severity ratings over 4 weeks. Three ECW peppers of each subplot were inoculated with one of 15 strains and repeated for each race (1, 3, 4, and 6). Each bar represents the least-square means of data pooled for a strain over three replications.

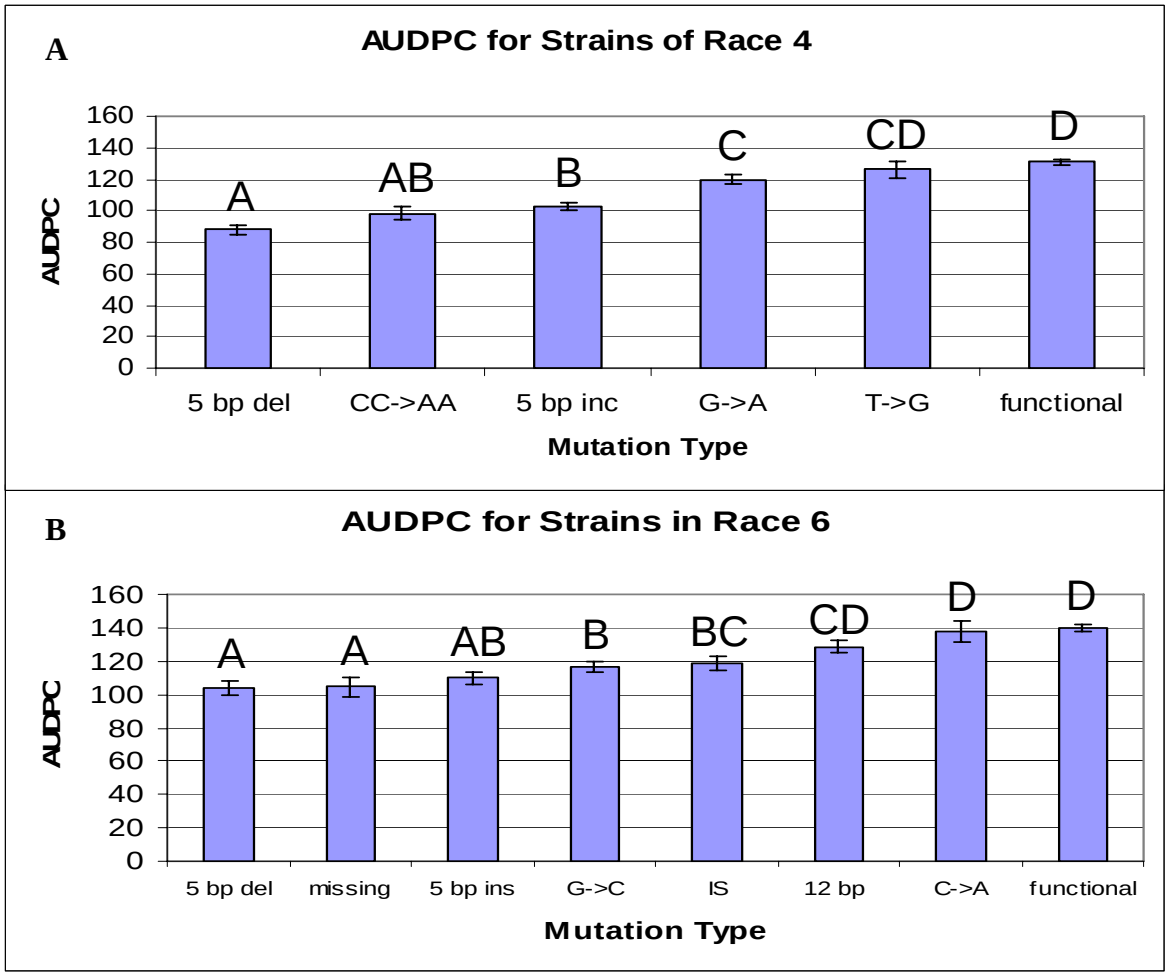

Figure 7. 2006 Area under disease progress curve (AUDPC) for bacterial aggressiveness on ECW peppers determined from biweekly disease severity ratings over 4 weeks. ECW peppers were inoculated with strains of races 4 and 6 containing different mutations in the *avrBs2* gene. **A:** Race 6 mutations (5-bp deletion, missing, 5-bp insertion, G to C, IS1646, 12-bp, C to A) were compared to strains of race 3 with non-mutated *avrBs2* genes. **B:** Race 4 mutations (5-bp deletion, CC to AA, 5-bp insertion, G to A and T to G) were compared to strains of race 1 with non-mutated *avrBs2* genes. Columns for strains of a certain mutation within a race shown with different letters are significantly different (P=0.05) according to the Tukey-Kramer least-square means adjustment for multiple comparisons. Each bar represents the least-square means of data pooled for a mutation type over three replications.