Quantification of bacteria in *Xanthomonas citri* pv. *malvacearum* infection and identification of virulence factors in the emergent strain Xcm4

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1 Abstract

The pathogen *Xanthomonas citri* pv. *malvacearum* (Xcm) is widely known as the causal agent of Cotton Bacterial Blight (CBB), which causes millions of dollars of crop loss in the United States annually. Understanding the molecular mechanism underlying this disease is vital for engineering resistant cotton varieties and preventing crop failures worldwide. In 2011, novel virulent strains of Xcm appeared in the southwestern United States which lacked previously identified key virulence factors but could infect varieties of cotton which were resistant to historical strains of Xcm, suggesting that the pathogen had evolved a new mechanism for effecting virulence. This thesis describes an efficient method of quantifying this bacteria's proliferation *in planta* in a laboratory setting and utilizes this method to test this pathogen for a primary mode of virulence through systematic gene knockouts. The data from this study suggests that light emission can be used to efficiently track bacterial populations *in planta*, and that novel strains of Xcm may be using a new type III effector named Tal7b as a primary virulence factor. Development of a method to track bacterial infections enables a more precise understanding of how bacteria proliferate during the infection process, which can be used to understand with more depth how this pathogen causes disease. Additionally, laboratory studies of Tal7b may yield potential routes for future crop editing and resistance to these emergent strains.

2 Introduction

2.1 *Xanthomonas citri* pv. *malvacearum*, the cotton pathogen

The pathogen *Xanthomonas citri* pv. *malvacearum* (Xcm) is a gram-negative, rod-shaped bacterium of the *Xanthomonas* family with a high GC content genome. Over 300 pathogenic interactions of *Xanthomonas* with plants have been discovered and associated with a variety of diseases and field rots in crops using Koch's postulates and genetic tools (Vauterin et al. 2000), and collectively these pathogens cause incalculable loss to an array of crops annually. Xcm is well known as the causal agent of Cotton Bacterial Blight (CBB), and previous studies on the pathogen have been performed to discover avirulence genes in the pathogen (De Feyter et al. 1993). Cotton Bacterial Blight causes an estimated 5 to 35 percent crop loss annually (Delannoy et al. 2005), and thus presents a massive impedance to the cotton industry. Usually, the disease takes the form of "water soaking", where the tissue darkens and undergoes soft rot while the leaves develop a darkened, "wet" appearance. As the disease has re-emerged in the 21st century, new strains and isolates have appeared that have overcome previous resistance engineered through breeding. "Virulence" in Xcm refers to the ability to the quantitative damage done to the cotton by the pathogen, measured through Water soaking. This contrasts with pathogenicity, which is the qualitative ability of the pathogen to effect disease on its host (Sacristán and García-Arenal 2008). Among the emergent strains is Xcm4, an isolate collected from Texas that exhibits strong virulence on cotton, but does not contain some of the previously established critical virulence factors for cotton infection (Cox et al. 2017). Thus, studying this isolate may yield information on how Xcm has evolved new methods of virulence.

Cotton is an important agricultural crop both in the United States and abroad, accounting for 1/3 of the world's fiber and generating 7 billion dollars of value annually in the US alone (United States EPA 2021). Furthermore, cotton provides a source of both biofuel and a potential food crop for developing nations (Sunilkumar et al. 2006; Li et al. 2009; Alhassan et al. 2014), making it a burgeoning crop for future agricultural development and production. Given its economic and developmental role, maximizing cotton production thus serves as a key objective for agronomic and scientific endeavors. The most commonly grown varieties are from the species *Gossypium hirsutum*, an allotetraploid that resulted from a genomic fusion between *G. arboreum* and *G. raimondii*, which makes it a challenge to work with and interpret on a molecular level (Li et al. 2015). Given the massive crop loss that CBB can present annually (Delannoy et al. 2005), it is therefore a high priority that the molecular mechanisms of CBB development and Xcm virulence be understood in order to enhance the ability to develop resistant cotton varieties.

2.2 Key limitations and knowledge gaps

2.2.1 Quantification of bacterial populations during infection

One of the primary challenges which comes from understanding the pathogenesis of *Xanthomonas* bacteria is the development of efficient methods of quantification of bacterial populations in plant tissues. Previous studies have primarily utilized simple scoring systems, rating virulence on a human-designed scale and averaging phenotype to assess differences (Hussain 1984). Complimentary to the assessment of disease has been direct measurement of

bacterial presence in order to assess whether plants are tolerant of bacterial propagation; most commonly, this involves collecting bacterial samples from inoculated leaves and dilution plating the bacteria for quantification (Haq et al. 2020). However, these methods each come with several downsides. Phenotypic ratings constitute a highly subjective scale, which has a large chance for human error in experimentation and requires blinding of scorers. Similarly, creating a consistent scale for disease symptoms, which can vary greatly from one infected sample to another, introduces a margin of error. Likewise, the process of obtaining the massive number of technical and biological replicates necessary for meaningful data collection of bacterial populations is time consuming and labor intensive, particularly for large-scale experiments. Furthermore, the individual samples suffer from internal variability with individual colony counts varying by up to an order of magnitude within a sample, which compounds with the multiplicative factor of deducing bacterial counts from increasingly small dilutions to create large discrepancies in bacterial counts. Given these issues, finding a more efficient and precise method for quantifying Xcm in plant tissue emerges as a technical imperative for the field to create ease and expedience of experimental procedures, as well as remove some of the individual variation and human error associated with these methods.

The *luxCDABE* operon is a series of five protein-encoding genes naturally found in *Photorhabdus luminescens*. Two of these protein products, LuxA and LuxB, combine to perform bioluminescence without needing a substrate. The light produced from these proteins has previously been demonstrated to be quantifiable in bacteria, and has been adapted to a high-GC form of the construct for expression in a wide variety of species (Craney et al. 2007) The use of *lux* has been previously demonstrated in *Ralstonia solanacearum* and *Pseudomonas syringae* to enable detection of bacterial presence *in planta* without affecting pathogenesis (Fan et al. 2007;

Cruz et al. 2014). While it is possible that the production of *Lux* by bacteria during infection may be affected by metabolic differences, the use of a constitutive promoter such as *Lac* without a relevant repressor means the bacteria has little means by which to shut down the production of *Lux* in response to the environment, and thus is unlikely to have high variability in response to the metabolic conditions within the leaf. *Lux* thus serves as a useful tool for quantifying bacterial presence, and its stable expression is an indicator of overall bacterial proliferation. Given this information, **I hypothesized that expressing the *luxCDABE* operon from a stable plasmid would allow the detection of Xcm *in planta*, and by quantifying this luminescence, I would be able to efficiently quantify bacterial populations through light emission from the pathogen.**

2.2.2 Virulence mechanisms used by the novel strains of Xcm

Key to understanding how pathogens function to cause disease in their hosts is the study of virulence factors, or genes in the bacteria which, when expressed, enable infection to take place. *Xanthomonas* species express a wide range of effector proteins that assist in disease induction, and though most are translocated through a specific secretion system (type III), these virulence factors have multivariant effects on basal plant defense (Büttner and Bonas 2010). Even within pathovars of *Xanthomonas,* massive variation in the effecter repertoire is seen (Potnis et al. 2015), suggesting a diverse suite of virulence factors is necessary for disease, although some are conserved between species. These novel virulence factors are likely to be the source for disease reemergence, and their identification and perturbation thus serves as the primary target for investigation. In the case of novel strains of Xcm, one of the most important

virulence factor classes to study are the novel Transcription Activator-Like (TAL) effectors present in these emergent strains.

One of the most common methods of disease enhancement exhibited by *Xanthomonas* species is the use of TAL effectors. These proteins are translocated into the plant cell by the type-III secretion system, where they are localized to the nucleus and act as sequence-specific transcriptional activators. Their target sequence in plant genome promoter regions is determined by the identity of two variable amino acids in a highly conserved repeat sequence (Boch et al. 2009; Moscou and Bogdanove 2009). It is worth noting that, on rare occasion, evidence has supported TAL effectors being able to act as transcriptional repressors rather than activators (Pereira et al. 2014). Based on the sequence of these amino acids (called Repeat Variable Diresidues, or RVDs), the binding sites of these TALs in the host genome can be predicted using software such as TALE-NT 2.0 or TALgetter (Doyle et al. 2012; Grau et al. 2013).

In addition to simply being fascinating biological elements, TAL proteins often play a vital role in bacterial pathogenesis. In rice the effects of TALs are well studied, as proteins such as Tal2g and PthXo1 induce the expression of specific genes in the the rice genome to enhance disease (Römer et al. 2010; Cernadas et al. 2014). This effect has also been demonstrated in numerous other plant hosts, such as citrus (Swarup et al. 1992), pepper (Marois et al. 2002), and soybean (Kladsuwan et al. 2017). Although TALs are not a necessity for all *Xanthomonas* species to effect disease, they function as a common mode of action in a variety of hosts. In particular, TALs have previously been demonstrated to play a vital role in CBB development, with some strains of Xcm utilizing TALs as major virulence factors during infection (Cox et al. 2017).

Major TAL effector targets in the host are referred to as either Susceptibility (S) or Resistance (R) genes. The former constitutes genes which, once TALs bind their promoter region and induce their expression, facilitate infection. Conversely, R genes, when activated by TAL effectors, activate plant resistance mechanisms or otherwise inhibit infection, commonly in the form of a hypersensitive response (HR), a form of localized programmed host cell death meant to prevent infection from spreading to the rest of the plant. Resistance genes are less common than susceptibility targets, and usually involve host protein recognition of a TAL, such as Xo1 recognizing *Xanthomonas oryzae* pv. *oryzicola* (Xoc) TAL effectors to activate an immune response (Triplett et al. 2016). This has likewise contributed to the selective evolution of resistance loci, with bacterial proteins such as Tal2h helping to evade the effects of Xo1 and thus allow disease progression (Read et al. 2016).

Central to these interactions between *Xanthomonas* and its host through TALs, however, are the induction of S genes. Susceptibility gene-encoded proteins can have a variety of cellular functions, including acting as transcription factors (Sugio et al. 2007) or utilizing transcription factors to activate other classes of gene, like pectin lyases, downstream which ultimately act as susceptibility factors (Schwartz et al. 2017). Most commonly, however, S genes encode nutrient transporter proteins, including sulfate transporters such as SULTR 3;6 (Cernadas et al. 2014), and, importantly, SWEET (Sugars Will Eventually be Exported Transporter) proteins (Chen et al. 2010; Cohn et al. 2014; Cox et al. 2017). The latter are the most common and are widely considered efficient targets of TALs for disease induction, although the method by which SWEETs and other nutrient transporters assist disease development is yet unknown. Additionally, studies in rice have demonstrated that multiple members of the SWEET family can

promote disease (Oliva et al. 2019), suggesting that SWEETs are prime candidates as targets for TALs in the emergent strains of Xcm.

Previous studies in cotton have determined that certain SWEET genes, such as *GhSWEET10*, serve as primary susceptibility targets for Xcm infection (Cox et al. 2017). However, genomic studies of *G. hirsutum* have identified over 55 SWEET genes, which provide ample potential targets of this type for Xcm to take advantage of through this pathway (Li et al. 2018). Early data involving Xcm4 has previously indicated that two closely linked SWEET genes, *GhSWEET14a* and *GhSWEET14b*, are upregulated during infection, but whether this contributes to disease induction is yet unknown. Furthermore, prediction of the binding sites for the TALs in Xcm4 have shown that one effector, Tal7b, has a putative binding site upstream of these two SWEETs (Cox et al. 2017). Based upon this evidence both in Xcm and other pathovars of *Xanthomonas*, **I hypothesized that at least one of the TAL effectors present in Xcm4 plays a major role in its ability to enhance CBB.**

3 Results

3.1 Bacterial populations and virulence are unlinked in Xcm

To establish whether understanding of bacterial populations is necessary for a detailed understanding of pathogenesis, I performed a series of inoculations with both a well-studied lab strain of Xcm lacking TAL effectors derived from the virulent H1005, HM2.2 (Cox et al. 2017) as well as several novel isolates collected from the southeastern United States outbreak. If virulence and bacterial populations are strongly correlative, then the avirulent strain HM2.2 should exhibit a marked reduction in bacterial population relative to the virulent strains, and likewise varying virulence from the strains should be correlative overall to bacterial populations. If this hypothesis is true, then measuring bacterial populations will be unnecessary and disease phenotype will be a relatively informative method of determining overall bacterial propagation.

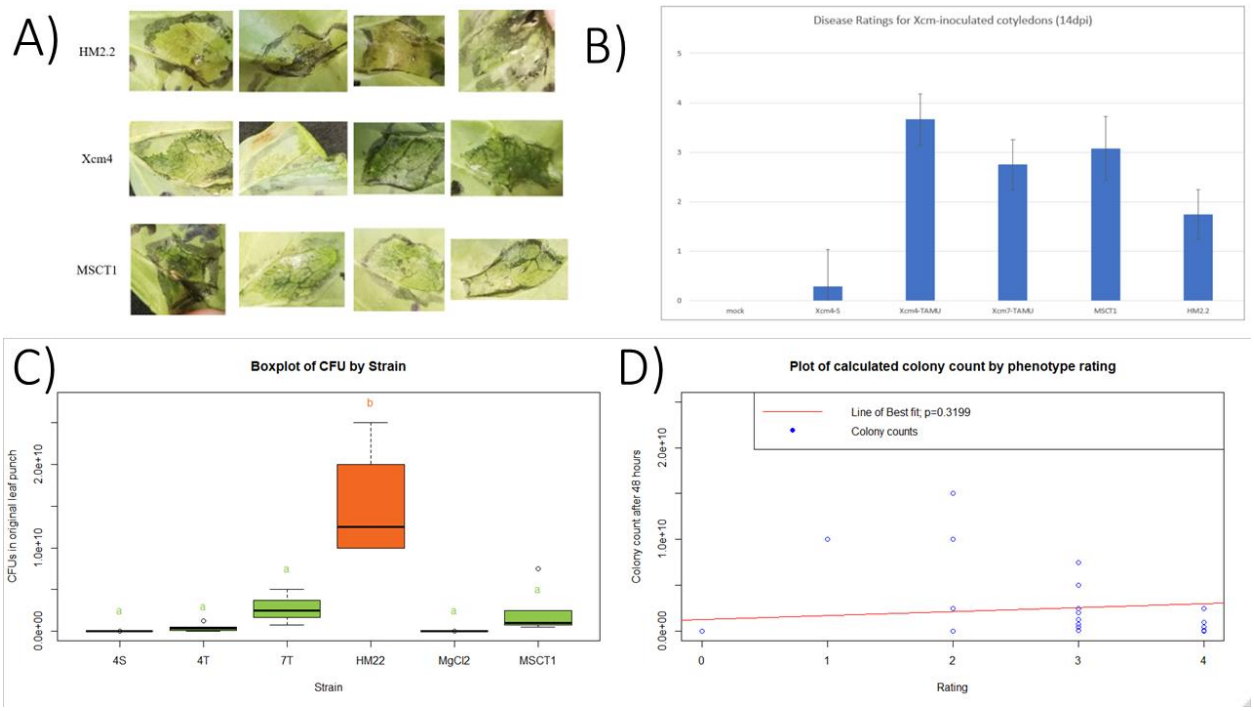


Figure 1. Various strain inoculations in cotton reveal a lack of correlation between disease phenotype and bacterial populations. A) Representative photos of water soaking symptoms from the major strains of relevance; HM2.2 (A strain of XcmH1005 with at least 7 TAL genes removed, low virulence), Xcm4 (The novel isolate of interest in this study), and MSCT1 (a virulent strain of Xcm). B) Bar graph of average phenotype ratings taken at 14 DPI. "Mock" represents a 10mM MgCl2 inoculation, and each previously undescribed strain another individual isolate from the outbreak in the southeastern United States. "-S" represents individual subcultures of Xcm4 and Xcm7 isolates that underwent whole genome sequencing but displayed inconsistent phenotypes on plates (See supplementary figure 1), and "-TAMU" denotes subcultures taken after this point that are representative of originally observed phenotype upon isolation. C) Boxplot of colony counts taken from samples through dilution plating at 14 DPI. All inocula demonstrated some level of Water soaking, as seen in A) and B). Colony counts were taken at 48 hours after plating. D) Dotplot of collected colony counts by phenotypic rating, and the appropriate line of best fit. P value calculated through chi-square test.

Inoculations were performed with these strains, and phenotype assessed after 14 days using a visual scoring system (Supplementary Figure 2). At this point, samples were additionally ground and plated to measure bacterial populations. The water soaking phenotype was strongest in a previously studied virulent strain, MSCT1 and similarly strong in one of the novel strains, while HM2.2 exhibited marked reduction in water soaking, as expected (Figure 1a, 1b). However, HM2.2 had a significantly higher population than all other inocula (Figure 1c),

indicating a disconnect between the decreased symptoms seen (Figure 1b) and the bacterial population. Likewise, the correlation between these two factors was not statistically significant (P=0.3199, Figure 1D), demonstrating that the degree of water soaking and the size of the bacterial populations are not inherently related. Based on these results, I concluded that measuring water soaking alone was not meaningful for understanding how the pathogen is multiplying *in planta*.

While these results were informative for determining a lack of correlation between water soaking and bacterial populations, these experiments also reinforced the need for a more efficient method of bacterial quantification, as data collection for this one trial's bacterial population was very labor intensive. Thus, given the need to measure bacterial populations and the strong incentive to find a more efficient and accurate method of doing so, I elected to pursue the use of luminescence as a proxy for bacterial populations.

3.2 Bacterial populations can be measured using the *lux* operon

In order to measure the effective virulence of these strains through *lux* emission, a stable vector encoding the operon was necessary. I elected to utilize the shuttle vector pHM1, as it contains a multiple cloning site (MCS) into which I could put gateway recombination sites for *lux* insertion as well as a *lac* promoter, which is expressed constitutively in *Xanthomonas* species (Wengelnik et al. 1996) (see Supplementary Table 1: Strains and Plasmids). I first used the restriction enzymes KpnI and HindIII to cut the MCS of pHM1, and used the same enzymes to remove the R1, R2, ccdB, and chloramphenicol resistance genes from pSLR2 and ligated this

fragment into pHM1, creating the gateway destination vector pTSB1. This vector was then gateway cloned (Thermo-Fisher) with the *luxCDABE* operon cloned into a gateway entry vector, creating pTSB2. This plasmid is capable of undergoing triparental mating, is stably preserved in *Xanthomonas*, and can express *lux* constitutively in Xcm, making it an ideal vector for use in assay development. I elected to use a plasmid rather than chromosomal integration described previously (Fan et al. 2007; Cruz et al. 2014) because it A) eliminates the possibility that the integration of lux into the genome will cause an ectopic mutation that affects the phenotype, and B) enables easy use of *lux* in other pathosystems through the broad host range of the pSa origin found on pHM1.

To test the efficacy of *lux* at detecting bacterial population differences *in planta*, I performed an assay to see whether lux expression could meaningfully correlate to the earlier established methods of phenotype scoring and bacterial counts. pTSB2 was transformed into wildtype Xcm, and then inoculated at different ODs before light emission was measured.

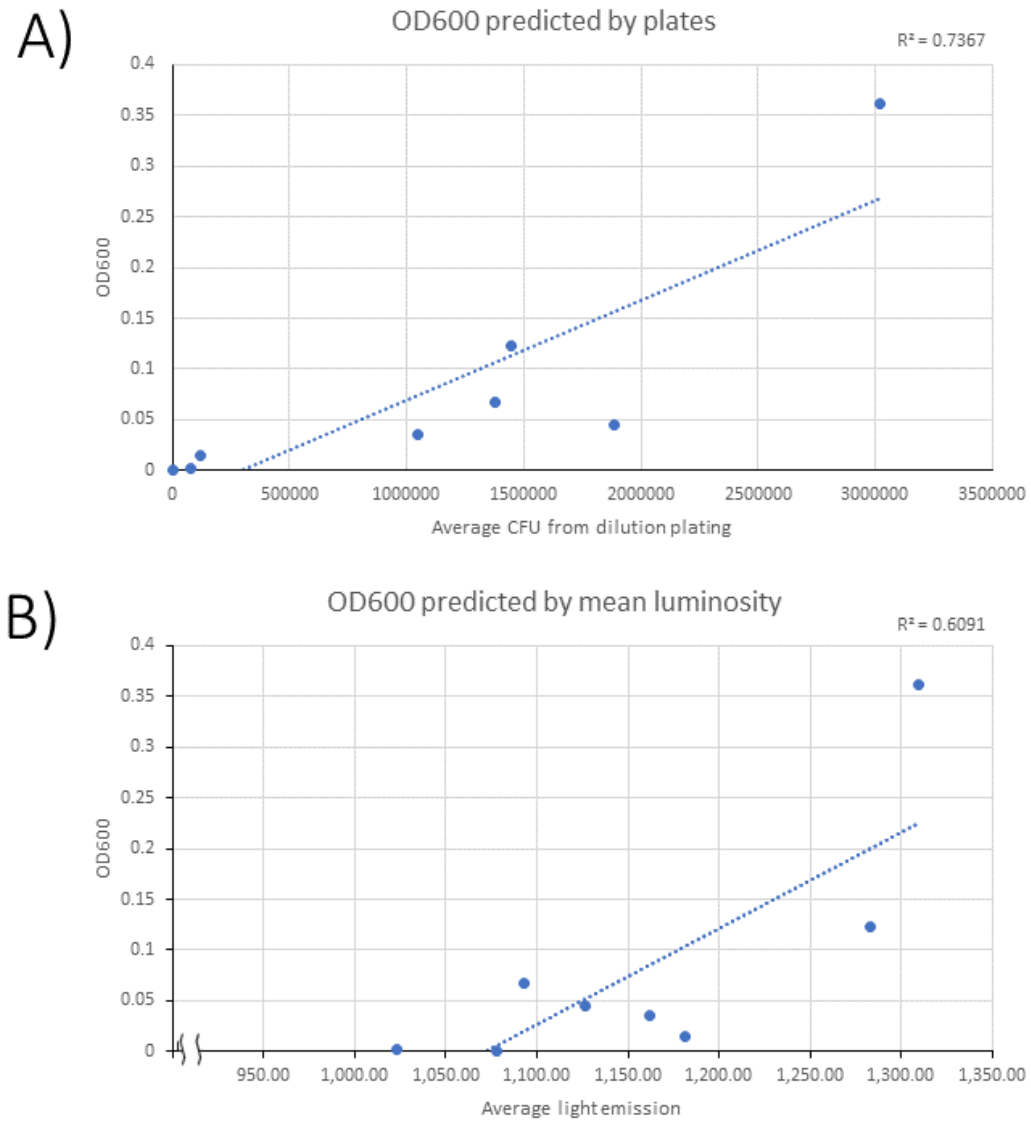


Figure 2. Graphical representation of predictiveness of quantification methods of bacterial population as measured through OD600, with regression. A) Plot representing a regression between a dilution of various inocula (3 technical replicates of sampling of inoculum, and each was subjected to 3 technical replicates of plating) and their measured OD600. R^2=0.7367. B) Plot representing a regression between the mean luminosity scores (n=4 to n=17) collected from inoculated plants. R^2=0.6091.

Although the light emission was found to have a weaker correlation to the original OD than dilution plating (Figure 2), the existence of a moderate correlation between the two suggests that using luminosity to approximate the population of bacteria present in the leaf is not an

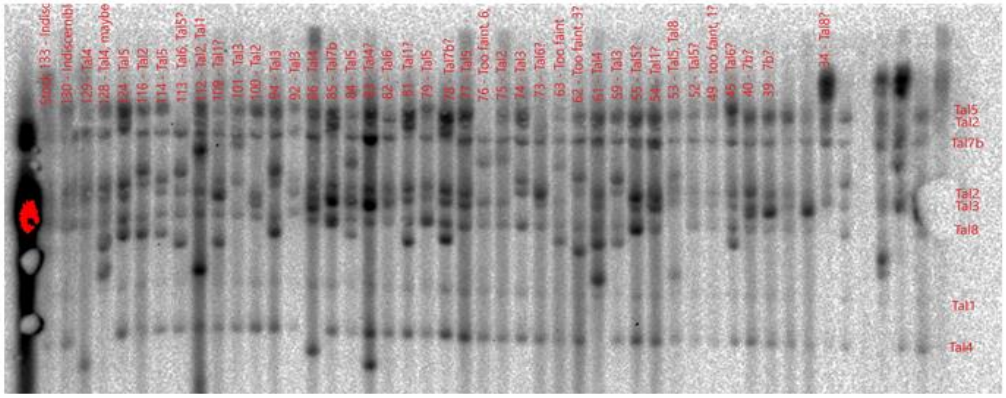
invalid strategy. Additionally, because this experiment sampled directly from inocula rather than taking samples from ground tissue samples, it does not account for another major source of potential inconsistency in grinding and plating, that of grinding the leaf tissue in order to obtain bacterial samples. Given these factors, I concluded that *lux* could be used to approximate bacterial population and elected to use the *lux* operon as a method of measuring bacterial populations in subsequent experiments.

3.3 Xcm4ΔTal7b exhibits a reduction in disease phenotype

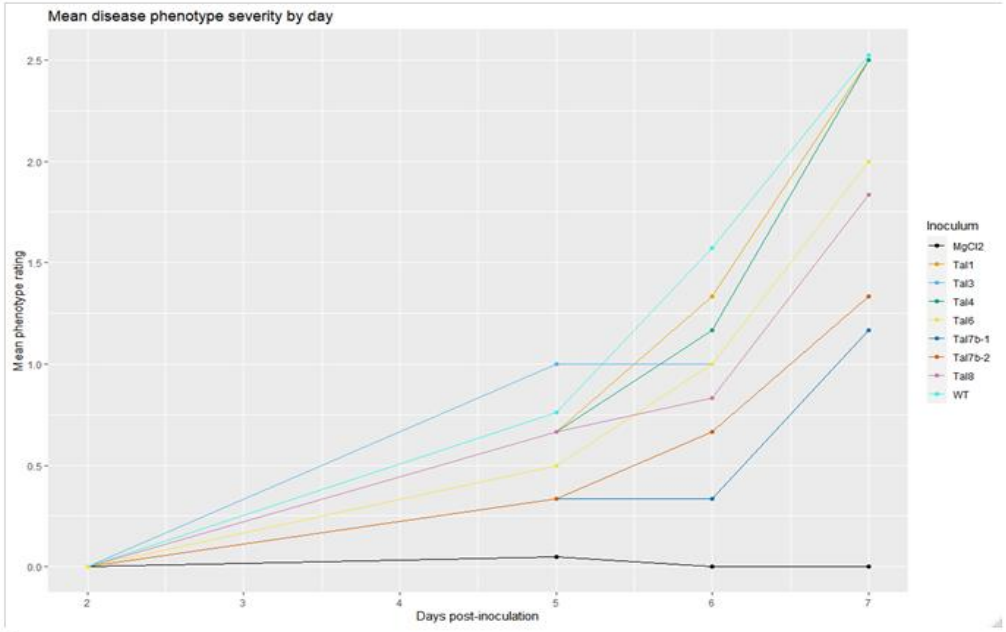
In order to test whether the TALs present in Xcm4 have any direct effect on the bacterial virulence, I used a knockout assay to systematically test the importance of individual TAL effectors in virulence on susceptible cotton (*G. hirsutum* Ac44E) and observe phenotype using the above outlined protocol. To create these plasmids, I transformed the suicide vector pTeM5 into wildtype Xcm4. pTeM5 contains a naturally occurring TAL effector – PthXo1 – interrupted by the Tn5 transposon carrying a kanamycin resistance gene, as well as an origin of replication that cannot be recognized by *Xanthomonas*. By transforming it into the bacteria and selecting on kanamycin, it selects only for cells which have undergone homologous recombination at this site, integrating the Tn5 transposon into one of the native TAL effectors, and thus generating an effective knockout. This recombination preferentially happens in TAL effector genes due to Tn5 being encoded within PthXo1 in this construct, as the region around Tn5 that allows it to undergo Homologous Recombination with the Xcm genome has an extremely high degree of homology with TAL effectors and little else within the genome. Over 100 Xcm4 knockout strains were generated from this experiment, and their genomic DNA was isolated and digested

to perform a Southern blot for identification (Figure 3A). Mutants were identified solely using a Southern blot due to the difficulty of obtaining accurate sequencing data for TAL effector genes. Their repetitive structure makes it extremely difficult for high-throughput or sanger sequencing to obtain accurate reads (Booher et al. 2015), meaning Southern blotting is the most efficient and cost-effective method of mutant identification for a library of this size.

A)


Tal5
Tal2
Tal7b
Tal2
Tal3
Tal8
Tal1
Tal4

B)


Mean disease phenotype severity by day
Mean phenotype rating
Days post-inoculation
Inoculum
MgCl2
Tal1
Tal3
Tal4
Tal6
Tal7b-1
Tal7b-2
Tal8
WT

C)

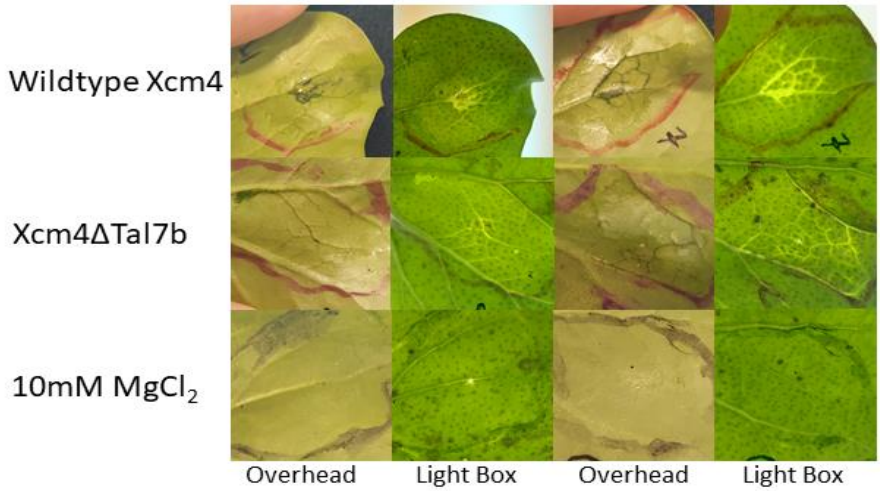

Wildtype Xcm4
Xcm4ΔTal7b
10mM MgCl2
Overhead
Light Box
Overhead
Light Box

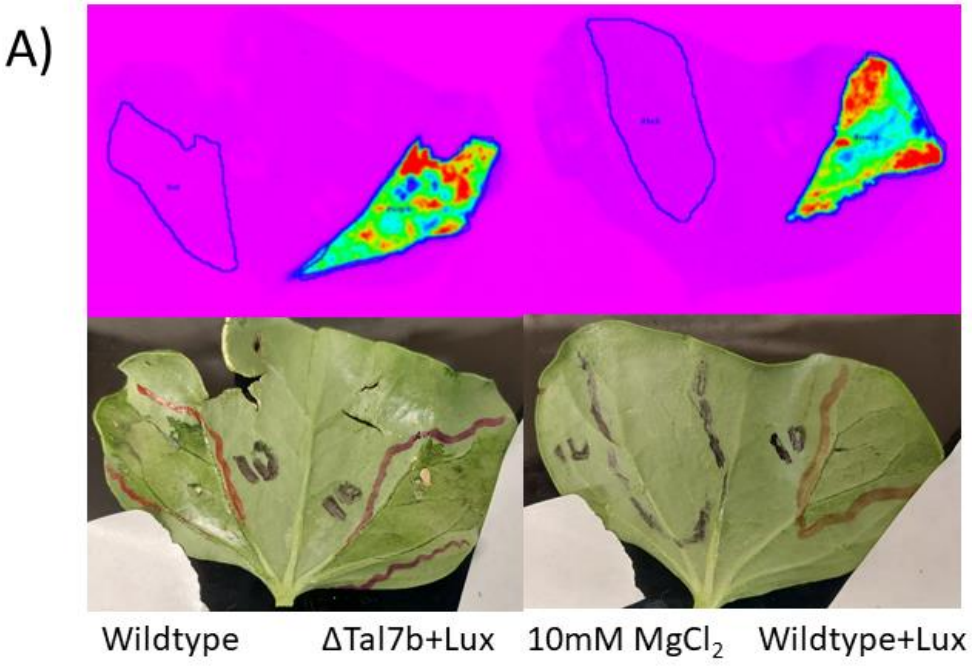
Figure 3. Creation and assessment of Xcm4 TAL effector knockout strains. A) Southern blot probed with a designer TAL effector against genomic DNA extractions from various knockout strains, each of which is numbered. TAL effector band locations are present on the right, interpreted from the (at this exposure, saturated) control DNA on the left. Shifts or absences of bands indicates a given TAL has been knocked out. B) Timescale graph of average phenotype recording by tested strain. A preliminary trial removed TALs 2 and 5 from consideration for this experiment (data not shown). Phenotype scale is recorded in supplemental figure 2. At 7 DPI, both independent 7b knockout lines showed a statistically significant reduction in phenotype from the wildtype, while no other strains did (for specifics on significance test, see supplemental figure 3). C) Exemplary leaves of the wildtype, one Tal7b knockout line, and the Magnesium Chloride blank. The same leaves are shown both with an overhead light to visualize water soaking, and a backlight from a light box to better visualize damage to the vasculature.

Once the mutants were identified, their ability to induce disease was systematically tested. Each line was inoculated into cotton and the resulting phenotype was observed. Of these knockout lines, only Tal7b knockouts showed a significant reduction in virulence relative to wildtype (Figure 3B). The phenotypic difference was large, as the Tal7b knockout was barely able to induce water soaking whereas the other knockout lines did not have a statistically significant reduction in virulence relative to the wildtype. Likewise, the vascular damage evident of more advanced water soaking seen in the wildtype strains was near absent in the Tal7b knockout lines, leading to the conclusion that the loss of Tal7b reduces Xcm4's ability to induce water soaking and effect disease in cotton.

3.4 The loss of Tal7b reduces bacterial proliferation *in planta*

Having assessed that Tal7b results in a decrease in bacterial capacity to induce water soaking in cotyledons, I next set out to investigate whether the loss of Tal7b simply allows the plant to become tolerant of bacterial infection, or if the loss of Tal7b prevents the bacteria from proliferating within the plant tissue at all. To investigate this difference, I transformed both the

wildtype Xcm4 and the knockout line with pTSB2, the *lux*-encoding stable plasmid, and inoculated these transformants into plant tissue to monitor bacterial growth. At 10 DPI, these plants had their luminescence measured to elucidate differences in bacterial populations (Figure 4).



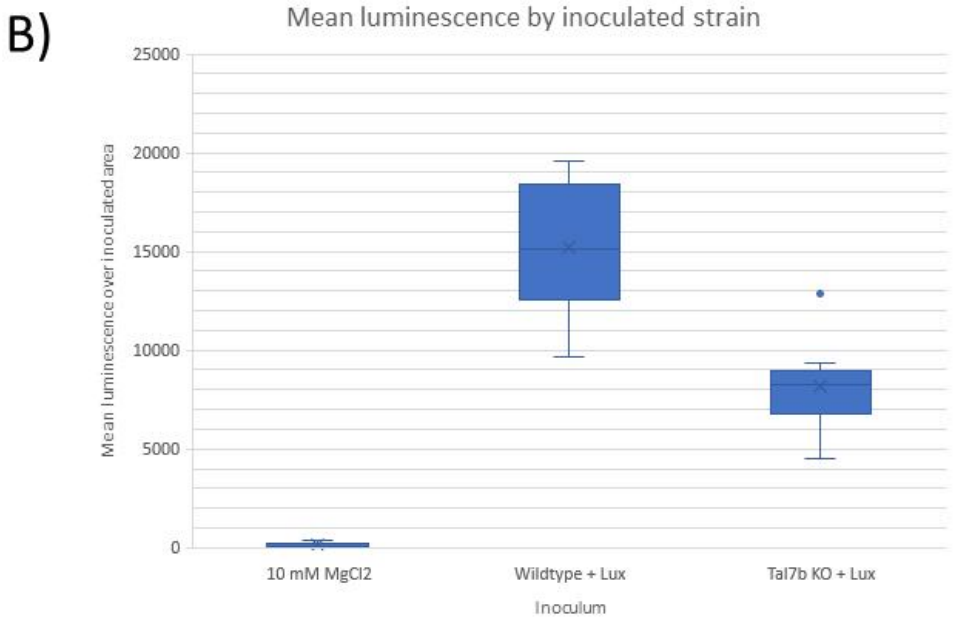


Figure 4. Luminescence of a Tal7b knockout and the relevant wildtype strains. A) Representative photos of inoculated areas at 7DPI showing water soaking, and their correlated area of bioluminescence detected under a CCD camera. Manually outlined inoculation areas for luminescence calculation are shown on the digital scan image. Wildtype Xcm was included as a negative control, to ensure no measurable luminescence was coming from the bacteria naturally. B) Luminescence over the area of infection was averaged, and background then removed for each data point. Student's unpaired t-test revealed $p<0.0001$ for every experimental group pairing. Luminescence measured in photons per pixel with CCD camera.

The results from this experiment suggest that the loss of Tal7b results in a decreased ability of Xcm4 to proliferate in the plant tissue. Combining this information with the phenotypic results presented earlier, the data collected suggest that Tal7b serves as a vital effector for both the progression of disease and bacterial proliferation within the infected tissue.

3.5 One tested Xcm4 Tal7b knockout strain fails to complement

After finding evidence that Tal7b resulted in knockdown of both virulence and bacterial proliferation *in planta*, I elected to perform a complementation assay to verify the singular effects of Tal7b on this system. I transformed a Tal7b knockout strain with pTSB3, a low-copy expression vector in *Xanthomonas* (pKEB1; Cox et al. 2017) that had Tal7b gateway cloned into the vector. This would allow stable, transient expression of Tal7b in the knockout in a manner unaffected by microbiological phenomena like quorum sensing, enabling accurate assessment of phenotype. Plants were assessed for phenotype at 7 days old, and the results were graphed (Figure 5).

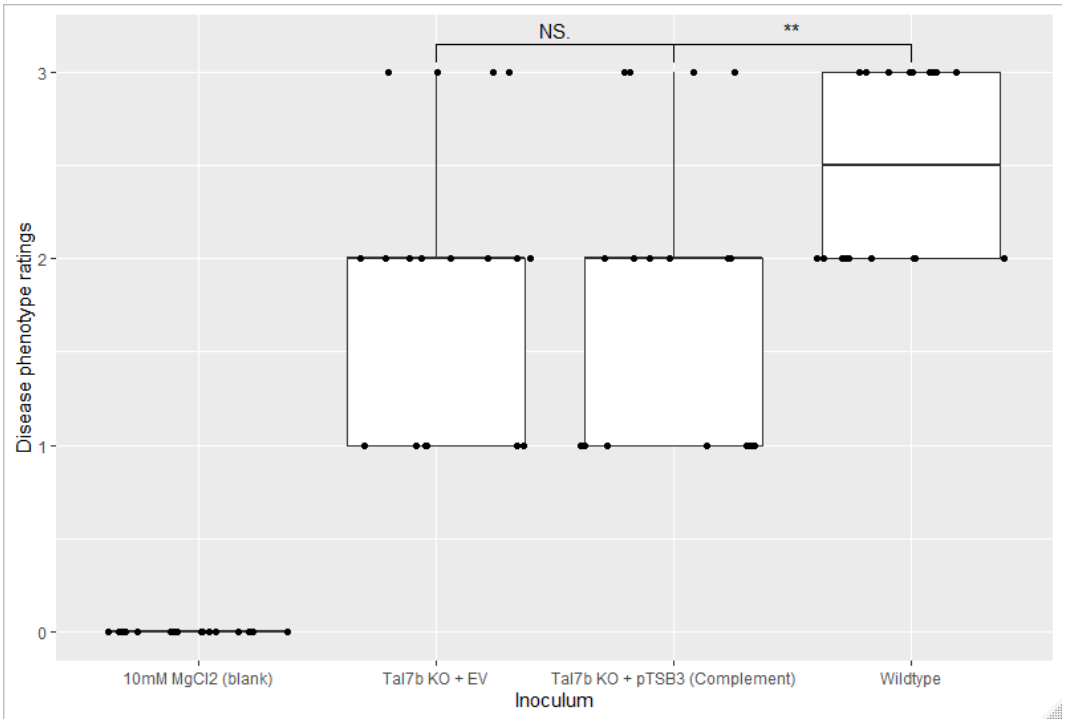


Figure 5. Boxplot of assessed phenotype in complementation assay. Phenotypes for individual samples were recorded 7dpi and graphed. Student's unpaired t-test used to assess statistical significance, shown above the boxes. The Tal7b knockout with empty vector was found to be near identical to the complement line, while the complement line's phenotype was significantly less than that of the wildtype ($p<0.001$).

The lack of complementation was evident upon assessment and drew concern for the hypothesis that Tal7b acts as a primary virulence factor in Xcm4 infection. Although these data do not support the hypothesis, it does not inherently reject the hypothesis either and merits further investigation in light of the previously collected evidence that Tal7b acts as a major virulence factor.

4 Discussion

The primary goals of this study were to: 1) test whether use of the *lux* operon was viable as a means for assessing bacterial populations *in planta*, and 2) test whether any of the TAL effectors found in an emergent strain of Xcm played a critical role in the development of disease. Testing of the *lux* operon yielded a meaningful correlation between measured luminescence and bacterial population, one which was comparable to the previous inefficient system of grinding and plating. Likewise, this method proved useful in a practical setting, quantifying a meaningful difference in bacterial population between a knockout strain and the wildtype Xcm4. This same knockout strain showed a reduction in virulence *in planta* as well, and its phenotype supported the second major hypothesis. While complementation of Tal7b did not restore virulence, these results do not automatically rule out the hypothesis that Tal7b acts as a major virulence factor.

Given the ease with which luminescence data can be collected (requiring only 10 seconds of CCD camera exposure; see Methods) and data processing, the correlation presented by these data – as well as their practical use – suggests that the adoption of *lux* serves as a viable way to expedite future experimentation and allow for more meaningful data collection in future. As mentioned above, the correlation between luminescence and OD600 (as a proxy for exact bacterial counts) being comparable to the same correlation for dilution plating also fails to account for the inefficiencies presented by the process of grinding leaf tissue to obtain bacterial counts, which adds in an extra source of variability which may make *lux* emission a more consistent measurement by comparison. The increased ease of data collection provided by *lux* opens the door for future scientific inquiry, as it enables investigators to have more time to either increase sample size or investigate other pertinent questions. The increased sample size presents

the ability as well to test for subtle phenotypes and differences more easily, in a manner which would be too inefficient with bacterial plating. With this tool more precise answers on subtle differences may be obtained, and more of the potential effects of various conditions on bacterial proliferation may be assessed. Finally, for vascular pathogens, this tool may present an easier method by which to track bacterial progress through the leaf. For example, in *Xanthomonas oryzae pv. oryzae*, *lux* usage may better elucidate bacterial progress through the leaf than the previous established methods of measuring lesion length, as the bacterial movement may be faster than disease phenotype shows (Yang et al. 2000). The addition of *lux* onto a stable plasmid also enables easier testing through experimental procedure, avoiding the problems and experimental slowdown that may appear through previous *lux* testing methods in bacteria which relied on a dual recombination into a safe harbor site (e.g. Cruz et al. 2014). Likewise, *lux* usage on a plasmid to track a microorganism has not been tested in a plant pathogenic setting before, and this study's use of *lux* encoded on a broad host range vector serves as a benchmark for testing *lux* and pTSB2 in other systems as a viable method of measuring host-microbe associations and interaction patterns in plants and other contexts.

The data on the role of TAL effectors as virulence factors, likewise, is encouraging. The potential of Tal7b as a virulence factor enables further investigation into how to engineer resistance to emergent strains of Xcm. In particular, because TALs are DNA binding proteins, software such as TALE-NT 2.0 or TALgetter (Doyle et al. 2012; Grau et al. 2013) can be used to predict where this TAL may be binding in the host genome. From there, plant breeders and geneticists may be able to further investigate whether engineering new lines of cotton which carry mutations in these binding sites may enable pathogen resistance. This has been done in response to pathogens in other crops like rice for the gene *OsSWEET14* or other S genes to

effectively generate resistance to disease development (Li et al. 2012; Oliva et al. 2019). Likewise, engineering crops with a binding site for a major virulent TALE in the promoter region of a major *R* gene can enable widespread resistance to the pathogen, using a device called a "promoter trap" (Römer et al. 2009; Hummel et al. 2012; Boch et al. 2014). Using these engineering strategies on Tal7b may yield new lines of cotton which are broadly resistant to emergent disease, mitigating the damaging effects of CBB on growers across the southeastern United States.

One of the major limitations of this study is the negative data presented for complementation of Tal7b. Although this seems to suggest that Tal7b is not responsible for the reduction in virulence presented by the knockout lines, there are caveats which restrict our interpretation of this result. Only one knockout line transformation was tested, meaning any ectopic mutation which may have been present in the given transformed cell may confound the ability of Tal7b to complement the knockout phenotype. This same ectopic mutation principle applies to the Tal7b gene reintroduced on pTSB3, which may have undergone a nonsense mutation or otherwise been changed during transformation or shortly thereafter. TAL effectors being highly repetitive makes them prime candidates for self-recombination in nature (Wilkins et al. 2015) and this may further explain why the reintroduction of Tal7b failed to induce complementation. In particular, the presence of two independent mutations in Tal7b as evidenced by differential patterning on a Southern blot (Figure 3A) resulting in a reduction in virulence (Figure 3B) serves as evidence that the failure to complement may be a limitation relating to the individual transformant strain rather than a biologically relevant conclusion. Thus, the experiment bears repeating, particularly with each independent Tal7b knockout strain to further parse out the biological relevance of this experiment.

The limitations of the *lux* operon experiments center around the untested nature of *lux* in other microbial interaction systems. Although *lux* was demonstrated to be a useful tool for detecting bacterial population differences in a non-destructive manner *in planta*, this was only tested in cotton and remains to be tested in a variety of other contexts, plant and otherwise. Additionally, the validation of *lux* correlation depended upon comparison with OD600, which – although roughly proportional to bacterial population per the Beer-Lambert law – is not universally precise in detecting bacterial populations (Stevenson et al. 2016), which may have an impact on the reliability of the established correlation between population and luminescence in a linear manner. In a similar fashion, the indistinguishable nature of the *lux* data collected immediately upon infection at extremely low bacterial titers (Figure 2B) suggests this assay may lack sensitivity at low bacterial concentrations, though this may be overcome with longer exposure times under the CCD camera. This longer exposure time, however, runs the risk of eliminating one of the primary benefits of using *lux* – the large reduction in time necessary to conduct an experiment with a given sample size. Further elucidation of *lux*'s usefulness at low bacterial titer and in other biological contexts will be necessary before the tool can see widespread usage in the field.

This study serves as a further investigation of principle studies into the use of *lux* to detect bacteria *in planta* (Fan et al. 2007; Cruz et al. 2014), as well as the role which TAL effectors play in the development of CBB (Cox et al. 2017). This study elaborates upon the former by demonstrating the meaningful correlation between bacterial luminescence and population, turning *lux* detection into a viable tool for elucidating differences in populations *in planta* during infection. Likewise, it demonstrates that *lux* encoded onto a stable cloning vector pTSB1 can work efficiently like the previous chromosomal insertions, making transformation

and testing a more expedited process. This broad-range vector, functioning in *Xanthomonas*, demonstrates that the plasmid may be useful in a broader array of contexts and help further our understanding of host-microbe interactions. Likewise, though the experiments presented in this manuscript do not unilaterally suggest that TAL effectors are playing a role in the development of CBB from Xcm4, the preponderance of evidence still supports this idea and has the potential to lead to further understanding of emergent strain pathogenic mechanisms, similar to what was previously discovered with AvrB6 and *GhSWEET10* for older Xcm strains (Cox et al. 2017).

Future studies should seek to elaborate on the presented data and attempt to elucidate their potential for major impact. As stated previously, the investigation of *lux* as a useful tool at low bacterial titer will broaden the range of applicability for this tool, potentially turning it into a broadly useful instrument for microbial detection in host-microbe interaction systems. Testing of *lux* in systems outside of plant pathogenesis will also be imperative for determining its use long-term as a reporter of bacterial presence. Well studied bacterial-host interactive systems, such as *Burkholderia* and *Rhizopus* in fungi (Kwak et al. 2012) or *Acropora millepora* and *Zooxanthellae* (Cervino et al. 2003) may serve as prime candidates to test the diversity of hosts in which this system may be useful for detecting microbial associations, and may show the widespread impact which luminescence quantification may have on organismal studies in a variety of fields.

The data presented in this manuscript on Tal7b's role in virulence will likewise serve as the basis for future experimentation. If mutants of Tal7b are found to be able to complement, then investigators may be able to test the sequence of Tal7b for binding sites in the cotton genome, and potentially investigate the mechanism by which Tal7b results in a reduction in virulence and bacterial populations. Confirmation through the use of Designer TAL effectors

(Cermak et al. 2011) will enable validation of the target. This information would vastly increase our understanding of how CBB may be effected in cotton, and furthermore will serve as a launching point for future engineering of resistance and the reduction of Xcm's impact on cotton growers in the United States and abroad. The economic impact of this investigation and resistance generation cannot be understated and serves as a prime area for scientific investigation to serve a practical good to those outside the field.

5 Methods

5.1 Bacterial and plant growth conditions

E. coli strains for cloning were grown in Luria-Bertani (LB) medium at 37C. *Xanthomonas* strains were grown in GYE medium at 28C. Bacterial strains used in this study are listed in Supplementary Table 1. All experiments were conducted in the Acala Ac44E line of *Gossypium hirsutum*. Plants were grown in 3.5-inch square pots with Lambert mix at 23C, with 16 hour days and 87% humidity.

5.2 Plant inoculation

Xcm strains were grown in GYE medium overnight introduced via syringe inoculation at a controlled OD_{600} of 0.1±0.01. All inocula were suspended in 10mM $MgCl_2$. Infiltrated area was marked with a sharpie on the underside of the leaf for downstream reference. Symptoms were monitored for water soaking phenotype up to 10 days after inoculation (see Supplementary Figure 2). 10-day old cotyledons were used as disease vectors.

5.3 Bacterial transformation

All *E. coli* transformations were performed using chemically competent bacteria and the NEB-described protocol. *Xcm* transformations were performed using either electroporation or tri-

parental mating (Figurski and Helinski 1979) with a modifier strain containing pUFR054 to increase efficiency (Feyter and Gabriel 1991). Transformants were selected using species-specific media (see above) and appropriate antibiotic.

5.4 Southern blotting

Southerns were performed using an XmnI and EcoRI digest, yielding unique fragments for each TAL effector as predicted by genome sequencing of wildtype Xcm4 and allowing mutants to confidently be mapped using Southern blotting. A synthetic TAL effector generated through Golden Gate cloning (Cermak et al. 2011) was used as the probe for each blot to identify the TALs, and the kanamycin resistance gene was excised from pTeM5 and used as a probe to test for double knockouts (data not shown). Blots were allowed to hybridize overnight in each case. GE Southern hybridization kits were used to perform hybridizations and imaging.

5.5 Bacterial population measurement

Bacterial measurement was primarily performed using quantification via the *Lux* operon. *Lux* expression was measured using optical density from a CCD camera (Bio-Rad) with 10 second exposure, and areas of infection manually outlined using ImageLab software (Bio-Rad) with detected luminescence averaged over the area. For knockout assays, software-calculated background luminescence was removed for calculation of final luminescence. Colony counts were generated by taking 21mm^2 standard sterile punches of infected leaf tissue before

suspending and grinding in 10mM $MgCl_2$ and dilution plating at various orders of magnitude from 10^{-1} to 10^{-8}. Individual colonies were then counted at dilution, and back-calculated to density measurements for comparison.

5.6 Statistical analysis

Statistical analysis was performed using RStudio software. The package ggplot2 from Tidyverse was used to generate several figures for this thesis. All other figures were generated using the Microsoft Office Suite (Microsoft).

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7 Supplementary figures

7.1 Supplementary Table 1: Strains and Plasmids

Strain or Plasmid	Relevant details	Source
E. Coli		
DH5α	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ$^-$	
Xanthomonas citri pv. malvacearum		
Xanthomonas citri pv. malvacearum 4 (Xcm4, Xcm4-TAMU)	Wild-type, induces Cotton Bacterial Blight. Also referred to as "Xcm4-TAMU" during early experiments to differentiate from the sequenced substrain, Xcm4-s.	(Cox et al. 2017)
Xanthomonas citri pv. malvacearum 4 sequenced (Xcm4-s)	Subculture of Xcm4 that was sent for whole genome long-read sequencing. Exhibited weak EPS production on plates and significantly reduced virulence *in planta*, leading future investigation to center on Xcm4.	(Cox et al. 2017)
Xanthomonas citri pv. malvacearum 7 (Xcm7, Xcm7-TAMU)	Wild-type, induces Cotton Bacterial Blight. Also referred to as "Xcm7-TAMU" during early experiments to differentiate from the sequenced substrain, Xcm7-s. Not pursued in these experiments at behest of collaborators at Texas A&M University.	(Cox et al. 2017)
Xanthomonas citri pv. malvacearum 7 sequenced (Xcm7-s)	Subculture of Xcm7 that was sent for whole genome long-read sequencing. Not pursued in these experiments at behest of collaborators at Texas A&M University.	(Cox et al. 2017)

Xanthomonas citri pv. malvacearum MSCT1	Virulent strain of Xcm used as a positive control to confirm plant growth conditions allow for CBB development.	(Cox et al. 2017)
Xcm4ΔTal7b	Tal7b knockout strain of Xcm4, KmR	This study
Xcm4ΔTal7b::pTSB2, pTSB3	Tal7b knockout line with the gene complemented in *trans*, as well as a plasmid transformation of a *lux*-encoding plasmid, SpR, KmR, GmR, ApR	This study
Xcm4ΔTal7b::pKEB1::dTALSWEET14	Tal7b knockout line with a designer TAL effector encoded on pKEB1 to test whether phenotype could be rescued. KmR, GmR	This study
Plasmids		
pKEB1	Low-copy gateway destination vector, ApR, GmR	(Cox et al. 2017)
pHM1	Broad host range cosmid, IncW, pSa ori, oriV, oriT, parA, cos, Smr, Spr	(Innes et al. 1988)
pSLR2	Gateway destination vector	(Lewenza et al. 1999)
pTSB1	pHM1::gwR_1R_2, CmR, ccdB	This study
pTSB2	pTSB1::gw(*luxCDABE*), SpR, SmR	This study
pTSB3	pKEB1::gw(Tal7b), GmR, ApR	This study
pTeM5	pSM7::Tn5, a suicide-vector copy of PthXo1 encoding the Tn5 transposon with the Kanamycin resistance gene. KmR, ApR.	(Cox et al. 2017)

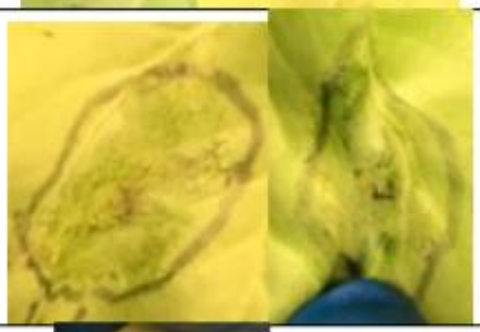
pUFRO54	Modifier plasmid for Tri-parental mating; M·XmaI, M·XmaIII, TcR	(Feyter and Gabriel 1991)

7.2 Supplementary Figure 2: Phenotype scoring scale used for assessment

DISEASE SCORING CARD

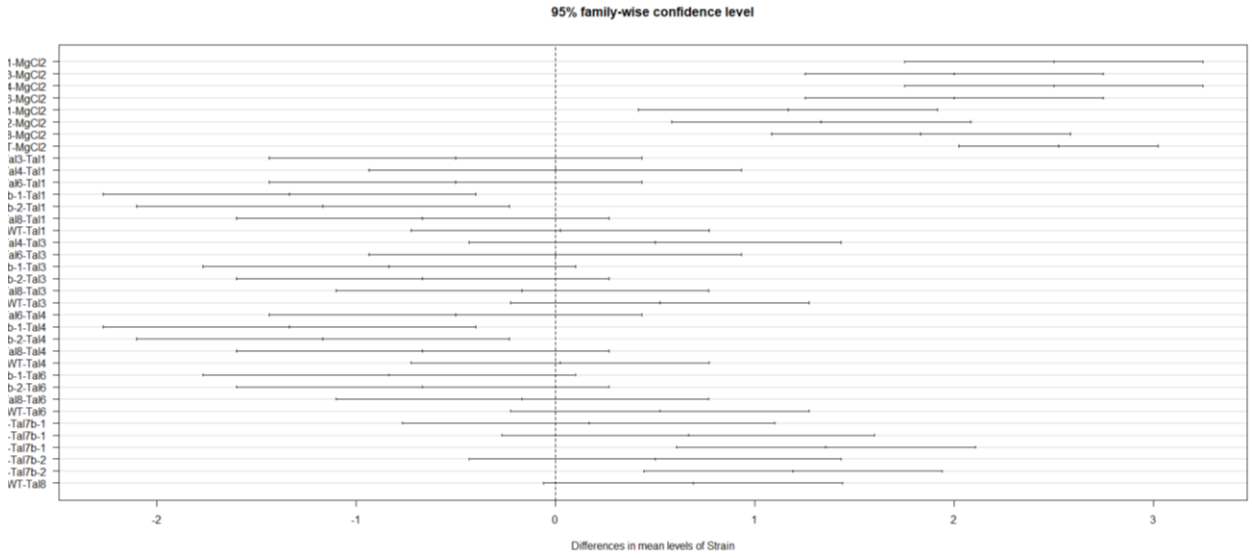
Generated by Taran Bauer and Sara Carpenter, 10/26/20

Photos credit to Sara Carpenter

	Rating level 0 • No evidence of disease present • Resembles negative control and the surrounding leaf tissue
	Rating level 1 • Disease is present, but weak • Very small patches of watersoaking (image right), or pervasive but very light disease (image left)
	Rating level 2 • Disease shows similar severity to 3, but patchy/incomplete appearance over the course of the leaf
	Rating level 3 • Disease symptoms present in most, if not all, of the leaf • Disease is darker in color, but with vasculature still evident
	Rating level 4 • Inoculated area shows complete watersoaking • Watersoaking is deep and dark in colour • Disease may also spread outside of the original inoculated area • Appearance resembles water leaking out of the leaf, with almost a bubbly appearance

Phenotype scores used to assess severity of disease. Scale is based on prior literature (e.g. Zachowski and Rudolph 1988) and assesses severity of water soaking in cotyledons. Representative photos of each level of phenotype are attached, alongside diagnostic criteria.

7.3 Supplemental Figure 3: ANOVA test for knockout phenotype



Supplementary Figure 3: ANOVA of knockout assay. Plot of Tukey's Honest Significant Difference test for each pairwise combination of TAL knockout lines present in the knockout assay. Of importance are the significant difference found between the Tal7b knockout strains and the MgCl2 blank, as well as the significant difference found between the Wildtype and the Tal7b knockout lines.

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