ABSTRACT

WANGSOMBOONDEE, TEERADA. Competitiveness and Aggressiveness of *Xanthomonas axonopodis* pv. *vesicatoria* with Mutations in *avrBs2* Locus. (Under the direction of Dr. David F. Ritchie.)

Strains with mutations in the *avrBs2* locus of *Xanthomonas axonopodis* (syn. *campestris*) pv. *vesicatoria*, which causes bacterial spot in pepper (*Capsicum* spp.), were detected and caused disease in pepper containing the *Bs2* resistance gene. Aggressiveness and competitiveness of the mutant strains was evaluated under laboratory and field conditions. Three strains each of races 3, 4, and 6 were separately inoculated on susceptible pepper plants (cv. Camelot) in field plots with one race/plot. Inoculum naturally spread within plots to bordering rows of pepper plants of ECW (*bs2/bs2*) or ECW-20R (*Bs2/Bs2*). A specific strain within each race predominated on either ECW or ECW-20R plants. Races 3 (functional *avrBs2*) and 6 (non-functional *avrBs2*) had similar AUDPC values, and caused similar disease severity on ECW plants. Races 4 (non-functional *avrBs2*, but functional *avrBs3*) and 6, which defeat the *Bs2* gene caused severe disease on ECW-20R, but severity was less compared to pepper plants of ECW lacking the *Bs2* gene. Frequency of recovery of strains in field plots was associated with bacterial multiplication and lesion efficiency results in the laboratory. Although, expression of the non-functional *avrBs2* gene in strains of races 4 and 6 was detected, AvrBs2 protein translation may not occur or be in an inactive form as indicated by no hypersensitive reaction (HR) and disease in plants carrying the *Bs2* resistance gene. Loss of *avrBs2*

activity apparently results in less fitness cost in race 6 than in race 4 strains; however, there is variability in competitiveness among strains within races.

Mutations in the 5-bp short-sequence repeat region of the avirulence gene *avrBs2* were detected in field strains that defeat the *Bs2* gene. The *avrBs2* has both avirulence and virulence activities. We hypothesized that if the *avrBs2* gene is required for full virulence and fitness of Xav, in the absence of selection pressure by the *Bs2* resistance gene, functional *avrBs2* should be favored. Seven strains representing races 4, 5, and 6 with 5-bp mutations (5-bp addition and 5-bp deletion) and resistant to rifampicin were tested for the potential to switch from non-functional to functional *avrBs2*. This hypothesis was tested in the laboratory using broth cultures and potted pepper plants and in a field experiment. No strains with functional *avrBs2* were detected in the laboratory experiments. Although 3 (0.15%) of 1,961 colonies from the field experiment had functional *avrBs2*, these strains did not result in a detectable population shift. This suggests that the selection pressure for functional *avrBs2* in the absence of resistance gene *Bs2* is weak or absent.

Results presents here indicate that pathogen strains with loss of avirulence gene functions (i.e., fail to elicit an HR) can be less aggressive under field conditions and that competitiveness varies among strains. However, such strains retain the ability to cause significant, unacceptable disease loss if no other controls are used. Studies have shown that aggressiveness is not the only factor contributing to pathogenic fitness. Other *avr* genes or other genes and environmental factors may contribute to overall pathogenic fitness.

COMPETITIVENESS AND AGGRESSIVENESS OF *XANTHOMONAS AXONOPODIS* PV. *VESICATORIA* WITH MUTATIONS IN *AVRBS2* LOCUS

by

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BIOGRAPHY

Teerada Wangsomboondee was born on February 9, 1969 in Bangkok, the capital of Thailand. She received primary, secondary, and high school education in Bangkok. In 1988, she began her undergraduate education in the Faculty of Agriculture, Khon Kaen University (KKU), located in northeast Thailand. After graduation, she worked as a research assistant at KKU for a few years.

In 1995, she received a scholarship to study for her M.S. and Ph.D. degrees in the United States. She was accepted in the Department of Plant Pathology, North Carolina State University in the fall 1996. In spring 1997, she began her M.S. research program under the direction of Dr. Jean B. Ristaino and graduated in summer 1999. She continued her Ph.D. program under the direction of Dr. David F. Ritchie in fall 1999 in Plant Pathology, NCSU.

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Bacterial spot caused by *Xanthomons axonopodis* (syn. *campestris*) pv. *vesicatoria* (Doigde 1920) Vauterin, Hoste, Kersters, and Swings 1995 and *Xanthomonas vesicatoria* (Doigde 1920) Vauterin, Hoste, Kersters, and Swings 1995 can cause serious economic losses of peppers (*Capsicum annuum* L.) and tomatoes (*Lycopersicum esculentum* Miller), especially in warm and humid environments (45). Necrotic spots on leaves, stems, peduncles, and fruits are distinctive symptoms of the disease (15). In favorable environments, bacterial spot may cause severe defoliation of the plants resulting in reduced yield and fruit quality.

These bacterial spot pathogens can be placed into three groups based on host range. The tomato group (XcvT) is only pathogenic on tomato, the pepper group (XcvP) is only pathogenic on pepper, and the tomato-pepper group (XcvTP) is pathogenic on both tomato and pepper (30). In addition, race-cultivar specificity has been used to differentiate the pathogens into several races including three tomato races (T1-3) and 11 pepper races (P0-10) (19,22,30,36,42). Strains have been divided into four distinct genomic groups, designated A, B, C, and D representing three species (17). The two groups, A and B were differentiated based on amylolytic and pectolytic activity, expression of unique protein bands, serology, DNA restriction profiles, and DNA homology (5,6,46). Reclassification among the pathovars of *X. campestris* on the basis of DNA-DNA hybridization, carbon utilization pattern (49, 50), and rep-polymerase chain reaction (rep-PCR) (29) separated the two previous *X. campestris* pv. *vesicatoria* groups into two species, *X. axonopodis* pv. *vesicatoria* (group A strains) and *X. vesicatoria* (group B strains). Group C strains may be a subspecies of the group A strains and is comprised of only tomato race T3 strains (5). Group D consists of *X. gardneri,* which is genetically distinct from A and B groups (17). Strains within groups A and B are widely distributed geographically, whereas groups C and D are very limited in distribution (17).

To date, 11 pepper races (designated 0 to 10) of *X. axonopodis* pv. *vesicatoria* (Xav) have been differentiated on the basis of the hypersensitive response (HR) in three near-isogenic lines of Early Calwonder (ECW-10R, ECW-20R, and ECW-30R) with three major resistance genes (*Bs1*, *Bs2*, and *Bs3*, respectively), and *C. pubescens* a plant

introduction (PI) 235047, which carries a major resistance gene (6,22,30,36,40,42). ECW is susceptible to all pepper races, ECW10-R is resistant to races 0, 2, and 5, ECW20-R is resistant to races 0, 1, 2, 3, 7, and 8, and ECW30-R is resistant to races 0, 1, 4, 7, and 9. PI 235047 is resistant to races 0, 1, 3, 4, and 6 (Table 1). Strains previously designated as race 6 but that defeat a major gene in PI 235047 are designated race 10 (36). Recently, two recessive genes designated *bs5* and *bs6* derived from PI 271322 and Pep13, respectively, also were identified and determined to provide non-hypersensitivity resistance to all currently known races (16).

In the early1980s, race 1 was reported to be the most common race in pepper worldwide except in Florida where race 2 was widely distributed (9). Since the 1990s, pepper race 3 has increased in the United States (17,38,41), Caribbean (17), Taiwan (12), and Korea (43). Race 4 was first identified in Australia (14) and also found in the southeastern United States (22), Barbados (18), and Florida (17). More recently, races 5 and 6 were identified in the United State (22,34,40). Races 7 and 8 were discovered within races 1 and 3 (36,42) and races 9 and 10 were discovered within races 4 and 6 (36) as a result of the recently identified resistance gene in PI 235047. Where cultivars carrying *Bs2* gene are grown, races 4 and 6 are the most commonly detected (34).

Expression of genotype-specific disease resistance is controlled by avirulence genes in the pathogen corresponding to specific resistance genes in the plant, consistent with the gene-for-gene hypothesis (10). Avirulence (*avr*) genes are defined by their ability to elicit an hypersensitive reaction (HR), and disease resistance in host plants containing the corresponding resistance (*R*) gene (26). It is believed that the products of *avr* genes serve as the signals that are recognized by the corresponding *R* gene products, and this recognition subsequently activates resistance pathways. The isolation and sequencing of plant *R* genes against a variety of pathogens has been a major advance in the analysis of plant-pathogen interactions (2,44)

One *avr* gene of interest is *avrBs2*, which responds to pepper plants carrying the cognate *Bs2* resistance gene (13,21,30). Laboratory and greenhouse experiments have shown that the *avrBs2* gene has both avirulence and virulence activities (11,21,47). It is proposed that *avrBs2* functions to increase bacterial fitness, which leads to increased

aggressiveness on susceptible pepper plants lacking *Bs2* gene, and that loss of *avrBs2* activity results in attenuation of pathogen virulence and fitness and thus reduced aggressiveness in susceptible plants. The role in virulence in the form of promoting pathogen aggressiveness or fitness has been described for other *avr* genes (28,37).

Sequencing of the *avrBs2* gene revealed that it encodes a putative protein that shares homology to agrocinopine synthase of *Agrobacterium tumefaciens* and glycerophosphoryl diester phosphodiesterase (UgpQ) of *Escherichia coli*. These polypeptides have an enzymatic role in pathogenicity (47). The AvrBs2 protein may have an enzymatic activity that helps the bacterium adapt to the host environment while providing signals that mediate early bacterium-plant recognition events (47). The *avrBs2* locus is found in the bacterial chromosome and is present in many *X. campestris* pathovars, representing a diverse host range (21). Recently, the *Bs2* resistance gene corresponding to *avrBs2* was cloned, sequenced, and shown to be a member of the nucleotide binding site-leucine-rich repeat class of resistance genes (48). Although, the resistance gene *Bs2* was originally considered to be highly durable, new strains of Xav have evolved containing non-functional *avrBs2*, which can defeat the *R* gene and cause economical disease losses in the field (23,34,40,41). A recent study showed that the *Bs2* gene also is temperature sensitive (39). Therefore, resistance in *Bs2* pepper plants infected with Xav carrying *avrBs2* can be defeated when temperatures are near to or greater than 32°C, thus resulting in disease.

The products of *avr* genes, together with other effector proteins, are transferred from the bacterial cytoplasm to the plant cytosol via a type III secretion system (7,8,27,31). The AvrBs2 protein alone is sufficient for the activation of disease resistance-mediated cell death and the secretion is type III (*hrp*) dependent (31). Interaction of the *avrBs2* and *Bs2* gene products for elicitaion of a HR was confirmed by *Agrobacterium*-mediated transient coexpression assay (48). Coinfiltration of an *A. tumefaciens* strain containing the 35S-*avrBs2* construct and a strain containing the 35S-*Bs2* construct into leaves of susceptible pepper plants resulted in a HR. Plants infiltrated with strains containing either the 35S-*avrBs2* or the 35S-*Bs2* construct alone did not exhibit any response on susceptible plants (48). Furthermore, direct biochemical evidence

by increase in cAMP levels in plants 24 hr after inoculation with Xav carrying a plasmid containing *avrBs2* fused with calmodulin-dependent adenalate cyclase domain (*avrBs2:cya*) showed that *avrBs2* is translocated into plant cytosal (7). A mutant deficient in *hrcV* did not result in increased cAMP levels in plants indicating that type III secretion system is important for *avrBs2* translocation (7).

Race shifts in populations of Xav have been shown to nullify the effectiveness of resistance genes within a single cropping season in the field (24,35). In Florida, a shift in the prevalent race 2 to race 1 occurred in the early 1990s and was thought to have resulted from introduction of race 1 strains on seeds (35). With plasmid born *avrBs1* and *avrBs3*, plasmid loss or a transposon insertion inactivating the *avr* gene results in mutation and potentially a subsequent race shift in the pathogen population (20,24,30). Mutations in *avrBs1* and *avrBs3* from races 2 and 1, respectively, shift Xav to race 3 to which only *Bs2* confers resistance. Races 4, 5, and 6 that overcome *Bs2* have been isolated from commercial pepper fields in the U.S. (22,23,34,40) and Barbados (32,33). These races were shown to cause economic losses of pepper in field research plots (23) and in commercial fields in Florida (34).

Mutations in the chromosomal-borne *avrBs2*, resulting in compatible interactions (no HR) on pepper containing *Bs2* gene, have been identified from field and laboratory strains of Xav (11,25,47). Eight classes of mutations in *avrBs2* have been detected; 1) an 1163-bp insertion element (IS1646) in the putative promoter region of *avrBs2*, 2) a 97-bp deletion between base pairs 230 and 326, 3) a 12-bp addition of GGAGCACGTGCT at bp 1305, 4) a 5-bp addition of CGCGC at bp 1526, 5) a 5-bp deletion of CGCGC at bp 1521, 6) a point mutation of G to C at bp 1386, 7) a point mutation of C to A at bp 1407, and 8) the apparent complete deletion of the *avrBs2* locus (11,25,47) (Fig.1). Loss of avirulence activity of this gene also results in growth rate reduction of the pathogen on susceptible plants lacking the *Bs2* gene (11,21). Several strains have been isolated with mutations in *avrBs2* that cause disease similar to a highly aggressive race 3 strain, which has a functional *avrBs2* (23,25). Races of Xav that overcome the *Bs2* resistance gene are routinely recovered from commercial pepper fields (23,34,40,41). These races cause disease on pepper plants with or without the *Bs2* resistance gene.

Molecular analysis of gene expression of some *avrBs2* mutants has been studied (11). The deletion of a repeat unit of 5 nucleotides (CGCGC) from a repeat region described above resulted in low AvrBs2 expression due to early truncation of the protein. This low accumulation of protein led to loss of *avrBs2* activity-inducing resistance in *Bs2* pepper plants and decreased fitness of the pathogen on susceptible plants (11). Strains with point mutations in *avrBs2* had slightly less or similar gene expression as functional *avrBs2*, with minimally reduced virulence function on susceptible plants (11).

Previous studies of *avrBs2* mutants showed that several classes of *avrBs2* mutations caused almost similar disease severity in susceptible plants (23,25). It was hypothesized that the classes of mutation may not be directly associated with aggressiveness of the mutant strains (25). Other genes may be involved in the overall aggressiveness of the bacteria or other mutations (ie, compensatory mutations) may have occurred elsewhere in the bacterial genome to compensate for the mutation in *avrBs2* locus. It has been shown that antibiotic-resistant avirulent bacteria can accumulate compensatory mutations that restore fitness and virulence without loss of the antibiotic resistance phenotypes (3,4). Changes in *avrBs2* activity resulted in decreased bacterial multiplication in plants without resistance genes (11,21); however, these strains were able to cause disease similar to the highly aggressive strains (23,25).

Host-plant resistance is an effective method for plant disease management. However, pathogen evolution due to selection pressure may result in loss of resistance. Mutations in or loss of avirulence genes in Xav allow the pathogen to evolve new, virulent races. For instant, races 4, 5, and 6 caused greater disease on plants carrying *Bs2* gene than races 0, 1, 2, and 3 (23,34). Recently, a serious epidemic of race 6 in commercial pepper fields was reported in southern Florida (34). Race 6 was also predominant in experimental plots and had potential to cause severe disease in pepper plants with or without *Bs2* resistance gene (23).

A penalty for gaining virulence or the loss of fitness in Xav and other plant pathogens from mutations in *avr* genes has been reported in laboratory studies (1,11,21,). Data for a fitness penalty of Xav under field conditions is lacking. We have addressed this question by studying aggressiveness and competitiveness of natural field mutant

strains compared with strains having functional *avrBs2* both in laboratory and field plots. Interaction between plant *R* gene products and *avr* gene products results in plant defense response, while *avrBs2* mutant strains fail to induce plant defense and thus cause disease in plants carrying *Bs2* gene. Gene expression of *avrBs2* mutant strains was also examined. The wide commercial use of pepper containing *Bs2* gene is considered to be a strong selection pressure for mutations in *avrBs2* locus. The mutations in 5-bp repeat region in *avrBs2* locus called short-sequence DNA repeats or DNA microsatellite are hypothesized to be able to switch to functional *avrBs2* in the absence of selection pressure by the *Bs2* gene if the gene is necessary for full virulence and fitness of the pathogen. This hypothesis was examined under laboratory and field conditions. Understanding of the evolutionary pathway of *avrBs2* locus may be useful as a model for studying evolution in *avr* genes of other plant pathogens.

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Table 1. Race differentiation within strains of *Xanthomonas axonopodis* pv. *vesicatoria*.

Race	Avirulence gene(s)	Differential pepper lines and resistance gene(s)				
		ECW (none)	ECW-10R (*Bs1*)	ECW-20R (*Bs2*)	ECW-30R (*Bs3*)	PI 235047 (*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	HR
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

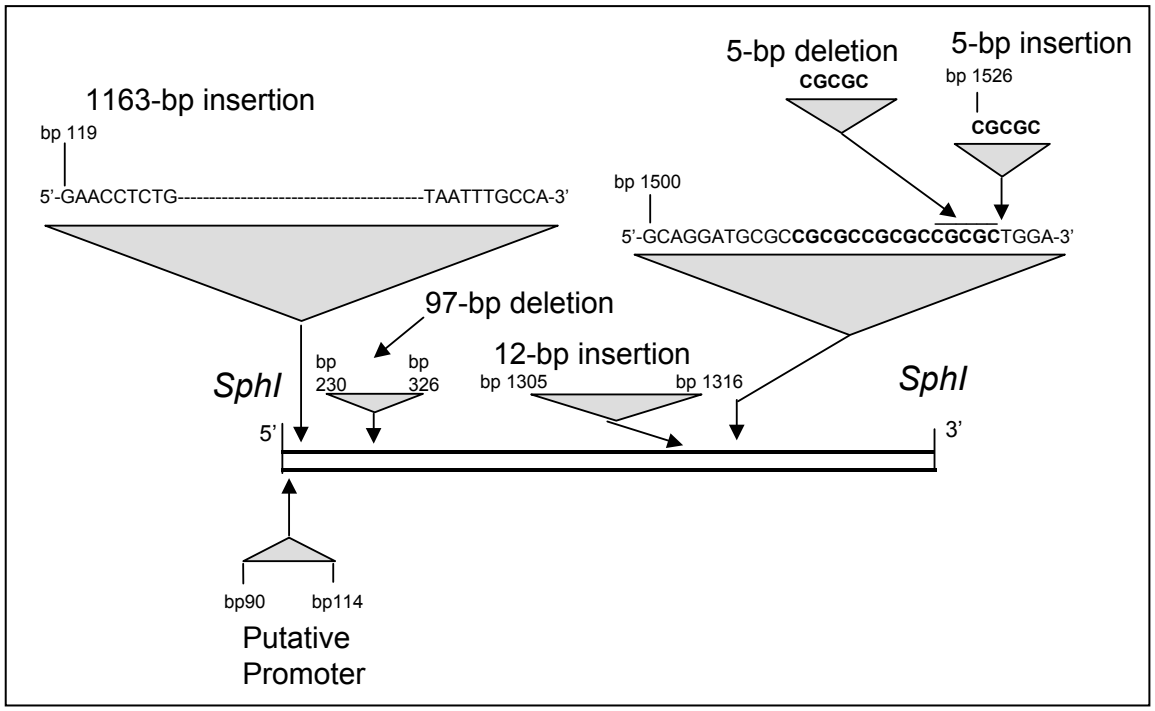

Fig 1. Mutations in the *avrBs2* locus (ORF1), which abolish the hypersensitivity reaction in pepper carrying resistance gene *Bs2*.

Chapter 1

Competitiveness and aggressiveness of *Xanthomonas axonopodis* pv. *vesicatoria* with mutations in *avrBs2* locus

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Competitiveness and aggressiveness of *Xanthomonas axonopodis* pv. *vesicatoria* with mutations in *avrBs2* locus

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ABSTRACT

Three strains each of races 3, 4, and 6 of *Xanthomonas axonopodis* (syn. *campestris*) pv. *vesicatoria* were separately inoculated on susceptible pepper plants (cv. Camelot) in field plots with one race/plot. Inoculum naturally spread within plots to bordering rows of pepper plants of ECW (*bs2/bs2*) or ECW-20R (*Bs2/Bs2*). A specific strain within each race predominated on either ECW or ECW-20R plants. Races 3 (functional *avrBs2*) and 6 (non-functional *avrBs2*) had similar AUDPC values, and caused similar disease severity on ECW plants. Races 4 (non-functional *avrBs2*, but functional *avrBs3*), and 6, which defeat the *Bs2* gene caused severe disease on ECW-20R, but severity was less compared to pepper plants of ECW lacking the *Bs2* gene. Frequency of recovery of strains in field plots was associated with bacterial multiplication and lesion efficiency tested in the laboratory. Although, expression of the non-functional *avrBs2* gene in strains of races 4 and 6 was detected, AvrBs2 protein translation may not occur as indicated by no hypersensitive reaction (HR) and disease in plants carrying the *Bs2* resistance gene. Loss of *avrBs2* activity apparently results in less fitness cost in race 6 than race 4 strains; however, there is variability in aggressiveness among strains within races.

INTRODUCTION

The plant-pathogen interaction is often characterized by a gene-for-gene relationship (5). Resistance-inducing activity of bacterial pathogens in plants is controlled by an avirulence (*avr*) gene in the pathogen and the corresponding resistance (*R*) gene in the host (16). It is hypothesized that the products of *avr* genes serve as the signals that are recognized by the corresponding *R* gene products, and this recognition subsequently

activates resistance pathways. The mechanisms by which the plant and pathogen genes interact to initiate resistance are becoming clearer as results of the isolation and characterization of several plant *R* genes and pathogen *avr* genes (1,27).

Plant pathogens can evolve to overcome host resistance by evading host recognition and subsequence responses. Bacterial spot of pepper and tomato is caused by *Xanthomonas axonopodis* (syn. *campestris*) pv. *vesicatoria* (Xav) and *X. vesicatoria* can be a major limiting factor in fruit production. There are three known independent dominant resistance genes (*Bs1*, *Bs2*, and *Bs3*) in pepper that correspond to *avr* genes in the pathogen (*avrBs1*, *avrBs2*, and *avrBs3*, respectively) (8,21). A fourth source of resistance has been identified in PI 235047 but not yet used in commercial pepper cultivars (26). Xav mutants can overcome resistance in pepper, *Capsicum annuum*, by transposon-induced mutation in the *avrBs1* gene (9) and by deletions in the repetitive region of the *avrBs3* gene that cause mutations in the *avrBs3* locus or loss of the plasmids carrying these loci (7). Similarly, mutations in the *avrBs2* locus result in both loss of *Bs2* recognition and attenuated bacterial growth in susceptible plants (6,10,28).

Laboratory and greenhouse experiments have shown that the *avrBs2* gene is a dual-function *avr* gene having both avirulence and virulence activities (6,10,28). Thus, it has been proposed that the *avrBs2* gene functions to increase bacterial fitness on susceptible pepper plants lacking the *Bs2* gene, and that loss of *avrBs2* activity results in attenuation of pathogen aggressiveness in susceptible plants (6,10,28). The role in virulence in the form of promoting pathogen aggressiveness or fitness has been described for other *avr* genes (16,20,24).

Mutations in the chromosomal-born *avrBs2* locus, resulting in a compatible interaction (i.e., no HR, disease) on pepper containing the *Bs2* gene, have been identified in strains of Xav isolated under field and laboratory conditions (6,14,28). Strains of Xav also have been isolated with mutations in *avrBs2* that cause disease similar to aggressive race 3 strains (carry the functional *avrBs2* gene) (12,14). Since the mid-1990s, races of Xav that overcome the *Bs2* resistance gene have been recovered from bacterial spot diseased pepper plants grown in commercial production fields (11,23,25). Races carrying non-functional *avrBs2* continue to cause disease severity that results in economically

important crop losses in peppers. Thus, determining the importance of the loss of fitness by the pathogen from the gaining of virulence has remained elusive under field conditions.

There are few studies for bacterial phytopathogens monitoring the natural evolution of avirulence and its corresponding relationship to pathogen fitness in field studies. Field experiments have not been done for testing the dual function of the *avrBs2* gene. For the last several years, our laboratory has been attempting to answer the question using pathogen strains that evolved and were selected for under natural conditions in commercial pepper fields. We determined competitiveness and aggressiveness between functional *avrBs2* and *avrBs2* mutant strains on pepper plants with and without the *Bs2* gene under laboratory and field conditions. Aggressiveness was measured as the degree of the disease severity caused by the pathogen as measured by area under disease progress curve (AUDPC). The ability of strains to compete with each other for dominance in the field was defined as competitiveness. The *avrBs2* gene expression in mutant strains also was examined. This study indicated the fitness penalty of some *avrBs2* mutant strains in the field experiments. The evolution of *avrBs2* gene for gaining virulence to maintain fitness on *Bs2* plants could contribute to a prediction of durability of the *Bs2* resistance gene in commercial pepper fields.

MATERAILS AND METHODS

Laboratory experiments

Bacterial multiplication: Bacterial strains (Table 1) were cultured on sucrose peptone agar (SPA) plates (20 g sucrose, 5 g peptone, 0.5 g K_2HPO_4, 0.25 g $MgSO_4$, and 15 g agar in 1 liter H_20) and bacterial suspensions (10^3 CFU/ml) were infiltrated with a needleless syringe into Early Calwonder (ECW) plant leaves. Leaves of three plants of ECW were infiltrated with each strain with each plant being a replication. Samples were collected daily from days 0-8. One section of leaf tissue infiltrated with each strain was removed from each replication using a cork borer (1 cm diameter) and placed into a 1.5 ml microcentrifuge tube containing 500 µl sterile distilled water. The leaf tissue was macerated in the tube and additional 500 µl of sterile distilled water added. Serial

dilutions were performed for each sample and 100 µl of suspension were spread on SPA plates for colony development. Colonies were counted 48-72 h later.

Lesion efficiency: To determine the ability of different strains of Xav to produce lesions, strains (Table 1) were cultured on SPA plates and bacterial suspensions (10^3 CFU/ml) were vacuum infiltrated into leaves of ECW and ECW-20R plants at the 4- to 5-leaf growth stage. Plants were incubated in a growth chamber with 12 hr of light at 24-28°C. Bacterial spot lesions were counted 14 days after infiltration using 1.0 cm^2 grid. Lesions within five 1.0-cm^2 grids per leaf and three leaves per plant were counted. Three to four plants were infiltrated with each strain and each plant served as a replication. The experiment was repeated at least twice.

***avrBs2* gene transcription:** *avrBs2* gene product is hypothesized to be an elicitor that interacts with the *Bs2* gene product in plant defense mechanism. Inactivation of *avrBs2* gene results in no HR and disease in pepper plants containing *Bs2* gene. Thus, whether or not *avrBs2* gene expression and subsequent AvrBs2 protein translation occurs in these mutant strains is questionable. To answer the question, the gene expression of Xav strains used in field plots was studied. Strains were cultured in sucrose peptone (SP) broth overnight. RNA extraction of each strain was performed following MasterPure™ Complete DNA and RNA Purification Kit's protocol (Epicentre Technologies, Madison, WI). Titan One Tube RT-PCR system (Roche Molecular Chemicals, Mannheim, Germany) was used for transcription detection. The RT-PCR reaction comprised approximately 5 µg of RNA in master mix 1; 1 µl of each forward and reverse primer, 1 µl RNase inhibitor, 4 µl dNTPs (10mM), 2.5 µl DTT solution (100 mM), and sterile distilled H_2O up to the total of 25 µl, and then added master mix 2; 14 µl sterile distilled H_2O, 10 µl 5x RT-PCR buffer, and 1 µl Enzyme mix. Two sets of primers were used in this experiment (Table 2); rpoN primers (amplify a region within transcription factor sigma 54 gene) to confirm the total mRNA transcription and PS6 primers (amplify a region within *avrBs2* locus) to detect the transcription of the *avrBs2* gene. The thermal cycling parameters were initiated for transcriptase reaction at 42°C for 30 min and denaturation at 94°C for 5 min followed by 40 cycles consisting of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 2 min. A final

extension at 72°C for 5 min followed. Amplified products were subjected to electrophoresis in 1% agarose gels containing 0.5 μg/ml ethidium bromide with 1X TBE running buffer. Translation of AvrBs2 protein was also examined in pepper ECW and ECW-20R with the expectation of an HR in ECW-20R within 24-36 hr if AvrBs2 functions properly. Plant leaves were infiltrated with suspensions of $5x10^8$ CFU/ml of each strain. Reactions on the leaves were recorded from 1-4 days after infiltration.

Field experiments

Field experiments were conducted at the Sandhills Research Station, Jackson Springs, North Carolina, to compare competitiveness and aggressiveness of strains with and without a mutation in *avrBs2* locus. Pepper plants of genotypes ECW and ECW-20R, and cultivar Camelot, were grown in the greenhouse for 7-8 weeks and transplanted as bacterial spot-free plants to the experimental field plots.

Plot design and bacterial inoculation: The experiments were conducted in a single field in the 2000-growing season, whereas two fields were utilized in 2001 each as a replication. Each field was divided into three plots separated by 10 m of rye planted the previous autumn. Each plot comprised of four subplots each of which contained two pepper genotypes, ECW and ECW-20R, and the susceptible cultivar Camelot. Each subplot was divided into two sets of three rows with five plants per row with 1.5 m apart for ECW and ECW-20R plants as shown in Fig 1. Plants within rows were 0.36 m (14 in) apart and row spacing was 1.1 m (44 in). Pathogen races were the major treatment representing a whole plot with split-split plot design. Into each plot, Xav strains of either races 3, 4, or 6 (Table 1) were introduced by inoculating alternate Camelot plants in the center row with one of three strains per race. These inoculated plants subsequently served as the initial inocula foci for each plot. The top third of Camelot plants to be inoculated was bent and submerged into a cup containing the bacterial suspension (10^8 CFU/ml) mixed with 0.02% Silwet L-77 just prior to inoculation.

Disease data and fruit yield: Data on disease development for each plant, based on a 0 (no disease lesions observed) to 9 (total defoliation) scale as previously described (12), were recorded weekly. Yield data for each plot were collected by doing a single harvest at the end of the experiment.

Sampling: Leaf samples were collected from ECW and ECW-20R plants approximately every 2 weeks. Two replications from each plot were sampled by arbitrarily excising one diseased leaf (having multiple lesions) from each plant and pooling leaves as a composite sample per cultivar per subplot. Thus, there were 10 diseased leaves per sample and four samples per plot. Samples were collected at three different times during the experiment.

Bacterial isolation and strain identification: For each sample, three bacterial spot lesions from each leave were excised, combined, and macerated in 200 µl of sterile water. Ten-microliters of this bacterial suspension were streaked on SPA plates, then 50 colonies with Xav morphology were selected. Each single colony was suspended in 30 µl sterile water and 2 µl of the suspension were spotted on plates of SPA, SPA amended with streptomycin (100 µg/ml), copper sulfate (200 µg/ml), or nutrient agar amended with 2% starch. These semi-selective media were used to differentiate strains within a race except strains Xcv314 and Xcv316 of race 4, which were differentiated using PCR primers to detect the 5-bp mutations. From the SPA plate, approximately eight representative colonies from each sample were selected and infiltrated into pepper differential lines (21) for race confirmation.

DNA templates for PCR reaction were derived by a freeze-thaw DNA extraction method. Approximately, a loop full of bacterial cells was added into 1.5 ml microcentrifuge tubes containing 200 µl sterile H_2O. Suspensions were vortexed and frozen at -80°C for 15 min, then immediately thawed at 65°C for 15 min. PCR was conducted in a 12.5 µl reaction volume. This consisted of 1.5 µl of DNA template added to an 11 µl master reaction mixture containing 1.25 µl 10X PCR buffer, 7.238 µl sterile distilled H_2O, 0.375 µl 10 mM $MgCl_2$, 0.25 µl 2 mM dNTPs, 0.25 µl 10 µM PS4F primer (Table 2), 0.25 µl 10 µM PS4R primer (Table 2), 0.5 µl 100% DMSO, 0.825 µl 75% glycerol, and 0.0625 µl Taq polymerase. The thermal cycling parameters were an initial denaturation at 80°C for 5 min and 94°C for 1 min 30 sec, followed by 25 cycles consisting of denaturation at 94°C for 1 min 10 sec, annealing at 64°C for 1 min 10 sec, and extension at 72°C for 2 min. A final extension at 72°C for 5 min followed. Amplified

products were subjected to electrophoresis using 4% agarose gels containing 0.5 μg/ml ethidium bromide with 1X TBE running buffer. A 100-base-pair (bp) DNA ladder was included in each gel as a size marker.

Statistic analyses: Lesion efficiency data of each strain was compared using mixed model of SAS software (SAS Institute, Carey, NC). Means were separated with least squares estimates of marginal means (LSMEANS statement). Disease rating was used to calculate AUDPCs as describe by Campbell and Madden (3). The mixed model with least square means was used to compare pairwise disease ratings for a single date among races. Logistic regression model was used for comparison of the estimated maximum disease rating during the epidemic phase of races 3, 4, and 6 strains. The model is nonlinear mixed model with random inflection point proportional for each plot and with within-plot variance proportional to the mean (19).

The logistic regression model is

$$y_{ij} = \frac{a}{1 + e^{b_i - ct_{ij}}} + \varepsilon_{ij}$$

y_{ij} = mean disease rating for plot i at sampling time j

i = plot (i = 1, 2)

j = sampling time (j = 1,…, 7)

t = disease rating date

a = maximum disease rating

c = controls steepness of increase

b/c = inflection point

b_i is random (different plots have different inflection points)

$\varepsilon_{ij} \sim N(0, \sigma^2 \mu_{ij})$ (variance increase with mean)

$\mu_{ij} = E(y_{ij})$

RESULTS

Bacterial multiplication: Differences in bacterial multiplication within and among races of Xav strains were observed. Strains of race 3 multiplied to the highest

population numbers compared with strains of races 4 and 6, which had similar population numbers (Fig 2). Among the strains of race 3, Xcv135 and Xcv224 had higher multiplication than strain Xcv246. Strain Xcv376 had the highest multiplication level among race 6 strains and Xcv310 had the lowest multiplication. The three strains of race 4 multiplied similarly.

Lesion efficiency: The ability to elicit lesions (Fig 3) by strains of races 3, 4, and 6 on ECW and ECW-20R plants varied. However, all strains of races 3 and 4, and Xcv376 of race 6 statistically elicited similar lesion numbers on ECW (Fig 4). Strains of race 4 and Xcv376 of race 6 also elicited similar lesion numbers on ECW-20R. ECW-20R plants infiltrated with strains of race 3 failed to elicit lesions as the result of the *Bs2* resistance gene. Xcv310 and Xcv378 of race 6 elicited fewer lesions on both ECW and ECW-20R than Xcv376 (Fig 4).

***avrBs2* gene transcription:** Xav strains used in field plots and representing races 3, 4, and 6 showed mRNA transcriptions of transcription factor sigma 54, which is a positive control and also of the *avrBs2* gene except for strain Xcv310 of race 6, which does not contain the *avrBs2* locus (Table 3, Fig 5). Production of active AvrBs2 protein was associated with an HR within 24-36 hr in ECW-20R plants containing *Bs2* gene infiltrated with race 3 strains. Strains of races 4 and 6 did not elicit an HR on ECW-20R. Instead, they elicited a compatible reaction with different levels of response (Table 3). Xcv376 and Xcv378 produced a stronger and faster compatible reaction than Xcv310 and strains of race 4.

Recovery frequency of the strains from field plots: Xcv135 was the predominant strain recovered from ECW in the race 3 plots both in 2000 and 2001 (Fig 6). Xcv135 was also isolated from suspected HR lesions (light brown tissues between leaf veins) around the edges of ECW-20R leaves. In the race 4 plots, Xcv182 predominated on both ECW and ECW-20R in 2001, whereas strain Xcv314 predominated in 2000. In both years, strain Xcv376 predominated on ECW and ECW-20R in the race 6 plots. In contrast, Xcv310 was rarely recovered from race 6 plots. The results of these recovery frequencies correlated with the multiplication rates of the bacterial strain in planta (Fig 2).

Bacterial spot ratings: In 2000, disease ratings for race 3 and 6 strains were similar on ECW and Camelot, both lack the *Bs2* resistance gene. Strains of race 3 produced the highest disease rating on ECW and Camelot in 2001 (Fig 7). As expected, ECW-20R, which carries the *Bs2* gene had the lowest disease rating for strains of race 3 in both years (Fig 7). Race 6 was associated with higher disease ratings than race 4 on both ECW and ECW-20R plants.

The logistic regression model was used to predict mean of disease rating (Fig 8). The overall disease rating during the epidemic phase of Xav in 2001 field experiment showed that strains of races 3 and 6 statistically caused similar disease severity both in ECW and Camelot plants (Table 4). Disease severity of strains of race 4 was similar to race 6 but significantly different from strains of race 3 both in ECW and Camelot plants. However, stains of races 3, 4, and 6 statistically presented similar disease severity in ECW-20R plants (Table 4).

Fruit yield: Yields of all pepper genotypes and cultivars were negatively associated with disease severity as indicated by the area under disease progress curve (AUDPC) (Fig 9). High AUDPC values correlated with low yield for Camelot inoculum plants and a very susceptible cultivar. ECW-20R plants in the race 3 plots produced the greatest fruit yields, being resistant to race 3 strains. Plants in race 4 plots had lower AUDPC values in both ECW and ECW-20R than did race 6 plots and produced greater yields than did plants in race 6 plots.

DISCUSSION

Previous studies of the *avrBs2* gene of Xav have shown both avirulence and virulence activities of the gene in which loss of *avrBs*2 activity decreases fitness of the pathogen on susceptible plants (6,10,28). Xav strains containing mutations in *avrBs2* gene (races 4 and 6) were compared with strains containing a functional *avrBs2* (race 3) under laboratory and field conditions. Isogenic strains of Xav were not used in these experiments. However, the strains used were isolated from diseased pepper plants from the field and thus represented the natural pathogen population.

In field experiments, high concentration of strains of race 3 in the plots resulted in HR (light brown tissues between leaf veins) around the edges of ECW-20R leaves. The strains of race 3 were recovered from these HR tissues thus the high AUDPC for race 3 strains on ECW-20R plants likely resulted from defoliation caused by HR. Strains of race 6 were more aggressive than races 3 and 4 on ECW-20R plants (carries *Bs2*). However, lesion efficiency of strains of race 4 was higher than Xcv310 and 378 of race 6 on ECW-20R plants in the laboratory. Differences in plant and bacterial growth conditions in laboratory and field may effect the aggressiveness of Xav. Strains of race 3 were the most aggressive race on susceptible plants, ECW and Camelot. It seems likely that loss of *avrBs2* activity affected the virulence and aggressiveness of Xav on susceptible plants as mentioned by other studies (6,10,28). Gassman *et al.* (6) showed an aggressive strain with a point mutation in *avrBs2* that had fitness advantage for Xav on *Bs2* plants. A mutant strain (Xcv376) with IS insertion in *avrBs2* locus in this experiment was very aggressive on *Bs2* plants. Under selection pressure of *Bs2* gene, this mutant strain predominated in epidemics of Xav in pepper fields. Similarly, *avrXa10* mutant strains of *X. oryzae* pv. *oryzae* showed high aggressiveness in rice plants carrying *Xa10* gene without fitness penalty (2,29). Although fitness loss occurred in strains of race 6 on susceptible hosts, they caused severe disease and significant fruit loss on *Bs2* plants in field experiments and also in commercial pepper fields in Florida (12,23).

Competitiveness of strains in field experiments was associated with lesion efficiency and bacterial multiplication demonstrated in the laboratory. Within each race, a particular strain predominated. This trend was especially pronounced in the strains of races 3 and 6 tested. Strain Xcv376 of race 6 competed better in the field, produced more lesions, and also had higher bacterial multiplication than Xcv378 and Xcv310. A race 6 strain (Xcv310) lacking *avrBs2* was rarely recovered from field plots and was less aggressive than the other two strains of race 6. Similar observations were found for strain Xcv135 of race3, which predominated in ECW field plots. This suggests the competitiveness and aggressiveness functions of the *avrBs2* gene. However within a race, which is defined by the functional avirulence gene(s) carried, competitiveness and possibly aggressiveness vary significantly, especially under field conditions. These

results are interpreted as suggesting that the cost of the gaining of virulence by this pathogen is influenced by multiple genetic and environmental factors. Interestingly, factors driving the mutation of *avr* genes other than host *R* genes have been reported in *X. oryzae* pv. *oryzae* in that virulent strains occurred before a resistance gene was introduced into the field (17). This also is the situation with race 10 strains, which defeat the resistance in PI 235047 plants although PI 235047 has not been grown in commercial pepper fields (15).

Assays of diseased peppers in plots inoculated with strains of races 3, 4, and 6 confirmed that strains of these races were associated with disease in the respective plots and that cross contamination did not occur. On susceptible ECW and Camelot, both of which lack any known major resistance genes, bacterial spot and fruit yield loss was greatest in the race 3 plots followed by the race 6 plots. Disease severity and yield loss was least in the race 4 plots. Thus, there is an association of reduced aggressiveness and decreased fruit loss for plants lacking a known major resistance gene inoculated with strains carrying a non-functional *avrBs2* gene. Although, strains of both races 4 and 6 carry non-functional *avrBs2*, race 4 strains had less AUDPC than race 6 strains in field experiments. Race 4 strains carry *avrBs3* gene, which may have residual effects in interaction among the genes in planta, if compared to race 6 stains without any functional *avr* genes.

The products of *avr* genes, together with other effector proteins, are transferred from the bacterial cytoplasm to the plant cytosol via a type III secretion system (4,18,22). The AvrBs2 protein alone is sufficient for the activation of disease resistance-mediated cell death and the secretion is type III (*hrp*) dependent (22). Transcription of the *avrBs2* gene was detected from non-functional (fail to elicit HR) *avrBs2* strains tested except for the strain not containing the gene (Xcv310). However, all mutant strains failed to elicit HR on *Bs2* plants within 24-30 hr after infiltration. This result indicates that AvrBs2 protein may not be translated or may be in an inactive form, which can not be recognized by the *Bs2* gene thus not triggering the defense responses. A mutation caused by a 5-bp addition was previously studied and it was showed that truncation of AvrBs2 protein resulted in no HR on *Bs2* plants (28). In contrast, a strain with a point mutation in *avrBs2*

was shown to induce a partial plant resistance response (HR) on *Bs2* plants three days post inoculation (6).

In this study, variation in competitiveness and aggressiveness of strains with a mutation in *avrBs2* gene were detected, however tested strains still caused severe disease in field plots indicating less fitness cost to retention of *avrBs2* function. This suggests the inefficiency of the *Bs2* gene in controlling bacterial spot in pepper fields, which was confirmed by the severe outbreak of race 6 in Florida (23). The combination of field and laboratory studies indicates that *avrBs2* evolves to decrease detection by the *Bs2* gene while at the same time maintains its aggressiveness function. Thus, the *Bs2* gene alone should not be expected to provide effective control of bacterial spot in commercial pepper fields. Even though the three *R* genes in pepper (*Bs1*, *Bs2*, and *Bs3*) have been defeated, the use of pepper cultivars containing some *R* gene combinations exhibits resistance proposed to be due to residual effects of the individual *R* genes and possibly due to the interaction among the genes (13).

Knowledge of the aggressiveness functions of *avr* genes in bacterial pathogens may be used to understand and develop durable resistance in plants (16). Further research needs to be done to thoroughly understand the evolution of Xav that compensate the mutations in *avrBs2* locus and regain fitness of the pathogen. This may include studying of secondary mutations that are hypothesized to gradually restore the fitness of the pathogen caused by a mutation in *avrBs2* gene (10). Persisting of *avrBs2* mutant strains in pepper fields under the absent of selection pressure of the *Bs2* gene or under various host genetic backgrounds is still questionable. Studies have shown that aggressiveness is not the only factor contributing to pathogenic fitness. Unknown *avr* genes or other genes and environmental factors may contribute to overall pathogenic fitness.

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Table 1. Characteristics of *Xanthomonas axonopodis* pv. *vesicatoria* strains used in field experiments.

	Strain	Selective characteristics[a]			*avrBs2* mutation	Reaction on plants[b]	
		Cu	Sm	Amy		ECW	ECW-20R
Race 3	Xcv135	S	S	+	Active *avrBs2*	C	HR
	Xcv224	R	S	-	Active *avrBs2*	C	HR
	Xcv246	S	S	-	Active *avrBs2*	C	HR
Race 4	Xcv182	R	S	-	5-bp addition	C	C
	Xcv314	S	S	-	5-bp deletion	C	C
	Xcv316	S	S	-	5-bp addition	C	C
Race 6	Xcv310	S	S	-	No *avrBs2*	C	C
	Xcv376	S	S	+	IS insertion	C	C
	Xcv378	R	S	-	5-bp deletion	C	C

[a] Cu = copper sulfate, Sm = streptomycin, and Amy = amylolytic activity
S = sensitive, R = resistant, + = amy activity, and - = no amy activity

[b] HR = Hypersensitive reaction; incompatible (resistance) C = no HR elicited; compatible (disease develops)

Table 2. Primer sequences used for detecting expression of the *avrBs2* and transcription factor sigma 54 genes and for detecting 5-bp repeat region in *avrBs2* locus.

Primer	Sequence (5′ to 3′)	Product size (bp)
PS6F	TGGACTGCAAGGAAAACACC	348 of *avrBs2*
PS6R	GCATCTTGGTCCAGCTGTC	
rpoNF	GAGCTCAAGCGTTCCACTG	397 of transcription
rpoNR	ATCTCGCGCAGCGTCAAC	factor sigma 54
PS4F	CTACAC GGGCGGCTT TGACCAATTCCTGTCC	171,176, and 181
PS4R	AGAAGCCGCGCAAGCGCTCGTCGTTCAACAT	

Table 3. Transcription of *avrBs2* gene in *Xanthomonas axonopodis* pv. *vesicatoria* strains used in field experiments and reaction on pepper ECW and ECW-20R (carries *Bs2*) leaves infiltrated with 10^8 CFU/ml of the strains.

Xav strain	RT-PCR reaction with primers		Reaction on pepper ECW-20R				Reaction on pepper ECW			
	rpoN*	PS6 (*avrBs2*)	24 hr	48 hr	72 hr	96 hr	24 hr	48 hr	72 hr	96 hr
Xcv135 (normal *avrBs2*)	Yes	Yes	HR	HR	HR	HR	-	+	++	+++
Xcv224 (normal *avrBs2*)	Yes	Yes	HR	HR	HR	HR	-	+	++	+++
Xcv182 (5-bp addition)	Yes	Yes	-	-	+	++	-	-	+	++
Xcv314 (5-bp addition)	Yes	Yes	-	-	+	++	-	-	+	++
Xcv316 (5-bp deletion)	Yes	Yes	-	-	+	++	-	-	+	++
Xcv310 (no *avrBs2*)	Yes	No	-	-	-	+	-	-	-	+
Xcv376 (IS addition)	Yes	Yes	-	+	++	+++	-	+	++	+++
Xcv378 (5-bp deletion)	Yes	Yes	-	+	++	+++	-	+	++	+++

HR = hypersensitive reaction, - = no reaction, + = level of compatible reaction

* rpoN primers were used as positive control for mRNA.

Table 4. Comparison of the estimated maximum disease rating (parameter a from model) during the epidemic phase of *Xanthomonas axonopodis* pv. *vesicatoria* in 2001 field experiment.

Comparison	Pr > | t |		
	ECW	ECW-20R	Camelot
Race 3 vs Race 4	0.0159*	0.8843	0.0254*
Race 3 vs Race 6	0.1094	0.8706	0.1306
Race 4 vs Race 6	0.1795	0.3844	0.2452

Bonferroni was used for the pairwise comparisons of values among races with $\alpha = 0.05$.
*significantly different

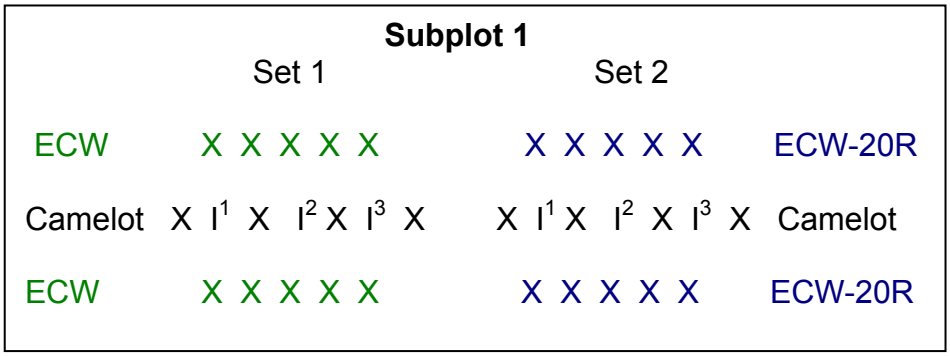

Fig 1. Planting pattern of a subplot containing two sets of plants (ECW and ECW-20R) with Camelot as the inoculum row. Xs represent a pepper plant and I^1-I^3 are plants inoculated with three different strains per race.

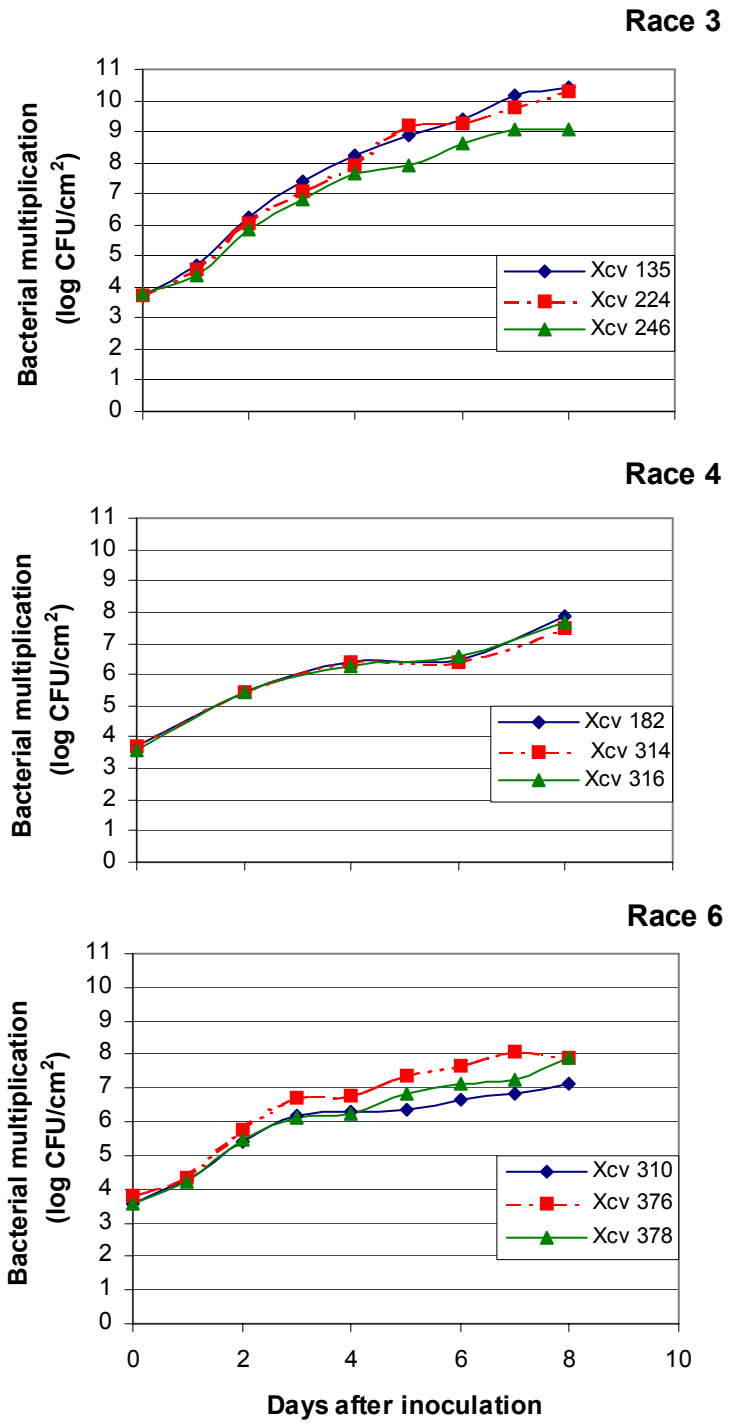

Fig 2. Bacterial multiplication in ECW leaves infiltrated with *Xanthomonas axonopodis* pv. *vesicatoria* strains of races 3, 4, and 6 and incubated in the laboratory at room temperature (24-28ºC).

Fig 3. Bacterial spot lesions on a ECW leaf vacuum infiltrated with a strain of *Xanthomonas axonopodis* pv. *vesicatoria*.

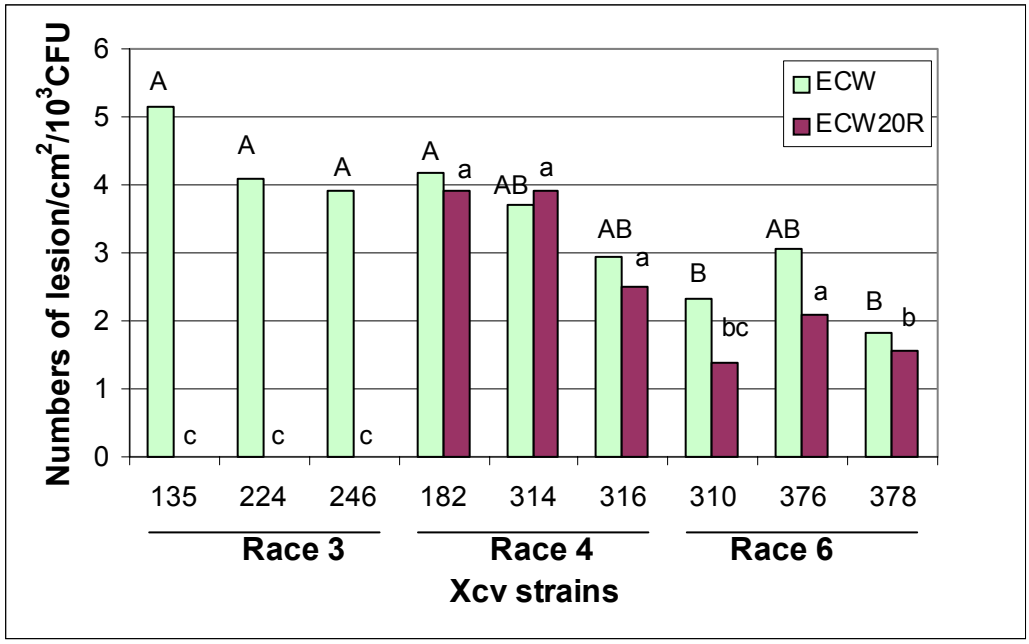

Fig 4. Lesion efficiency of *Xanthomonas axonopodis* pv. *vesicatoria* strains represented by numbers of lesion/cm^2 of leave/10^{3}CFU/ml of bacterial inoculum on two pepper genotypes ECW and ECW-20R. Means followed by the same letter of each pepper genotype are not significant based on the LSD ($\alpha = 0.05$).

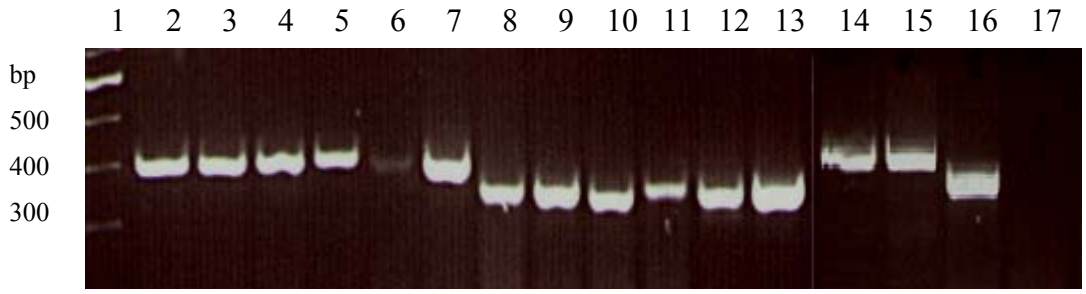

Fig 5. *avrBs2* gene expression of functional and mutant *avrBs2* strains represented by RT-PCR with two primer sets. Lane 1 is 100 bp ladder. Lanes 2-7, 14, and 15 are amplified products with rpoN primers and lanes 8-13, 16, and 17 are amplified products with PS6 primers of Xcv224, 182, 314, 316, 376, 378, 135, and 310, respectively.

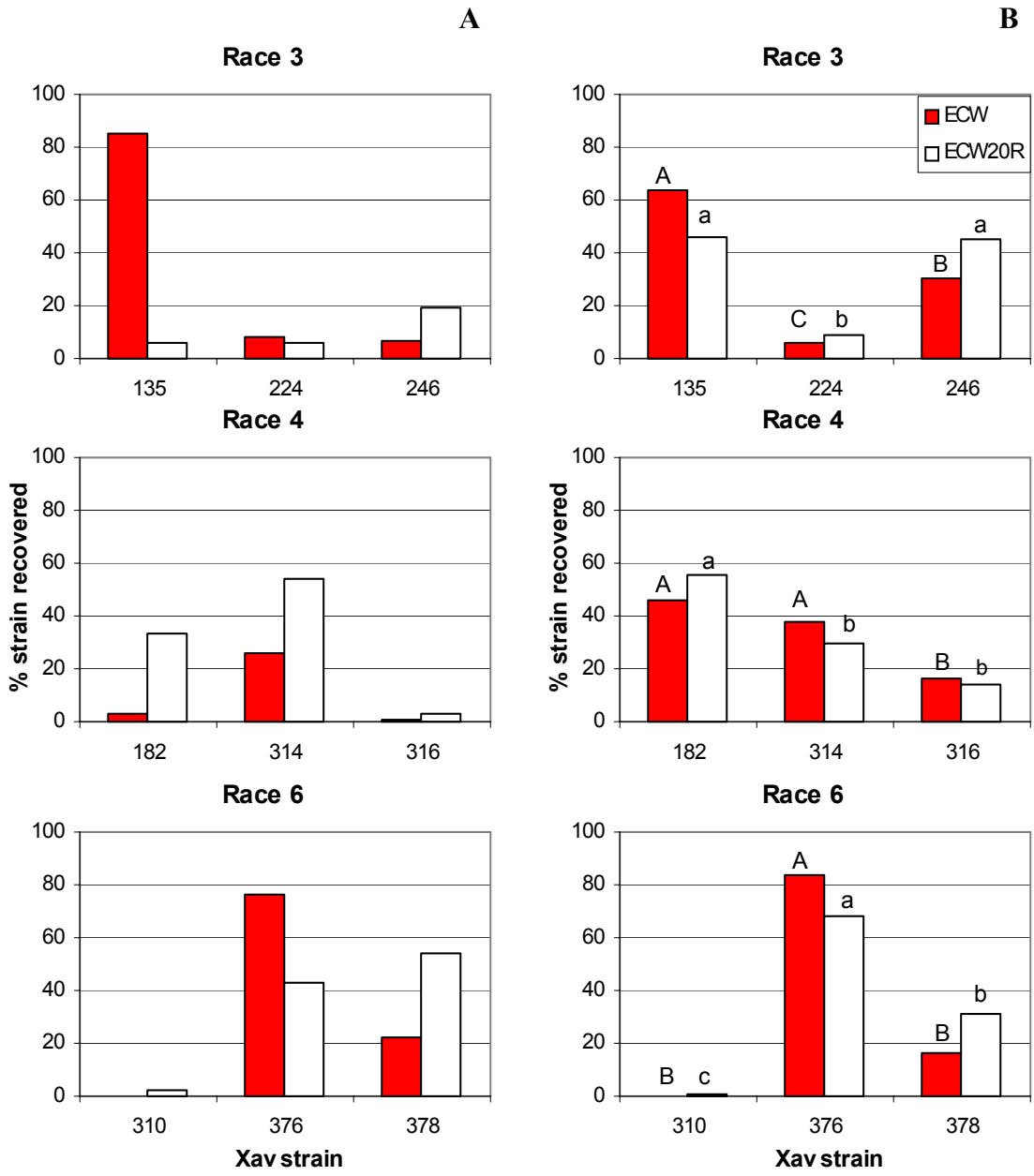

Fig 6. Recovery percentages of *Xanthomonas axonopodis* pv. *vesicatoria* strains in the 2000 (A) and 2001 (B) field experiments from two pepper genotypes ECW, which lacks a major *R* gene and ECW-20R, which carries the major *R* gene *Bs2*. Means followed by the same letter of each pepper genotype are not significant based on the LSD ($\alpha = 0.05$).

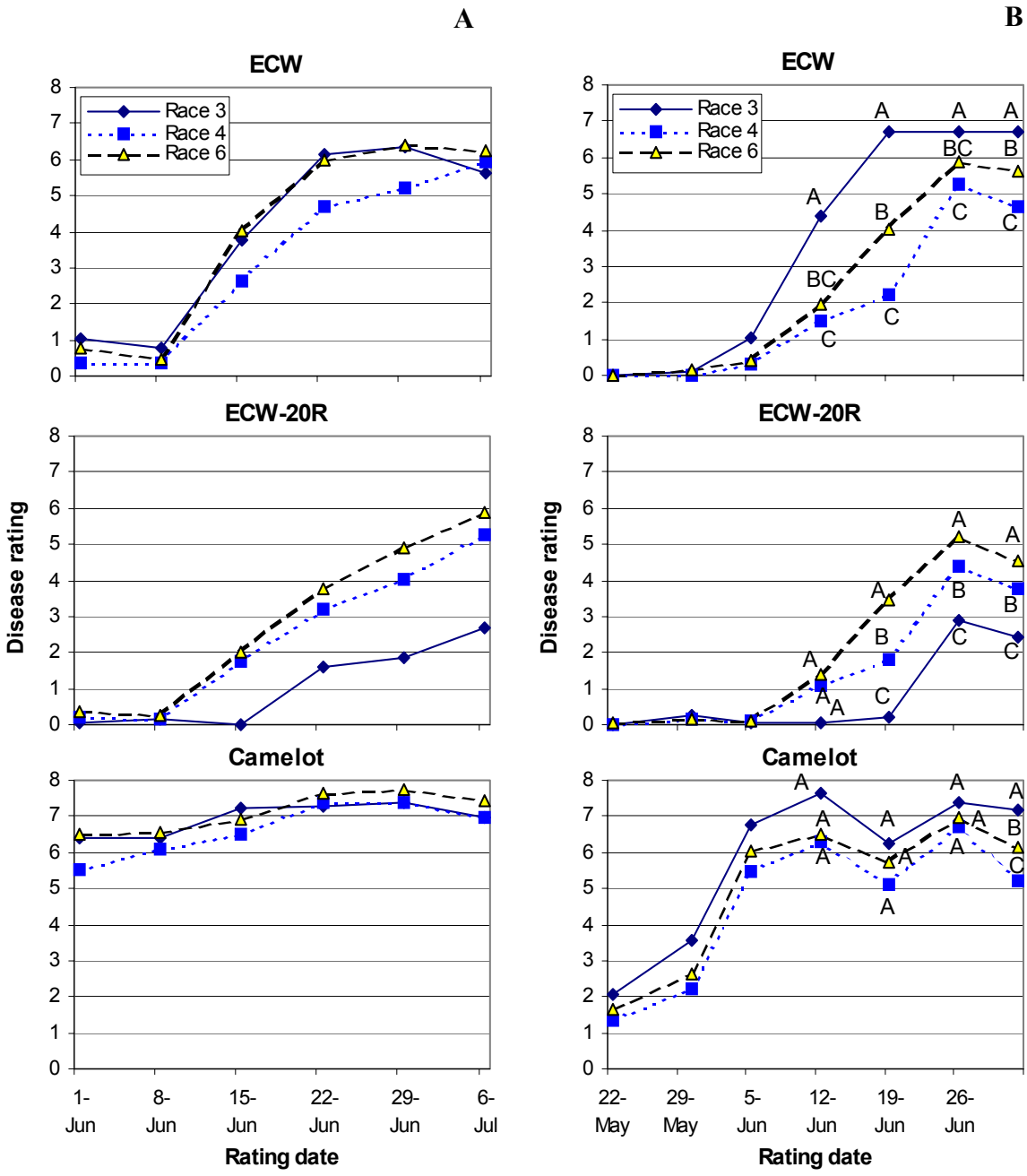

Fig 7. Disease rating for ECW, ECW-20R, and Camelot (inoculum rows) in 2000 (A) and 2001 (B) field experiments. The ratings were done weekly using a 0 to 9 scale. Means followed by the same letter of each pepper genotype are not significant based on the LSD ($\alpha = 0.05$).

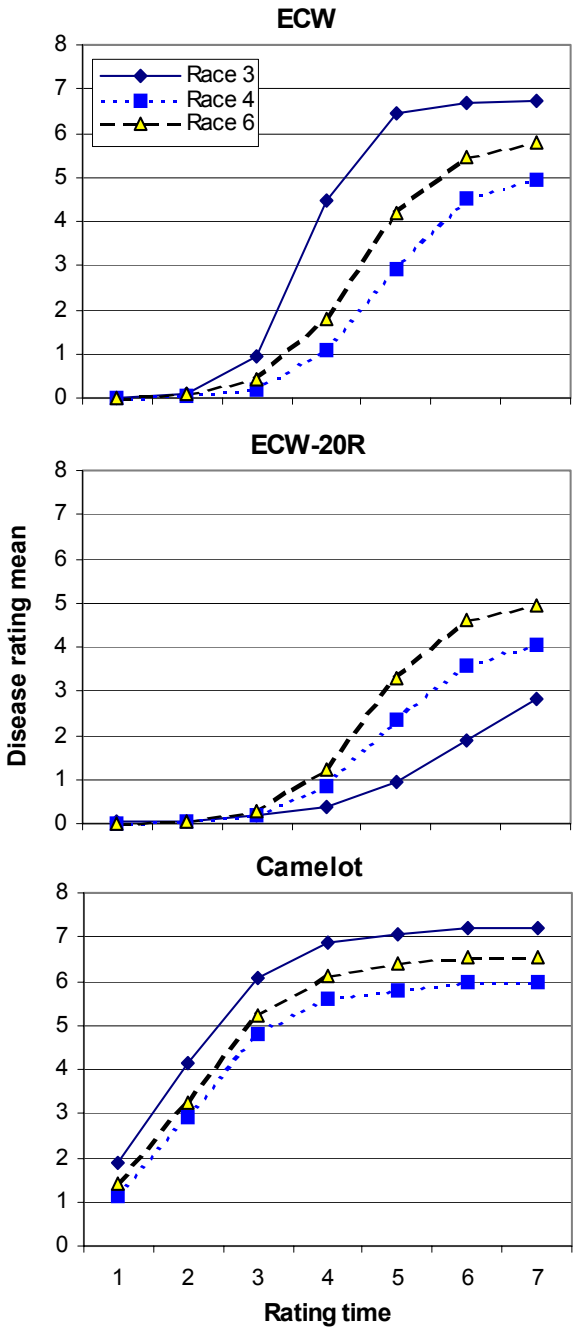

Fig 8. Predicted mean of disease rating from logistic regression model with nonlinear mixed model according to 2001 disease rating data.

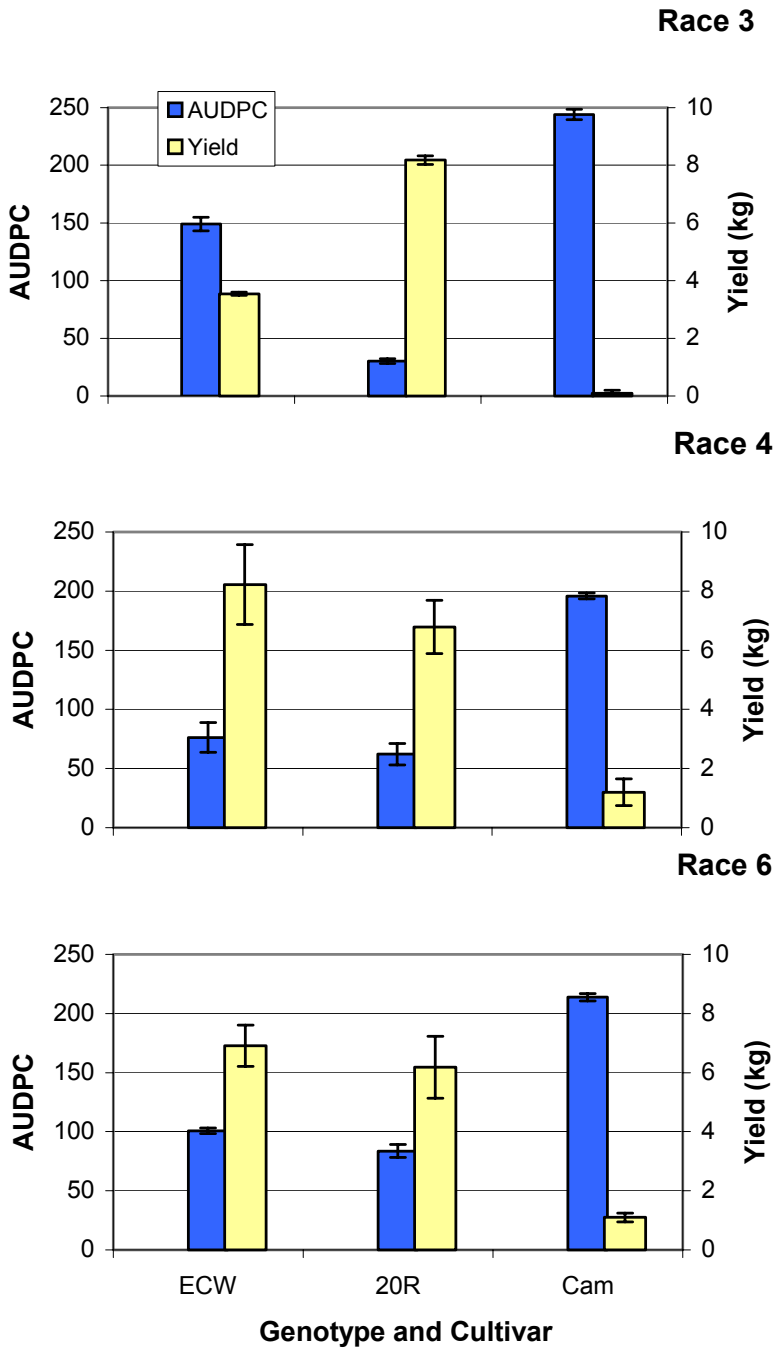

Fig 9. Area under disease progress curves (AUDPC) and yield of ECW, ECW-20R, and Camelot plants from field plots in 2001 into which strains of races 3, 4, and 6 were introduced.

Chapter 2

Apparent lack of strong selection pressure on non-functional *avrBs2* for change to a functional *avrBs2*

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Apparent lack of strong selection pressure on non-functional *avrBs2* for change to a functional *avrBs2*

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ABSTRACT

Mutations in the 5-bp short-sequence DNA repeat region of the avirulence gene *avrBs2* were detected in field strains of *Xanthomonas axonopodis* (syn. *campestris*) pv. *vesicatoria* (Xav) that defeat the *Bs2* resistance gene. We hypothesized that if the *avrBs2* gene is required for full virulence and fitness of Xav, in the absence of selection pressure by the *Bs2* resistance gene, functional *avrBs2* should be favored. Seven strains representing races 4, 5, and 6 with 5-bp mutations (5-bp addition and 5-bp deletion) and resistant to rifampicin were tested for the potential to change from non-functional to functional *avrBs2*. This hypothesis was tested in the laboratory using broth cultures and potted pepper plants and in a field experiment. No strains with functional *avrBs2* were detected in the laboratory experiments. Although 3 (0.15%) of 1,961 colonies from the field experiment had functional *avrBs2*, these strains did not result in a detectable population shift. This suggests that the selection pressure for functional *avrBs2* in the absence of resistance gene *Bs2* is weak or absent.

INTRODUCTION

All organisms must possess mechanisms to maintain their fitness in responding to environmental changes. Pathogenic bacteria have evolved allowing certain phenotypic traits to respond rapidly to natural selection and unpredictable changes in the environment, while also ensuring the conservation of essential functions encoded by other genes (11). This hypothesis relates to the co-existence of highly mutable loci (contingency genes) and loci with much lower mutation rates (housekeeping genes). Short-sequence DNA repeats (SSRs), or DNA microsatellites, have been found in many prokaryotic genomes and are hypothesized to be agents of bacterial evolution (11,12,16).

The repeat regions in a gene with precise deletion or addition of repeat units are thought to be caused by slipped-strand mispairing (11,12,16), and may be generated by mutational pressure (2,11). Functional SSRs in bacteria have been found to be involved in gene regulation of virulence factors (2). Increases or decreases in SSRs can create mutations in genomes thus change gene function resulting from alteration of the translational reading frame (4,11,12,16). DNA microsatellites found in contingency genes, the product of which interacts with unpredictable environments can show high mutation rates allowing the bacteria to act swiftly upon encountering deleterious environmental conditions (11). In some human pathogens, strand-slippage mutation can add or delete DNA repeats and change non-pathogenic bacteria to pathogenic bacteria depending on selection pressure of survival conditions of the bacteria such as in *Haemophilus influenzae* and *Neisseria gonorrhoeae* (12,17,18).

Mutations in the chromosomal-born avirulence gene, *avrBs2*, of *Xanthomonas axonopodis* pv. *vesicatoria* (Xav), the causual agent of bacterial spot of pepper, have been found and examined (4,10,15). Field strains with mutations in the *avrBs2* gene can defeat the *Bs2* resistance gene in pepper and result in economical disease loss (7,8,13,14). Mutant strains with addition or deletion of a repeat unit of five nucleotides (CGCGC) in the *avrBs2* locus (Fig 1) were often detected in strains from fields containing pepper carrying the *Bs2* gene. The mutations due to the addition or deletion of a 5-bp repeat unit result in non-functional *avrBs2* gene and strains with these mutations fail to elicit hypersensitive reaction (HR) in pepper plants carrying the *Bs2* gene. Molecular analysis of 5-bp mutations in *avrBs2* gene showed low AvrBs2 protein expression due to early truncation of the protein (4). This low accumulation of protein led to loss of *avrBs2* activity-inducing resistance in pepper plants carrying *Bs2* and decrease fitness of the pathogen on susceptible plants (4).

The *avrBs2* gene is considered to have both avirulence and virulence activities (4,6,15). Studies showed that the *avrBs2* functions are to increase bacterial fitness on susceptible pepper plants lacking the *Bs2* gene, and that loss of *avrBs2* functions resulted in attenuation of pathogen virulence in susceptible plants. We propose that if the *avrBs2* gene is essential for full virulence and fitness of the pathogen, in the absence of selection

pressure by the *Bs2* resistance gene, functional *avrBs2* should be favored for the pathogen. Thus, there would be a propensity for 5-bp mutations to switch and create a functional *avrBs2* thus gaining full virulence function. This hypothesis was tested both in the laboratory and in field experiments.

MATERIALS AND METHODS

Laboratory experiments

Detection of mixed populations by PCR: Mixed bacterial suspensions at 10^4 CFU/ml of Xcv135 (functional *avrBs2*), Xcv314 (5-bp deletion), and Xcv316 (5-bp addition) were grown in 25 ml of sucrose peptone broth (SPB) (20 g sucrose and 5 g peptone, 0.5 g K_2HPO_4, and 0.25 g $MgSO_4$ in 1 liter H_20) at the following ratios; 1:0, 1:1, 1:1:1, 10:1, 10:1:1, 100:1, and 100:1:1. Mixed cultures were incubated with shaking at 28ºC for 24 hr. Five replications of 1.0 ml of bacterial cultures were aliquoted into 1.5 ml microcentrifuge tubes from each flask. Pellets were collected then 200 µl of sterile distilled H_2O added to each tube. Bacterial suspensions were vortexed, frozen at –80ºC for 15 min, and immediately thawed at 65ºC for 15 min. The suspensions were used as DNA templates for PCR. PCR was conducted in a 12.5 µl reaction volume. The PCR reaction mixture was 1.5 µl of DNA template added to an 11 µl master reaction mixture containing 1.25 µl 10X PCR buffer, 7.238 µl sterile distilled H_2O, 0.375 µl 10 mM $MgCl_2$, 0.25 µl 2 mM dNTPs, 0.25 µl 10 µM PS4F primer (5′-CTACACGGGCGGCTTTGACCAATTCCTGTCC-3′), 0.25 µl 10 µM PS4R primer (5′-AGAAGCCGCGCAAGCGCTCGTCGTTCAACAT-3′), 0.5 µl 100% DMSO, 0.825 µl 75% glycerol, and 0.0625 µl Taq polymerase. This primer set amplifies the 5-bp SSR region of *avrBs2*. The thermal cycling parameters were initial denaturation at 80°C for 5 min and 94°C for 1 min 30 sec, followed by 25 cycles consisting of denaturation at 94°C for 1 min 10 sec, annealing at 64°C for 1 min 10 sec, and extension at 72°C for 2 min. A final extension at 72°C for 5 min followed. Amplified products were subjected to electrophoresis in 4% agarose gels containing 0.5 µg/ml ethidium bromide with 1X TBE

running buffer. A 100-base-pair (bp) DNA ladder was included in each gel as a size marker.

Reversibility of 5-bp mutations in broth cultures: To test for the effects of temperatures and bacterial starvation on spontaneously reversing the 5-bp mutations, strains of races 4 and 6 with 5-bp addition and deletion (Table 1) and a strain of race 3 with a functional *avrBs2* were cultured in 25 ml SPB with shaking at 24, 28, 32, and 36°C. The optimum growth temperature of Xav is 28°C. Bacterial suspensions of each treatment were sampled at 24 and 48 hr after incubation. Five replications of 1.0 ml of bacterial cultures from each flask were aliquoted into 1.5 ml microcentrifuge tubes. Pellets were collected then 50-200 µl of sterile distilled H_2O were added to each tube. DNA templates were extracted using the freeze-thaw method and used for PCR to detect changes in the 5-bp repeat region as described above. The experiment was repeated twice.

Reversibility of 5-bp mutations in potted pepper plants: Xav strains of races 4, 5, and 6 with 5-bp SSR mutations (Table 1) at 10^3 CFU/ml were infiltrated with a needleless syringe into leaves of pepper plants, Early Calwonder (ECW) and ECW-20R (under non-selection and selection pressures of the *Bs2* resistance gene, respectively). Each strain was infiltrated into leaves using three plants as replications. Plants remained at room temperature for 5 weeks. At 3, 4, and 5 weeks after infiltration, 1.0-cm diameter of leaf tissues/plant/strain from the infiltrated area were excised. Bacteria were isolated and single colonies selected. This was done by placing three pieces of tissue infiltrated with each strain in a 1.5 ml microcentrifuge tube containing 500 µl sterile distilled H_2O. The tissues were macerated and an addition 500 µl sterile distilled H_2O added. Serial dilutions of the tissue suspension were performed and 100 µl of the diluted suspension spread on SPA plates. Plates were incubated at 28°C for two days. Composition of 15-20 bacterial colonies per tube was collected and used for PCR as described above. Five replications of composite colonies were tested per strain. The experiment was repeated twice.

Field experiment

A field experiment for testing reversibility of 5-bp mutations was conducted at Sandhills Research Station. The field plot consisted of different pepper genotypes and for sampling purposes was divided into four quadrants (Fig 2, Table 2). Seven strains of races 4, 5, and 6 with 5-bp mutations and rifampicin resistance (Table 1) were inoculated to ECW-20R plants located in the middle of the field plot and which served as initial source of inoculum. Mutant strains were inoculated to pepper plants carrying the *Bs2* gene (ECW-20R) to assure that the initial inoculum consisted of the non-functional *avrBs2* strains. Pepper lines and cultivars from two quadrants were sampled after the disease spread to plants at the borders of the field (i.e. all susceptible plants expressed symptom). Infected leave samples (1-2 leaves per plant) were collected twice two weeks apart from each pepper line and cultivar. Bacterial isolations were performed. Three bacterial spot lesions from each leave were excised, combined and macerated in 200 μl of sterile water. Ten-microliters of bacterial suspensions were streaked on SPA plates amended with 50 μg/ml rifampicin and 20 to 30 Xav colonies were selected arbitrarily for plant infiltration to identify races. Identification of changes in *avrBs2* mutants was evaluated in three near-isogenic pepper lines of ECW for race 1 (from race 4), race 2 (from race 5), and race 3 (from race 6). Bacterial isolation was repeated twice for both sample sets with a total of 1,961 colonies assayed.

RESULTS

Detection of mixed populations by PCR: PCR primers were developed to detect the gene sequence area of the 5-bp mutations in the *avrBs2* locus. The expected PCR products are 171, 176, and 181 bp for 5-bp deletion, functional *avrBs2*, and 5-bp addition, respectively. A PCR band larger than 181 bp was also amplified and observed only when mixed populations were detected. Mixed populations of 5-bp mutant and functional *avrBs2* strains were detected by observing multiple bands of the PCR products of the previous mention sizes. The primers detected the mutations in mixed populations at the ratios of 1:1, 1:1:1, 10:1, and 10:1:1 (Fig 3), but failed to detect the mutations in mixed populations at the ratios of 100:1 and 100:1:1. Only a single band of the higher

ratio populations was amplified for 100:1 and 100:1:1 ratios (figure not shown). Sequence analysis of the extra bands showed homology with the *avrBs2* region containing 5-bp repeats. This band may result from unspecific binding of the primers in the mixed populations.

Reversibility of 5-bp mutations in broth cultures and potted pepper plants: Bacterial starvation, temperature stress, and plant genotypes were used to select 5-bp reversibility. A total of 112 PCR samples for broth culture and 420 PCR samples for potted pepper plants were tested. Spontaneous changes in reversibility of 5-bp mutations could not be detected in broth cultures nor in potted pepper plants. The PCR products exhibited only a single band indicating a single population of bacteria. Although very low mutant populations may have been presented, the PCR primers failed to detect them.

Field experiment for reversibility of 5-bp mutations: A total of 1,961 Xav colonies were evaluated from diseased leaves of the pepper genotypes and cultivars from the field experiment. Reversibility of the 5-bp repeat region in *avrBs2* locus was detected by the recovery of race 1 strains in ECW and PI 235047 and a race 3 strain in ECW (Table 3). Strains of race 1 should derive from a change in 5-bp repeat region to functional *avrBs2* from race 4 and a strain of race 3 from race 6. Although the changes in 5-bp mutations occurred, the percentages of the recovery were very low, 0.10 and 0.05, respectively. The recovery of races 4, 5, and 6 were 17%, 6%, and 76%, respectively (Table 3).

DISCUSSION

Here we studied the possibility of mutations in the 5-bp repeat region in *avrBs2* locus to switch to functional *avrBs2* in the absence of host *R* gene selection, over a range of temperatures, and during bacterial starvation. The laboratory experiments for bacterial starvation with high density of bacterial populations and growth temperatures below and above the optimum temperature for bacterial growth did not result in detectable changes in the frequency of 5-bp mutations to functional *avrBs2*. A very low ratio of reversibility of 5-bp mutations may have occurred but could not be detected. An extra PCR product that is larger than the expected products could be used as an indicator for mixed

populations if they are in a detectable ratio. Mutations in bacterial populations may occur in long-term culture and during prolonged starvation in which there is intense selective pressure for any mutation that confers a competitive advantage (3). Mutations in *avrBs2* gene under starvation are possible but the mutation types may not be specific only for 5-bp mutations. Other insertion or deletion types of mutations in *avrBs2* locus may possibly occur (4,10).

With the rifampicin-resistant phenotype of strains used in the field experiment, it was possible to confirm that the recovered strains were those placed into the field. A low percentage of strains having the 5-bp switch from non-functional *avrBs2* (races 4 and 6) to functional *avrBs2* (races1 and 3, respectively) was detected after the disease spread to the borders of the field. Strains with functional *avrBs2* did not result in a detectable population shift within a single cropping season. In contrast for mutations with plasmid-born *avrBs1* and *avrBs3* under *R* genes selection pressure, populations of race 3 shifted from race 1 by losing plasmid carrying *avrBs1* and from race 2 by IS*476* inactivating *avrBs3* and rapidly predominated within a cropping season (9). Mutation at *avrBs1* locus was estimated to occur at a frequency of $5x10^{-4}$ (1,5) that was higher than a spontaneous mutation frequency at *avrBs2* locus, less than 10^{-6} (15).

Since the first detection of races that defeat the *Bs2* gene, strains containing non-functional *avrBs2* have been continuously found in commercial pepper fields (13), however the types of mutation in *avrBs2* locus were not thoroughly evaluated. Results from the field experiment reported here indicate that although a 5-bp mutation switch to a functional *avrBs2* was detected, populations of these strains did not increase and compete with the original populations carrying a non-functional *avrBs2*. It seems likely that gaining *avrBs2* function of Xav in this field experiment may not increase competitiveness and aggressiveness functions of the strains under very high density of non-functional *avrBs2* strains.

Populations of race 6 with 5-bp mutations predominated in the field experiment followed by strains of races 4 and 5, respectively. The higher aggressiveness of strains of race 6 than of race 4 previously was reported from field experiments (T. Wangsomboondee, Ph.D. dissertation Chapter 1). Only strains of races 4 and 6 were

recovered from field experiments (8) and these races have been associated with severe disease in commercial pepper fields (13). Strains of race 5 are rarely detected in commercial pepper fields. Factors that cause strains of races 4 and 5, both containing non-functional *avrBs2* and another *avr* gene to be less aggressive than race 6 strains containing only non-functional *avrBs2* remain elusive. Loading of functional *avrBs1* and *avrBs3* genes in strains of race 5 and 4, respectively, may somehow have residual effects that are thought to result from the *R* genes in the plant to the pathogen fitness resulting in less aggressiveness and competitiveness in plants not carrying the corresponding *R* genes.

The selection pressure of strains carrying 5-bp mutations in the *avrBs2* gene may be the wide use of pepper plants containing the *Bs2* gene (13). However, we suggest that the selection pressure for reversion to functional *avrBs2* in the absence of resistance gene *Bs2* is weak or of no consequence. It is possible that the absence of *Bs2* gene may not be the only factor or selection pressure for reversion in the 5-bp SSR region to functional *avrBs2.* In this study, functional *avrBs2* may not have been necessary for full virulence and fitness of the pathogen. Studies showed that some strains with mutations in *avrBs2* locus cause severe disease in the field (4, T. Wangsomboondee, Ph.D. dissertation Chapter 1). In addition, the aggressiveness of mutant strains was as high as functional *avrBs2* strains (8, T. Wangsomboondee, Ph.D. dissertation Chapter 1). Therefore, there is a potential of strains with mutations in *avrBs2* gene to be favored for the pathogen survival in the future under the lack of *Bs2* selection pressure. More experiments should be established to evaluate factors such as overwintering that may play a role as selection pressure of the mutant and functional *avrBs2* strains in the field.

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Table 1. *Xanthomonas axonopodis* pv. *vesicatoria* strains with a 5-bp mutation in the *avrBs2* locus used in laboratory and field experiments.

Race (*avr* gene)	Strain	Type of 5-bp mutation
4 (*avrBs3*)	Xcv314	Deletion
4 (*avrBs3*)	Xcv316	Addition
5 (*avrBs1*)	Xcv206	Addition
5 (*avrBs1*)	Xcv293	Deletion
6 (none)*	Xcv201	Deletion
6 (none)	Xcv312	Deletion
6 (none)	Xcv459	Addition

* no functional *avr* genes corresponding to *R* genes *Bs1*, *Bs2*, and *Bs3*.

Table 2. Majors resistance gene(s) of pepper cultivars and genotypes used in 5-bp field experiment.

Cultivar or genotype	Major resistance gene(s)
ECW	None
ECW-10R	*Bs1*
ECW-20R	*Bs2*
ECW-30R	*Bs3*
ECW-123R	*Bs1, Bs2,* and *Bs3*
Camelot	None
X3R Camelot	*Bs2*
Boynton Bell	*Bs1* and *Bs2*
CNPH 703	Quantitative resistance
PI 235047	*Bs4*

Table 3. Numbers of strains of pathogen races recovered from different pepper genotypes in field experiment.

Cultivar	Number of colony recovered in the field plot					
	Race 1	Race 2	Race 3	Race 4	Race 5	Race 6
Camelot	0	0	0	40	26	375
X3R Camelot	0	0	0	9	1	260
ECW	0	0	1	60	1	403
ECW123R	0	0	0	0	0	122
Boynton Bell	0	0	0	13	0	107
CNPH 703	0	0	0	41	0	69
PI 235047	1	0	0	129	74	82
ECW/R4[a]	1	0	0	45	0	1
ECW/R5	0	0	0	0	20	30
ECW/R6	0	0	0	0	0	50
Total	2	0	1	337	122	1499
Percentage	0.10	0	0.05	17.19	6.22	76.44

[a] ECW plants between inoculum row of race 4

gcaggatgcgc **cgcgc cgcgc cgcgc** tggatcgcttgctg	functional *avrBs2*
gcaggatgcgc **cgcgc cgcgc cgcgc cgcgc** tggatcgcttgctg	5-bp addition
gcaggatgcgc **cgcgc cgcgc** tggatcgcttgctg	5-bp deletion

Fig 1. Sequence of 5-bp repeat region in *avrBs2* locus.

	3	4	5	6	7	8	9	10	11
1	CNPH	123R	X3R	PI	BB	123R	CNPH	PI	BB
2		C	X	X	X	X	X	C	
3	CNPH	123R	X3R	PI	BB	123R	CNPH	PI	BB
4		C	X	X	X	X	X	C	
5	CNPH	123R	X3R	PI	BB	123R	CNPH	PI	BB
6		C	X	X	X	X	X	C	X3R
7		C	X	X	X	X	X	C	X3R
8		C	X	X	X	X	X	C	X3R
9	10R	C	X	X	X	X	X	C	10R
10	20R	C	X	X	X	X	X	C	20R
11	30R	C	X	X	X	X	X	C	30R
12		C	X	X0	X0	X0	X	C	
13		C	X	X0	X0	X0	X	C	
14		C	X	X0	X0	X0	X	C	
15		C	X	X0	X0	X0	X	C	
16		C	X	X	X0	X	X	C	
17	10R	C	X	X	X	X	X	C	10R
18	20R	C	X	X	X	X	X	C	20R
19	30R	C	X	X	X	X	X	C	30R
20	BB	C	X	X	X	X	X	C	
21	BB	C	X	X	X	X	X	C	
22	BB	C	X	X	X	X	X	C	
23	X3R	PI	BB	CNPH	123R	PI	X3R	CNPH	123R
24		C	X	X	X	X	C	X	
25	X3R	PI	BB	CNPH	123R	PI	X3R	CNPH	123R
26		C	X	X	X	X	C	X	
27	X3R	PI	BB	CNPH	123R	PI	X3R	CNPH	123R

Fig 2. Field plot pattern of 5-bp mutations experiment. Each set of letter(s) represents pepper plants. X = ECW; C = Camelot; BB = Boynton Bell; CNPH = CNPH 703; X3R = X3R Camelot; 123R = ECW-123R; PI = PI 235047; 10R, 20R, and 30R = ECW-10R, ECW-20R, and ECW-30R, respectively; 0 = ECW-20R infiltrated with 5-bp mutant strains of races 4, 6, and 5 in column 6, 7, and 8, respectively.

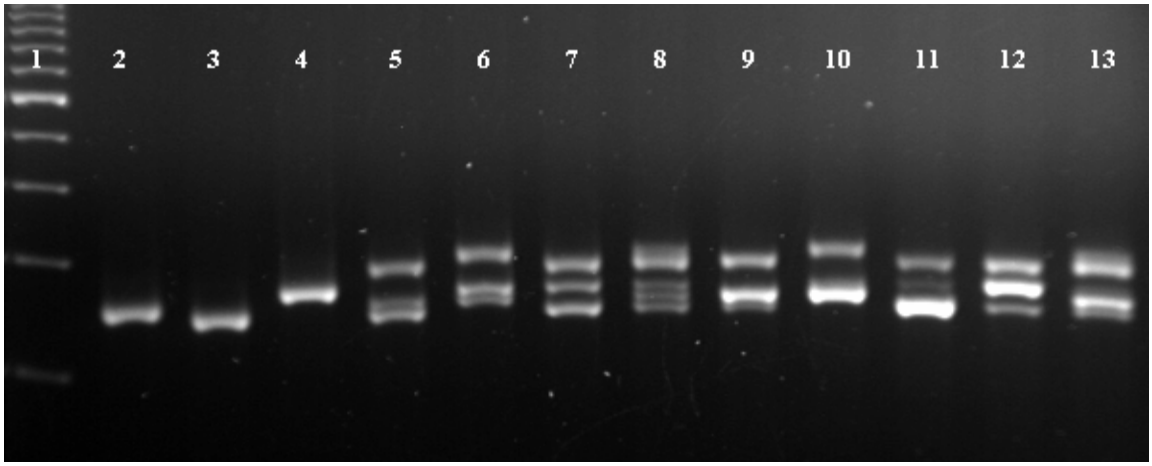

Fig 3. Detection of 5-bp addition (Xav316), 5-bp deletion (Xav314), and wild type (Xav135) strains in mixed cultures in 4% agarose gel. Lane1 is 100-bp DNA marker. Lanes 2-4 are single culture of Xav135, 314, and 316, respectively. Lanes 5-8 are mixed cultures of 1:1 and 1:1:1 ratio of Xav135:314, 135:316, 314:316, and 135:314:316 respectively. Lanes 9-13 are mixed cultures of 10:1 and 10:1:1 ratio of Xav135:314, 135:316, 314:316, 316:314, and 135:314:316 respectively. The highest molecular weight bands from lanes 5-13 are non-specific amplified products of mixed cultures.