Title

CRISPR/Cas9-mediated editing of pepper Bs5 homolog in tomato (Solanum lycopersicum) confers disease resistance to bacterial spot disease

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CRISPR/Cas9-mediated editing of pepper *Bs5* homolog in tomato (*Solanum lycopersicum*) confers disease resistance to bacterial spot disease

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TABLE OF CONTENTS

Abstract……1

CHAPTER 1: Introduction……2

CHAPTER 2: Establishing disease resistance in tomato using CRISPR/Cas9 system……..6

2.1 Introduction……6

2.2 Searching for the *Bs5* gene in Solanaceae family……..…9

2.3 Predicting the Bs5 protein structure……....11

2.4 Gene expression of Bs5/Bs5-like genes in tomato……....12

2.5 Cas9-mediated gene editing of Bs5/Bs5-like in tomato……....13

2.6 Discussion……...15

2.7 Materials and Methods……...16

2.8 Additional figures……18

CHAPTER 3: Evaluating the resistance phenotype in bs5 tomato mutants……20

3.1 Introduction……..……..…..20

3.2 Bacterial spot resistance phenotype evaluation of mutant tomato lines.……..……21

3.3 Biochemical evidence of bacterial effector translocation in bs5 resistant tomatoes……..……25

3.4 Field trials of *Slbs5-2* line in Florida……...…….26

3.5 Discussion……28

3.6 Materials and Methods…….……..29

Chapter 4: Concluding remarks……..31

REFERENCES……..32

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Abstract

CRISPR/Cas9-mediated editing of pepper *Bs5* homolog in tomato (Solanum lycopersicum) confers disease resistance to bacterial spot disease

Xanthomonas spp. are the causative pathogens of bacterial spot and have caused major yield losses in commercial varieties of tomato and pepper. Bacterial spot disease is a threat to worldwide production of Solanaceous crops, especially in warm and humid environments. The *Bacterial Spot 5 (Bs5)* gene, which is conserved among Solanaceae, is composed of three exons, and encodes a small proline-rich putative tail-anchored protein with an unknown function. *Bs5* is flanked by its paralog, *Bs5-like*, in a head-to-head orientation. The Bs5 protein is a putative member of the cysteine-rich transmembrane module (CYSTM) protein family, a diverse family found to be expressed in response to biotic and abiotic stress in many eukaryotes. A naturally occurring recessive allele, *bs5*, was found in pepper to confer disease resistance against a hypervirulent strain of *Xanthomonas euvesicatoria* in pepper (*Capsicum annuum*). The *bs5* resistance phenotype is characterized by restricted pathogen growth and an absence of necrotic lesions characteristic of bacterial spot susceptibility. Pepper varieties containing the *bs5* allele have shown durable resistance, impeding the emergence of *bs5*-resistant strains in commercial fields. Deployment of the *bs5* allele in tomato has the potential to confer disease resistance against bacterial spot. The causative mutation of *bs5* is an in-frame six nucleotide deletion in the third exon. It was unknown whether the precise six nucleotide deletion is the sole mutation that confers the resistance phenotype against bacterial spot. We set out to investigate whether introducing nucleotide insertions or deletions to the coding region of tomato *Bs5* would be sufficient to introduce resistance. Using CRISPR/Cas9-mediated gene editing, we established two distinct mutant lines with mutations in exon 2 of *Bs5* and *Bs5-L.* We also established a mutant line with mutations in exon 3, the location of the *bs5* allele mutation in pepper. Our objective was to establish *bs5* resistance in tomatoes using CRISPR/Cas9 based genome editing. We were able to generate several mutant tomato lines with alleles distinct from *bs5* in the pepper allele. These tomato lines exhibited resistance against several species of xanthomonads.

CHAPTER 1. Introduction

Classical plant breeding was extremely successful in generating high yielding crop varieties[1]. Despite this, there is a lack of genetic diversity in several crops which would otherwise allow for the sourcing of genetic resistance to abiotic and biotic stressors. This lack of diversity is impeding the development of novel traits in elite germplasms. New precise gene editing tools have allowed for the ability to expand genetic diversity in crops[2]. By targeting homologs present also in closely related crop species, this further enables the possibility of deploying specific traits which would otherwise be impossible to introduce through sexual reproduction.

Gene functional studies have typically been done using labor intensive, long-term, and imprecise methods: *Agrobacterium* transformation, chemical mutagenesis, and virus induced gene silencing[3,4,5]. Typically, mutating specific genetic sequences with these strategies required screening of large populations of mutants without the guarantee of success[6]. In the past 10 years, crop genetic engineering has undergone a paradigm shift due to the emergence of a novel technology that employs clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems derived from bacteria/archaea. CRISPR/Cas systems are used by bacteria/archaea to stave off infection from bacteriophages[7]. They are adaptive immune systems employed to confer resistance to invading viral genomes. CRISPR immunity is primed by integration of short sequences (spacers) from the viral genome into the bacterial genome to form CRISPR arrays. These spacers are typically flanked by repeat sequences[8].

CRISPR/Cas systems are comprised of two classes: Class 1, which include multi-subunit effector protein complexes, and Class 2, which include single protein effector complexes[9,10]. In the past decade, several Class 2 CRISPR/Cas systems have been employed for crop genome editing, with the CRISPR/Cas9 from *Streptococcus pyogenes* being the most widely used in plants thus far[2]. Typically, CRISPR array transcription is initiated upon viral invasion, resulting in the production of a CRISPR RNA precursor called pre-crRNA. The pre-crRNA contains the acquired spacer sequences flanked by repeat sequences, and these pre-crRNAs are subsequently processed and matured into CRISPR RNAs (crRNAs)[10]. Upon immunity activation triggered by viral invasion of the cell, crRNA-guided Cas effector nucleases (ribonucleic protein complexes) seek, target, and cleave invading sequences from viral genomes in a sequence-specific manner[7].

The CRISPR/Cas9 complex is comprised of the Cas9 endonuclease, the crRNA, and a trans-activating crRNA (tracrRNA)[7,11,13]. The crRNA and tracrRNA components hybridize due to their partial complementarity to each other. The the tracrRNA also contains a stem-loop structure that facilitates interaction with the Cas9 endonuclease. The crRNA contains a 5' end spacer sequence, also known as the "guide" sequence[7,13]. The spacer sequence is derived from captured invading nucleic acid sequences stored in host genome CRISPR arrays[12-14]. For recognition and cleavage of invading viral DNA by Cas9 complexes, canonical base pairing is essential between the spacer sequence in the

crRNA and the complementary protospacer sequence from the invading viral DNA. Additionally, a sequence proximal to the 3' end of the protospacer, called the protospacer adjacent motif (PAM), is required for Cas9 cleavage activity. Protospacer sequences are first integrated into host genome CRISPR arrays through a process called protospacer acquisition[12-15]. Protospacer sequences integrated into CRISPR array loci lack PAM sequences to prevent auto-cleavage of the CRISPR array by Cas9. During viral DNA invasion, Cas9 seeks PAM sequences through a process involving rapid random association with DNA and rapid disassociation from non-PAM sequences[16-19]. When Cas9 finds its complementary genomic target, it unwinds duplexed DNA to allow access to nuclease domains within the Cas9 enzyme. Once unwound, the duplexed DNA is cleaved by the RuvC and HNH domains, RuvC cleaving the non-target DNA strand, meanwhile HNH cleaving the target DNA strand[11,17]. These two domains leave a blunt double stranded break (DSB) about three base pairs upstream of the PAM sequence. After the Cas9 complex recognizes and cleaves viral DNA, it dissociates[16-22].

Typically, when the CRISPR/Cas9 system is repurposed for gene editing in research, the crRNA and tracrRNA are fused into a single guide RNA (sgRNA)[23-25]. This fusion does not hinder the three-dimensional folding of the sgRNA, nor does it hinder targeting or cleavage of DNA by Cas9[20-23]. Initially, Cas9-mediated genome editing was done in human and mouse cells[25,26,104]. Gene mutagenesis in eukaryotic cells was done by inducing Cas9-mediated double-stranded breaks (DSBs) in targeted DNA sequences. Since repair of DSBs in eukaryotic cells is usually through non-homologous end joining (NHEJ), this results in either insertions or deletions (indels) due to NHEJ being an error prone DNA repair mechanism[27-31]. Usually, indels disrupt gene function due to the generation of pre-mature stop codons or significant abrogation of the wild-type amino acid sequence that is subsequently translated from the mutant transcript. Soon after Cas9 was first used for editing of human and mouse genomes, it was used for gene editing in plants[32-38].

CRISPR/Cas9 is distinct from previous precise genome editing tools like Zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs)[39-41]. These older methods typically took more time and labor to implement and were more difficult to design for specific targets. They were thus less cost effective to produce and required specific expertise than more modern alternatives. Also, Cas9-mediated DSBs results in higher mutation efficiency, usually leading to homozygous mutations that could be more easily passed on to subsequent generations[39]. Usually, the gene editing methods such as ZFN/TALENS/CRISPR resulted in unwanted off-target mutations in other parts of the genome[42]. Cas9-mediated gene editing typically led to less off-target mutations than ZFN and TALENs[39,42,43].

In order to achieve efficient plant genome editing with the CRISPR/Cas9 system, high levels of Cas9 and sgRNA expression are necessary. *Agrobacterium tumefaciens* is a Gram-negative bacterium that is the causative agent of crown gall disease in plants. Disease symptoms are caused by integration of segments of DNA, also known as the

transfer DNA (T-DNA), from plasmid to host plant cell[44]. The standard method for introduction of Cas9-sgRNA expression systems in plants has been the *Agrobacterium* T-DNA binary system[44,45]. Transformation of transgenes into plant genomes is mediated by *Agrobacterium tumefaciens* containing a binary vector system. The system is comprised of a pair of plasmids, one known as the T-DNA binary vector, and the other known as the *vir* helper plasmid (hence the term "binary")[44]. A binary vector (for example pPVP2000 or pCAMBIA2300) is composed of left and right T-DNA borders which usually contain multiple cloning sites (MCS) and a plant selectable marker[44,45]. Outside of the T-DNA borders, the binary vector contains sites for replication in both *Escherichia coli* and *Agrobacterium tumefaciens* as well as bacterial selectable markers. The *vir* helper plasmid contains *vir* genes that are derived from the original Ti plasmid of *Agrobacterium*. This set of genes expresses proteins that excise the T-DNA from the binary vector by cleaving the left and right border sequences flanking the T-DNA[44]. Some of these vir proteins subsequently shuttle the T-DNA into plant cells and randomly integrate the T-DNA into the plant genome. The snRNA U6 Pol III promoter is typically used for high and constitutive expression of transcripts to be introduced into plants such as sgRNA[35]. Also, an optimized mutated sgRNA scaffold sequence has previously been shown to increase transcription and editing efficiencies[34,35]**.** To achieve high expression levels of the Cas9 gene, the 35S constitutive promoter from Cauliflower Mosaic Virus has been used previously in many dicotyledonous plant species[35].

For the many plant species that are recalcitrant to *in planta Agrobacterium* transformation, co-cultivation of *Agrobacterium* with explants, followed by tissue culture and regeneration, is required for establishment of stable transformants expressing integrated Cas9-sgRNA transgenes[34,35,45,47]. After regeneration, Cas9-sgRNA integration into the plant genome is confirmed by genotyping using PCR amplification at the gene target site. Typically, Agrobacterium-mediated transformations result in the transgene being integrated hemizygously. The hemizygous transgene will be absent in some of the subsequent progeny in the next generation due to Mendelian inheritance. This can be achieved by either cross-pollination with a wild-type plant or self-pollination.

NHEJ is an error prone repair pathway as well as the most prevalent DSB repair pathway in plant cells[48]. NHEJ usually leads to introduction of relatively small deletions or insertions (~1-30 bp) at the DSB site[30,48,49]. If the sgRNA target site is within an exon, introduction of indels could lead to shifting of the open reading frame of a coding sequence (CDS) which could lead to significant changes in the amino acid sequence of the translated protein. If the target site is within the first or an early exon, another possible outcome is creation of a premature stop codon, which would result in a complete loss in gene function (gene knockout) (KO). The closer the target site is to the 3' end, the less likely a complete gene KO will be conferred.

Cas9-based plant genome editing has been employed for gene functional studies in plants for a variety of traits and outcomes: developmental, disease resistance, drought resistance, improved yield, sterility[49]. NHEJ repair pathway is facilitated by several

molecular factors[50]. It does not require a DNA template for repair, such as in homology-directed repair, and it is prevalent in all cycles of the cell. Due to this, NHEJ is the most ubiquitous repair pathway used by plant cells to repair DSBs[48]. When a DSB is repaired by NHEJ, two proteins, KU70 and KU80, interact with the DSB blunt ends to inhibit the initiation of resection of these ends (which inhibits initiation of HDR)[27,50]. Although introducing a donor DNA template for HDR results in more precise gene edits after Cas9-mediated DSBs, HDR rates are low in plants, and HDR repair outcomes are difficult to establish using conventional *Agrobacterium*-based methods[48-50]. Exploiting the NHEJ pathway is more advantageous since NHEJ is both more prevalent in plant cells, especially when the mutation or genotype of interest and does not need to be precise (as with gene function studies).

Employing Cas9 to establish novel traits through gene mutations in tomato crops has been done before[51,54]. In this study, we used the *Agrobacterium* transformation system to introduce Cas9 in tomato for the purpose of editing a specific gene, *Bs5*. The goal was to establish disease resistance against a bacterial spot disease by mutating the tomato *Bs5* gene, which is a homolog of a resistance gene previously identified in pepper, a close relative of tomato in the Solanaceae family. In Chapter 2, we report the successful establishment of Cas9-sgRNA T-DNA integration in a tomato variety. An allelic series of alterations at various sites in this gene was successfully generated in these mutant tomatoes, and progeny from these mutants were subsequently cultivated for future experiments. In Chapter 3, progeny from Cas9 gene edited tomatoes were evaluated for their resistance phenotype in the lab and in field trials. We devised a series of experiments to evaluate their resistance phenotype in leaves, fruits, and at the cellular level. Although there is precedence for use of Cas9 to edit tomato genomes for resistance, with this work, we further bolstered the usability and practicality of establishing disease resistance in tomato using Cas9-mediated gene editing[52-54].

CHAPTER 2: Establishing disease resistance in tomato using CRISPR/Cas9 system

2.1 Introduction

Tomato (*Solanum lycopersicum*) is commonly used as a fleshy fruit model system in crop research. It is a member of the Nightshade (Solanaceae) family that includes tobacco, pepper, potato, eggplant, etc. The commercial value of tomato production is valued over a billion dollars a year in the United States[55,56]. Although not a staple crop like rice or wheat, tomato fruits are a rich source of nutrition and play a central role in many types of cuisines around the world. Tomato is a major dietary source of vitamin A and C, as well as phenolic antioxidants[56].

The tomato genome was sequenced in 2012, culminating years of work by the Tomato Genome Consortium and is well documented in several genomic databases[57]. The tomato genome sequencing project produced a high-quality genomic sequence of the tomato cultivar, Heinz 1706. The tomato genome is diploid, composed of 12 chromosomes (2N=24) with an estimated size of around 950 Mb. Members of the Solanaceae family share similar genetic content and significant synteny, especially between closely related species like *Capsicum anuum* (pepper) and *S. lycopersicum*[57,58].

Tomato crops are currently susceptible to infection by many different bacterial, viral, and fungal pathogens. Diseases in tomato have become a burden for growers over the years, which have led to increasing yield losses[53-55,59,60]. In the future, this could lead to even further commercial yield loss of tomato crops due to increased disease pressure. Bacterial spot disease, one of the most important diseases, occurs on pepper and tomato worldwide causes necrotic lesions on the leaves, stems, and fruits (Fig. 1).

Disease pressure from bacterial spot disease can increase intensity in commercial fields whenever hypervirulent strains of a pathogen arise. Increased disease pressure from bacterial spot disease can lead to unmarketable fruit, plant death, and even loss of entire harvests[55,59]. A potential factor influencing disease pressure is global climate change which may exacerbate weather volatility resulting in a warmer and wetter local environment that is more conducive to pathogen proliferation. Since commercial tomato cultivars have limited genetic resistance, disease pressure necessitates growers to use chemical treatments to inhibit pathogen proliferation. Chemical treatments can be detrimental to the health of both growers and consumers. For that reason, in the past 20 years, genetic sources of resistance to disease have been increasingly employed in order to combat diseases in commercial crops[4,51,53,59,60].

In the past, tomato breeding programs have introduced disease resistance genes (also known as R genes) from other species, including wild tomato species into commercial tomato cultivars by breeding[51,52,59]. Two examples are the Rx3 and Bs2

genes, which confer resistance against bacterial spot disease[61,62]. *Rx3/Bs2* genes are part of a large gene family that encodes proteins containing an N-terminal coiled coil domain, nucleotide binding domain, and a C-terminal a leucine rich repeat domain (NLR). NLR genes typically induce the hypersensitive response (HR) upon molecular pattern recognition of a pathogen[63-65]. HR usually leads to rapid cell death at the site of infection which prevents subsequent systemic infection of the whole plant. Plants do not have an adaptive immune system. In general, plants employ two major disease resistance mechanisms to attack pathogens. One is the nucleotide-binding leucine-rich repeat (NLR) protein resistance pathway, and another is the transmembrane pattern recognition receptor (PRR) pathway[63-66].

Historically, most of the disease resistance genes deployed in commercial crops were either genes encoding NLR proteins or PRR proteins[64]. These resistance genes are typically inherited dominantly, which facilitates their introgression gene by hybridization if one of the parents harbors the resistance allele. Establishing disease resistance with this approach is not durable, and often leads to the pathogen overcoming resistance through mutations that disrupt the R gene within a few years after its deployment [63]. Several dominant disease resistance genes effective against bacterial spot disease have been deployed in agronomic varieties of pepper and tomato[61,67,68,69]. Despite these efforts, bacterial spot disease continues to affect commercial peppers as hypervirulent strains of the bacteria have evolved to overcome dominant resistance genes such as: Bs1, Bs2, and Bs3 by alteration of the effectors which are recognized by these R genes[70-72]. Bacterial populations of Xanthomonas species capable of overcoming bacterial spot disease resistance is a major problem in commercial tomato production since other control strategies are

Various *Xanthomonas* species are the causative pathogens of bacterial spot disease. These diseases cause major yield loss in commercial varieties of Solanaceous crops such as tomato and pepper[73]. Four closely related bacteria cause bacterial spot disease: *Xanthomonas vesicatoria*, *X. euvesicatoria*, *X. gardneri*, and *X. perforans*. *Xanthomonas spp*. invade plants through wounds or natural openings in leaves, fruits, or stems with the most prevalent symptom being necrotic lesions at site of infection. Upon invasion and subsequent population growth, they secrete virulence effectors through their type three secretion system (T3SS), causing susceptibility of the plant cell by modulating molecular components needed for plant immunity[74,75]. Climate change threatens to exacerbate yield loss in major food crops in the upcoming years. Due to changing weather patterns and increasing average temperature in certain regions, pathogen populations may shift, and pathogen disease pressure may increase, necessitating novel forms of genetic resistance.

Genetic modification of food crops has emerged as a primary method to improve the productivity of many crops suffering from declining productivity[1,2,54]. It is imperative to establish new varieties of food crops that are resistant to pathogens in sustainable ways in order to meet the food demands of the 2 billion more people across the world

expected in the next three decades[2]. Genetic sources of disease resistance isa promising alternative to the use of chemical intervention.

The *Bacterial Spot 5* (*BS5*) gene is conserved among Solanaceae, is composed of three exons, and encodes a proline-rich putative transmembrane protein with an unknown function[76,77]. The encoded protein is small (~92 amino acids) and part of a class of small (70-110 amino acids) proteins called cysteine-rich transmembrane domain (CYSTM) tail-anchored proteins found among different phyla (although absent in prokaryotes) and implicated in abiotic and biotic stress responses. These proteins typical have an N-terminal region containing a varying number of repeating motifs including glycine, tyrosine, proline, and glutamine (GYPQ), and in the case of Bs5, these motifs are arranged as five GYPXX repeats[78-81]. The proline-rich N-terminus may form a polyproline II helical structure which serves to solvate adjacent amino acids to promote protein-protein interactions[80-82]. In plants, the 15-16 residues that span the transmembrane domain in CYSTM tail-anchored proteins are highly conserved and tend to have four consecutive cysteines in the transmembrane domain, followed by two hydrophobic amino acids (leucine or isoleucine)[80]. In *Arabidopsis thaliana*, double knockouts of the BS5 homologs, *WIH1* and *WIH2*, lead to loss of development of megasporogenesis[83].

In pepper and tomato, the *BS5* gene is flanked by a nearly identical gene in a head-to-head orientation (*BS5-like*). A recessively inherited resistance allele, *bacterial spot 5* (*bs5*), has been reported to confer disease resistance against different strains of *Xanthomonas* species[76,84]. The *bs5* allele was discovered in a pepper accession in breeding field studies and thus is a naturally derived allele in pepper[76]. The *bs5* allele conferred disease resistance even with hypervirulent strains of *Xanthomonas*. The resistance phenotype it confers lacked the hypersensitive response prevalent in other dominantly inherited NLR resistance genes (*Rx3*, *Bs2*)[85]. The phenotype of bs5 resistance is characterized by lack of symptoms, faint chlorosis at the site of infection, and a reduction in bacterial growth. Commercial pepper varieties containing the *bs5* allele have shown durable resistance, impeding the emergence of hypervirulent strains in commercial fields[71,84]. Deployment of the *bs5* allele in tomato has the potential to confer disease resistance against bacterial spot disease.

The causative mutation of the *bs5* allele in pepper is an in-frame deletion of six nucleotides in the third exon[77]. This mutation causes the deletion of two consecutive leucines within the putative transmembrane domain of the Bs5 protein. This deletion may perturb the tertiary structure of the transmembrane domain, which could lead to alterations in its putative membrane anchoring or facilitation of protein-protein interactions. It is unknown whether the double leucine deletion causes the abrogation of a molecular interaction between the Bs5 protein and other endogenous protein(s). Also, it is unknown whether the double leucine deletion might alter the interaction between the Bs5 protein and a secreted bacterial virulence protein. Although, the direct molecular cause of disease resistance is unknown, altering the nucleotide sequence of tomato *Bs5*

seemed feasible due to the close relatedness of tomato and pepper, as well as both crops being susceptible to infection by the same species of *Xanthomonas*.

It was unknown whether the precise six nucleotide deletion is the sole mutation responsible for conferring resistance against bacterial spot disease or merely mutating other parts of the gene could confer similar resistance. Additionally, it was unknown whether bs5 resistance is broad against other bacteria with similar modes of infection or similar type three secretion machinery. Since pepper and tomato belong to the same family and the need for novel forms of bacterial spot disease resistance in commercial tomato crops, we sought to test *bs5* resistance in tomatoes. We investigated whether introducing nucleotide insertions or deletions in the coding region of tomato *Bs5/Bs5-like* (*SlBs5/SlBs5-L*) would be sufficient to establish resistance.

Our objective was to establish *bs5* resistance in tomatoes using Cas9-based genome editing. By employing the *Agrobacterium*-based transformation method, we stably introduced Cas9-sgRNA into the tomato variety FL8000. We were able to generate a series of gene edited tomato lines with deletions and insertions distinct from the pepper *bs5* allele. Since we did not introduce Cas9 into tomato along with a DNA donor template for the *bs5* allele, we were unable to generate Cas9 edited tomato lines with the precise six base pair deletion found in the pepper *bs5* allele. This is because DSBs produced by Cas9 typically are repaired by error prone NHEJ[29,30]. We were, however, able to generate several distinct gene edited tomato lines with an array of different indel mutations in exon 2 and exon 3 of tomato *Bs5/Bs5-like*. Subsequent generations of these gene edited tomatoes retained homozygosity of their respective mutations. These mutant lines were further studied for their resistance phenotype as well as any detrimental phenotypes due to pleiotropic effects.

2.2 Searching for the *Bs5* gene in Solanaceae family

In order to establish *bs5* disease resistance in tomato, we searched the tomato genome for the homolog to the pepper *BS5* gene. We used the BLAST program in the Sol Genomics Network database to search the total genome (build SL3.0) of tomato for *BS5* homologs[86]. Tomato *Bs5/Bs5-like* genes were found to have the same head-to-head orientation as their homologs from pepper. In both pepper and tomato, *Bs5/Bs5-like* share a putative promoter region. Additionally, sequence alignment in Solanaceae species of putative protein sequences for Bs5/Bs5-like was conducted (Fig. 2).

Bs5/Bs5-like protein sequences were found to be highly conserved between pepper and tomato and other solanaceous species. One key difference between pepper Bs5-like and tomato Bs5-like was a single absent GYPXX motif in the pepper Bs5 amino acid sequence. Since pepper *bs5* disease resistance only required the *bs5* allele (double leucine deletion) in the *Bs5* gene, and not the *Bs5-like* gene, we posited that resistance

might be due to the single absent GYPXX motif in the pepper Bs5-like protein (which may render it non-functional).

Figure 1. Bacterial spot disease symptoms. Formation of dark brown or yellow necrotic lesions on tomato fruit and pepper leaves.

```
SlBs5   MSYYNQQQPPVGVPPPQGYPPEGYSKDAYPPPGYPQQGYPPQGYPQQGYPPPQYAPQYGAPPPQQHQQQSSSTGLMQGCLAALCCCCLLDACF
SlBs5L  MSYYNQQQPPVGVPPPQGYPPEGYPKDAYPPPGYPQQGYPQQGYPPQGY-PPQYAPQYGAPPPHQQQQQS-GTGFMEGCLAALCCCCLLDACF
StBs5   MSYYNQQQPPVGVPPPQGYPPEGYSKDAYPPPGYPQQGYPPQGYPQQGYPPPQYAPQYGAPPPQQQQQQSSSTGLMQGCLAALCCCCLLDAIF
StBs5L  MSYYNQQQPPVGVPPPQGYPPEGYPKDAYPPPGYPQQGYPPQGYPPQGY-PPQYAPQYGAPPP--QQQQSGSTGFMEGCLAALCCCCLLDACF
SmBs5   MSYYNQQQAPVGVPPPQGYPPEGYSKDAYPPPGYPPQGYPQQGYPPQGY-PPQYAPQYGAPPPQQQHQSSSNTGLLQGCLAALCCCCLLDACF
SmBs5L  MSYYNQQQPPVGVPPPQGYPPEGYPKDAYPPPGYPQQGYPQQGYPPQGY-PPQYAPQYGAPPP--QQQQSGSTGFMEGCLAALCCCCLLDACF
JsBs5   MSYYNQQQTPVGVPPPQGYPPEGYSKDAYPPPGYPQQGYPQQGYPPQGY-PPQYAPQYGAPPPQQHQQQSSTTGLMQGCLAALCCCCLLDACF
JsBs5L  MSYYNQQQPPVGVPPPQGYPPEGYPKDAYPPPGYPQQGYPPQYAPQYGA-PP---PQYGAPPPHQQQQQSGSSGFMEGCLAALCCCCLLDACF
CaBs5   MSYYNQQQPPVGVPPPQGYPPEGYPKDSYPPPGYPQQGYPQQGYPPQGY-PPQYAPQYGAPPPQQQHQSSSSTGLLQGCLAALCCCCLLDACF
CaBs5L  MSYYNQQQPPVGVPPPQGYPPEGYPKDAYPPPGYPQQGYPP-----QGY-PPQYAPQYGAPPSQQQQQ-SGSSGFMEGCLAALCCCCLLDACF
ItBs5   MSYYNQQQPPVGVPPPQGYPPEGYPKDAYPPPGYPQQGYPQ-----QGY-PPQYAPQYGAPPPQQQQQSSSSSGLMQGCLAALCCCCLLEACF
ItBs5L  MSYYNQQQPPVGVPPPQGYPPEGYPKDAYPPPGYPQQGYPQ-----QGY-PPQYAPQYGAPPPQQQQQ-SSSGGFMEGCLAALCCCCLLDACF
```

Figure 2. Multiple amino acid sequence alignment of Bs5/Bs5-like proteins in Solanaceae. Clustal OMEGA multiple amino acid sequence alignment of putative Bs5 proteins from Nightshade family. The multiple sequence alignment was constructed with Bs5 and Bs5L from *Solanum lycopersicum* (tomato; Sl), *Solanum tuberosum* (potato; St), *Solanum melongena* (eggplant; Sm), *Jaltomata sinuosa* (Js*), Capsicum annuum* (pepper; Ca), and *Iochroma cyaneum* (Ic). The conserved GYPXX motif is underlined in red while and cysteine-rich transmembrane module (CYSTM) which includes the double leucine is highlighted in yellow.

2.3 Predicting the structure of Bs5 protein

The CYSTM superfamily of proteins has been implicated in biotic and abiotic stress response across different eukaryotes including plants[80]. The Bs5 protein family is part of the CYSTM superfamily and is found in different plant species even outside of the Solanaceae family. Tail-anchored membrane prediction was done using the TMpred program[87]. The TMpred program makes a prediction of membrane-spanning regions and their orientation. The algorithm is based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins. Results found that amino acids 74-88 in Bs5 protein sequence were strongly favored to be integrated into a membrane (Fig. 3a). RoseTTAFold is a "three-track" neural network that distinguishes patterns in protein sequences, predicts amino acid residue interactions, and solves putative three-dimensional structure of proteins[88]. The network enables rapid generation of protein structure models from amino acid sequence information alone. The predicted Bs5 protein was modelled using the RoseTTAFold program. The RoseTTAFold program predicted a highly disordered pepper Bs5 protein with a predominantly random coiled N-terminal and a C-terminal helix in the putative transmembrane domain (Fig. 3b).

Although the Bs5 protein is part of a family of tail anchored CYSTM proteins, the molecular mechanisms involved in conferring bacterial spot disease resistance in *bs5* pepper plants are not understood. Due to the small size and abundance of prolines in the amino acid sequence of the Bs5 protein (~92 amino acids), it was predicted that protein purification, solving of the three-dimensional structure, and biochemical functional studies would be quite difficult.

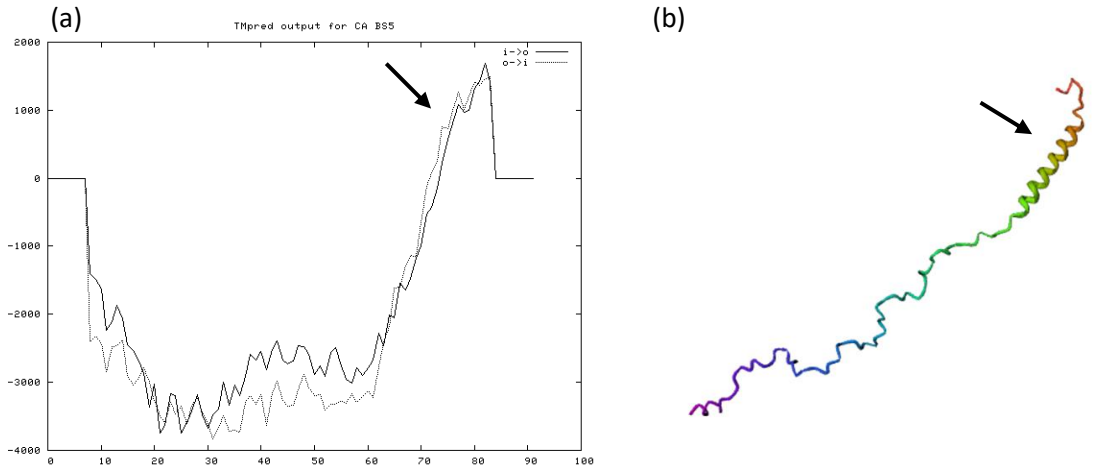

Figure 3. Results for pepper Bs5 protein from TMPred and RoseTTAFold programs. (a) Hydrophobicity plot of Bs5 obtained using TMPred Server. X axis represents amino acid sequence from N- to C-terminal and Y axis represents hydrophobicity scores computed by server. Scores above 500 are considered significantly hydrophobic. Figure shows which of the inside-> outside (i-> o) helices correspond to which of the outside-> inside (o-> i) helices**. (b)** Predicted protein-folding structure using RoseTTAFold server shows disordered random coil N-terminus and a single helical structure at the putative transmembrane region. The putative membrane-spanning region is marked by an arrow.

2.4 Gene expression of *Bs5/Bs5-like* genes in tomato

It was unknown whether the *Bs5/Bs5-like* genes in tomato were expressed and whether transcriptional changes in *Bs5/Bs5-like* genes occurred upon *Xanthomonas* infection. The RNA-seq database, TomExpress, provides gene transcriptional data for an array of conditions and different tomato plant tissues[89]. Transcriptional data from this database not only showed that *Bs5* and *Bs5-like* genes are expressed in tomato, but that are differentially expressed in a tissue-dependent manner (Figure 4a). Data showed that *Bs5* is more highly expressed during fruit development than *Bs5-like,* and that *Bs5* and *Bs5-like* are both expressed at similar levels in leaves and flowers.

We also conducted RNA-seq analysis in the FL8000 tomato variety. Tomato plants were inoculated with *X. gardneri* or mock control and transcript levels of *Bs5/Bs5-like*, along with paralogs, were quantified. Upon *Xanthomonas* inoculation, tomato leaves underwent at least 3 orders of magnitude increase in transcript abundance of *Bs5*, while there was no significant change in transcript abundance of *Bs5-like*. One thing to note is that the *Solyc05g01606.4* paralog of *Bs5* also increased in transcript abundance (although modestly compared to *Bs5)*.

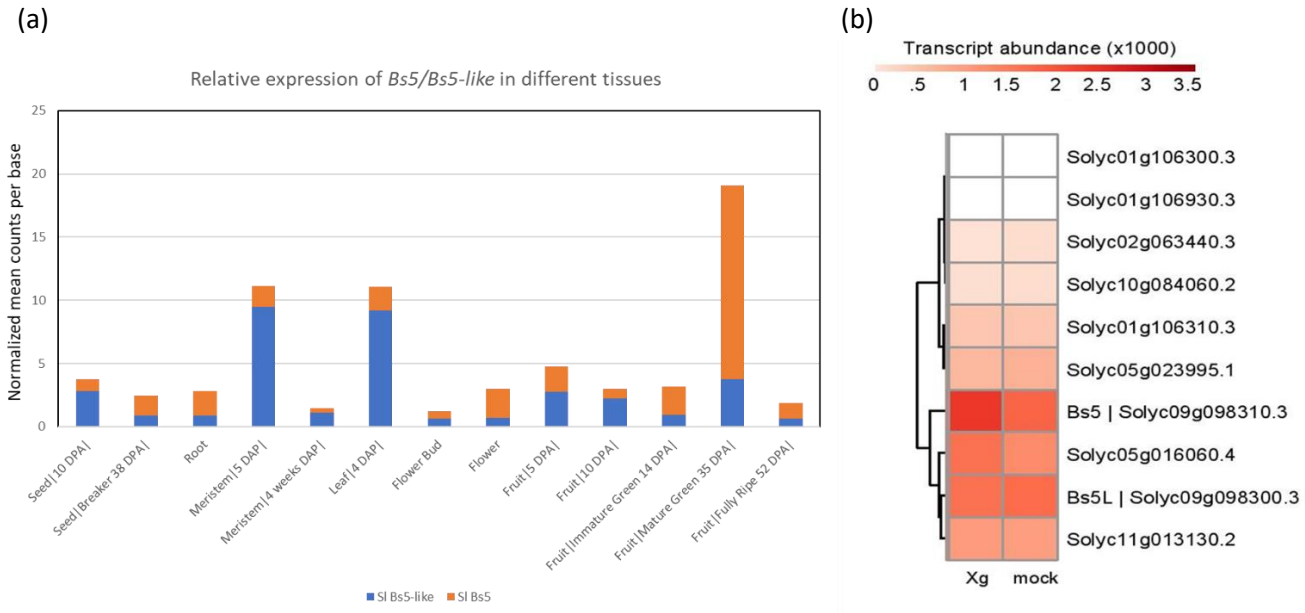

Figure 4. Expression of Bs5/Bs5L and paralogs in tomato. (a) Relative expression of *Bs5* and *Bs5L* in tomato leaves, flowers, and fruits at different stages of development. Expression data was obtained from the TomExpress RNA-seq database. DAP, days after planting; DPA, days post anthesis. **(b)** Upregulation of *Bs5* gene expression in in tomato in response to inoculation with *Xanthomonas gardneri*. Other tomato paralogs of *Bs5/Bs5-like* also included.

2.5 Cas9-mediated gene editing of Bs5/Bs5-like in tomato

The *Bs5/Bs5-like* locus is flanked by a transcription factor, *WD40*, on one side and a receptor-like kinase flanking, *RLK*, on the other side (Fig. 5/Fig. S2). The *Bs5-like* gene is upstream of the *Bs5* gene, and they are separated by only ~3 kb. The transcription factor WD40 is downstream of *Bs5* while the RLK is upstream of *Bs5-like*. *Bs5* and *Bs5-like* also share a promoter region in a head-to-head manner with *Bs5* expressing in the opposite direction of *Bs5-like*. The *Bs5/Bs5-like* locus is in chromosome 9 in tomato while it is in chromosome 3 in pepper.

In order to establish *bs5* disease resistance in tomato, we designed and implemented a Cas9-mediated gene editing strategy (Fig. 6). A binary vector containing Cas9 driven by the CaMV 35S constitutive promoter, along with U6-expressing sgRNA, was introduced into tomato (FL8000 variety) using *Agrobacterium*-mediated transformation. We implemented a sgRNA targeting exon 2 of *Bs5* and *Bs5-like* simultaneously and an sgRNA targeting exon 3 of *Bs5* and *Bs5-like* simultaneously (Fig. 7). Targeting *Bs5* and *Bs5-like* simultaneously was possible because target sequences for both sgRNAs were conserved in both *Bs5/Bs5-like* genes. Tomato transformants were subsequently regenerated under antibiotic selection using in vitro plant tissue culture. Afterward, regenerants were transferred to soil for propagation and self-pollination.

Genomic DNA from tomato leaf tissue from T_0 transformants and T_1 progeny from self-pollination was extracted and fragments of Cas9 transgene and gene target sites in *Bs5/Bs5*-like were amplified using PCR (Fig. 8). Transformants contained an array of different mutations at the sgRNA target site that include insertions and deletions (Fig. 8/Fig. S1). These mutations resulted in perturbation of the subsequent amino acid composition of the translated protein. In some cases, such as those altered at exon 2, mutations led to the formation of a pre-mature stop codon (Fig. 8/Table 1).

In subsequent generations, the T-DNA containing Cas9-sgRNA was either segregated away from the mutant line or the mutant line was cross-pollinated with a wild-type FL8000 variety to ensure removable of the T-DNA in subsequent generations.

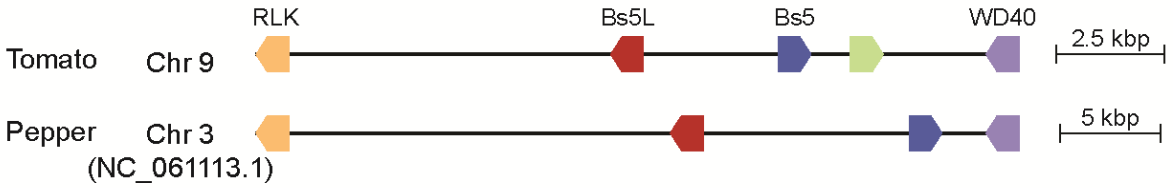

Figure 5. The genomic organization around Bs5 and Bs5L in Solanaceae species. The simplified genomic organization around *Bs5* and *Bs5L* in Solanaceae species. The schematics reflects the positions of the center of the genes but not the size of the genes. The putative orthologs are indicated with the blocks in the same colors. *Bs5* and *Bs5-L* both flanked by RLK and WD40.

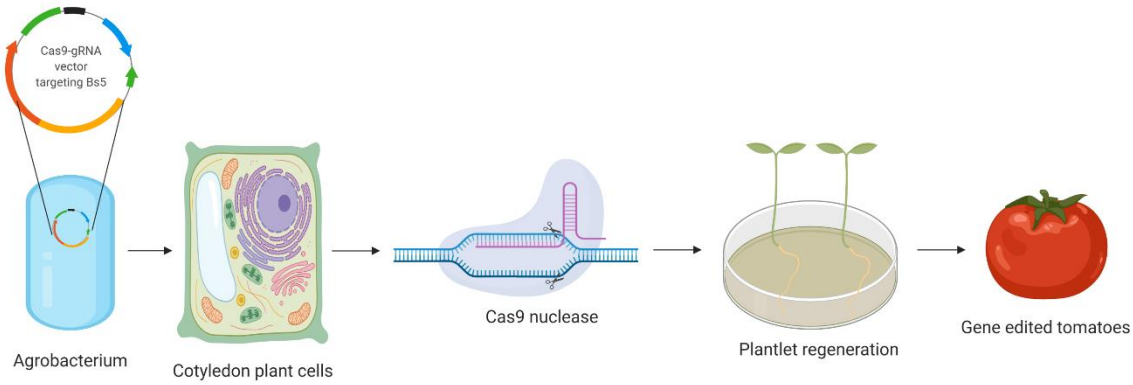

Figure 6. Diagram of Cas9-mediated gene editing strategy of tomatoes. Cas9-sgRNA was introduced into plant cells via Agrobacterium-mediated transformation. Transformants were regenerated and fruit was harvested for subsequent genotyping and experiments.

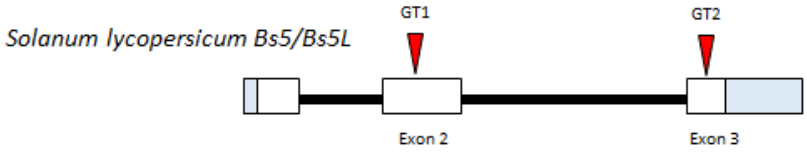

Figure 7. CRISPR/Cas9 gene targeting. Schematic diagram showing CRISPR/Cas9 genetic targets in exon 2 and exon 3 of both *Bs5* and *Bs5L* in tomato. Target sites are marked by red arrows. Exon 2 from both *Bs5* and *Bs5L* was simultaneously targeted by sgRNA, GT1, and exon 3 from both *Bs5* and *Bs5L* was simultaneously targeted by sgRNA, GT2.

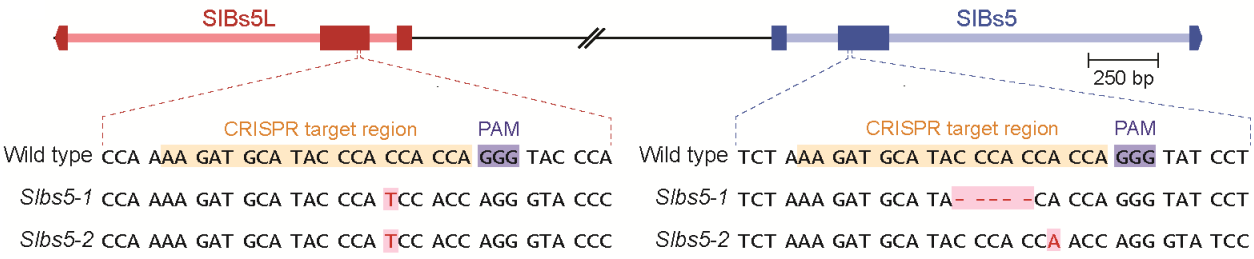

Figure 8. CRISPR/Cas9 exon 2 gene targeting and genotyping. Schematic diagram showing CRISPR/Cas9 genetic targets in exon 2 for both *Bs5* and *Bs5L* in tomato. There was a 1 base pair insertion mutation in *SlBs5-L* of both mutants, while in *SlBs5*, a 5 base pair deletion in *Slbs5-1* and a 1 base pair insertion in *Slbs5-2*.

Table 1. Primers used for genotyping of tomatoes transformed with Cas9

Amplicon target	Forward primer	Reverse primer
SlBs5 exon 2	GGCTTAGTCCTTGTTTGACT	TCATAACAAAACATCACGAGTG
SlBs5-like exon 2	CACACACATAGTAACAGACAAGAC	CCACCACAAGGTAAATCACTT
SlBs5 exon 3	GCTGTACAATATCATGTTTCTC	GTAACCAGAGCAAGGTGC
SlBs5-like exon 3	TTGTCTATAGCTATAGGTTTTCC	TTGGCAAATAGACACTTGAATTC

Table 2. Summary of mutant tomato lines generated by Cas9 gene editing. Changes in amino acid content marked by red letters.

Line	Bs5 allele	Bs5-L allele	Bs5 protein	Bs5-L protein
Wildtype	--	--	MSYYNQQQPPVGVPPPQGYPPEGYSKDAY PPPGYPQQGYPPQGYPQQGYPPPQYAPQY GAPPPQQHQQQSSSTGLMQGCLAALCCCC LLDACF*	MSYYNQQQPPVGVPPPQGYPPEGYPKDAYPPP GYPQQGYPQQGYPPQGYPPQYAPQYGAPPPH QQQQQSGTGFMEGCLAALCCCCLLDACF*
Slbs5-1	5 bp deletion, exon 2	1 bp insertion, exon 2	MSYYNQQQPPVGVPPPQGYPPEGYSKDAY TRVSSARVSTTGLSSTRVSTSTVCTSVWCST SSTTSTAI*	MSYYNQQQPPVGVPPPQGYPPEGYPKDAYPPT RVPTAGLPSTRLPTSRVPSTVCTSVWCSTSSST TTAIWYWFHGRMFGCSVLLLSLGCMLLML*
Slbs5-2	1 bp insertion, exon 2	1 bp insertion, exon 2	MSYYNQQQPPVGVPPPQGYPPEGYSKDAY PPTRVSSARVSTTGLSSTRVSTSTVCTSVWC STSSTTSTAI*	MSYYNQQQPPVGVPPPQGYPPEGYPKDAYPPT RVPTAGLPSTRLPTSRVPSTVCTSVWCSTSSST TTAIWYWFHGRMFGCSVLLLSLGCMLLML*
Slbs5-3	7 bp deletion, exon 3	1 bp insertion, exon 3	MSYYNQQQPPVGVPPPQGYPPEGYSKDAY PPPGYPQQGYPPQGYPQQGYPPPQYAPQY GAPPPQQHQQQSSSTGLMQGCLAALCCSW MHAFEGVNDLCHVLMAKVY*	MSYYNQQQPPVGVPPPQGYPPEGYPKDAYPPP GYPQQGYPQQGYPPQGYPPQYAPQYGAPPPH QQQQQSGTGFMEGCLAALCCCCLLGCMLLML*

2.6 Discussion

We found that the orthologs of pepper *Bs5/Bs5-like* genes were also present in the tomato genome. Gene expression data collected from RNA-seq databases and from our own experiments showed that the tomato *Bs5* gene is expressed and upregulated both during fruit development and *Xanthomonas* infection in leaf. We were successful in generating an allelic series of mutant tomato lines (Table 2) with frameshift mutations in *Bs5/Bs5-like* exon 2 (*Slbs5-1* and *Slbs5-2*) and exon 3 (*Slbs5-3*). For *Slbs5-1*, a five base

pair deletion in exon 2 of *Bs5* leads to a premature stop codon which could considerably perturb the function of the Bs5 protein. We thus consider this mutation a putative gene knockout of *Bs5*. In exon 2 of *Bs5-like*, a one base pair insertion also leads to a frameshift but does not lead to a premature stop codon. Nevertheless, the amino acid content of the mutant sequence is significantly different from that of the wildtype.

As for *Slbs5-2*, the mutation in exon 2 of *Bs5-like* is identical to that of *Slbs5-1*. The difference between the two mutant lines is in the *Bs5* gene, *Slbs5-2* has one base pair insertion. This mutation also leads to a premature stop codon and significant changes the amino acid sequence which we would consider a gene knockout. For *Slbs5-3*, the mutation is in the third and last exon of *SlBs5/SlBs5-L*. Exon 3 contains the codons for the amino acids that comprise the putative cysteine-rich transmembrane domain. In fact, we were fortunate to have found a PAM sequence adjacent to the nucleotides encoding the double leucine. For *SlBs5* in *Slbs5-3*, the 7 base pair deletion introduced by Cas9 resulted in deletion of both the two leucines, as well as two cysteines, within the putative transmembrane module. Since this gene target is in the last exon, any mutations introduced into this region would not lead to significant upstream frameshifts or changes in the N-terminal region of the protein. We hypothesized that mutating the putative transmembrane domain would lead to a resistance phenotype like that of *bs5* in pepper since the causative mutation of pepper *bs5* resistance is in the transmembrane domain.

2.7 Materials and Methods

Identification of Bs5 orthologs in Solanaceae

We collected genome assemblies and annotations for available Solanaceae species (Table S1). From each species, we identified the genomic region syntenic with Chromosome 9 of *S. lycopersicum* that contains *SlBs5* and *SlBs5L*. We used reciprocal best BLAST search with BLASTP v2.9.0+ to identify orthologs *Bs5L* (Solyc09g098300.3.1), *Bs5* (Solyc09g098310.3.1), (ITAG 4.0) [91,92]. The genomic region containing putative orthologs of all or most of these queries were examined. We used Exonerate v2.4.0 to confirm the absence of the putative orthologs in this region[93]. If the orthologs were present without being annotated, their gene models were annotated. The orders and structures of the genes and genome architecture in this syntenic region were collectively compared to the tomato genome to corroborate the presence of *Bs5* and *Bs5L* orthologs.

Clustal Omega, TMPred, and RoseTTAfold programs

Clustal Omega Multiple Sequence Alignment Tool was accessed via the EMBL-EBI tools web-interface server, Clustal Omega[87]. TMpred Tool was accessed via the Oxford web-interface server, Transmembrane Prediction Server[88]. RoseTTAFold was accessed via the RosettaCommons web-interface server, Robetta[89].

TomExpress transcriptional data

Tomato transcriptional data was acquired from TomExpress RNA-seq database[90].

RNA-seq analysis

Wildtype FL8000 plants were used for RNA sequencing. Leaves from each plant were syringe infiltrated with mock solution (10 mM $MgCl_2$) or a *X. gardneri* suspension (OD_{600nm}=0.25). Six hours after syringe infiltration, leaf disc samples were collected, and total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma, STRN250). A total of 24 RNA sequencing libraries were prepared using the Illumina TruSeq Stranded mRNA Library Prep (illumina 20020594). All libraries were sequenced as 50bp single-end reads on a HiSeq 4000 sequencing platform. Illumina adapters and low-quality reads were removed from the sequenced libraries using Trim Galore v0.6.4 (--illumina -q 20) (bioinformatics.babraham.ac.uk). The filtered reads were aligned to the reference genome (SL 4.0) using STAR v2.6.1c[94]. We used primary alignments for gene counting using FeatureCounts v1.6.3, and edgeR to analyze differentially expressed genes (DEGs)[95,96]. For two compared conditions, DEGs were defined to have |log2 Fold Change| >= 1 and false discovery rate (FDR) < 0.05. We employed eggNog-mapper to obtain functional annotations for ITAG 4.0, and clusterProfiler to perform gene ontology enrichment tests using the functional annotations of the DEGs[97,98].

Cas9-mediated inactivation of *SlBs5* and *SlBs5-like* genes

Two single guide RNAs (sgRNAs) were used to target SlBs5 (Solyc09g098310.3) and SlBs5-like (Solyc09g098300.3) simultaneously. One sgRNA targeted both *SlBs5* and *SlBs5-like* in exon 2 (GT1), and another sgRNA targeted both *SlBs5* and *SlBs5-like* in exon 3 (GT2). Each guide was independently cloned into a pENTR/D-TOPO-based entry plasmid containing the *Arabidopsis* U6-26 promoter to drive gRNA expression and an enhanced double 35S promoter driving Cas9 expression. A gateway LR reaction (Thermo Fisher Scientific) was used to move the gRNA and Cas9 cassette into a pPZP200-based binary vector[99]. Using the Agrobacterium tumefaciens co-cultivation method, the binary construct was used for transformation into the FL8000 variety at the Innovative Genomics Institute Transformation Core Facility. Kanamycin-resistant plants were genotyped, and the selected mutants were self-pollinated for the use in subsequent experiments. All primers are listed in Table 1.

2.8 Additional figures.

Figure S1: CRISPR/Cas9 gene targeting and genotyping. Genotype for three mutant lines of tomato aligned with the wild-type sequence (top). Gene target sequence for exon 2 or exon 3 underlined. PAM sequence written in blue.

Table S1: Solanaceae species used for genomic analyses for Bs5/Bs5L homologs

Species	Common name	Version	Annotation file
Solanum lycopersicum	Tomato	ITAG 4.0	ITAG4.0_proteins.fasta
Solanum tuberosum	Potato	DM 1-3 516 R44 v6.1	DM_1-3_516_R44_potato.v6.1.repr_hc_gene_models.pep.fa.gz
Solanum melongena	Eggplant	V4.1	Eggplant_V4.1_protein.function.fa
Jaltomata sinuosa		GCA_003996215.1_ASM399621v1	N/A (Genome assembly only)
Capsicum annuum	Pepper	GCF_002878395.1_UCD10Xv1.1	GCF_002878395.1_UCD10Xv1.1_protein.faa.gz
Iochroma cyaneum		V1.0	IC_v1.0_gene_models.gff
Lycium barbarum	Wolfberry	GCA_019175385.1_ASM1917538v1	Lba.Final.coding.gene.pep (From the authors)
Nicotiana benthamiana		V261	Niben261_genome.annotation.proteins.fasta.gz

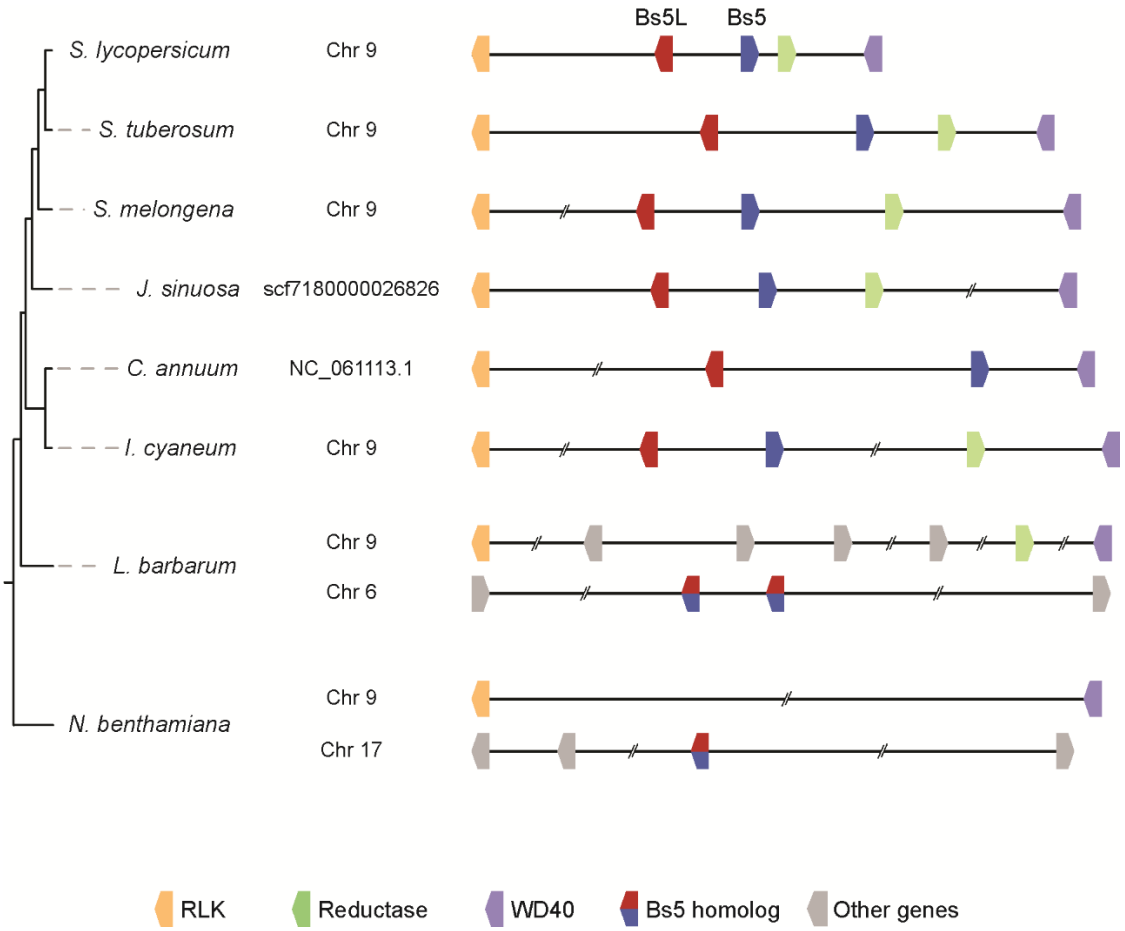

Figure S2: Genomic organization of *Bs5/Bs5-L* in Solanaceous species. Aligned genomic organization schematic for several Solanaceous species. Bs5/Bs5-L is flanked by WD40 and RLK genes in most species except for *L. barbarum* and *N. benthamiana*.

CHAPTER 3: Evaluating the resistance phenotype in bs5 tomato mutants

3.1 Introduction

Infection of plants by *Xanthomonas* starts with the translocation of effector proteins into the plant host cytoplasm through the T3SS. Once inside the plant cell, these effector proteins promote virulence and susceptibility, causing eventual disease symptoms. Bacterial spot disease symptoms are characterized by yellow, dark brown, or black spots on leaves and fruit. Older spots tend to be of a darker color or black. Large leaf blotches may also occur, especially in cases of high disease severity. In severe cases, these spots lead to necrotic lesions and can lead to unmarketable fruit or plant death.

The *bs5* resistance gene has been successfully deployed in commercial pepper varieties for the last decade[77,84]. In commercial fields, *bs5* peppers exhibited robust, durable resistance and impeded the selection of hypervirulent strains of *Xanthomonas* species after several years. The resistance phenotype in *bs5* peppers consists of a minimum of a 10-fold reduction in internal bacterial population size as well as lack of necrotic lesion formation. The *bs5* resistance gene has been introgressed into pepper varieties containing the NLR resistance gene, *Bs2*. The *Bs2* resistance gene detects the virulence effector, *avrBs2*, secreted by *Xanthomonas*. Upon detection of *avrBs2*, *Bs2* induces a hypersensitive response at the site of infection. Cell death at the site of infection is an immune mechanism that is employed by plants in order to reduce the risk of systemic movement of pathogens[85]. Pepper varieties containing both *Bs2/bs5* lacked the hypersensitive response phenotype upon *Xanthomonas* infection but retained a robust resistance phenotype due to less growth and thus less likelihood of necrotic lesion formation. Introduction of the *bs5* gene into varieties of crops like pepper or tomato containing other disease resistance genes (gene stacking) may help impede the emergence of hypervirulent strains and extend the durability of NLR and PRR genes since there will be much lower population sizes of the pathogen to select upon.

In order to test the possible utility of the *bs5* resistance gene in other tomato crops, we devised a series of experiments to test the resistance phenotype of our Cas9 gene edited tomatoes. Since it was unknown whether the six base pair deletion in the third exon of pepper *Bs5* was the only allele sufficient to confer resistance, we opted to target two different sites in tomato *Bs5/Bs5-like* genes (exon 2 and exon 3). Due to the low rates of homology-directed repair in tomato, we did not attempt a precise edit of the tomato Bs5 gene to establish a six base pair deletion.

These Cas9-edited mutant tomato lines showed resistance phenotypes like the phenotype seen *bs5* in pepper against several species of *Xanthomonas*. No apparent physiological defect or phenotype detrimental to growth or yield was seen in *bs5* tomatoes in either growth chamber or field trial studies.

3.2 Bacterial spot resistance phenotype evaluation of mutant tomato lines

We tested the progeny of our T_0 Cas9-edited tomato lines by conducting bacterial growth assays using three species of *Xanthomonas (X. gardneri, X. perforans, and X. euvesicatoria)*. *Xanthomonas spp.* bacteria were cultured on media and then infiltrated into the leaves of our gene edited tomato lines. As a control, the susceptible wildtype, FL8000 variety, was also infiltrated with bacteria. Quantification of internal bacterial populations was done over a course of a week. Every gene edited tomato line exhibited a similar resistance phenotype to that of pepper *bs5* (Fig. 9, 10, 11). Internal bacterial populations were reduced by an order of magnitude in all gene edited tomato lines. Infiltrated leaves also exhibited a significant reduction of necrotic lesions compared to that of susceptible wildtype, FL8000. We also tested the virulence of *Pseudomonas syringae pathovar tomato* strain R2 that also uses the type three secretion system to our *bs5* resistant tomatoes. Unfortunately, our *bs5* mutant tomato lines showed no resistance to this pathogen, exhibiting similar symptoms and allowing similar bacterial growth as that of susceptible wildtype, FL8000 (Fig. 12).

Our gene edited tomato lines were tested for susceptibility bacterial spot disease symptoms from topically applied inoculum, more typical of natural infections. Instead of using the leaf infiltration method to inoculate tomato leaf with *Xanthomonas*, we used a dip inoculation method in which cells are suspended in a low concentration of a surfactant. Dip inoculation was done in order to conserve the integrity of the tomato leaf as syringe infiltration typically leads to more abrasions than dip inoculation. Similar to that seen in *bs5* resistant pepper varieties, our mutant tomato lines showed fewer bacterial spot disease symptoms compared to the susceptible wildtype plant (Fig. 13).

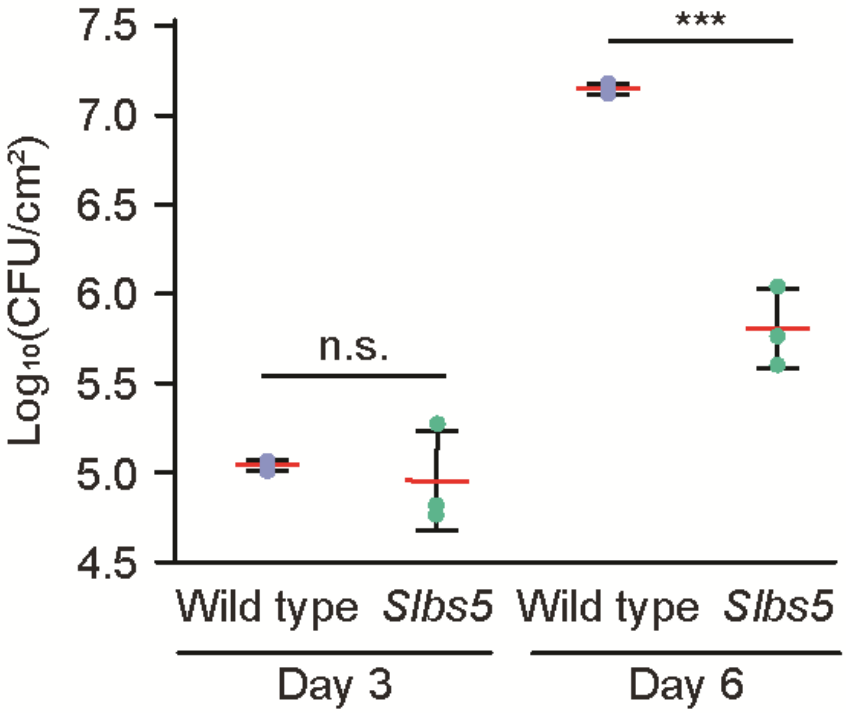

Figure 9. Growth of *X. perforans* GEV485 on *Slbs5-1* line. Mutant line *Slbs5-1* showed significant reduction (>10-fold) in internal bacterial populations in leaf over the course of 6 days. Significant differences between population sizes were determined by using a one-tailed t-test. Asterisks represent significant differences in bacterial population values from t tests: $P < 0.001$. No significant difference in population sizes as denoted by n.s.

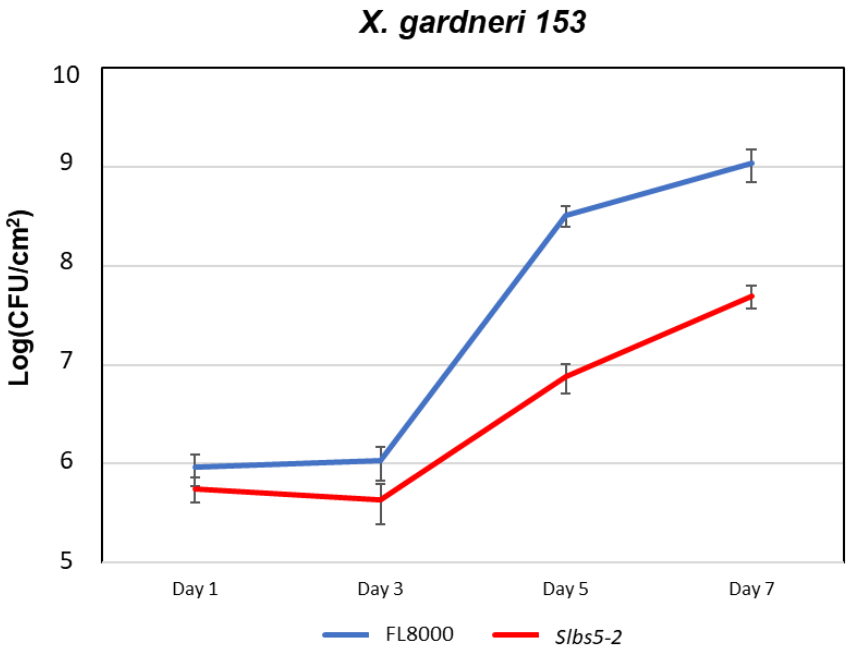

Figure 10. Internal growth of *X. gardneri 153* assessed over 7 days. *Slbs5-2* was found to support 10-fold lower internal bacterial populations after 7 days in fully expanded leaves.

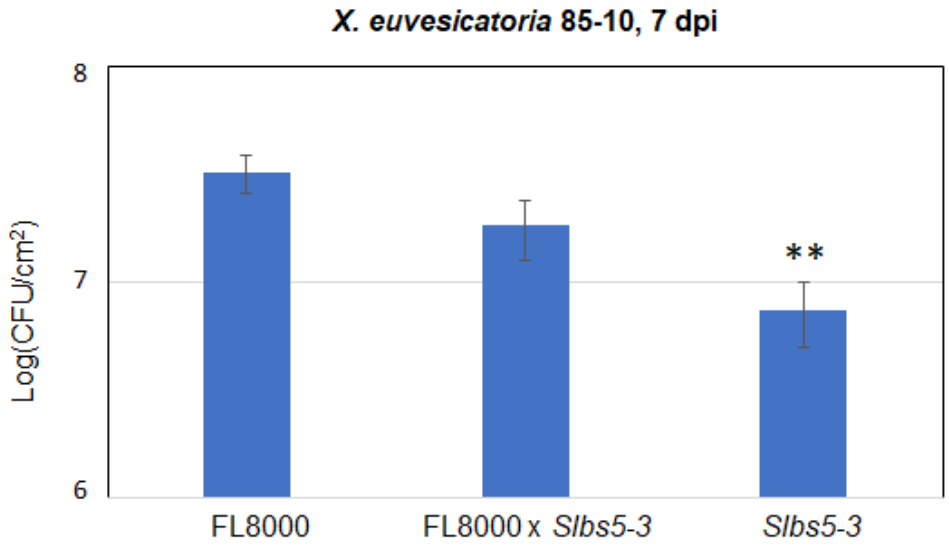

Figure 11. Internal growth of *X. euvesicatoria 85-10* after 7 days. Internal bacterial populations were significantly reduced in *Slbs5-3* mutant tomatoes. A heterozygous cross of wildtype FL8000 and *Slbs5-3* was also tested. Significant differences between population sizes were determined by using a one-tailed t-test. Asterisks represent significant P values from t tests: $P < 0.01$.

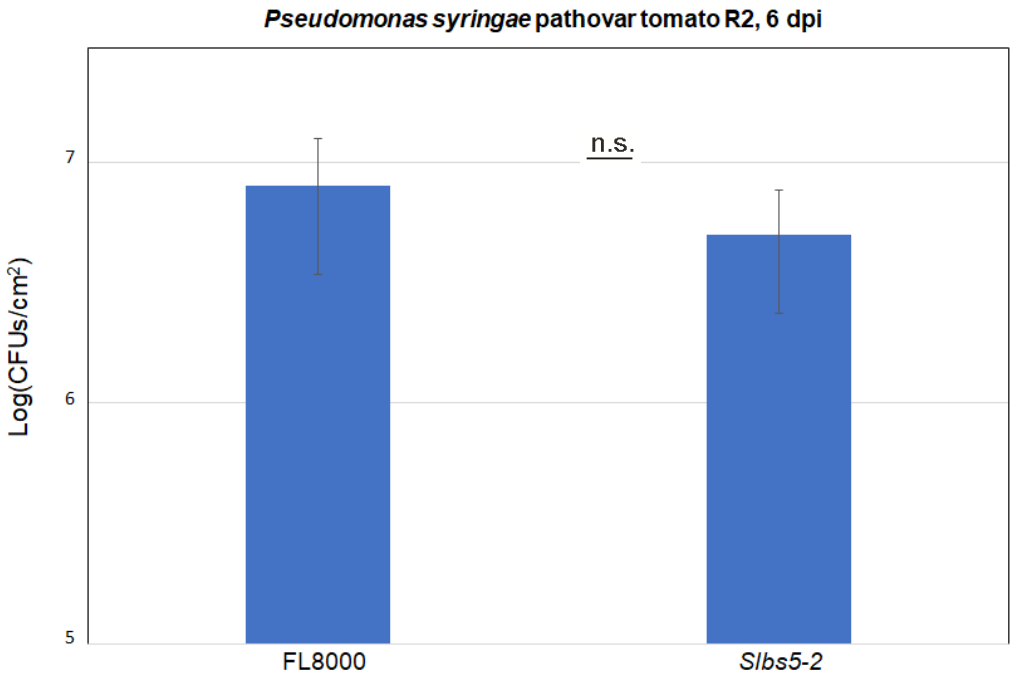

Figure 12. Growth of *P. syringae* pv. tomato R2 in *Slbs5-2* line. No significant reduction of internal bacterial growth is seen in *Slbs5-2* 6 days post infiltration. Asterisks indicate significant differences between population sizes as determined by using a one-tailed t-test. No significant difference in population sizes as denoted by n.s.

Figure 13. Bacterial spot disease symptoms in *Slbs5* tomato lines inoculated by dipping in bacterial suspension. Susceptible wildtype variety, FL8000, showed significant more necrotic lesions 20 days post inoculation than FL8000, *Slbs5-3* and *Slbs5-1*.

3.3 Biochemical evidence of bacterial effector translocation in *bs5* resistant tomatoes

In a previous study in which the *bs5* allele was introgressed into peppers containing the *Bs2* resistance gene, no hypersensitive response phenotype developed after bacterial inoculation[84]. Typically, *Bs2* will confer the hypersensitive response upon detection of the translocated avrBs2 effector from *Xanthomonas spp*. Since *bs5/Bs2* exhibited no hypersensitive response, it was posited the *bs5* resistance allele somehow impeded the T3SS translocation of effector proteins from the bacterium. The inhibition of T3SS translocation may be one of the mechanisms by which *bs5* confers resistance against *Xanthomonas,* since secreted effector proteins are generally required by bacterial pathogens to increase grow in plants.

We thus assess whether our Cas9-edited *bs5* mutant tomato lines inhibited bacterial effector translocation. In order to biochemically measure effector translocation from *Xanthomonas* bacteria into plant cells through the T3SS, the calmodulin-dependent adenylate cyclase (Cya) assay was used in our experiments[100]. We used the Cya domain of the *Bordetella pertussis* cyclolysin as a reporter protein to detect the translocation of the *Xanthomonas* effector protein, AvrBs2, into our Cas9-edited *bs5* tomato lines. Adenylate cyclase activity leads to the production of cAMP and is dependent on the presence of plant calmodulin. Adenylate cyclase activity can only occur upon translocation from the prokaryotic cell into the plant host cell. Translocation of the fusion reporter protein is dependent on the T3SS. This is due to mutations in the hrcV gene, which render the T3SS system non-functional, resulting in no fusion reporter protein translocation.

The experiment employed the use of a strain of *Xanthomonas gardneri* expressing a fusion protein comprised of amino acids 1-100 of avrBs2[100]. The first 100 N-terminal amino acids of the avrBs2 protein contain the secretion and translocation signals that are necessary for successful translocation into plant cells via the T3SS. We hypothesized that our Cya assays would show a marked reduction in effector translocation of $avrBs2_{1\text{-}100AA}$:Cya in our *bs5* mutant lines compared to wildtype FL8000. As expected, our allelic series of resistant mutant tomato lines all showed a significant reduction in translocation of effector proteins when compared to susceptible, wildtype tomatoes (Fig. 14).

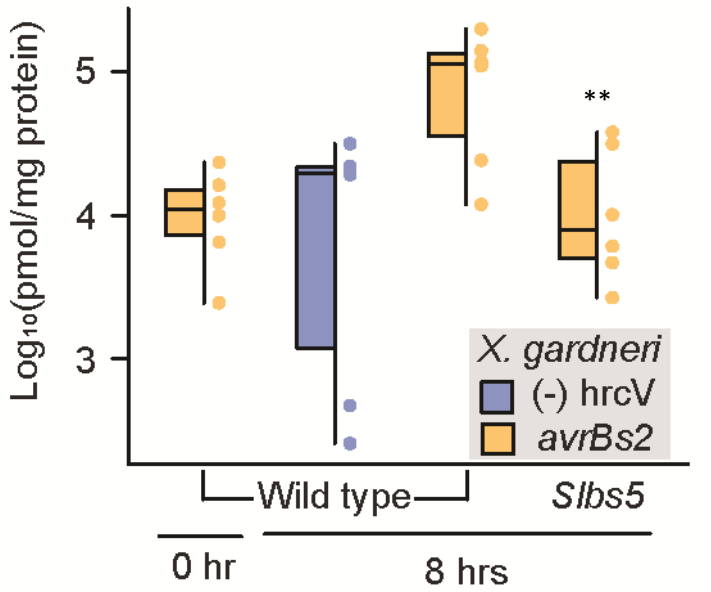

Figure 14. Cya reporter assay on *bs5* resistant tomato lines. Biochemical reporter assay shows that *bs5* resistant tomato line *Slbs5-1* impedes translocation of avrBs2$_{1\text{-}100AA}$: Cya 10-fold 8 hours post infiltration. Box and whisker plot generated using the mean and standard deviation of cAMP levels with individual counts superimposed. Significant differences between cAMP levels were determined by using a one-tailed t-test. Asterisks represent significant P values from t tests: $P < 0.01$.

3.4 Field trials of *Slbs5-2* line in Florida

Although the results from our initial bacterial growth and disease symptom assays were promising in showing lower bacterial growth within leaves and thus lower numbers of lesions, we wanted to further evaluate our tomato lines subject to natural infection in commercial tomato fields. Conducting disease symptom assays in growth chambers in which parameters like temperature and humidity were controlled would not have informed us about the practicality of *bs5* mutant tomatoes in commercial fields.

Demonstrating the usefulness of *bs5* tomatoes in the field would establish the potential for its use as a genetic source of resistance in commercial tomato varieties and as an addition to an already existing repertoire of disease resistance genes. We therefore conducted field trials in Florida where the climate is generally humid and conducive for disease and where commercial tomato fields are naturally challenged with inoculum of *Xanthomonas spp*.

Four field trials were conducted with the *Slbs5-2* mutant tomato line at the Gulf Coast Research and Education Center during in 2018 and 2019. After greenhouse propagation from seeds, plants were transplanted to the field and were inoculated with a four-isolate cocktail of *Xanthomonas perforans* race T4. *Slbs5-2* was rated lower in disease severity compared to wild type FL8000 and disease severity on leaves was found

to be lower in *Slbs5-2* plants (Fig. 15) although the difference was not significant (P value > 0.05). There was no significant difference in marketable yield of fruit between *Slbs5-2* and FL8000 either (Fig. 16).

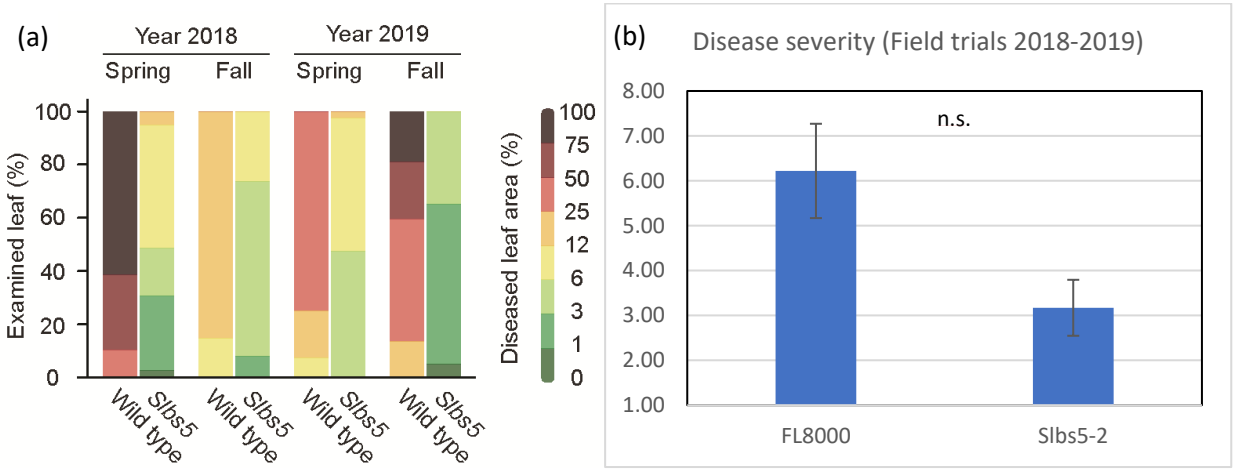

Figure 15. Disease severity of *bs5* tomato mutants in field trials. (a) Examined leaf percentage and diseased leaf area percentage were lower in *Slbs5-2* than FL8000. **(b)** *Slbs5-2* tomato line exhibited less % disease severity compared to wild type FL8000 across several seasons. Despite lower disease severity, the differences in disease severity were not significant (n.s.). Statistical significance was determined using a one paired t-test (P = 0.5).

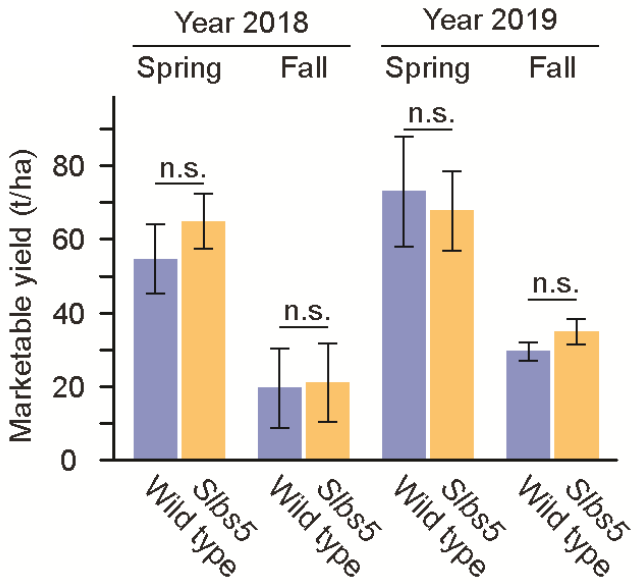

Figure 16. Marketable yield of *bs5* tomato fruits in field trials. Across several growing seasons, *Slbs5-2* did not show a significant difference in marketable yield of tomato fruit compared to wildtype FL8000. Statistical analysis to determine significant differences was done using a one-tailed ANOVA with Bonferroni Correction (P > 0.05).

3.5 Discussion

Our study demonstrates the utility of *bs5* for disease resistance gene in tomato. We employed Cas9 to establish mutations in the homolog of *Bs5* in tomato and observed disease resistance of our mutant lines. Our three *bs5* mutant tomato lines showed a significant lack of necrotic lesion symptoms characteristic of bacterial spot disease infection. Additionally, the internal populations of *Xanthomonas* inside leaves was reduced by 10-fold when measured several days after infiltration into leaves. This reduction in internal growth of the pathogen is similar to that seen in *bs5* resistant peppers.

Impeding growth populations in *Xanthomonas* within leaves is crucial to prevent systemic infection in young plants. Infection during the early stages of plant development is particularly damaging and can lead to yield loss in commercial fields. Our results indicate that not only do *bs5* resistant tomatoes inhibit internal population growth of *Xanthomonas*, but they also inhibit the translocation of effector proteins from bacterium to plant cell. The blocking of effector secretion into the plant cell may be part of the mechanism that inhibits internal bacterial growth in the leaf since effectors are required for modification of the apoplast such as to increase water soaking[103]. The molecular mechanisms in which the *bs5* resistance gene inhibits bacterial growth and effector translocation remains unknown.

Deploying *bs5* with other disease resistance genes (*Bs2*) has the potential to inhibit the emergence of hypervirulent strains of *Xanthomonas*. Hypervirulent strains tend to emerge after deployment of dominantly inherited disease resistance genes such as NLRs. Since these resistance genes confer such a strong inhibition of growth of bacteria within the plant there is strong selection for plant variants that would enable pathogen growth. After several years, *Xanthomonas* bacteria can overcome resistance if a mutant strain with sufficient mutation(s) in the gene that the NLR detects is selected. The reduced selection for such mutants in bs5 plants due to the proliferation of the pathogen reduced the selection pressure on the pathogen.

Despite success in establishing a similar resistance phenotype in tomato, our field trial results showed lack of increase in marketable yield of tomato fruits. We tested a single mutant line, *Slbs5-2*, although testing our two other lines might have yielded better results. We posited that it may be useful in future experiments to also test disease pressure in fruits from our *bs5* mutant lines. Perhaps the *bs5* mutant alleles only confer significant resistance to bacterial spot in the leaves and stems while not conferring the same resistance levels in fruits, which explains why marketable yield was not significantly different. Despite these results, the *bs5* gene may be useful in commercial tomatoes when used simultaneously with other genetic sources of resistance. We also demonstrated the potential for *bs5* deployment in not only Solanaceous crop species, but other commercial crops targeted by bacterial pathogens.

3.6 Materials and Methods

Bacterial and Plant material

For all experiments, we used the wild type tomato Florida 8000 (FL8000), which is susceptible to *Xanthomonas spp.*[68]. Wild type and mutant plants were grown on soil (Miracle-Gro Supersoil Potting Soil) in a growth chamber at 25°C under a 16-h light/8-h dark photoperiod and 50% relative humidity. Experiments were performed with six-week-old plants. *Xanthomonas perforans* GEV485 (*Xp* GEV485), *X. euvesicatoria* 85-10 (*Xe* 85-10), *X. gardneri* 153 (*Xg* 153), *Pseudomonas syringae pv. tomato R2 (Pst R2)*, were used for plant inoculation. *Agrobacterium tumefaciens* strains LBA4404 and AGL1 were used for tomato transformation.

Pathogen assays

For pathogen assays, *Xanthomonas* bacterial cultures were grown in NYG (0.5% peptone, 0.3% yeast extract, 2% glycerol) with 100 µg/ml rifampicin for 18 h at 28°C on a shaker at 180 rpm. For *Pseudomonas* bacterial cultures PA media was used (2% peptone, 1% K_2SO_4, 0.14 % $MgCl_2$, 1.5 % agar). After centrifugation at 4,000 ×g for 15 min, cells were washed once with 10 mM $MgCl_2$, and diluted to OD_{600nm}=0.0003 for syringe infiltrated pathogen assays and OD_{600nm}=0.003 for dip inoculation symptom assays.

For bacterial growth assays, bacterial suspension (OD_{600nm}=0.0003, 10 mM MgCl2) was infiltrated into fully expanded leaves and plants were grown on soil (Miracle-Gro Supersoil Potting Soil) in a growth chamber at 25°C under a 16-h light/8-h dark photoperiod and 50% relative humidity. Leaf punches were collected, homogenized and then serially diluted. For quantification of bacterial populations, serial dilutions of leaf homogenates were plated onto NYGA (0.5% peptone, 0.3% yeast extract, 2% glycerol, 1.5% agar) with 100 µg/ml rifampicin and 50 µg/ml cycloheximide. After incubation at 28°C for 4 to 5 days, typical colonies of *Xanthomonas spp./P. syringae* were counted, and the bacterial population on each plant was estimated. Statistical significance was calculated using single-paired t-test.

Plants were inoculated by dipping three leaflets into the bacterial suspension (10 mM $MgCl_2$) amended with 0.02% Silwet L-77. Infected plants were grown for 14-20 days at 25°C until symptoms developed.

Calmodulin-dependent adenylate cyclase (Cya) assay

A *Xanthomonas gardneri* strain carrying a plasmid expressing an *avrBs2:adenylate cyclase* fusion reporter gene (avrBs2$_{1\text{-}100AA}$:Cya) was grown at 28°C until stationary phase in NYG containing 100 µg/ml of rifampicin and harvested by centrifugation[100]. Harvested bacteria were resuspended in sterile 10 mM $MgCl_2$ solution (OD_{600nm}=0.5) and infiltrated into fully expanded leaves. Cyclic AMP (cAMP) was extracted immediately after inoculation and after 8 hours and cAMP levels were measured with an enzyme

immunoassay kit (Cayman Chemical) according to the manufacturer's instruction and expressed as nmol of cAMP per mg of total protein. Statistical significance was calculated using single-paired t-test.

Field trial assays

Seeds were sown in July of 2018 for the initial Fall field trial and another set of seeds was sown in February 2019 for an additional field trial in the Spring. Single row plots of 10 30-day old plants were established at the Gulf Coast Research and Education Center (GCREC) in Balm, FL. Experimental plots were arranged in a randomized complete block design with four replications.

Tomato seedlings were transplanted into raised beds covered with reflective polyethylene mulch. Pic-Clor 60 fumigant was applied at a rate of 336,25 kg/ha. Between-bed spacing was five feet, and plants were spaced 18 inches within a row. Plants were staked and tied, and irrigation was applied through drip tape beneath the plastic mulch. A recommended fertilizer and pesticide program was followed throughout the growing season, excluding the use of SAR inducers, copper, and other bactericides. Plants were inoculated by spraying approximately 2 months after sowing with a four-isolate cocktail of *X. perforans race T4.* Sprayer inoculated with 5 gallons of inoculum across ~600 plants. 4-5 mL of inoculum was sprayed evenly on both side of each plant with an inoculum concentration of 10^6 CFUs/mL of each *Xanthomonas* strain (GEV904, GEV917, GEV1001, GEV1063).

Individual plants were evaluated for bacterial spot disease severity one month after inoculation using the Horsfall-Barratt rating scale[101]. Statistically significant differences of disease severity were calculated using single-paired t-test on the means of disease severity counts across four seasons of field trials. Vine-ripened (breaker stage through red) fruits were harvested two times from eight plants of each plot on two separate dates one- and two-months post-inoculation. Fruits were weighed and graded according to USDA standards (51.1859 of the US Standards for Grades of Fresh Tomatoes)[102]. Small fruits are 7x7 (unmarketable), and medium, large and extra-large fruits are 6x7, 6x6 and 5x6, respectively, according to the USDA specifications[102]. To calculate total marketable yield, only the medium, large and extra-large fruit categories were considered. Small fruits are unmarketable and, therefore, are not used to determine total marketable yield. Statistically significant differences in marketable yield across four seasons of field trials were calculated using ANOVA with Bonferroni Correction.

CHAPTER 4: Concluding remarks

The *bs5* resistance gene shows promise for disease control not only in pepper and tomato, but other Solanaceous species, and perhaps crops outside of the Solanaceae family[84]. Pyramiding is the establishment of several resistance genes into a single cultivar. Pyramiding several resistance genes (*Bs1/Bs2/Bs3/bs5*) into pepper or tomato lines has the potential of conferring robust, durable bacterial spot resistance in commercial fields.

The *bs5* resistance gene is an example of a novel mechanism of disease resistance that is distinct from previously elucidated mechanisms. The bs5 protein is neither part of the NLR or PRR resistance protein families. The molecular mechanisms of *bs5* resistance are currently unknown. However, proteins within the CYSTM proteins have been shown to be involved in an array of functions involved with stress tolerance and have been shown to interact with other transmembrane proteins or dimerize. Future experiments will elucidate the molecular mechanisms involving *bs5* resistance.

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