

SEED-BORNE DISSEMINATION OF THE COTTON BACTERIAL BLIGHT PATHOGEN
UNDER FIELD CONDITIONS AND STRATEGIES FOR TRANSLATIONAL
RESISTANCE

BY

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(Under the Direction of Brian H. Kvitko)

ABSTRACT

The reemergence of cotton bacterial blight (CBB) caused by *Xanthomonas citri* pv. *malvacearum* (Xcm) after a multi-decade absence raises questions regarding the factors underlying its resurgence and possible reservoirs of the pathogen. This study investigated seed-borne transmission of Xcm under field conditions and explored genetic modifications strategies to enhance cotton resistance. Leave sample testing and genotyping of recovered isolates from field trials conducted over two growing seasons revealed that CBB-resistant cultivars can serve as inoculum sources for the disease. In our first genetic modification strategy, we attempt to introduce the AtEFR pattern recognition receptor (PRR) construct into cotton but genotyped 136 EFR candidate plants lack the transgene. In our second strategy to disrupt susceptibility gene expression by Xcm Transcription-activator-like (TAL) effectors, we introduced and confirmed mutations in GhTFIIA γ that can prevent the interaction between Xcm effector and host protein however, infertility of 2 lines with high edit efficiency hindered progress.

KEYWORDS: *Xanthomonas citri* pv. *malvacearum*, Cotton, Seeds, Bacteria, Resistant

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DEDICATION

This thesis is dedicated to everyone who has been instrumental to my learning and development.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

JUSTIFICATION

Cotton (*Gossypium*), a member of the *Malvaceae* family serves as the fundamental pillar of the textile industry. This indeterminate crop holds exceptional economic significance within the botanical family and yields a boll encompassing white fibers that can be used in the manufacturing of garments, bags, denim trousers, and jackets. The utilization of cotton fiber in these products is attributed to its multitude of advantageous qualities which includes comfort, color retention, absorbency and strength. (Hegde et al., 2004).

Most of the cotton production in the United States (US) is concentrated within the region commonly referred to as the Cotton Belt, encompassing the southeastern states. Among these states, Georgia ranks second in terms of cotton production with Texas leading the way and contributing approximately 40% of the total cotton production in the United States in recent years. (Meyer, 2018) and (USDA, 2022b).

Cotton, like other agriculturally significant crops encounters various pathogenic threats from nematodes, fungi, viruses, and bacteria. Among these, cotton bacterial blight (CBB) induced by *Xanthomonas citri* pv. *malvaceum* (Xcm) stands as the predominant bacterial disease of paramount economic importance in cotton cultivation because it not only leads to yield reduction but also diminishes the marketable quality of cotton fiber (Rothrock et al., 2015). This pathogen has been controlled by classical resistance genes and acid delinting of seeds for more than 50 years but its re-emergence in the southeastern US instigated an evaluation of current cotton production practices (Phillips et al., 2017; Rothrock et al., 2015). Modern molecular and genomic technologies can now be used to deduce the underlying cause of this

disease re-emergence and pinpoint optimized routes towards the development of durable resistance. (Phillips et al., 2017; Rothrock et al., 2015).

The objective of this study is to incorporate resistance against cotton bacterial blight (CBB) into cotton lines by introduction of *EFR* and *TFIIA* γ -like disease resistance traits, employing transgenic and genome editing methodologies. Concurrently, this study seeks to examine the role of inoculated CBB-resistant seed in the dissemination of CBB and evaluate the potential for seed-to-seed transmission of the disease. By addressing these research aims, we aim to bridge existing knowledge gaps pertaining to the reemergence of CBB, its epidemiology, and contribute to the development of enduring, broad-spectrum resistance. The outcomes of this study will offer significant benefits to cotton growers, as it will mitigate yield losses and minimize disease management expenses, thereby augmenting their profitability.

INTRODUCTION

Host

Production, origin and traits of cultivated cotton

Cotton (*Gossypium* sp.) holds a preeminent position as the primary natural fiber utilized in global textile production, representing approximately 50% of the total fibers employed in the textile industry (Hegde et al., 2004) and (Krifa & Stevens, 2016). Taxonomically, it falls within the *Malvaceae* family and the *Gossypium* genus. The basic chromosome number of *Gossypium* is 13 and seven genomes of *Gossypium* species designated A, B, C, D, E, F, and G, have been identified according to chromosomal size and affinity at meiosis. (Acquaah, 2012).

The *Gossypium* (*G.*) genus comprises over 60 species with 45 of them being diploid and the remaining fifteen species being tetraploid (Emani, 2016). Furthermore, the genus can be categorized into two groups based on ploidy: diploid ($2n = 2x = 26$) and tetraploid ($2n = 4x = 52$)

(Wendel et al., 2012) and (Emani, 2016). The old-world cotton ($2n=26$) consists of diploids with A, B, E, or F genomes. The cultivated types have the AA genome and comprise *Gossypium herbaceum* which has five races that originated in Africa and Asia and *G. arboreum* which has six races of tree cotton found in India. (Acquaah, 2012).

New world cotton ($2n=52$) consists of tetraploids with the genome AADD (13 pairs of each of large and small chromosomes). The dominant species are *Gossypium barbadense* (Sea Island and Egyptian cotton) and *G. hirsutum* (upland cotton) of which 90 per cent of the current world production use this species. (Acquaah, 2012).

According to (Ritchie et al., 2007) wild cotton exhibits an indeterminate fruiting pattern wherein it continues to produce vegetative tissue even after transitioning into its reproductive development stage. This characteristic poses a disadvantage to the crop production system as it diverts valuable plant resources away from seed and lint production. To mitigate this issue, commercial cultivation of cotton typically adopts an annual crop approach. In terms of its growth characteristics, cotton plants can reach heights of 1.5 to 2.0 meters. The emergence of the first true leaf occurs approximately eight days after seedling establishment, while the appearance of the initial flower takes place around 59 days after planting. Harvesting of the cotton crop can be conducted approximately 128 days after the initial planting, as reported by (Chaudhry et al., 2003), The growth and development of cotton are significantly influenced by temperature.

Cotton growth rates were reported to be slower on cooler days compared to warmer days, highlighting the importance of temperature measurements throughout the crop's growing season to estimate developmental stages accurately. Cultivated cotton plants exhibit a well-defined developmental pattern under favorable conditions of moisture, temperature, and light as described by (Ritchie et al., 2007). Notably, a significant portion of the cotton plant's growth cycle is

dedicated to the development and maturation of cotton bolls. To achieve successful cotton cultivation, extended periods of abundant sunlight, high temperatures, and moderate rainfall between 60-120 cm has been reported as requirements for cotton plant growth by (Khan & Khaliq, 2004; Rahman et al., 2018; Usman, 2009).

Importance of cotton

Cotton production holds significant importance in numerous economies worldwide because it serves as a key contributor to economic growth and development. (Ahmad & Hasanuzzaman, 2020) highlights that cotton's annual economic impact is estimated to be around \$600 billion globally. This substantial figure underscores the fact that cotton is the foremost natural fiber produced and traded on a global scale. The economic influence of cotton production extends to various sectors and encompasses aspects such as agriculture, manufacturing, trade, and employment opportunities. According to the United States Department of Agriculture (USDA, 2022a), in the year 2022, the planted acreage of cotton in the United States was reported to be approximately 13.8 million acres. This extensive cultivation area produced a total of 14.7 million bales of cotton. Among the states within the US, Texas, Georgia, Mississippi, Oklahoma, Alabama, and Arkansas emerged as the leading cotton-producing states. These states were responsible for a significant portion of cotton production, highlighting their prominence in the cotton industry within the country. According to Food and Agriculture Organization (FAO., 2021), cotton plays a vital role in sustaining the livelihoods of over 100 million families globally by serving as a source of income and employment particularly in some of the world's most impoverished regions. In terms of its productivity, a single bale of cotton, weighing around 480 pounds of cleaned cotton lint holds immense potential as this amount of cotton can be utilized to produce more than 200 pairs of jeans or 1,200 t-shirts. (USDA, 2022b). These statistics emphasize

the significant economic and social contributions of the cotton industry, highlighting its capacity to support millions of families worldwide while also serving as a key raw material to produce various textile products.

In addition to its fiber, cotton provides valuable seed and byproducts that offer a range of feed ingredients which can effectively reduce the cost of beef cattle production. For instance, both cottonseed meal and whole cottonseed can be utilized as components in cattle rations across different classes of cattle (Mullenix & Stewart, 2021). This shows the versatility of cotton byproducts in the livestock industry particularly in supporting cost-effective and nutritious feed options for beef cattle.

Disease

CBB causal agent and taxonomy

Xanthomonas citri pv. *malvacearum* (Xcm) is the causal agent of cotton bacterial blight (CBB), a disease that encompasses various manifestations including seedling blight, black arm, stem canker, angular leaf spot, and bacterial boll rot. CBB was regarded as the most destructive disease of cotton and leads to substantial yield losses, particularly during the rainy season (Delannoy et al., 2005). The first reported occurrence of CBB dates to 1891 in Alabama, United States as documented by (Atkinson, 1891). Since then, the disease has been a significant concern for cotton growers due to its detrimental impact on crop productivity. The various manifestations of CBB affect different parts of the cotton plant, leading to reduced yields and quality of bolls thereby posing a significant threat to cotton cultivation.

This pathogen has undergone several name changes over time. In 1978, Dye named it as *Xanthomonas campestris* pv. *malvacearum* based on physiological and biochemical characteristics, DNA-DNA (deoxyribonucleic acid) hybridization, and host specificity (Dye,

1978). Later, (Vauterin et al., 1995) renamed it as *Xanthomonas axonopodis* pv. *malvacearum*, considering 16S rDNA and DNA-DNA hybridization. (Schaad et al., 2006), further revised the name to *Xanthomonas citri* subsp. *malvacearum* considering DNA-DNA hybridization, repetitive-PCR (re-PCR), serology, internal transcribed spacer (ITS), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Finally, (Ah-You et al., 2009) settled on the name *Xanthomonas citri* pv. *malvacearum*, based on DNA-DNA hybridization, amplified fragment length polymorphism (AFLP), and multilocus sequence analysis (MLSA) involving 16S rDNA, *gyrB*, *atpD*, and *dnaK*. These name changes reflect the advancements in molecular techniques and the refinement of taxonomic classification methods, allowing for a more accurate understanding of the bacterial pathogen's identity and its relationship with other *Xanthomonas* species.

Symptoms

CBB begins as small water-soaked lesions on leaves, seedlings, mature plants and most importantly boll rot. The lesions progress into characteristic angular shapes when leaf veins limit bacterial movement (Brinkerhoff, 1970). Unlike many other lesions on cotton leaves, those associated with bacterial blight are more triangular or rectangular, although the shape may be more difficult to distinguish with leaf aging. It can occur at any stage in the plant's life cycle and on any aerial organ. (Hillocks, 1992; Mohan, 1983; Rothrock et al., 2015; Verma, 1986a).

Bacterial lesions may appear on the upper surface of the leaf, however, the wet or “greasy” appearance of the lesions is often observed more clearly on the underside of the leaf. The bacteria can spread internally to the seed and survive for many years. (Ritchie et al., 2007).

Genus *Xanthomonas*

Xanthomonas, a gram-negative, rod-shaped bacterium belonging to the family *Xanthomonadaceae*, encompasses numerous pathovars primarily associated with infections in diverse plants, including 124 monocots and 268 dicots (An et al., 2020). Its impact is particularly significant in regions characterized by warm and humid climates (Chan & Goodwin, 1999; Leyns et al., 1984). This genus exhibits an extensive host range, demonstrating remarkable diversity in its ability to infect various plant species (Brunings & Gabriel, 2003). One distinctive feature of *Xanthomonas* is the production of a yellow pigment known as xanthomonadin which serves as an important chemotaxonomic and diagnostic marker for the identification and classification of the genus. The population structure and diversity of *Xanthomonas* pathogens are influenced by processes such as recombination and horizontal gene transfer across different *Xanthomonas* pathosystems (Mansfield et al., 2012). Several factors have been identified to play a role in host specificity and bacterial pathogenicity in various *Xanthomonas* species. These include the type III secretion system (T3SS) and its associated effectors, lipopolysaccharides, adhesins, transcription factors, and TonB-dependent receptors (Mansfield et al., 2012; White et al., 2009).

Xanthomonas spp. use the T3SS, encoded by the *hrp* (hypersensitive reaction and pathogenicity) cluster to translocate proteins referred to as type III secreted effectors (T3SEs) into plant host cells. (Rossier et al., 1999; White et al., 2009). *Xanthomonas* T3SEs are generally called *Xanthomonas* outer proteins (Xops), except for AvrBs1, AvrBs2 and AvrBs3, which are traditionally associated with their respective avirulence phenotype, recognized by corresponding R proteins from hosts, resulting in effector-triggered immunity (ETI) (Buttner & He, 2009; White et al., 2009). The T3SS plays a significant role in suppressing host defenses and facilitating disease

progression in *Xanthomonas* and other bacterial pathogens. However, other pathogenicity factors also contribute to the overall virulence and fitness of the pathogen. These factors include cell wall-degrading enzymes secreted by the type II secretion system (T2SS), type IV secreted effectors such as VirB1 to VirB11 and VirD4, the type VI secretion system (T6SS) and its associated effectors, adhesins, lipopolysaccharides, small RNAs, and various transcriptional regulators such as Rpf, HrpG, HrpX, HpaR, Clp, Zur, FhrR, and RsmA (Mansfield et al., 2012; Timilsina et al., 2020). Although not all these secretion systems and factors are directly involved in the pathogen's virulence, they can impact the overall fitness and success of the pathogen (Buttner & Bonas, 2010; Mansfield et al., 2012).

Disease cycle and Epidemiology

The life cycle of Xcm can be divided into active and quiescent stages. During the active stages, moisture plays a critical role in the spread of the bacterium and its ability to infect plants. In this stage, favorable environmental conditions including moisture facilitate the growth and dissemination of the pathogen (Weindling, 1948). On the other hand, during the quiescent stages, dry tissues and debris from previously harvested cotton crops provide the most suitable conditions for the survival of Xcm. The pathogen can persist in dry plant materials, such as crop residues, facilitating its survival between growing seasons (Schnathorst, 1968). The understanding to these active and quiescent stages of Xcm life cycle has been crucial in implementation of appropriate management strategies to control the disease, such as crop rotation, sanitation practices, and the use of disease-free seeds. However, the initial inoculum of the pathogen is seed-borne, meaning it can be present in infected seeds and serve as a source of infection for subsequent plantings (Mohan, 1983). Weeds can also serve as a potential source of inoculum for Xcm, this is evident from a field

survey conducted by (Koczan et al., 2017) where they reported the isolation of Xcm from weed samples. Their finding suggests that weeds can harbor the pathogen and contribute to its spread within agricultural ecosystems.

Mode of Transmission

Studies by (Faulwetter, 1917; Rolfs, 1915; Wickens, 1956) provide valuable insights into the transmission of the disease through seed contamination. (Rolfs, 1915) reported that the bacterium could be transmitted by cotton seeds that were externally and/or internally contaminated. This suggests that both surface contamination and internal colonization of the seeds can contribute to disease development in cotton seedlings. (Faulwetter, 1917) demonstrated that surface contamination of cotton seeds can lead to disease development in cotton seedlings. This indicates that the presence of Xcm on the seed surface can result in infection and subsequent disease symptoms in young plants. (Wickens, 1956) observed seed-to-seedling transmission of CBB by artificially inoculating cotton seeds with Xcm. The study demonstrated that when the seeds were inoculated with the pathogen, symptom development occurred in the resulting seedlings, confirming the ability of the bacterium to be transmitted from seed to seedling.

Secondary Spread

Many abiotic and biotic factors contribute to the secondary pathogen spread of Xcm. Including rain, wind, wind-blown rain, irrigation water, and plant-to-plant contact (Faulwetter, 1917). However, there is a discrepancy in the literature regarding the role of insects in the dissemination of Xcm. While (Faulwetter, 1917) reported that insects do not play a role in the transmission of Xcm, suggesting that they are not involved in the dissemination of the pathogen. A contrasting finding was reported by (Thaxton & El-Zik, 2001) they reported that insects can indeed

act as vectors for the transmission of Xcm. According to their research, insects are responsible for carrying and spreading the pathogen, contributing to its dissemination within and between cotton plants. Observations made during the 1945 experiments by (Weindling, 1948), indicated that most of the natural spread of bacterial blight in the seedling stage in the field was brought about by movement of inoculum in drainage water at the time of washing rains late in May.

Factors affecting Infection and Disease enhancement.

The aperture of stomata, which regulate gas exchange in plants plays a crucial role in the infection process of Xcm. It has been observed that the successful invasion of mature cotton leaves by Xcm occurs predominantly when stomata are open (Weindling, 1948). Regardless of whether bacterial suspensions were applied to the upper or lower leaf surface, the pathogen was able to invade the host through stomata. Additionally, (Innes, 1983) noted that Xcm can enter the host plant through natural openings or wounds and rapidly disseminate throughout the vascular tissues, causing localized vascular infections in any aerial parts of cotton plants at any growth stage.

Furthermore, the presence of water congestion in intercellular spaces, which are normally filled with air and the initial concentration of bacteria in these intercellular spaces are additional factors that can influence the duration of the incubation period and the progression of the disease. (Weindling, 1948). The ability of the pathogen to penetrate the host through stomata, as well as other natural openings and wounds allows it to access the vascular tissues and rapidly spread within the host. The presence of water congestion and the initial bacterial concentration in the intercellular spaces might further contribute to the disease development. Susceptibility of cotton to bacterial blight is also affected by the stage of development and the condition of leaves and plants. (Smith, 1921) in his recognized that juicy or succulent tissues favor disease development.

Environmental factors

(Voloudakis et al., 2006) conducted a study on cotton bacterial blight and observed that the severity of the disease was more pronounced in subhumid regions compared to semiarid regions. They found that regions with higher wind activity, rainfall ranging from 25.4 to 76.2 mm and dust events during the growing season were more susceptible to the disease. Additionally, (Kirkpatrick et al., 2001) reported that disease infestation was higher in areas with high humidity as it created favorable conditions for the growth and spread of the pathogen.

Asymptomatic infection

Asymptomatic infection refers to the presence of a pathogen within a host plant without any visible symptoms or signs of disease. Research conducted by (Stoughton, 1930) provided insights into the phenomenon of asymptomatic infection in cotton plants by Xcm. Stoughton's work suggested that infected plants may remain symptomless until environmental conditions become favorable for the development of visible lesions (Stoughton, 1930). Additionally, Stoughton's report proposed that these types of infections can lead to the presence of the pathogen in internally infected seeds. (Wickens, 1956) expanded on the understanding of Xcm's infection process by observing the movement of bacteria from symptomatic cotyledons through the petiole and documenting the progression of symptoms. Recent studies, such as the one conducted by (Gluck-Thaler et al., 2020) have indicated that Xcm is considered a non-vascular pathogen of cotton. This conclusion is supported by the absence of the *cbsA* gene, which is associated with a vascular colonization lifestyle, as well as the lack of clear evidence demonstrating vascular colonization and spread by Xcm which has now been demonstrated by (Mijatović et al., 2021).

Morphology and physical properties of *Xanthomonas citri* pv. *malvacearum*

Xcm is classified as a gram-negative bacterium. As a characteristic feature of gram-negative bacteria, it possesses a lipopolysaccharide cell wall constituent that serves as a selective barrier, offering protection against certain antimicrobial compounds (Innes, 1983). Morphologically, Xcm appears as rod-shaped cells with a single flagellum located at one end, enabling the bacteria to exhibit motility and move short distances within a liquid environment through rotational motion. (Showmaker et al., 2017).

The colonies of *Xanthomonas* spp., including Xcm, exhibit a distinct appearance characterized by a yellow coloration, fluidity, stickiness, and sliminess which can be attributed to the abundant production of extracellular polysaccharides (EPS). EPS are high-molecular-weight carbohydrates that adhere to the outer surface of bacterial cells and play a significant role in promoting the disease process (Schumann & D'Arcy, 2010).

The identification and characterization of Xcm strains have revealed the existence of multiple physiological races. Based on their reaction to a set of 11 host differential strains, a total of 22 physiological races of Xcm have been identified (El-Zik & Thaxton, 1994; Hunter et al., 1968). Among these races, Race 18 has been observed to be the most virulent and prevalent in various countries including the United States (Thaxton & El-Zik, 2001; Verma & Singh, 1975).

Pathogenicity and virulence factors of *Xanthomonas*

Xanthomonas were predicted to employ a range of virulence factors to enhance its pathogenicity and adapt to host environments. These factors include exopolysaccharides, lipopolysaccharides, adhesins, protein secretion systems, siderophores, quorum sensing, biofilms, chemotactic sensors, and degradative enzymes (Buttner & Bonas, 2010). The type III secreted effector (T3SE) proteins have been found to play a crucial role in bacterial pathogenicity. In the

case of *Xanthomonas* spp., T3SE proteins have been identified and implicated in their pathogenicity (Melotto & Kunkel, 2013). These proteins are believed to contribute to the ability of *Xanthomonas* spp. to colonize host tissues, manipulate host immune responses, and facilitate disease development.

The Type III secretion system (T3SS) plays a crucial role in the pathogenicity of *Xanthomonas* spp. by facilitating the translocation of effector proteins from the bacterial cytosol into host cells (Ghosh, 2004). These effector proteins are responsible for interfering with immune responses in the host, including the recognition of Microbe-Associated Molecular Patterns (MAMPs), thereby promoting a favorable environment for bacterial proliferation within host tissues (Gala'n & Collmer, 1999). *Xanthomonas* spp. possess hrp genes that encode a functional hrp T3SS, which is essential for their pathogenic behavior. Studies by (Cornelis & Van Gijsegem, 2000) demonstrated that mutation of any of the hrp genes resulted in a complete loss of pathogenicity, highlighting the critical role of these genes in encoding components of the T3SS. Among the T3SEs, transcriptional activator like effector (TALE) proteins have been extensively studied.

These TALE proteins are delivered into the plant cell via the needle-like T3SS and contribute to the pathogenicity and manipulation of host cellular processes by interacting with specific host targets. (Gala'n & Collmer, 1999). TALE proteins, functionally resemble eukaryotic transcription factors (Buttner & Bonas, 2010) are translocated to the host plant nucleus where they bind to specific promoter sequences known as effector-binding elements (EBEs) and regulate host gene (UPA20, Os8N3, OsTFIIAg1, or OsTFX1) expression (Boch & Bonas, 2010; Boch et al., 2009). TALEs belong to the *avrBs3/pthA* gene family which is highly conserved among different *Xanthomonas* spp. However, closely related proteins have been found in several biovars

of *Ralstonia solanacearum*. (Boch et al., 2014a). TALEs contain an N-terminal T3S signal domain, a central repeat region (CRR), C-terminal nuclear localization signals (NLS) that guide the protein's translocation into the nucleus of the host cell (Boch et al., 2009; Huang et al., 2017; Kay & Bonas, 2009) and an acid activation domain (AD) that allows the TAL effector protein to start transcription (Boch et al., 2009; Buttner & Bonas, 2010; Huang et al., 2017). CRRs contain tandem repeats of 33–35 amino acids that differ only at residues 12 and 13; these are designated repeat variable di-residues (RVDs) and determine the specificity of DNA binding (Boch & Bonas, 2010; Boch et al., 2009; Deng et al., 2012). TALE-mediated activation of EBEs can induce host susceptibility (S) or resistance (R) genes (Boch & Bonas, 2010).

Resistant varieties

Resistance to CBB is currently characterized by the hypersensitive response (HR), a rapid localized cell death at the sites of infection. A significant number of resistance genes, known as B genes, have been identified in cotton to confer resistance against Xcm. Currently, over 20 major BB resistance genes have been identified, including B1, B2, B3, B4, B5, B6, B7, B8, B9K, B9L, B10K, B10L, B11, B12, BIn, Bn, Bs, and four unnamed genes. These resistance genes have been documented in various studies such as (Brinkerhoff, 1970; Innes, 1983; Jalloul et al., 2015; Knight, 1948; Knight & Clouston, 1939; Verma, 1986a; Zhang et al., 2020), among these resistance genes, B12 is of particular significance as it confers resistance to all races of Xcm, including the highly virulent HV1 strain from Africa and Race 18. The broad-spectrum B12 resistance gene was initially identified in the upland cotton cultivar S295, which originated from Africa, as reported by (Wallace & El-Zik, 1989). Minor genes, such as Bsm and Dsm, which consist of polygene complexes, also contribute to resistance against Xcm, albeit in a more specific manner, targeting different races of Xcm. (Bird & Hadley, 1958). Disease resistance gene(s) in plant cultivars can

break down in the field due to the evolution of pathogens under high selection pressure. Thus, the protection of crop plants against pathogens is a continuous arms race (Kankanala et al., 2019).

Xcm strains can evolve to overcome the resistance conferred by a single gene but cotton possesses a limited amount of resistant genes within its genome underscoring the imperative for the development of enhanced strategies to ensure adequate protection of the plant. For example, the pyramiding of multiple B genes to enhance resistance against multiple races of Xcm through the combination of B2 with B3 and other polygenic complexes has proven effective in providing significant protection against broad races of Xcm in the United States, as documented by (Essenberg et al., 2014).

Cotton defense against Xcm

Microscopic examination of hypersensitive response (HR) tissues revealed distinct cellular changes at the infection sites. These changes included the rapid collapse of cells with retracted plasmalemma, condensed cytoplasm, disorganized organelles such as chloroplasts and nuclei, and the accumulation of electron-dense material. These observations has documented by (Al-Mousawi et al., 1982), provided insights into the cytological responses associated with Xcm-cotton interactions.

Reactive oxygen species (ROS) are generated through the oxidative burst, which is involved in various physiological processes throughout the life of a plant. In cotton, ROS production has been observed during fiber elongation, as documented by (Mei et al., 2009). In the context of HR-like resistance, the oxidative burst is considered a crucial event. For instance, in cotyledons of the Réba B50 cultivar containing the B2B3 genes and challenged by Xcm race 18, a significant peak in the burst was observed at 3 hours post-inoculation (hpi). It has been suggest by (Jalloul et al., 2002) that the oxylipin pathway likely plays a central role in the defense strategy

of cotton. They suggest that jasmonic acid (JA) mediates HR cell death in response to Xcm by regulating several defense responses, including the transcriptional activation of GhLox1 gene.

Work done by (Wang et al., 2020) identified *Gossypium hirsutum* GhWAK7A, a wall-associated kinase, that positively regulates cotton response to both *Verticillium dahliae* (Vd) and *Fusarium oxysporum* sp. vasinfectum (Fov) Vd and Fov infections by directly interacting with both GhLYK5 and GhCERK1 to promote chitin-induced GhLYK5-GhCERK1 dimerization and suggest that GhWAK7A might perceive an unknown ligand from Vd and Fov to activate defense signaling. (Li et al., 2020; Saud & Wang, 2022) also report that cotton shows a specific response and resistance to the threat of non-biological adversity with a response pathway from perception to expression of resistance-associated genes.

Management

In addition to seed treatments and resistant varieties, various agronomic practices have been implemented in certain regions to control bacterial blight of cotton. These practices include crop rotation to reduce the amount of inoculum that survives between cotton crops, appropriate land preparation to ensure adequate drainage, balanced fertilization, efficient irrigation, destruction of infected plant parts and deep soil processing to bury plant residues (Hussain et al., 2014; Kemeraït et al., 2017). These strategies aim to minimize the availability of favorable conditions for the pathogen and reduce disease incidence. The presence of Xcm in weeds has reported by (Koczan et al., 2017) underscores the importance of implementing effective weed management practices as part of integrated disease management strategies for cotton bacterial blight. Controlling weeds in and around cotton fields can help reduce the potential reservoirs of the pathogen and minimize its transmission to cotton plants, thereby aiding in disease prevention and

control because, a single infected plant in cotton field can create wild spread diseases within that particular area under favorable environmental conditions (Jalloul et al., 2015).

To manage the presence of cotton diseases, farmers have adopted various strategies to keep disease levels under control. Two practical methods for controlling bacterial blight of cotton are seed treatments like acid delinting and treatment with specific germicidal dusts and the utilization of resistant varieties.

Acid delinting

One commonly employed seed treatment approach is acid delinting, which involves the removal of fuzz from ginned cottonseed and surface sterilization (Verma, 1986a). Acid delinting systems can be categorized into three major types: wet-acid, gas-acid, and dilute wet-acid (Delouche, 1981). The wet-acid and gas-acid systems yield lint-free seed with excellent flowability, while the dilute wet-acid process results in lint-free "black" seed or partially-but uniformly-delinted seed. (Duggar & Cauthen, 1914) observed that treating the seed coat with concentrated sulfuric acid, a process known as "charring," reduced the percentage of cotton bolls infected with "boll rot" or anthracnose from 11.3 to 5.9 percent. (Mooers et al., 1926; Young, 1942), also reported the beneficial effects of acid delinting in controlling various diseases.

In contrast to earlier research suggesting that acid delinting effectively removes Xcm and other bacteria from the seed surface, subsequent studies by (Alexander et al., 2012; Hunter & Brinkerhoff, 1964) have indicated that the bacterium can persist at low inoculum levels following these treatments, thus, complete elimination of Xcm through acid delinting may not always be achieved.

Reemergence of CBB in USA

Prior to 2011, CBB was effectively controlled in the United States through various measures such as seed sterilization, classical resistance genes, and agricultural practices including crop rotation and equipment sterilization (Alexander et al., 2012). These strategies successfully prevented the disease from posing significant economic concerns. However, during the 2011 growing season, an outbreak of CBB was observed in Missouri, Mississippi, and Arkansas, marking a re-emergence of the disease.

(Phillips et al., 2017) conducted a study shedding light on the re-emergence of CBB as an agronomic problem in the United States. Their research indicated that the resurgence of CBB was not attributed to a substantial genetic change or race shift in the bacterial pathogen. Instead, agricultural factors were identified as the likely cause, specifically the extensive planting of susceptible cultivars. Their study highlighted the importance of deploying new resistance loci to prevent further spread of the disease as many of the popular cultivars preferred by farmers lacked resistance traits. These findings of (Phillips et al., 2017) underscored the need for proactive measures in managing CBB and suggested the incorporation of novel and durable resistance loci into cotton breeding programs. By utilizing varieties with enhanced/new resistance traits, the plant can be prepared for when new Xcm strain emerges that is no longer recognized by the current single locus resistance and curtail the impact and spread of CBB within agricultural systems. These recommendations are crucial for addressing the re-emergence of CBB as an ongoing agronomic concern in the United States.

Biotechnological improvement of cotton

Crop improvement through conventional breeding methods, such as mass selection, recurrent selection, top crossing and pedigree method has traditionally played a vital role in

agriculture. However, these methods are associated with significant challenges, including the lengthy duration of around 7-8 years making the process tedious, laborious, time-consuming, and costly (Hussain et al., 2012; Minhas et al., 2018). In contrast, genetic engineering, a biotechnology approach that involves the direct manipulation of an organism's genetic material offers several advantages over conventional breeding methods. One key advantage is the ability to introduce, remove, or modify specific genes of interest while minimizing undesired changes to the rest of the crop genome. This targeted approach allows for precise genetic modifications, potentially accelerating the development of desired traits in crops (Christou, 2013).

As a result, crops exhibiting desired agronomic traits can be obtained in fewer generations compared with conventional breeding. Secondly, genetic engineering allows for interchange of genetic material across species. Thus, the raw genetic materials that can be exploited for this process is not restricted to the genes available within the species. Furthermore, plant transformation during genetic engineering allows the introduction of new genes into vegetatively propagated crops such as banana (*Musa* sp.) (Adero et al., 2023), cassava (*Manihot esculenta*) (Schöpke et al., 2001), and potato (*Solanum tuberosum*) (Bradshaw, 2021).

These features make genetic engineering a powerful tool for enhancing resistance against plant pathogens. Most cases of plant genetic engineering rely on conventional transgenic approaches or the more recent genome-editing technologies i.e. clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated protein (CRISPR-Cas9). In conventional transgenic methods, genes that encode desired agronomic traits are inserted into the genome at random locations through plant transformation (Lorence & Verpoorte, 2004). In contrast, genome editing allows changes to the endogenous plant DNA, such as deletions, insertions, and replacements of DNA of various lengths at designated targets (Barrangou & Doudna, 2016).

In the context of cotton, transgenic methods have been widely employed to transfer genes that confer resistance to biotic and abiotic stresses. Numerous studies by (Farooq et al., 2019; Hao et al., 2018; Mishra et al., 2017; Wang et al., 2015; Zhang et al., 2008; Zhang et al., 2017) have demonstrated the success of this approach in enhancing stress tolerance in cotton.

The CRISPR-Cas9 system has emerged as a powerful tool for gene editing and is among the latest methods for engineering plant traits. Its application in cotton transformation has shown promising results particularly in the areas of disease resistance and gene function determination (Gao et al., 2017). One notable advantage of the CRISPR-Cas9 system is its effectiveness in inducing mutations in homoeologous cotton genes. Cotton been an allotetraploid crop contains multiple sets of homoeologous genes derived from its ancestral species. The ability to precisely edit these homoeologous genes is crucial for achieving desired trait modifications in cotton.

By leveraging the CRISPR/Cas9 system, we can introduce specific genetic modifications, such as gene knockouts or targeted mutations in cotton, thereby providing a more efficient and precise approach to engineering desirable traits in cotton varieties like enhancing its disease resistance and improved understanding of gene functions.

RESEARCH OBJECTIVES

The overall goals of this project is to investigate seed-borne dissemination of CBB under field conditions and explore strategies for translational resistance in cotton. Findings from these studies will provide further knowledge on the relationship between Xcm and cotton and aid in the management of CBB.

Two objectives are:

1. Recovery and characterization of homozygous cotton lines with the EFR and xa5-like disease resistance traits

2. Test if resistant cotton seeds can serve as an inoculum source for *Xanthomonas citri* pv *malvacearum* under field conditions.

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CHAPTER 2

RESISTANT COTTON SEEDS CAN SERVE AS AN INOCULUM SOURCE FOR COTTON BACTERIA BLIGHT UNDER FIELD CONDITIONS¹

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ABSTRACT

The reemergence of cotton bacterial blight caused by *Xanthomonas citri* pv. *malvacearum* (Xcm) after a multi-decade absence raises questions regarding the factors underlying its resurgence and possible reservoirs of the pathogen. In our prior work we determined that Xcm can effectively colonizes CBB-resistant cotton after seed inoculation. In this study we investigated whether seed-inoculated CBB-resistant cotton cultivars could additionally serve as an inoculum source for CBB-susceptible plants under field conditions. Over two field seasons in Tifton, Georgia, we planted 24 plots with the CBB-susceptible DP 2141NR B3XF. Plots measured 25 feet containing eight rows with 36-inch row spacing and 4-inch seed spacing. Using a randomized complete block design, seedlings between 12 and 15 feet in the fourth and fifth rows were removed and replaced with Xcm inoculated CBB-resistant (PHY 411 W3FE) seed in 8 plots, Xcm inoculated CBB-susceptible (DP 2141NR B3XF) seed in 8 plots while 8 plots were left untouched. To facilitate inoculum source tracking, the CBB-resistant and CBB-susceptible seed were each inoculated with Xcm strains carrying distinct spontaneous antibiotic resistance mutations. Leaves from six plants were collected from each plot at 40, 60, and 80 days after planting (DAP) corresponding to two locations with seed-inoculated plants (rows IV and V) and four locations surrounding the inoculated plants. From each leaf sample, ten 0.2 cm diameter leaf punches were macerated and plated on selective media to recover Xcm. Colonies were confirmed as Xcm by PCR and source tracking was accomplished by antibiotic resistance profiling and genotyping. Over two years Xcm originating from the seed inoculated CBB-resistant plants and CBB-susceptible plants were recovered in roughly equal numbers from the non-inoculated DP 2141NR B3XF CBB-susceptible plants. In both years Xcm recovery was highest at 40 DAP and declined at both 60 DAP and 80 DAP. In addition, CBB symptoms were only observed sporadically and were more prevalent at 40

DAP. Our work supports the model that Xcm-infested CBB-resistant resistant cotton seed can serve as inoculum source for Xcm susceptible cotton and provides evidence for latent asymptomatic Xcm infection.

KEYWORDS: *Xanthomonas citri* pv. *malvacearum*, Cotton, Seeds, Bacteria, Resistant

INTRODUCTION

Cotton is an economically important crop in the United States and a major export commodity generating substantial revenue through global textile industries and contributing significantly to the overall agricultural sector. This significant crop encounters various pathogenic threats from nematodes, fungi, viruses, and bacteria. Among these, cotton bacterial blight (CBB) caused by *Xanthomonas citri* pv. *malvacearum* (Xcm) stands as the predominant bacterial disease of economic importance in cotton cultivation because it leads to both yield reduction and diminishes the marketable quality of cotton fiber (Rothrock et al., 2015).

The first reported occurrence of CBB dates to 1891 in Alabama, United States, (Atkinson, 1891). Since then, the disease has been a significant concern for cotton growers due to its detrimental impact on crop productivity and it is regarded as the most destructive disease of cotton leading to substantial yield losses particularly during the rainy season (Delannoy et al., 2005). CBB begins as small water-soaked lesions (spots) on the leaves of seedling and mature plants and can lead to boll rot. Unlike many other lesions on cotton leaves, those associated with bacterial blight are more triangular or rectangular, although the shape may be more difficult to distinguish with leaf aging. CBB can occur at any stage in the plant's life cycle and on any aerial organ. (Hillocks, 1992; Mohan, 1983; Rothrock et al., 2015; Verma, 1986a, 1986b). (Innes, 1983) noted that Xcm can enter the host plant through natural openings or wounds and rapidly

disseminate throughout the vascular tissues causing localized vascular infections in any aerial parts of cotton plants at any growth stage. Many abiotic and biotic factors contribute to the secondary pathogen spread of Xcm, including rain, wind, wind-blown rain, irrigation water and plant-to-plant contact (Faulwetter, 1917).

Seed infection is a critical factor in the spread, persistence and management of cotton bacterial blight. Previous studies have revealed that Xcm can infect cotton seeds and survive within seeds thereby facilitating the spread of the disease. and seed infestation has been proposed as primary means of transmission between seasons (Innes, 1983; Thaxton & El-Zik, 2001; Verma, 1986a, 1986b). The infection rates of cotton seed can vary widely from less than 1% to 10-20% of seeds (Archibald, 1927; Bain, 1939; Chester, 1938; Rolfs, 1915; Schnathorst, 1968).

Research by (Rolfs, 1915) demonstrated the transmission of CBB through externally and/or internally seed contamination, (Faulwetter, 1917) showed that surface contamination can lead to disease development in cotton seedlings while (Wickens, 1956) observed seed-to-seedling transmission of CBB by artificially inoculating cotton seeds with Xcm. These studies underscore the importance of preventing seed-borne transmission of CBB.

Historical records highlight the severe yield losses caused by Xcm in the early 20th century, which were mitigated with the development of resistant cultivars. Surprisingly, after an interval of approximately 60-70 years, the CBB has resurfaced in cotton fields. Research by (Phillips et al., 2017) uncovered a trend among growers to favor susceptible cotton cultivars over resistant ones. Additionally, their findings revealed that the reemergent Xcm isolates (MS14002 and MS14003) exhibited an unaltered repertoire of virulence proteins, making them indistinguishable from the historical race 18 isolate. Experimental studies focusing on seed-to-

seedling transmission of Xcm have indicated that both susceptible and resistant cotton cultivars can be effectively colonized by this pathogen (Mijatović et al., 2021).

The reemergence of the pathogen after a significant eclipse period raises several intriguing questions including the possible reservoirs of the pathogen during this time frame and the factors that facilitated its resurgence. If it can be concluded that neither the host, the pathogen nor the environment have undergone significant changes then it becomes apparent that our understanding of this system remains incomplete and limited. The available literature is deficient in terms of providing comprehensive insights into the potential transmission capabilities of Xcm by resistant cotton cultivars.

To address some of this knowledge gap, further research is needed to explore the potential reservoir role of covertly infected plants in pathogen dissemination and disease spread. Our objective is to investigate whether inoculated CBB resistant cotton seeds grown under field conditions can serve as a potential source of inoculum and spread to CBB susceptible plants. We conducted field experiments in Tifton, Georgia, over two growing seasons to investigate the spread of Xcm from inoculated cotton seed. Twenty-four plots of eight rows each were established, with susceptible cotton (DP 2141NR B3XF) planted in all plots. In eight plots, seedlings in rows IV and V were replaced with Xcm-inoculated resistant (PHY 411 W3FE), In another eight plots, seedlings in rows IV and V were replaced with Xcm-inoculated susceptible seeds (DP 2141NR B3XF). Leaf samples were collected in six locations per plot at 40, 60, and 80 days after planting (DAP) and analyzed for Xcm presence and symptoms. In both years Xcm recovery was highest at 40 DAP and declined at both 60 DAP and 80 DAP. Xcm originating from resistant plant were recovered from uninoculated susceptible plants while CBB symptoms were only observed sporadically and more prevalent at 40 DAP.

Our results support the interpretation that Xcm can persist within resistant cultivars which may have facilitated the rapid CBB reemergence coinciding with trends in planting CBB susceptible cultivars in the mid-to-late 2010s. We also explore the possible indications of vertical transmission of Xcm through the from seed to seed.

MATERIALS AND METHOD

Recovery of Xcm 4.02RfSm

To isolate Xcm 4.02 RfSm , dense culture suspension of spontaneous rifampicin resistant Xcm 4.02Rf that was previously isolated by (Mijatović et al., 2021) was plated onto LB agar plates augmented streptomycin at 40µg/ml and incubated for 48 hours at 30°C. Single colonies were recovered and streaked to isolation. Isolates were reconfirmed using Xcm-specific PCR (Wang et al., 2019). Cotton cotyledons inoculated with Xcm 4.02 RfSm developed water-soaking lesions similar to those observed on seedlings inoculated with the Xcm 4.02Rf.

Bacterial Inoculation

Xcm 4.02Rf and Xcm 4.02RfSm were streaked from glycerol stock onto LB agar plates supplemented with only 40µg/ml of rifampicin and LB agar plates supplemented with 40µg/ml of rifampicin and streptomycin respectively and incubated for 72 hours at 30°C. Xcm growth from this plate were scrapped with a sterile loop in diluted in water to make a suspension of Xcm with an optical density (OD₆₀₀) of 0.3. The seeds were then immersed in each suspension for 20 minutes according to experimental design ensuring thorough contact between the seeds and the Xcm solution. After the dipping process, the treated seeds were placed in glass petri dishes lined with sterile cotton cloth squares. Approximately 15-20 mL of sterile water was added to each petri dish to provide a suitable environment for seed germination.

Planting and Field Plot Design

The study was conducted at the intersection of Zion Hope road and Carpenter road in Tifton, Georgia in United States with geographical coordinates of 31.4945150 latitude and - 83.5429700 longitude. The first-year trial was between May 17th 2023 and October 3rd 2023 while the second year trial was between June 7th 2024 and September 17th 2024. The field plot layout in both years consisted of a total of 24 plots in a randomized complete block design. DP 2141NR B3XF (CBB-susceptible) and PHY 411 W3FE (CBB-resistant) were used in this field experiment. Each plot measured 25 feet in length with 8 rows. The entire field was planted with susceptible cotton cultivar DP 2141NR B3XF, 2 weeks later, plants between 12ft and 15ft in the 4th and 5th rows of 8 plots (see figure 2.1) each are uprooted and supplied with resistant seeds PHY 411 W3FE and susceptible seeds DP 2141NR B3XF inoculated with either Xcm 4.02Rf or Xcm 4.02RfSm.

The planting method for inoculated seed involved creating three 1-inch-deep holes in the soil, spaced 3 to 4 inches apart. The seeds were then placed into these holes and covered with soil. Two seeds were planted per hole during this replanting process, two weeks after the replanting, the plants were thinned, and additional inoculated seeds were planted in place of non-germinated seeds to ensure expected inoculated plant density.

Sampling

Samples were collected by randomly selecting two leaves from both the upper and lower regions of a plant from surrounding inoculated plants i.e. a plant from row 3 (beside row 4), row 6 (beside row 5), front of inoculated plots, back of inoculated plots, inoculated plants from rows 4 and 5 in 16 plots, and uninoculated plants from rows 4 and 5 of the remaining 8 plots. A total

of 6 plants were sampled from each of the 24 plots. Sampling took place approximately 40 days, and 80 days after the initial planting of inoculated seeds. At maturity, bolls were harvested from each plot in the field. The bolls were carefully collected to ensure accurate representation of all treatments and plots by removing the mature bolls from the plants.

Imaging

Before processing each leave sample, front and back images of the leaves were captured for analysis of symptoms. This step aimed to visually assess and identify any water-soaked lesion characteristics that may indicate symptoms of cotton bacterial blight (CBB). The leaves were carefully placed on a flat surface, ensuring that both the front and back sides were clearly visible. High-resolution images were taken using a smartphone camera, under suitable lighting conditions. The captured images provided a visual record of the leaves condition before maceration, these images were subsequently analyzed and compared to determine the presence and severity of CBB symptoms in different plots using the standard in figure 2.5 and correlating it with the other data collected such as colony growth, PCR result and seed to seed extraction results.

Xcm isolation, identification and genotyping

10 leave punches/discs (~0.1-cm radius) were collected per sample using a biopsy punch. These leaf discs were then placed in plastic maceration tubes containing 400µl of sterilized water. Three high-density zirconium beads measuring 3 mm in diameter (Glen Mills) were added to each tube. Maceration was performed once for 2 minutes using a speedmill (analytikjena) operating at a frequency of 50/60 Hz. After maceration, 100ul of the maceration fluid was plated onto LB agar media supplemented with 40µg/ml of rifampicin and 200 µg/ml of cycloheximide (CHX) to select for Xcm. After incubating the plates for 72 hours in 30°C incubator, plates with

growth circular, mucoid with glistening yellow colonies consistent with Xcm were selected for further analysis. Colonies were streaked to isolated to pure culture on fresh LB agar plates amended with CHX 200µg/ml and rifampicin 40µg/ml, Xcm candidates was aseptically collected using a cocktail stick and diluted in 20 µL of sterile water. The resulting suspension was boiled for 10 minutes to obtain an Xcm solution from which 1-2 µL was utilized as a template for polymerase chain reaction (PCR) amplification. previously described by(Wang et al., 2019). For PCR amplification, a standard protocol was employed. The PCR reaction mixtures with a total volume of 25 µl, comprised 1-2 µl of the Xcm solution, 12.5 µl of a 2x Green GoTaq DNA polymerase mix containing GoTaq DNA polymerase, MgCl₂, and dNTPs, 1.5 µl of each specific primer (MSCT1-P2F: TATTATTTATCCCACCAGAGG, MSCTI-P2R: TCAGAGTATTCAGAGTAAGTGCC) targeting the noncoding region of the MSCT1 chromosome, and 8.5 µl of sterilized water. The PCR reactions were conducted using FlexCycler2 PCR Thermal Cyclers manufactured by Analytik Jena. Aliquots of 5 µL from the PCR reactions were subjected to electrophoresis in a 1.5% (w/v) agarose gel prepared in TAE buffer. Following electrophoresis, the agarose gels were stained with invitrogen syber safe, a DNA-specific fluorescent dye. The stained gels were visualized using the Syngene PXi gel imager which enables the capture of high-resolution images of the DNA bands in the gel.

Antibiotic resistance phenotyping and genotyping

All isolates from years 1 and 2 were patch plated into 3 plates containing LB, LB and rifampicin, LB, rifampicin, and streptomycin to check for their markers. Isolates from sampling at 41DAP in year 1 were confirmed for the presence serine (TCC) to phenylalanine (TTC) mutation on codon 559 of *rpoB* gene which has been reported to be responsible for rifampicin resistant bacteria using customed designed primers (forward: 5'-CGCGATCAAGGAATTCTTCGGC-3', Reverse: 5'-GTGCAGACGCGGCCGTAATG-3'). The isolates were also genotyped for lysine

(AAG) to AGG (arginine) mutation at codon 43 or 88 in *rpsL* gene which has been reported to induce streptomycin resistance in *Xanthomonas oryzae* pv. *oryzicola* and *Xanthomonas oryzae* pv. *oryzae* by (Zhang et al., 2015a) using primers 5'-CGGACGAGGAGTAAGCG-3' and 5'-AT GAAGC G GGCAATGGT-3' published by (Zhang et al., 2015a). PCR products from both genotyping were sequenced by sanger sequencing and resulting DNA sequence were then analyzed using Geneious prime software to check for specific mutations in the genes. Information from antibiotic resistance phenotyping and genotyping was used to plot Xcm recovery maps in figures 2.2 and 2.3.

Seed Testing

Bolls of plants from susceptible and resistant inoculated seeds were harvested, ginned and seeds planted out on flat trays in a plant growth room. At two weeks, cotyledons were tested for Xcm contamination using the previously described isolation method. Afterwards, PCR confirmed Xcm from harvested seeds from resistant inoculated seeds were sequenced to check for mutations on codon 559 of their *rpoB* gene using customized designed primers (forward: 5'-CGCGATCAAGGAATTCTTCGGC-3', Reverse: 5'-GTGCAGACGCGGCCGTAATG-3') and mutations on codons 43 and 88 of the *rpsL* gene using primers 5'-CGGACGAGGAGTAAGCG-3' and 5'-AT GAAGC G GGCAATGGT-3' published by (Zhang et al., 2015a).

RESULTS

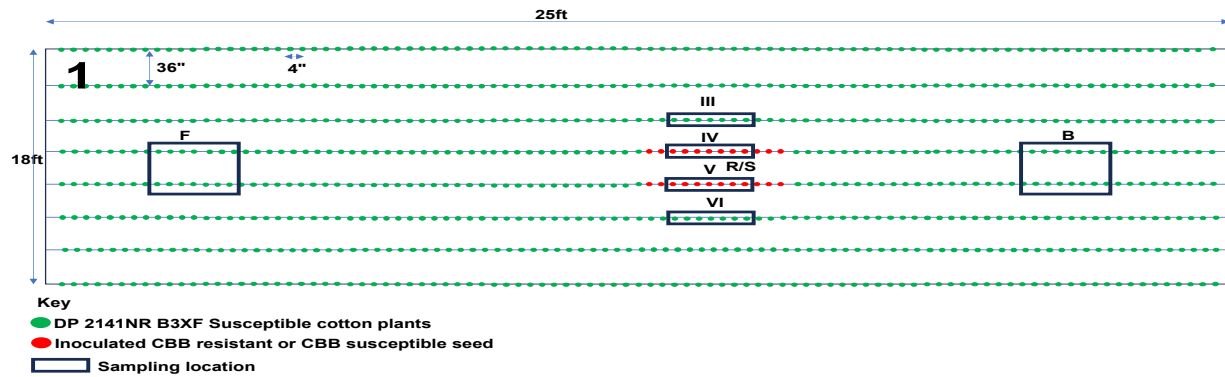


Figure 2.1: Idealized figure of 16 out of 24 treated plots, entire field was initially planted with DP 2141NR B3XF susceptible cotton plants at 4 inches spacing between plants 36 inches row spacing, each plot was 25ft long and 18ft wide. Established plants between 12ft and 15ft in rows IV and V of 16 plots were uprooted after 2 weeks and replaced with resistant or susceptible seeds already inoculated with Xcm 4.02 RfSm or Xcm 4.02 Rf with an optical density (OD_{600}) of 0.3 while the remaining 8 plots were left untouched. pseudorandom leaf samples were taken around the inoculated plants at four locations III, VI, F and B and on inoculated plants on rows IV and V. This sampling pattern was also done for uninoculated plots.

Plant Growth

In the first year (2023) of this experiment, we noted lower germination rates in some plots inoculated with susceptible (DP 2141NR B3XF) seeds. To address this, we replanted these plots with inoculated seeds. In the second year, this issue did not arise. However, in both years, we observed a general reduction in plant height among the inoculated plants compared to the uninoculated plants.

Xcm recovery rates and distribution

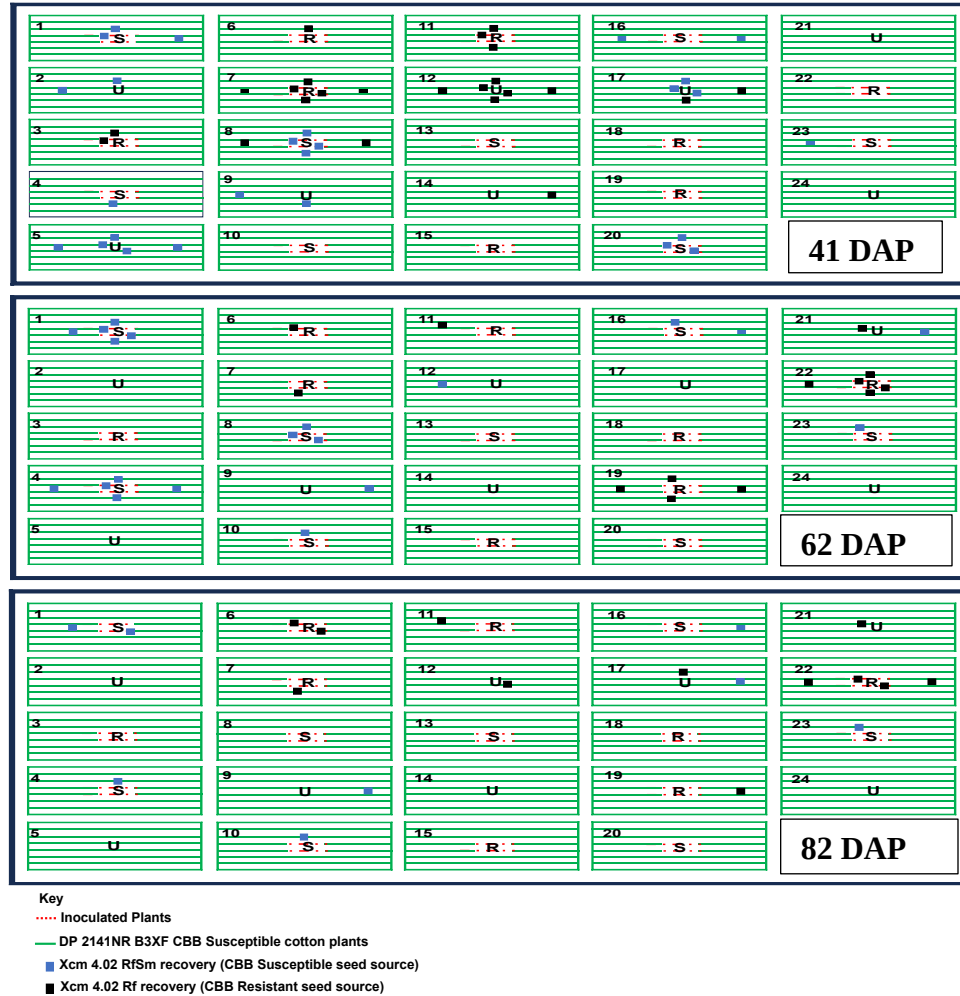


Figure 2.2: Year 1 Xcm recovery map showing which Xcm strain was confirmed by PCR and antibiotic marker profiling in each sampling location at 40 DAP, 60 DAP and 80 DAP in all 24 plots when resistant seeds were inoculated with Xcm 4.02 Rf and susceptible seeds with Xcm 4.02 RfSm. Locations where no Xcm was confirmed were left empty.

Twenty-four plots were established each measuring 25 feet by 18 feet with 36-inch row spacing and 4-inch seed spacing. The susceptible cotton cultivar DP 2141NR B3XF was initially planted in all plots. Two weeks later, in eighteen plots, established plants between 12 and 15 feet in rows IV and V were replaced after with Xcm-inoculated seeds, CBB resistant seeds PHY 411 W3FE inoculated with Xcm 4.02 Rf in year 1 and Xcm 4.02 RfSm in year 2 and CBB susceptible seeds DP 2141NR B3XF inoculated with Xcm 4.02 RfSm in year 1 and Xcm 4.02 Rf

in year 2. The remaining eight plots served as uninoculated controls. Leaf samples were collected at six locations per plot, from inoculated plants in rows IV and V and four locations (III, VI, F and B) surrounding the inoculated plants at 41, 62, and 82 days after initial planting of inoculated seeds for year 1 and 44, 62 and 83 days after initial planting of inoculated seeds for year 2. An idealized plot map is shown in Figure 1.

In 2023 (Y1), CBB-resistant seed was inoculated with Xcm 4.02 Rf while CBB-susceptible seed was inoculated with 4.02 RfSm. We collected leaf samples 41, 62 and 82 days after planting to monitor the spread of our inoculum strains under field conditions. We recovered and confirmed Xcm from 49 samples collected 41 DAP (See Figure 2.2 and Table 2.1). Of these isolates, 40 were recovered from un-inoculated plants (see figure 2.4) with 23 identified as Xcm 4.02 Rf indicating that they originated from resistant inoculated seeds while 26 Xcm 4.02 RfSm indicating that they originated from susceptible inoculated seeds. A total of 33 Xcm isolates were confirmed during the second sampling 62 DAP. Out of these 33, 13 were Xcm 4.02 Rf and 20 Xcm 4.02 RfSm. Out of these same 33 isolates, 25 were recovered from non-inoculated plants. Among the 13 Xcm 4.02 Rf, 10 were from un-inoculated susceptible plants. Out of the 20 Xcm 4.02 RfSm 15 were recovered from un-inoculated susceptible plants. At 82 DAP, a total of 20 Xcm isolates were confirmed of which 15 were recovered from non-inoculated plants Among these 8 were Xcm 4.02 Rf, were from and 7 were Xcm 4.02 RfSm,. Notably, Xcm recovered from inoculated plants always correlated with their respective inoculated strains.

We collected 12 bolls produced by CBB-resistant seed inoculated plants from each plot. Bolls were harvested, ginned and seeds planted out on flat trays in a plant growth room. At two weeks, cotyledons were tested for Xcm colonization using the previously described isolation and genotyping method. Result of Xcm recovery from this experiment is shown in Table 2.4 where

out of 8 plots where Xcm 4.02 Rf inoculated CBB-resistant seeds were planted in year 1, we recovered 4 Xcm 4.02 Rf in two plots. Two isolates from plot 7 where Xcm 4.02 Rf was recovered from leave samples of seed-inoculated plant taken 41 DAP and two isolates from plot 19 where no Xcm was recovered from leave samples of seed-inoculated plant taken throughout the 3 sampling periods in year 1. The overall Xcm recovery rate was 0.13% of 3011 cotyledons tested in 8 plots where Xcm 4.02 Rf inoculated CBB-resistant seeds were planted in year 1.

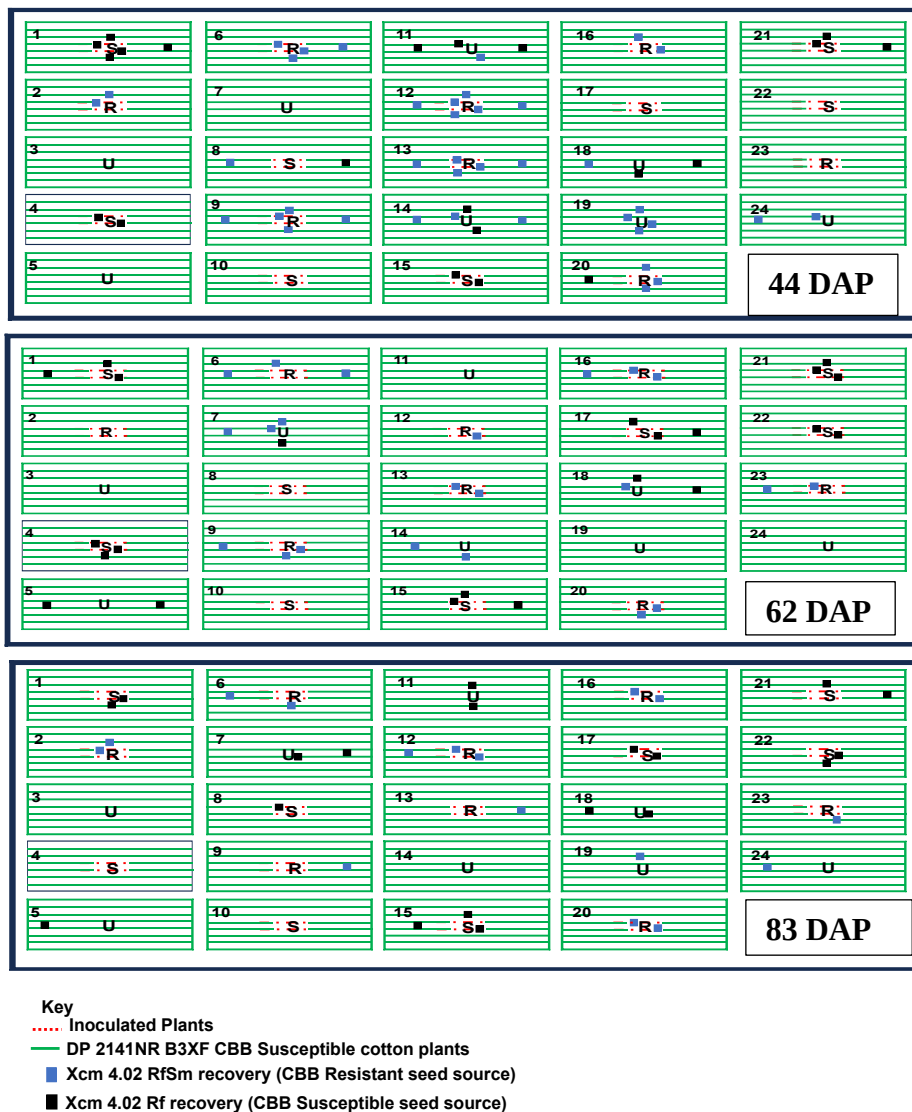


Figure 2.3: Year 2 Xcm recovery map showing which Xcm strain was confirmed by PCR and antibiotic marker profiling in each sampling location at 44 DAP, 62 DAP and 83 DAP in all 24 plots when resistant seeds were inoculated with Xcm 4.02 RfSm and susceptible seeds with Xcm 4.02 Rf. Locations where no Xcm was confirmed were left empty.

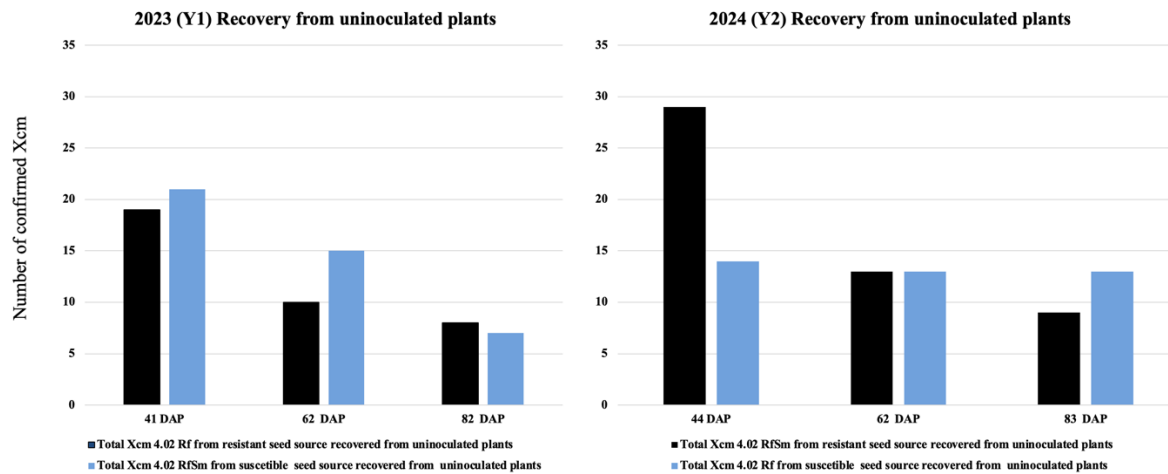


Figure 2.4: Xcm recovery from uninoculated plants at ~ 40, 60 and 80 DAP in 2023 and 2024 field trials

To address potential biases associated with specific strain characteristics, we strategically alternated the strains between the two cultivars in the second year of the experiment. This approach allowed us to assess whether the observed recovery trends were primarily influenced by the strains themselves or by other factors.

In 2024 (Y2), the Xcm strains were inverted with CBB-resistant seed being inoculated with Xcm 4.02 RfSm while CBB-susceptible seed was inoculated with 4.02 Rf. We collected leaf samples 44, 62 and 83 days after planting to monitor the spread of our inoculum strains under field conditions. We recovered and confirmed Xcm from 60 samples collected 44 DAP (See Figure 2.3 and Table 2.2). Of these isolates, 43 were recovered from uninoculated plants (see figure 2.4) with 39 identified as Xcm 4.02 RfSm indicating that they originated from resistant inoculated seeds while 21 Xcm 4.02 Rf indicating that they originated from susceptible inoculated seeds. A total of 44 Xcm isolates were confirmed during the second sampling 62 DAP. Out of these 44, 22 were Xcm 4.02 Rf and 22 Xcm 4.02 RfSm. Out of these same 44 isolates, 27 were recovered from non-inoculated plants. Among the 22 Xcm 4.02 RfSm, 13 were

from un-inoculated susceptible plants. Out of the 22 Xcm 4.02 Rf 13 were also recovered from un-inoculated susceptible plants. At 83 DAP, a total of 35 Xcm isolates were confirmed of which 22 were recovered from non-inoculated plants. Of these 13 were Xcm 4.02 Rf and 9 were Xcm 4.02 RfSm. notably, as in Y1, Xcm recovered directly from seed-inoculated plants always corresponded with their respective inoculated strains. CBB symptoms were observed in only 16 samples in Y1 (8, 6, and 2 sample at 41, 62 and 82 DAP respectively) and 28 samples in Y2 (15, 6, and 7 samples at 44, 62 and 83 DAP respectively). Of these, 10 were associated with recovery of Xcm in year 1 and 18 in year 2 (see table 2.3 and figure 2.6).

Table 2.1: Xcm recovery and disease score sheet overlap for year 1

Plot	Location	41 DAP		62 DAP		82 DAP	
		Recovery	Symptoms	Recovery	Symptoms	Recovery	Symptoms
1	III	RfSm		RfSm			
1	IV	RfSm		RfSm	1	RfSm	
1	V			RfSm			
1	VI			RfSm			
1	F			RfSm			
1	B	RfSm				RfSm	
2	III	RfSm					
2	IV						
2	V						
2	VI						
2	F	RfSm					
2	B						
3	III	Rf					
3	IV	Rf					
3	V						
3	VI						
3	F						
3	B						
4	III			RfSm		RfSm	
4	IV			RfSm			
4	V				1		
4	VI	RfSm		RfSm			
4	F			RfSm			
4	B			RfSm			
5	III	RfSm	1				
5	IV	RfSm					
5	V	RfSm					
5	VI						
5	F	RfSm					
5	B	RfSm					
6	III	Rf					
6	IV			Rf		Rf	
6	V					Rf	
6	VI						
6	F						
6	B						
7	III	Rf					
7	IV	Rf					
7	V	Rf					
7	VI	Rf		Rf		Rf	1
7	F	Rf					
7	B	Rf					
8	III	RfSm		RfSm			
8	IV	RfSm		RfSm			
8	V	RfSm		RfSm			
8	VI	RfSm					
8	F	Rf					
8	B	Rf					
9	III						
9	IV						
9	V						
9	VI	RfSm	3				
9	F	RfSm					
9	B			RfSm		RfSm	
10	III			RfSm	1	RfSm	
10	IV						
10	V						
10	VI						
10	F						
10	B						
11	III	Rf					
11	IV	Rf					
11	V						
11	VI	Rf					
11	F			Rf		Rf	
11	B						
12	III	Rf	1				
12	IV	Rf					
12	V	Rf		RfSm		Rf	
12	VI	Rf					
12	F	Rf					
12	B	Rf					

Table 2.1 Contd.: Xcm recovery and disease score sheet for year 1

Plot	Location	41 DAP		62 DAP		82 DAP	
		Recovery	Symptoms	Recovery	Symptoms	Recovery	Symptoms
13	III						
13	IV						
13	V						
13	VI						
13	F						
13	B						
14	III						
14	IV						
14	V						
14	VI						
14	F						
14	B	Rf					
15	III						
15	IV						
15	V						
15	VI						
15	F						
15	B						
16	III			RfSm			
16	IV						
16	V						
16	VI						
16	F	RfSm					
16	B	RfSm		RfSm		RfSm	
17	III	RfSm				Rf	
17	IV	RfSm					
17	V	RfSm					
17	VI	Rf					
17	F						
17	B	Rf				RfSm	
18	III						
18	IV						
18	V						
18	VI						
18	F						
18	B						
19	III			Rf			
19	IV						
19	V						
19	VI			Rf			
19	F			Rf			
19	B			Rf		Rf	
20	III	RfSm					
20	IV	RfSm	3				
20	V	RfSm	4				
20	VI						
20	F						
20	B						
21	III						
21	IV			Rf		Rf	
21	V						
21	VI						
21	F						
21	B			RfSm			
22	III			Rf			
22	IV			Rf	1	Rf	
22	V			Rf		Rf	
22	VI			Rf			
22	F			Rf		Rf	
22	B					Rf	
23	III			RfSm		RfSm	
23	IV						
23	V						
23	VI						
23	F	RfSm					
23	B						
24	III						
24	IV						
24	V						
24	VI						
24	F						
24	B						

Table 2.2: Xcm recovery and disease score sheet overlap for year 2

Plot	Location	44 DAP		62 DAP		83 DAP	
		Recovery	Symptoms	Recovery	Symptoms	Recovery	Symptoms
1	III	Rf	3	Rf			
1	IV	Rf					
1	V	Rf		Rf		Rf	
1	VI	Rf	3			Rf	
1	F			Rf			
1	B	Rf					
2	III	RfSm				RfSm	
2	IV	RfSm				RfSm	
2	V						
2	VI						
2	F						
2	B						
3	III						
3	IV						
3	V						
3	VI						
3	F						
3	B						
4	III						
4	IV	Rf	3	Rf			
4	V	Rf	3	Rf			
4	VI			Rf			
4	F						
4	B						
5	III						
5	IV						
5	V						
5	VI						
5	F			Rf		Rf	
5	B			Rf			
6	III			RfSm			
6	IV	RfSm	3				
6	V	RfSm					
6	VI	RfSm				RfSm	
6	F			RfSm		RfSm	
6	B	RfSm		RfSm			
7	III			RfSm			
7	IV			RfSm			
7	V					Rf	
7	VI			Rf			
7	F			RfSm		Rf	2
7	B						
8	III						
8	IV					Rf	
8	V						
8	VI						
8	F	RfSm					
8	B	Rf					
9	III	RfSm					
9	IV	RfSm					
9	V			RfSm			
9	VI	RfSm		RfSm			
9	F	RfSm		RfSm			
9	B	RfSm				RfSm	
10	III						
10	IV						
10	V						
10	VI						
10	F						
10	B						
11	III					Rf	
11	IV	Rf					
11	V						
11	VI	RfSm				Rf	
11	F	Rf					
11	B	Rf					
12	III	RfSm					
12	IV	RfSm				RfSm	
12	V	RfSm		RfSm		RfSm	
12	VI	RfSm					
12	F	RfSm				RfSm	
12	B	RfSm					

Table 2.2 Contd: Xcm recovery and disease score sheet overlap for year 2

Plot	Location	44 DAP		62 DAP		83 DAP	
		Recovery	Symptoms	Recovery	Symptoms	Recovery	Symptoms
13	III						
13	IV	RfSm		RfSm			
13	V	RfSm		RfSm			
13	VI	RfSm					
13	F	RfSm					
13	B	RfSm				RfSm	
14	III	Rf					
14	IV	RfSm					
14	V						
14	VI	Rf		RfSm			
14	F	RfSm		RfSm			
14	B	RfSm					
15	III			Rf		Rf	
15	IV	Rf	3	Rf			
15	V	Rf	1			Rf	
15	VI						
15	F					Rf	
15	B			Rf			
16	III	RfSm					
16	IV	RfSm		RfSm		RfSm	
16	V	RfSm	4	RfSm		RfSm	
16	VI						
16	F			RfSm			
16	B						
17	III			Rf	1		
17	IV					Rf	
17	V			Rf	1	Rf	2
17	VI						
17	F						
17	B			Rf			
18	III			Rf			
18	IV			RfSm			
18	V					Rf	
18	VI	Rf					
18	F	RfSm				Rf	
18	B	Rf		Rf			
19	III	RfSm				RfSm	
19	IV	RfSm	3				
19	V	RfSm					
19	VI	RfSm					
19	F						
19	B						
20	III	RfSm	1				
20	IV					RfSm	
20	V	RfSm		RfSm		RfSm	
20	VI	RfSm		RfSm			
20	F	Rf					
20	B						
21	III	Rf		Rf		Rf	
21	IV	Rf		Rf	1		2
21	V			Rf			2
21	VI						
21	F						
21	B	Rf				Rf	
22	III						
22	IV			Rf			
22	V			Rf		Rf	
22	VI					Rf	
22	F						
22	B						
23	III						
23	IV			RfSm			2
23	V						
23	VI					RfSm	
23	F			RfSm			
23	B						
24	III						
24	IV	RfSm					
24	V						
24	VI						
24	F	RfSm				RfSm	
24	B						

Table 2.3: Analysis table of year 1 and 2 Xcm recoveries and disease scoring

		Year 1			Year 2				
		41 DAP	62 DAP	82 DAP	44 DAP	62 DAP	83 DAP		
Total Rf Recovered		23	13	12	21	22	19		
Total RfSm Recovered		26	20	8	39	22	16		
Total Rf + RfSm Recovered		49	33	20	60	44	35		
Total Rf Recovered from inoculated plants	RF	4	3	4	7	9	6		
Total RfSm Recovered from inoculated plants	RfSm	5	5	1	10	8	7		
Total Rf Recovered from uninoculated plants	RF	19	10	8	14	13	13		
Total RfSm Recovered from uninoculated plants	RfSm	21	15	7	29	14	9		
Key		Disease symptom ratings	Frequency in each sampling			Disease symptom ratings	Frequency in each sampling		
		41 DAP	62 DAP	82 DAP	44 DAP	62 DAP	83 DAP		
Rf = Xcm with only Rifampicin marker was recovered		1	2	4	1	1	2	3	0
Rfsm= Xcm with Rifampicin and streptomycin marker was recovered		2	0	0	0	2	0	0	5
0 = no disease symptom was observed		3	2	0	0	3	7	0	0
N= no Xcm was confirmed		4	1	0	0	4	1	0	0
		Total symptomatic samples associated with recovery of Xcm	5	4	1	Total symptomatic samples associated with recovery of Xcm	10	3	5
		Total asymptomatic samples associated with recovery of Xcm	44	29	19	Total asymptomatic samples associated with recovery of Xcm	50	41	30

Disease symptoms scoring reference and rating

Figure 2.5 below shows the reference that was followed in scoring the sample leaves for symptoms of CBB, while figure 2.6 shows the ratings of samples associated with Xcm recovery in 2023 and 2024 trial, while figure 2.7 shows observed symptoms on the field on July 10th, 2023 (26 days after planting).

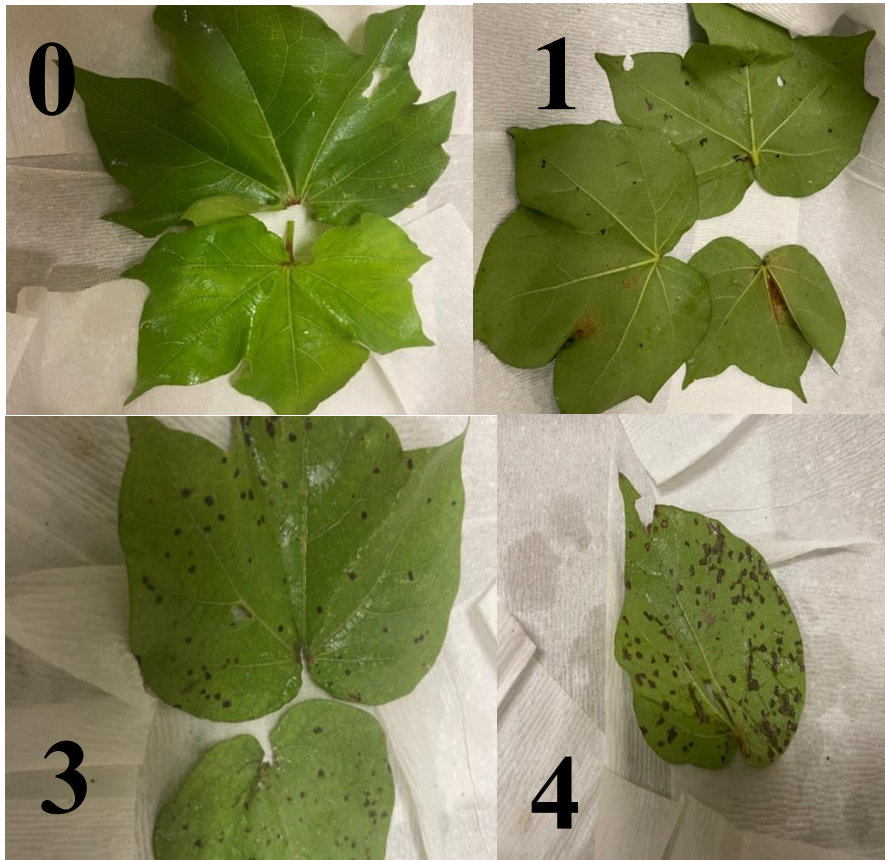


Figure 2.5: Disease symptom scoring reference, “0” when no visible symptom on the leaf, “1” when less than half of the leaf area with angular lesions, “3” when about half of the leaves with symptoms and “4” when more than half of the leaf with symptom but not entire leaf area.

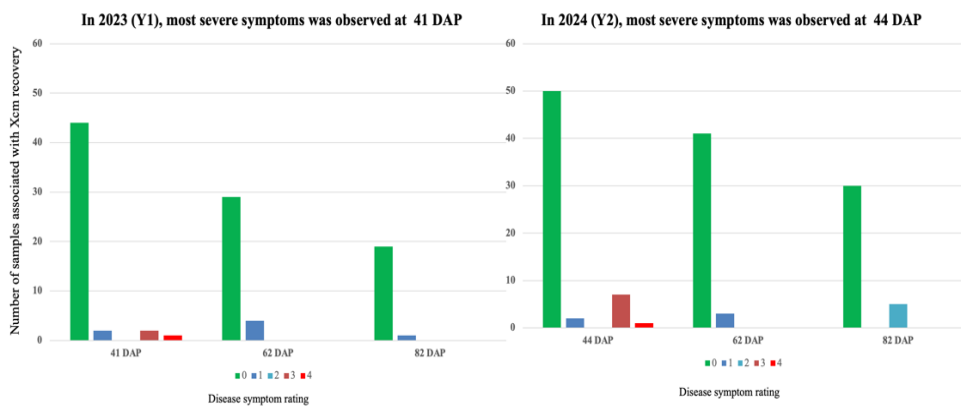


Figure 2.6: Disease symptom rating of samples associated with Xcm recovery in 2023 and 2024 trial



Figure 2.7: Observed symptoms on the field on July 10th, 2023 (26 days after planting).

Seed testing

Of the 3011 cotyledons tested from seeds harvested from 8 resistant seed-inoculated plots, Xcm was confirmed in only 4 cotyledons from plots 7R and 19R. Notably, Xcm was not detected in the parent leaf samples from plot 19R. The recovered Xcm strains exhibited the same mutations as the original inoculum suggesting seed-to-seed transmission. This represents a recovery rate of 0.13% (see Table 2.4).

Table 2.4: Xcm testing result of cotyledons from harvested seed of inoculated resistant plants

Resistant seed - inoculated plot	Xcm recovered from leaves of seed-inoculated plants in 2023 trial	Number of cotyledons with Xcm recovery	Number of cotyledons tested
6R	N	0	468 (0%)
15R	N	0	408 (0%)
22R	N	0	327 (0%)
19R	N	2	401 (0.5%)
11R	Y	0	376 (0%)
7R	Y	2	294 (0.68%)
18R	Y	0	360 (0%)
3R	N	0	377 (0%)
Total		4	3011 (0.13%)

Summary of *rpoB* and *rpsL* genotyping results

All 49 Xcm isolates sequenced from the sampling at 41DAP exhibited the S559P mutation on their *rpoB* gene, suggesting a common origin. 24 out of 26 Xcm 4.02 RfSm have the

Lysine (AAG) to AGG (Arginine) mutation at codon 43 of there *rpsL* gene while the remaining 2 isolates have it on codon 88. We confirmed the origin of these 2 isolates by sequencing 20 PCR-amplified colonies from our Xcm control (with rifampicin and streptomycin resistant) strain and discovered that 3 of them also carried the lysine (AAG) to AGG (arginine) mutation at codon 88. The four Xcm 4.02 Rf recovered from cotyledons from harvested seed of inoculated resistant plants also have the S559P mutation on their *rpoB* gene and lack the Lysine (AAG) to AGG (Arginine) mutation at codon 43 of there *rpsL* gene.

DISCUSSION

Cotton Bacterial blight caused by *Xanthomonas citri* pv. *malvacearum* (Xcm), is a major disease of cotton. While historically a chronic problem in the United States, the deployment of resistant cotton varieties and acid-delinted seed has significantly reduced its impact. However, outbreaks can still occur in susceptible varieties. Early-season rain has been proposed to promote pathogen spread, followed by heavy wind-driven rains after canopy formation, high humidity, and warm temperatures, all favoring disease development (Isakeit, 2016). Xcm survives on infected crop residue and can be disseminated by wind, water, and thunderstorm fronts beyond the initial infection point (Thaxton & El-Zik, 2001). The bacterium enters plants through stomata or wounds and blowing dust and sand events have also been linked to bacterial blight epidemics. Seedborne bacterial pathogens pose a significant threat to crop production, particularly due to the limited efficacy of available chemical control measures compared to fungal diseases.

Seedborne pathogens pose a significant and ongoing threat to agriculture. They can contribute to the resurgence of historical diseases and the introduction of novel pathogens to new regions. In today's globalized economy, seeds have become a major vector for the long-distance dispersal of plant pathogens, transcending geographical barriers. Bacterial pathogens present a

significant challenge because unlike seedborne fungal diseases, effective management strategies for bacterial diseases are limited. (Gitaitis & Walcott, 2007). While seedborne transmission of Xcm is well-established, we investigate whether CBB-resistant plants can contribute to Xcm spread under field conditions.

Xcm Recovery rates and distribution

We documented that Xcm disseminated under field conditions from resistant seed inoculated plants to surrounding susceptible plants. The use of different markers for susceptible and resistant inoculated seeds enabled the tracking of the bacterial source during recovery.

In year 1, In two plots during the first sampling, one plot in second sampling and third sampling, recovered Xcm isolates from these plots contained both were from both resistant and susceptible seed source

Similarly, in year 2, in four plots during the first sampling, two plots during the second sampling, Xcm 4.02 RfSm and Xcm 4.02 Rf were recovered from these plots, which suggests transmission from both resistant and susceptible inoculated plants in both years. In the year 1 first sampling at 40 DAP, out of 49 Xcm isolates recovered only 9 isolates (18.36%) were recovered from inoculated plants and this plants are in only 6 out of 16 inoculated plots this can be indicative of the pathogens ability to be vary in its establishment and attachment of the same host. In both years of this trial, Xcm recovered from inoculated plants corresponded with the source and we recovered from non-inoculated plants Xcm in roughly equal numbers from both resistant and susceptible inoculated plants. A gradual decline in recovery was observed over the sampling period from 49 in the first sampling to 33 in the second sampling and 20 in the last sampling for year 1 and similar pattern in second year. The observed gradual decline in Xcm recovery over the sampling period may suggests that the population of the pathogen is decreasing

or becoming less detectable. Several factors could contribute to this decline. For instance, Xcm populations may naturally decline over time due to environmental factors, competition with other microorganisms or internal factors such as senescence or as the plants grow and develop, they may become more resistant to Xcm infection, limiting the pathogen's ability to spread and multiply. Changes in environmental conditions, such as temperature, humidity, or rainfall can affect the survival and activity of Xcm. For example, extreme hot or cold weather conditions may reduce the pathogen's viability or interfere with its ability to infect plants. Our experiment supports this interpretation because, no disease management practice like fungicides or bactericides application was implemented during this period. Therefore, the host and or the environment are the most likely factors contributing to this decline in Xcm.

As (Kemerait et al., 2017) noted, Xcm can survive in infected crop residue and soil, potentially serving as a source of inoculum for future infections. However, the duration of survival is not well understood and maybe influenced by various factors including environmental conditions. Understanding the factors that influence Xcm survival and population dynamics may be crucial for developing effective disease management strategies.

It is also important to note that the inoculated areas (6ft) in each plot (200ft) represents only 3% of the plot area and this small percentage was responsible for the spread within and between plots. For example, all samples from plots 7, 8 and 12 in the year 1 first sampling (Figure 2) were positive for Xcm. Though plot 12 was a control and untreated plot, we still recovered 100% Xcm recovered. From the six samples collected randomly in the plot probably because of the plots proximity to inoculated resistant plants 7 and 11 where the Xcm recovered from the plots probably originated from because of the marker. similar thing was observed in

control plot 5 with 5 positive Xcm from 6 samples collected, the recovered Xcms also probably originated from surrounding inoculated susceptible plants in plots 4 and 10.

This might be an indication of the potency of the pathogen to spread from very few infected plants regardless of whether they are resistant or susceptible cultivar to surround healthy plants. Hence revealing that resistant and susceptible cultivar can almost equally spread the pathogen. For instance, In the year 1 first sampling, 82.61% Xcm 4.02 Rf originating from inoculated resistant plant were recovered from several uninoculated susceptible plants compared to 80.77% of Xcm 4.02 RfSm that originated inoculated susceptible plants recovered from several uninoculated susceptible plants. Similarly in the year 2 first sampling, despite swapping of the strains, 71.79% Xcm 4.02 RfSm originating from inoculated resistant plant were recovered from several uninoculated susceptible plants compared to 66.67% of Xcm 4.02 Rf that originated inoculated susceptible plants recovered from several uninoculated susceptible plants. This suggests that the resistance mechanisms present in the resistant cultivars may be compromised or overcome by certain Xcm pressure/population or the epidemics of Xcm strains may vary with some strains being more aggressive and capable of overcoming the resistance mechanisms of certain cultivars. Additionally, some resistant cultivar might exhibit partial resistance, allowing some Xcm strains to establish infections only at limited conditions or delaying the establishment of infection. These delayed infected plants could still serve as a source of inoculum for surrounding susceptible plants. For instance, in year 1 first sampling, no Xcm was recovered from plots 19 and plots 22 including from inoculated plants, but in second sampling at 60 days after planting, 4 Xcm originating from resistant plant were recovered from susceptible plants in plot 19 while 5 Xcm from resistant plant were recovered in plot 20 similarly in the 3rd sampling at 80 days after planting Xcm originating from resistant plant was recovered in both plots.

Similarly in year 2, even though no Xcm originating from resistant plant was recovered in both inoculated and uninoculated plants in plot 23 from the first sampling, they were later in the plot during second and third sampling. It is unknown if this pattern holds for other resistant cultivars of cotton.

Given our low limit of detection because we sampled very limited tissue of 10 leaf punches (each of ~0.1-cm radius), and plated only 100µl of the maceration fluid, the absence of Xcm in certain plots or tissues cannot be interpreted to mean absence of the pathogen, because might contain low-density Xcm populations below our limit of detection or missed by our random sampling techniques.

Disease Symptoms

Our observed symptoms primarily consisted of angular leaf spots with occasional water-soaked lesions, aligning with the descriptions of (Kemerait et al., 2017) who described bacterial blight symptoms as starting with small, water-soaked lesions on leaves, progressing to characteristic angular shapes as the bacteria spread, the lesions are typically more triangular or rectangular than lesions caused by other diseases. In our study, the highest symptom severity was observed during the first sampling at approximately 40 DAP in both years. For instance, in year 1, Only 5 of 24 samples exhibited visible symptoms (see figure 2.6 and Table 2.3). Two samples (5III and 12III) received a score of 1 (less than half of the leaves with symptoms). Samples from 9VI and 20IV received a score of 3 (half of the leaves with symptoms). The highest symptom severity (score of 4, indicating more than half of the leaves with symptoms) was observed in a susceptible inoculated plant (20V). Notably, the number of symptomatic samples declined throughout the study with only 4 samples showing symptoms during the second sampling at approximately 60 DAP and 1 sample from susceptible inoculated plant during the third sampling

at approximately 80 DAP. Similar decline in symptomatic samples was observed in year 2 from 10 symptomatic samples in at 44 DAP to 5 symptomatic samples at 83 DAP. Surprisingly, Xcm was recovered and confirmed in a higher number of samples (44, 29, and 19) that did not exhibit any visible symptoms during any of the sampling stages. Also, many samples from inoculated plants showed no symptoms of bacteria blight. This finding further suggests the possibility of latent Xcm infections within the plants.

The decline in visible symptoms over time could be attributed to several factors, including leaf age as (Kemerait et al., 2017) reported, symptom distinctiveness, particularly the angular shape might diminish as leaves age. This may explain the decrease in observed symptoms despite potentially ongoing infections. Environmental factors such as temperature, humidity, and rainfall can also influence disease progression and symptom expression. Cotton plants may also possess defense mechanisms that limit symptom development even in the presence of Xcm infection.

Our result and previous research suggests that asymptomatic infections in cotton might be more prevalent than previously thought and highlights the need for further research to understand the prevalence of latent Xcm infections of other pathogens in cotton, their impact on cotton yield and boll quality, strategies for managing latent infections and reducing the risk of disease spread, investigate the factors influencing symptom development and decline over time.

This study reveals the need to accurately detect CBB in the asymptomatic phase by convenient means. Hyperspectral technology, fluorescence imaging, and infrared thermal imaging have greatly improved the detection of other leaf pathogens like *Xylella fastidiosa* and *Ips typographus* in asymptomatic phase (Camino et al., 2021; Huo et al., 2021; Zarco-Tejada et al., 2018).

Xcm contaminated Seeds

Xcm is a well-established seedborne pathogen that can survive on cotton lint and be transmitted to emerging seedlings (Innes, 1983; Verma, 1986b). However, the exact location of Xcm within or on the seed coat remains a subject of debate. Some studies have reported its presence on the seed coat (Hunter & Brinkerhoff, 1964), while others haven't been able to detect it within the embryo (Hunter & Brinkerhoff, 1964; Verma, 1986b). This inconsistency highlights the need for further investigation into the precise location of Xcm within the seed. Our research provides some evidence for the potential of seed-to-seed transmission of Xcm. We successfully recovered by isolation and confirmed by PCR the pathogen from seedlings grown from seeds harvested from both susceptible and resistant inoculated plots. Notably, the recovery rate was lower (0.13%) in 3011 seedlings from resistant inoculated plants tested compared to 0.3% in 2355 seedlings from susceptible inoculated plants. The 4 PCR confirmed Xcms recovered from seedlings from resistant inoculated plants were sequenced for *rpoB* and *rpsL* mutations and they match in phenotype and genotype to the strain that was used to inoculate the plots i.e. they have Serine (TCC) to Phenylalanine (TTC) mutation on Codon 559 (S559P) of *rpoB* gene and no Lysine (AAG) to AGG (Arginine) mutation at codon 43rd or 88th of there *rpsL* gene. Indicating that they are likely from the plants that emanated from seeds that were inoculated with rifampicin only resistant and not from the susceptible inoculated once. While this is not sufficient evidence for seed-to-seed transmission, It suggests that Xcm can persist within and/or on cotton seeds, even in resistant cultivars and Xcm contaminated resistant and susceptible cotton seeds can contribute to the spread of bacterial blight in agricultural systems. This may imply that cotton growers who decides to keep seeds from previous years for planting in the next

growing season may be building CBB inoculum on their fields even if no symptom was observed on the parent plants.

Notably, no symptom was observed in any of the cotyledons tested for CBB making it evident that the ability of Xcm to be transmitted through seeds poses significant challenges for disease management because infected seeds acting as a hidden source of inoculum can facilitate the introduction of the pathogen into previously uninfected areas and compromising the effectiveness of traditional control measures. Therefore, understanding the location of Xcm within the seed is crucial for developing targeted strategies to prevent seed-to-seed transmission. Further research is necessary to determine if Xcm primarily colonizes the seed coat or can also reside within the embryo. This knowledge, combined with studies on the impact of seed-to-seed transmission on disease outbreaks in the field can guide the development of effective strategies to manage bacterial blight in cotton.

(Mijatović et al., 2021) demonstrated the ability of Xcm strains to systemically colonize both resistant and susceptible cotton seedlings. (An et al., 2020) also highlighted the lack of consistent data on the location of seedborne pathogens in different plant species. They discussed examples of both external and internal colonization by various *Xanthomonas* species.

This study highlights the importance of time in disease epidemiology, alongside the traditional disease triangle of host, pathogen, and environment. Plant age significantly influences the outcome of host-pathogen interactions (Hu & Yang, 2019). Throughout their life cycle, plants undergo dynamic changes that create distinct environments for pathogen interactions. Plants have evolved to integrate developmental signals with pathogen responses, optimizing defense timing and intensity (Hu & Yang, 2019). Our findings support this concept. We observed a gradual decline in Xcm recovery rates from cotton plants, with the highest recovery at 40 days

after planting (seedling stage) and the lowest at 80 days after planting (mature stage). This pattern was consistent in both years, regardless of the Xcm strain used. This suggests that plant age plays a crucial role in Xcm recovery, potentially due to changes in plant defense mechanisms. However, further investigation is needed to determine the impact on cotton yield and boll formation. The findings of this study highlights the importance of considering latent infections in disease management strategies because resistant cotton cultivars, despite showing no visible symptoms can harbor *Xcm* and act as a reservoir or source of inoculum thereby contributing to the spread of the pathogen.

Our study suggests that the re-emergence of bacterial blight (CBB) in the southern United States in 2015 may be attributed to a combination of factors among which is latent infections where resistant cotton cultivars could have harbored latent infections of the bacteria, which were transmitted to susceptible cotton cultivars. However, a potential limitation of our study was the variable growth of inoculated seedlings which could have led to reduced inoculum levels, delayed disease development and spread.

In conclusion, the understanding role of latent infections in spread of cotton bacterial blight is crucial for effective disease management because even when resistant cotton cultivars are planted the can still harbor the bacteria without showing symptoms. By implementing strategies to address latent infections and prevent the spread of Xcm from resistant cultivars, we can mitigate the impact of this pathogen and protect cotton crops.

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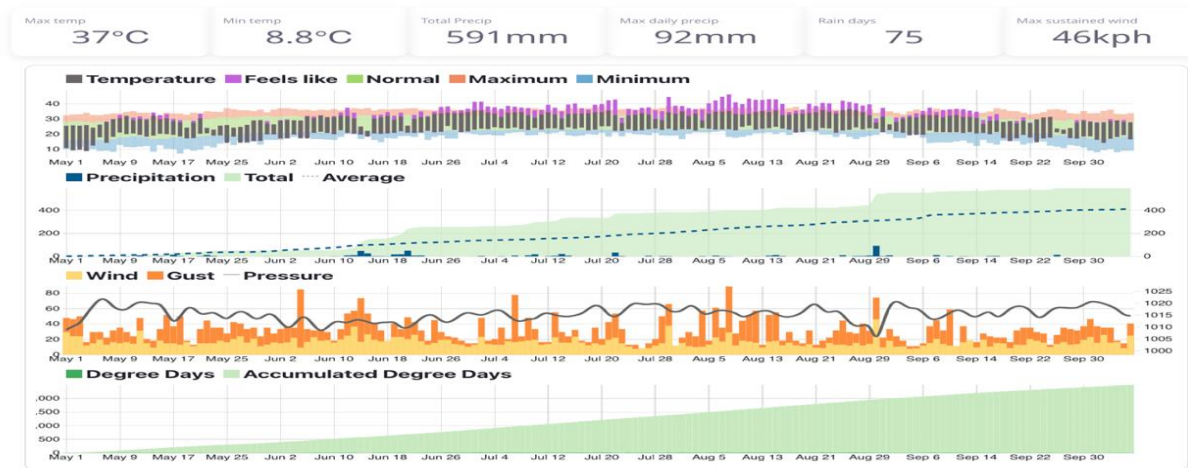
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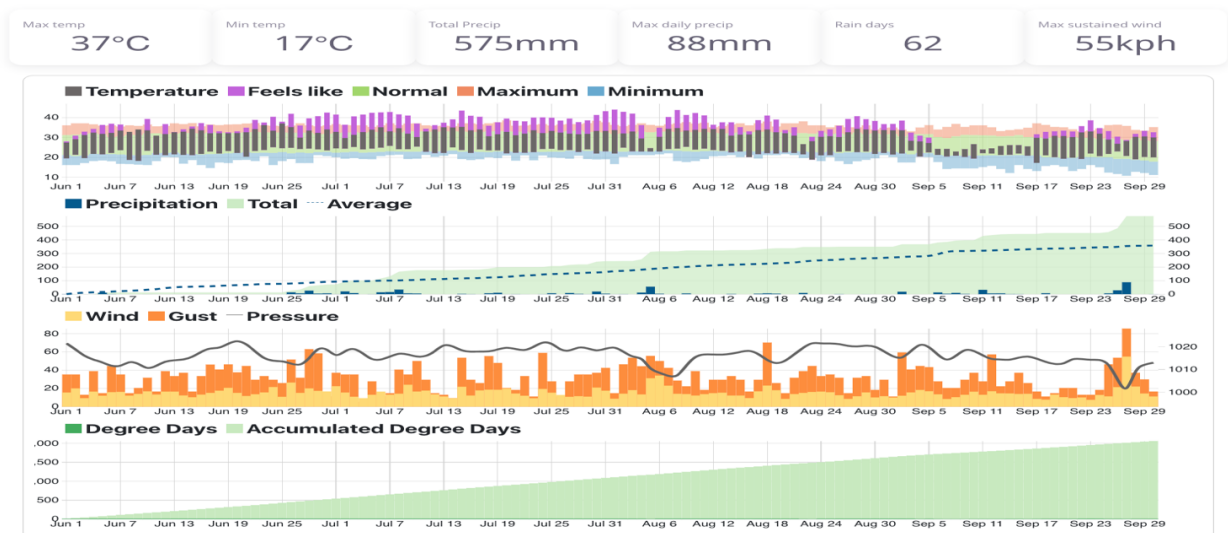
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Supplemental Figures



Supplemental figure 1: Historical weather report for year 1 showing weather history between 05/01/2023 and 10/07/2023 (duration of experiment)

Source: www.visualcrossing.com



Supplemental figure 2: Historical weather report for year 2 showing weather history between 06/01/2024 and 09/30/2024 (duration of experiment).

Source: www.visualcrossing.com

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CHAPTER 3

RECOVERY AND CHARACTERIZATION OF TRANSGENIC COTTON LINES WITH EFR AND XA-5 LIKE DISEASE RESISTANCE TRAITS ²

²Adepoju P.O, Kvitko B.H.. To be submitted to Molecular Plant Pathology

ABSTRACT

Cotton faces significant threats from various diseases including cotton bacterial blight (CBB) caused by *Xanthomonas citri* pv. *malvacearum* (Xcm). Here, we explored two strategies to improve cotton's resistance to Xcm.

First by introducing the *AtEFR* pattern recognition receptor (PRR) construct into cotton, this receptor confers broad-spectrum resistance by recognizing a wide range of pathogen-associated molecular patterns (PAMPs) and has been demonstrated to effective against *Xanthomonas* pathogens of other crops. 136 EFR candidate plants that were genotype lack the transgene implying lack of integration or expression of the transgene.

Our second strategy is to disrupt susceptibility gene expression by Xcm Transcription-activator-like (TAL) effectors, we introduced mutations in *GhTFIIA γ* to prevent the interaction between the bacterial effector and host protein thereby leading to disease resistance. 17 out of 23 genotyped candidate lines were found to have the transgene based on PCR genotyping but only 2 have a high edit efficiency. Unfortunately, the infertility of these 2 lines precludes their use and further development.

INTRODUCTION

The mitigation of infectious disease outbreaks remains a significant concern for cotton growers who strive to attain sustainable and profitable cotton production. Particularly, the absence of effective chemical control measures against cotton bacterial blight, a prevalent disease, has led to increased focus on strategies such as breeding for disease resistance. To develop enduring resistance to cotton bacterial blight, a comprehensive understanding of the genetic aspects governing the host-pathogen interactions is crucial in identifying vulnerable points within this system that can be exploited.

Plants possess a two-tiered immune system. The first layer involves transmembrane pattern recognition receptors (PRRs) that recognize conserved microbial-associated molecular patterns (MAMPs) like flagellin. The second layer relies on intracellular resistance (R) proteins encoded by R genes, which detect pathogen-derived effector proteins (Jones & Dangl, 2006). To successfully infect host plants, bacterial pathogens must adhere to plant surfaces, invade intercellular spaces, acquire nutrients, and suppress host defense responses. These processes often rely on bacterial protein secretion systems which deliver effector proteins into the extracellular milieu or directly into host cells a process that is referred to as translocation (Buttner & Bonas, 2010). Xcm uses a needle-like Type III Secretion System (T3SS) to inject a repertoire of virulence effector proteins directly into host cells. These effectors manipulate host cellular and or immune processes to facilitate infection and suppress host defenses in order to create conditions that is suitable for disease progression and proliferation (Buttner & Bonas, 2010). A prominent component of Xcm effector repertoire is Transcription Activator-Like (TAL) effectors, which can bind to specific DNA sequences within the host genome to alter gene expression (Phillips et al., 2017). For instance, the Xcm effector Avrb6 targets the *GhSWEET10* gene, encoding a sucrose transporter, to promote bacterial growth by providing a carbon source (Cox et al., 2017).

To defend against this attack, Plant R proteins activate defense responses upon direct recognition of effector proteins like Avrb6 or by detecting effector-induced modifications to host proteins. This effector-triggered immunity (ETI) often results in a hypersensitive response (HR), a localized cell death response that limits pathogen spread. Effector proteins that trigger HR in resistant plants are termed Avr proteins (Jones & Dangl, 2006). From this understanding of the pathogen's infection mechanism, it is evident that targeting conserved and stable pathogen traits,

such as T3SS or TAL effectors, can lead to more durable and sustainable disease resistance strategies partly because the pathogen cannot easily evolve from this protein without a fitness cost.

In this experimental study, we aim to employ two strategies at enhancing cotton resistance to *Xanthomonas citri* pv. *malvacearum* (Xcm). Our first approach involves the introduction of a surface immune receptor gene (*EFR*) from *Arabidopsis* into cotton plants to enhance the recognition of not only Xcm but other bacterial pathogens. EFR, which is specific to the Brassicaceae family, has been shown to recognize the N-terminal peptide motif elf18 found in bacterial elongation factor thermal unstable (EF-Tu). EF-Tu is an abundant protein in bacteria and serves a critical role in protein synthesis by facilitating the binding of aminoacyl transfer RNA to the ribosome (Sprinzl, 1994; Zipfel et al., 2006), and also present in the biofilm of *Xanthomonas* (Zimaro et al., 2013). Upon activation, EFR induces plant defense responses against a wide range of bacteria. This recognition and binding mechanism mediated by EFR has found significant applications in engineering crops with enhanced resistance against various bacterial diseases.

The potential of enhancing plant resistance through the expression of *AtEFR* has been demonstrated in several plant species, such as tomato (*Solanum lycopersicum*) and sweet orange (*Citrus sinensis*). In tomato, the introduction of EFR, has shown by (Lacombe et al., 2010) and (Kunwar et al., 2018) resulted in increased resistance against phytopathogenic bacteria from different genera including *Pseudomonas syringae* pv. *syringae* and *Ralstonia solanacearum*. Transgenic tomato plants expressing EFR were reported to exhibit greater resistance to *Xanthomonas perforans* when compared to wild-type tomato plants. Similarly, in sweet orange, the expression of EFR has been found to confer ligand-dependent activation of defense

responses, thereby improving resistance against two citrus bacterial pathogens, namely *Xanthomonas citri* and *Xylella fastidiosa* (Mitre et al., 2021).

Furthermore, in a study conducted by (Piazza et al., 2021) The stable expression of *EFR* in transgenic apple plants resulted in the activation of the PAMP-triggered immune response in apple leaves upon treatment with supernatant from *Erwinia amylovora*, a pathogenic bacterium causing fire blight. This immune response was evidenced by the production of reactive oxygen species and the induction of known defense genes. Moreover, the extent of tissue necrosis associated with *E. amylovora* infection was significantly reduced in the transgenic apple rootstocks compared to the wild-type plants.

Other crops where transgenic expression of *AtEFR* has been demonstrated to effectively enhance resistance against various bacterial pathogens include wheat (*Triticum aestivum*), (Schoonbeek et al., 2015), where the expression of *AtEFR* increased resistance against *Pseudomonas syringae* pv. *oryzae*, in rice (*Oryza sativa*), where *AtEFR* expression conferred enhanced resistance against *Xanthomonas oryzae* pv. *oryzae* (Schwessinger et al., 2015) and potato (*Solanum tuberosum*) where transgenic plants expressing *AtEFR* exhibited increased resistance to *Ralstonia solanacearum* (Boschi et al., 2017). These findings highlight the potential of utilizing EFR-mediated recognition and defense mechanisms to enhance resistance in various plant species against diverse bacterial pathogens. This interfamily transfer highlights the potential of utilizing pattern recognition receptors (PRRs) like EFR to increase resistance in different plant species.

Since our objective is to enhance cotton resistance against Xcm, the utilization of PRRs such as EFR, which have established functional roles in other plant families could prove to be an

effective strategy. This approach capitalizes on the knowledge gained from the documented function of PRRs in other plant species, providing a promising avenue to achieve improved resistance against Xcm in cotton.

The second strategy involves disrupting the host's susceptibility to TAL effector regulation. According to (Pessina et al., 2016), the concepts of resistance and susceptibility are inherently interconnected. Shifting the focus from resistance to susceptibility offers a distinctive perspective centering on S-genes. An S-gene is characterized as such when its loss-of-function results in recessively inherited resistance (Pavan et al., 2010).

Susceptibility genes (S-genes) represent the direct or indirect targets of pathogen effectors and in many cases they encode either negative regulators of host defenses (Engelhardt et al., 2018) or promoters of pathogen growth (Bai et al., 2000; Bezruczyk et al., 2018). The disruption of the S-genes can lead to induction of recessive resistance. However, since S-genes often play a crucial role in the host, their disruption may lead to pleiotropic effects in the host plant (Engelhardt et al., 2018) as in potatoes where the tetra-allelic *Stdnd1* mutant lines, although showed strong resistance phenotype, also showed reduced growth, long and thin stems, as well as necrosis of all leaves. (Engelhardt et al., 2018). It has been demonstrated that many *Xanthomonas* sp. and some other bacteria can directly induce the expression of S-genes of their hosts (Zhang et al., 2015b) like *GhSWEET* genes of cotton by (Cox et al., 2017) utilizing its TAL effectors just like bacteria in other genera e.g. *Ralstonia* (Bogdanove et al., 2010). TAL effectors have a modular structure (Huang et al., 2017).

Currently, two distinct mechanisms mediate the interaction between TAL effectors and host gene expression factors. First, the central repeat region of the TAL effector directly binds to specific DNA sequences in the host promoter via its repeat variable di-residues (RVDs) (Yuan et

al., 2016). Second, the ubiquitous transcription factor binding motif of the TAL effector interacts with the host's basal transcription factor IIA gamma (TFIIA γ) (Hui et al., 2019). TFIIA is a basal transcription factor of eukaryotes and it is essential for polymerase II–dependent transcription (Høiby et al., 2007). It consists of two subunits, the large subunit TFIIA $\alpha\beta$ and the small subunit TFIIA γ (Li et al., 1999) which is highly conserved among eukaryotes.

Rice TFIIA γ 5 has been implicated as a key factor in plant-pathogen interactions, particularly in response to *Xanthomonas* infections. While it was initially suggested to be a cofactor for TAL effector activity (Iyer & McCouch, 2004) either as a helper of TALE function (Boch et al., 2014b) or as a TALE-targeted host gene (Gu et al., 2009).

Rice TFIIA γ 5 has been suggested to be a cofactor that directly enables TALEs to induce host gene expression (Iyer & McCouch, 2004) either as a helper of TALE function (Boch et al., 2014b), or as a TALE-targeted host gene (Gu et al., 2009). In rice, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causes bacterial blight and *X. oryzae* pv. *oryzicola* (*Xoc*) causes bacterial streak both of which are highly devastating diseases.

The recessive resistance gene *xa5* is widely used to improve rice resistance to *Xoo* (Kottapalli et al., 2007). There are two copies of this gene, one on chromosome 1 and one on chromosome 5. *Xa5* is a natural allele of the gene for the transcription factor IIA gamma subunit 5 (TFIIA γ 5), changing a valine to a glutamine (TFIIA γ 5^{V39E}) ((Iyer & McCouch, 2004) (Iyer & McCouch, 2004) (Sugio et al., 2007)). Because The TFIIA γ V39E mutation did not lead to the loss of function of the TFIIA γ gene (Jiang et al., 2006). despite preventing its recruitment by *Xanthomonas* TAL effectors, *Xa5* also, presents a promising disease resistance strategy. Transcriptionally suppressing the TFIIA γ in citrus and tomato have led to an increase in resistance to *Xanthomonas citri* pv. *citri* and *Xanthomonas campestris* pv. *vesicatoria* respectfully and rice

carrying this resistance has been effectively deployed in the field (Huang et al., 2017). This finding further supports the idea that targeting *TFIIA γ* components can be a promising approach for developing durable disease resistance against Xcm and other pathogens in the *Xanthomonadaceae* family.

MATERIALS AND METHODS

Construct development

pBIN19 35S:EFR::HA vector, *GhTFIIA γ* guide RNA design, *Ghxa5* transgene construct design and cloning, assembly of pDIRECT_22A g4 and pDIRECT_22A g4 *Ghxa5* vectors were carried out by (Mijatovic et. al., 2022) (unpublished). pBIN19 35S:EFR::HA vector contained a EFR transgene, under a CaMV 35S promoter to drive the expression of EFR transgene, a kanamycin marker (*apha-3*), *aph(3')*-II that confers resistance to neomycin and kanamycin for the selection of *E.coli* DH5 α cells containing the vector under NOS Promoter and coding sequences for tetracycline resistance regulatory protein (TetR), origin of replication (OriV), *trfA* which produces a trans-acting replication protein that binds to and activates the origin of replication, CAP binding to bind the catabolite activator protein (CAP) and also influencing gene expression, lac operon and promoter for lactose metabolism, and NOS Terminator to signal the end of transcription while pBIN19 35S: Δ EFR:HA with the EFR gene deleted is a derivative of the original EFR vector described by (Lacombe et al., 2010) serves as a control. The pDIRECT_22A g4 *Ghxa5* vectors contained *xa5* transgene, coding sequence for Cas9 to produce Cas9 (Csn1) endonuclease from the *Streptococcus pyogenes* Type II CRISPR/Cas system responsible for generating RNA-guided double strand breaks expression of which is driving by CaMV 35S promoter, *xa5* transgene promoted by StUBI3, a kanamycin marker (*apha-3*), *aph(3')*-II promoted by CaMV 35S to confer resistance to neomycin and kanamycin for the

selection of cells containing the vector, origin of replication, CAP binding to bind the catabolite activator protein (CAP) and also influencing gene expression, lac operon and promoter for lactose metabolism, coding sequence for pVS1 RepA that codes for a replication protein pVS1 StaA that codes for stability protein and NOS Terminator to signal the end of transcription,

Management of transgenic lines

Candidate (T₁) cotton seeds putatively expressing *AtEFR* were received from our collaborators from University of North Texas, planted in flat trays and grown in growth room at Kvitko Laboratory for a month before DNA extraction. Twenty-one candidate cotton plants putatively expressing *Ghxa5* were received from our collaborators from Clemson university, additionally, twelve control plants out of which ten were putatively expressing only Cas9, and two putatively expressing only Cas9 and the guide. They were all subjected to acclimatization in the greenhouse for a period of three to five days. During this acclimatization phase, the plants were exposed to a controlled light environment with a photoperiod of 16 hours of light and 8 hours of darkness.

To supplement light when natural light levels dropped below 300 $\mu\text{mol. m}^{-2}\text{s}^{-1}$, the plants were provided with 16 hours of supplemental light. The greenhouse conditions were maintained at a temperature of 30°C during cooling periods and 16°C during heating periods to ensure optimal growth and development of the cotton plants.

Transplanting

Following the acclimatization period, the candidate cotton plants which had developed 4 to 5 leaves, were transplanted into pots containing promix BK25 potting medium after which the plants were watered daily in the early morning to ensure proper hydration and growth.

Fertilizer Application

Twice weekly applications of Peter's 15-5-15 Cal Mag with Iron and Peter's 20-20-20 fertilizers were carried out to provide essential nutrients to the plants. We selected this fertilizers for their balanced nutrient composition which includes elements such as nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and iron (Fe) that are important for plant growth and development.

Pest Management

To address the issue of thrips infestation, which was prevalent in the greenhouse, several measures were implemented for pest control. First, Triact 70, a product containing a clarified hydrophobic extract of neem oil was utilized as a treatment against thrips. Neem oil has been known for its insecticidal properties and is effective in managing various pests. Additionally, marathon 1% granular which contains the active ingredient imidacloprid, was employed as a systemic insecticide for thrips control. To complement these treatments, sticky traps were strategically placed to capture and monitor thrips activity. These traps are coated with a sticky substance that attracts and traps the pests, helping to reduce their population. Moreover, sticks intended for staking were thoroughly washed and disinfected before being used.

Controlled Pollination

To ensure controlled pollination and prevent cross-pollination in the cotton plants, a method using white pollen bags was employed. These bags were placed over the flowers at the candle stage, which is a critical stage of flower development before pollination occurs to minimize the chances of cross-pollination there is still a potential for natural cross-pollination to occur although cotton is primarily considered a self-pollinating crop.

DNA Extraction

The DNA extractions were carried out using modified hexadecyltrimethylammonium bromide (CTAB) protocol described by (Doyle & Doyle, 1987). Briefly, the process begins with weighing approximately 100-150 mg young leaves exhibiting a brighter green color, these tissue samples were immediately frozen in liquid nitrogen for 3-5 minutes. Subsequently, the frozen leaves were ground to a fine powder using a speed mill (analytikjena) operating at 50/60 Hz for approximately 10 minutes. Following leaf grinding, the tissue is partitioned into a 2ml tube, and 1mL of prewarmed (65°C) 2X CTAB was added. A brief vortexing of 10 seconds was performed before incubating the mixture for 30 minutes at 65°C. A midway vortexing step, lasting 10 seconds, was done to enhance the efficiency of the extraction process. After this, 800uL of chloroform was added and thorough vortexed to achieve homogenization of the sample followed by centrifugation at 15000 rpm for 10 minutes to separate the aqueous layer, which is carefully pipetted off and transferred to a new sterile, DNase-free 1.7mL tube. To precipitate the genomic DNA, 480uL (0.6X volume) of pre-cooled Isopropanol was added to the aqueous layer. Slow, progressive inversion by hand was done ensure the proper mixing of Isopropanol and supernatant until the DNA becomes visible followed by centrifugation at full speed for 5 minutes to facilitate the precipitation of genomic DNA, which was then carefully poured off. The next steps involved two rounds of washing the DNA pellet with 70% Ethanol. Finally, to eliminate RNA contamination, 100uL of 0.1X TE buffer, along with a 1:1000 dilution of an RNase A cocktail was added to the pellet and incubated at 37°C for 30 minutes to ensure thorough RNA digestion.

Genotyping

DNA samples extracted from both *xa5* and *EFR* candidate line was used as templates in the PCR reaction. The PCR amplification for *EFR* confirmation were expected to generate an amplicon of the expected size 262bp corresponding to the presence of the *AtEFR* transgene using the extracted plasmid from agrobacterium strain caring the plasmid as positive control and received *ΔEFR* lines (from University of North Texas) as negative control and previously reported primers by (Piazza et al., 2021) in Table 3.4. While The PCR amplification for *Ghxa5* confirmation was expected to generate an amplicon of the expected size of 750bp using *TFIIAγ* gene is positive control and pDIRECT_22A lines (Cas9 only) has negative control. In both experiments, a standard PCR protocol was followed for the amplification process where each PCR reaction mixture (25 µl) contained approximately 1-2 µl of template DNA at a concentration of approximately 50 µg/ml. Additionally, the mixture included 12.5 µl of 2× PCR mix, which consisted of Taq DNA polymerase, MgCl₂, and dNTPs. The specific primers for each target region were added at a concentration of 1-2 µl each.

Finally, sterilized water was added to bring the total reaction volume to 25 µl and the reactions were carried out using FlexCycler2 PCR thermal cyclers manufactured by Analytik Jena. The PCR cycling parameters, including denaturation, annealing, and extension temperatures and times, were optimized based on the specific primer sequences and target regions.

Gel electrophoresis

PCR product aliquots of 5 µl were subjected to electrophoresis using a 1.5% (w/v) agarose gel prepared in TAE buffer. The TAE buffer was prepared by diluting 40 ml of 50X

TAE buffer in 1960 ml of water. The gel electrophoresis was conducted at a constant voltage of 80 V for a duration of 40 minutes. Following electrophoresis, the agarose gels were stained with Invitrogen SYBR Safe, a fluorescent DNA stain commonly used for visualization of nucleic acids. The stained gels were then imaged using the syngene PXi gel documentation system, which allows for precise and accurate visualization of the gel and its contents. (see table 3 for genotyping results).

PCR Clean-up for Sequencing

After PCR confirmation of *Ghxa5*, we purify amplicons from the agarose gel using the Monarch spin PCR & DNA cleanup kit produced by New England Biolab (NEB). The purification process was carried out according to the instructions provided by the manufacturer using a column-based purification method to efficiently isolate and purify the DNA fragments of interest from the gel, remove unwanted impurities and contaminants. The PCR products were sent to Eurofins genomics for sanger sequencing and the received sequencing data were then compared to the expected sequences. To perform this comparison, the BLAST tool available on the National Center for Biotechnology Information (NCBI) website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was utilized.

TIDE Sequencing

The PCR products obtained from the candidate xa5 lines that tested positive for TF2Aγ, Cas9, guide and xa5 were sent to Eurofins Genomics for TIDE analysis. TIDE (Tracking of Indels by Decomposition) is a method developed by (Brinkman et al., 2014) that enables accurate identification and quantification of insertions and deletions (indels) resulting from the

introduction of double-strand breaks (DSBs). This method utilizes a pair of standard Sanger sequence traces from two PCR products.

To analyze the sequence traces, a TIDE analysis tool provided by the ShinyApps website at <http://shinyapps.datacurators.nl/tide/> was used. This tool performs chromatogram deconvolution of the edited and control samples allowing for the determination of the total editing efficiency and distribution of indels generated by Cas9. We used this to evaluate the efficiency and accuracy of the Cas9-mediated editing process in the candidate xa5 lines. (See table 3.1 for mutation efficiency results).

Crossing

Efforts were made to cross the flowering control/cas9 only lines with the non-flowering xa5 candidate lines to induce seed production. Unfortunately, the cross between these lines only lead to production of very small bolls in few plants and no bolls in most crosses.

Creating Additional edits by Viral-induced gene editing

To address the low editing efficiency observed in line 29, we employed a one agrobacterium/two-vector approach as described by (Uranga et al., 2023) and (Aragonés et al., 2022). This approach involves the use of T-DNA vectors namely pLX-TRV1 and pLX-TRV2 which have compatible replication origins. These vectors enable simultaneous agroinoculation of viral genomic components also known as multipartite virus components or joinTRV, using a single agrobacterium strain. In this approach, pLX-TRV1 serves as the replicase function while pLX-TRV2 contains an engineered TRV RNA2 sequence with a heterologous sub-genomic promoter derived from pea early browning virus (PEBV). This promoter is responsible for driving the expression of the desired insert. Both viral systems are based on compact T-DNA

binary vectors of the pLX series, which have been successfully utilized for initiating RNA and DNA virus infections through *Agrobacterium*-mediated inoculation, also known as agroinoculation. The recombinant viral replicons carrying the single guide RNA (sgRNA) constructs will be assembled and delivered into plants expressing the Cas9 protein through agroinoculation.

By utilizing this one *agrobacterium*/two-vector approach, we aim to enhance the editing efficiency in line 29 and improve the recovery of highly edited progeny. The use of these viral replicons and agroinoculation as a delivery system has been demonstrated to be effective in facilitating efficient genome editing in tobacco, making it a promising strategy for our experimental purposes. We obtained vectors pLX-TRV1 and pLX-TRV2 and cloned guide sequences for inactivation of TF2A γ into pLX-TRV2 and constructed vectors for editing the TF2A γ gene in line 29 (E10P2) cotton seedlings.

Plasmid construction

We used pLX-TRV2 as the backbone vector and designed cloning guide sequences to edit TF2A γ , using phytoene desaturase (PDS) as control because of easy identification of transformed plants (bleached phenotype). Gene fragments were ordered from IDT based on designs recommendation by (Uranga et al., 2023) while the PDS guide sequence was obtained from (Gao et al., 2017).

Plasmid Extraction and Linearization

Obtained pLX-TRV2 vector was streaked out on LB plate supplemented with Kanamycin at 50 μ g/ml and plasmid was extracted using thermo scientific genejet plasmid Miniprep kit according to manufacturer's recommendations. The extracted plasmid was linearized with BsaI-

HF enzyme by making a mix of 20 µl consisting of 1µl of BsaIHFv2 (added last), 2 µl of rCutSmart buffer, 1µg of extracted TRV2 plasmid and 16µl autoclaved ultrapure water. This linearization mix was incubated in water bath for 1 hour after which x4 loading dye was added and run on 1% agarose gel for 40 minutes after which expected band size of about 6kb was confirmed on gel imager. Viewed DNA was cut from gel and DNA extracted using Monarch DNA Gel Extraction according to manufacturer's protocol and used for Gibson assembly to transform *E. coli* MaHI cells.

Gibson Assembly

The eluted DNA and resuspended gene fragment was used for gibson assembly. we resuspended the DNA in filtered TE Buffer and the constructs were spinned for few seconds before suspending in 10ul TE Buffer. We then make a gibson-mix that contains 0.5 µl of the insert, 1ul of digested vector, 10 µl of gibson assembly master mix reaction and 9.5 µl of water. This reaction was run for 15minutes at 50°C. after which chemically competent *E. coli* maHi cells were used. we selected 3 mahi cells (2 for constructs and 1 for no DNA control). we then added 10µl of the gibson mix in the cells and leave on ice for 30 minutes after which they were incubated at 42 °C for 2 minutes and 30 seconds. 950 µl of LB was then added to cell plus gibson-mix mixture and incubated in 37 °C for 2 hours with continuous shaking after which 300ul of the mixture was plated on LB plate supplemented with kanamycin at 50µg/ml and incubated at 37 °C. After 48 hours plates were observed colonies.

Confirmation of recombinant plasmids

Liquid culture supplemented with kanamycin at 50µg/ml of recovery colonies were made and TRV2 plasmid was extracted from them using Thermo scientific genejet plasmid miniprep

kit according to manufacturer's recommendations and sent for whole plasmid sequencing to confirm insert.

Agrobacterium transformation by Electroporation

Agrobacterium tumefaciens EHA105 strain were grown in LB liquid culture supplemented with rifampicin and spectinomycin at 40µg/ml for 24 hours. The next day, the cells were made electrocompetent by four series of sucrose washing and resuspension with 1ml, 0.5ml (twice) and 0.4ml filter sterilized 300mM Sucrose. 10µl of 500ng of plasmid DNA and 100 µl of *Agrobacterium tumefaciens* EHA105 washed suspension were added to a 1mm gap electroporation cuvette and electroporated at 1.8 kV, 200 ohms, 2.5 uF (at time constant over 3.7) after which 900 µl of LB liquid were added to the cuvette and remove as much volume as possible to a labeled culture tube. We also electroporated 100µl of washed suspension with no added DNA as a control. The labeled culture tube was incubated at 30°C with continuous shaking for 3 hours after which 400 µl of the culture plated in LB plates supplemented with rifampicin, spectinomycin at 40µg/ml and kanamycin at 50µg/ml and incubated at 30°C for 3 days. After which plates were checked for recombinants.

TRV1 + TRV2

TRV1 plasmid was again transformed into successful transformants already carrying the TRV2 plasmid evident by growth on LB+RIF+SPEC+KM plates by the aforementioned method by remaking electrocompetent cell of *Agrobacterium tumefaciens* EHA105 strain with the TRV2 plasmid, electroporated with TRV1 plasmid and plating 400 µl of the electroporated cultures onto LB plates supplemented with rifampicin at 50 µg/ml, spectinomycin at 40 µg/ml, gentamycin at 40 µg/ml, kanamycin at 50 µg/ml and incubated at 30°C for 3 days. After which plates were

checked for recombinants. Single colonies of this transformed strains (treatment and control) was used to make liquid cultures and stored as 15% final concentration glycerol stocks.

ROS measurements

In order to assess the PAMP-triggered ROS burst induced by *elf18* perception and subsequent activation of defense signaling in candidate *EFR* expressing cotton lines, a method previously described by (Sang & Macho, 2017) was employed using *Arabidopsis thaliana* (Col-0) as positive controls as it can recognize both flg22 by FLS2 (Flagellin sensitive 2), and *elf18* using ΔEFR lines as negative control. Leaf discs of cotton, measuring 4 mm², were collected from 8-week-old plants and placed in individual wells of a 96-well plate. Each well contained 100 μ L of an elicitor master mix consisting of 50 nM elicitor peptide, 100 μ M luminol, and 20 μ g/ml horse radish peroxidase. The measurement of ROS was performed using a Spectramax ID3 multi-mode plate reader. A kinetics session was set up to measure luminescence in each well at regular intervals and measurements were taken every 2 minutes for a total duration of one hour. The luminescence data obtained from the measurements was represented as relative luminescence units (RLU). By implementing this experimental procedure, we aim to quantitatively evaluate the PAMP-triggered ROS burst in response to *elf18* perception and determine the activation of defense signaling pathways in candidate *AtEFR* expressing cotton lines.

Total RNA extraction

Total RNA was extracted from the candidate *EFR* expressing lines using a modified extraction protocol described by (Gambino et al., 2008) followed by purification to obtain high-quality RNA samples. Briefly, we grind approximately 50-150 mg of frozen plant tissue into a

fine powder using speed mill (analytikjena) operating at a frequency of 50/60 after which the ground tissue was transferred to a pre-cooled 2 mL microcentrifuge tube. 20 μ L of 2-mercaptoethanol was added to prevent RNA degradation and 1 mL of preheated extraction buffer (80°C) followed 0.8 mL of chloroform were added, we vortex the tube vigorously for 10-15 seconds and incubate at 62°C for 10 minutes with occasional vortexing and later centrifuge the tube at maximum speed for 30 minutes at room temperature. To Precipitation RNA, we transfer 800ul of supernatant to new tube and added 1/8 volume of 10M LiCl to the supernatant and mix well by inverting the tube. The tube was Incubated at -20°C for at least 2 hours and centrifuged at maximum speed for 20 minutes at 4°C. The supernatant was discarded and RNA pellets were washed twice with 70% ethanol and centrifuge at maximum speed for 1 minute after each wash. after which the pellets was briefly air-dried. To remove residual genomic DNA, the RNA samples were subjected an off-column DNase treatment using a TURBO DNA-free kit (Thermofisher Scientific) following the manufacturer's recommendations. Following DNase treatment, the samples were cleaned using New England Biolabs (NEB) Monarch RNA clean and concentrate kit following manufacturer's instructions. The cDNA library was then created using qScript cDNA supermix (Quantabio) according to manufacturer's instructions. All RNA and cDNA samples were tested for genomic DNA (gDNA) contamination during qPCR analysis using cotton *GhGAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) gene primers (see table 3.4).

Quantitative PCR

For the qPCR reaction, 1-5 ng of cDNA template was used (standardized to the same concentration per experimental replicate). Conditions of the qPCR were kept identical throughout all runs within experimental replicates following the protocol of (Smith et al., 2018).

Amplification of cDNA was done in 10 µl reactions using Luna Universal qPCR Master Mix (NEB), 0.25 µM primers and 2 µL of standardized cDNA. Master mixes and primers were pre-aliquoted for single use and stored at -20°C. All PCR reactions were run in triplicate wells and sample-well organization was kept identical between plates within experimental replicates. We followed the default thermal cycling protocol in the StepOne software v2.3 (Thermo Fisher Scientific) with real-time capture of SYBR green and ROX fluorescence as follows: 10 min at 90°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, with camera capture at the end of each cycle. A melt curve was generated after the 40th cycle, using the following parameters: 95°C for 15 s, 60°C for 1 min, then a slow ramp (0.3°C per second) to 95°C. All runs were conducted on the Step One Plus real-time PCR system (Thermo Fisher Scientific). For housekeeping gene controls, we used previously published *GhUBQ1* primers by (Cox et al., 2017) while for EFR amplification, *EFR* (qPCR) primers published by (Piazza et al., 2021) was used (see table 3.4).

RESULTS

Genotyping and Tide Analysis result

The presence of the xa5 transgene in the regenerated putative transgenic lines was confirmed by PCR analysis. Using *Ghxa5*-specific primers (see table 3.4), an amplicon of the expected size (750bp) was obtained in 17 out of the 23 candidate lines while no amplification was observed in the control lines. Among the 17 lines with successful amplification, only two lines exhibited a high editing efficiency of 97% and showed the expected 4 base pair snip (table 3.1). However, these lines did not produce flowers or seeds. On the other hand, one line (line 29/E10P2) displayed a lower editing efficiency of 29%, but it contained the xa5 transgene and

produce flowers and seeds. This line was chosen as the candidate to proceed with the experiment.

Table 3.1: Tide analysis result of *Ghxa5* candidate lines

Construct	Event Description	Lab analysis number	% Crisper Edit efficiency	bp. frequencies (+ = insertion, - = deletion)	comment
22g4 = pDIRECT_22 A g4	E1P1	11	29.1	+1	
Xa5 = pDIRECT_22 A g4 xa5	E1P1	13	27.4	-4	
	E1P2	14	27.1	-4	
	E2P1	15	0.2	0	
	E2P4	18	2.9	0	
	E2P5	19	2.6	+1	
	E3P1	20	54	+1(19.3%) and -1(30.3)	
	E4P1	22	97.2	-4	No flower/seed
	E4P2	23	95.2	-4	No flower/seed
	E5P1	24	2.1	0	
	E7P1	25	3.2	+1	
	E9P1	27	3.4	0	
	E10P1	28	3.2	-1	
	E10P2	29	29.4	-2(2.9%) and -4(21.1%)	Produced highest bolls
	E10P3	30	2.2	0	
	E11P1	32	88.9	+3 (64.7%) and -1(21.5%)	
	E11P2	33	33.2	-1 (12.8%) and -4(10.8%)	

Only two lines displayed a high edit efficiency of 97 % and 95% with the expected for base pair deletion at expected cut site 207 bp (figure 3.1) when compare with control samples where we don't expect any editing with 1% edit efficiency. Some lines had edit efficiency of 89%, 33%, 54%, 29%, 27%, 27% but did not produce flowers and or bolls except for line 29 with edit

efficiency of 29% and produced the highest bolls. Notably, some a significant edit efficiency of 29% was observed in line 11 without the transgene but only the guide (g4).

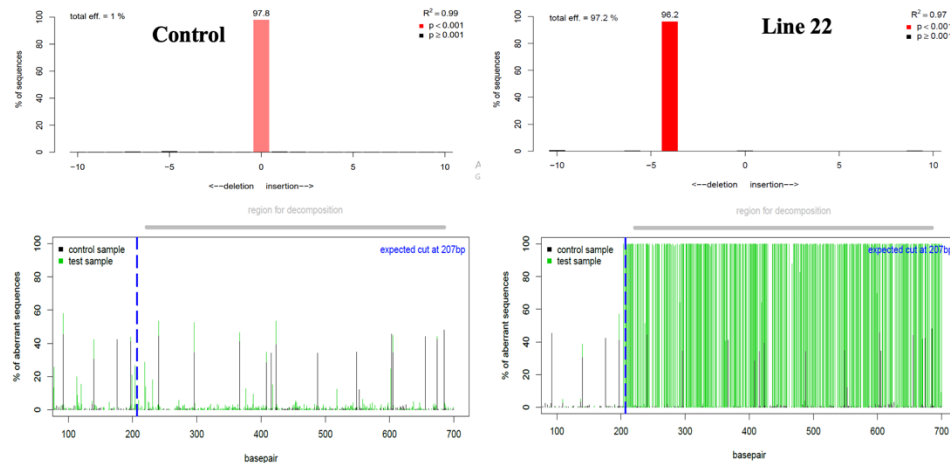


Figure 3.1: Comparing tide analysis result from line 22 (right) with edit efficiency of 97.2% and 4 base pair deletion to control line (left) expressing on Cas 9 with edit efficiency of 1% and no insertion or deletion.

Flowering and Seed production results

Among the 23 candidate lines containing the xa5 transgene, only 7 lines were able to produce seeds (See table 3.2) where line 29 produced the highest seed with a total seed weight of 66.697g. Similarly, out of the 10 control lines (carrying on Cas 9), only 7 lines produced seeds, additionally, both g4 lines carrying only Cas 9 and the guide RNA also produced seeds.

Table 3.2: T₁ seed weights at harvest

Construct	Lab.no	Treatment	Seed Weight (g)
22A(control)	2	E3P1	60.795
	5	E6P1	69.285
	6	E6P2	47.646
	7	E7P1	59.928
	8	E7P2	12.089
	9	E8P1	6.463
	10	E8P2	8.935
g4	11	E1P1	31.772
	12	E2P1	41.191
xa5	15	E2P1	1.203
	17	E2P3	35.736
	18	E2P4	47.47
	19	E2P5	22.55
	29	E10P2	66.697
	31	E10P4	57.5
	30	E10P3	11.63

Creation of additional edits by viral-induced gene editing.

Using Gibson assembly for the homology-based cloning, we successfully transformed *E. coli* MaHI cells with our vectors followed by electroporation to transform *Agrobacterium tumefaciens* EHA105 strain with same vectors. The insert sequence of the identified recombinant viral vectors was verified through sanger sequencing using whole genome sequencing. *Agrobacterium*-mediated transient transformation will be used to introduce the vectors into line 29 seedlings.

ROS measurement results

As expected, we observed flg22 and elf18-induced responses in the *Arabidopsis* Col-0 positive controls see (figure 3.2). Flg22 was also used as a positive control for responsiveness in cotton. However, only plant 3 of the seven EFR candidate plants exhibited responses to either flg22 or elf18 as measured by ROS production. Likewise, the wildtype unmodified Coker312 control samples plant did not show any response to both flagellin and elf18.

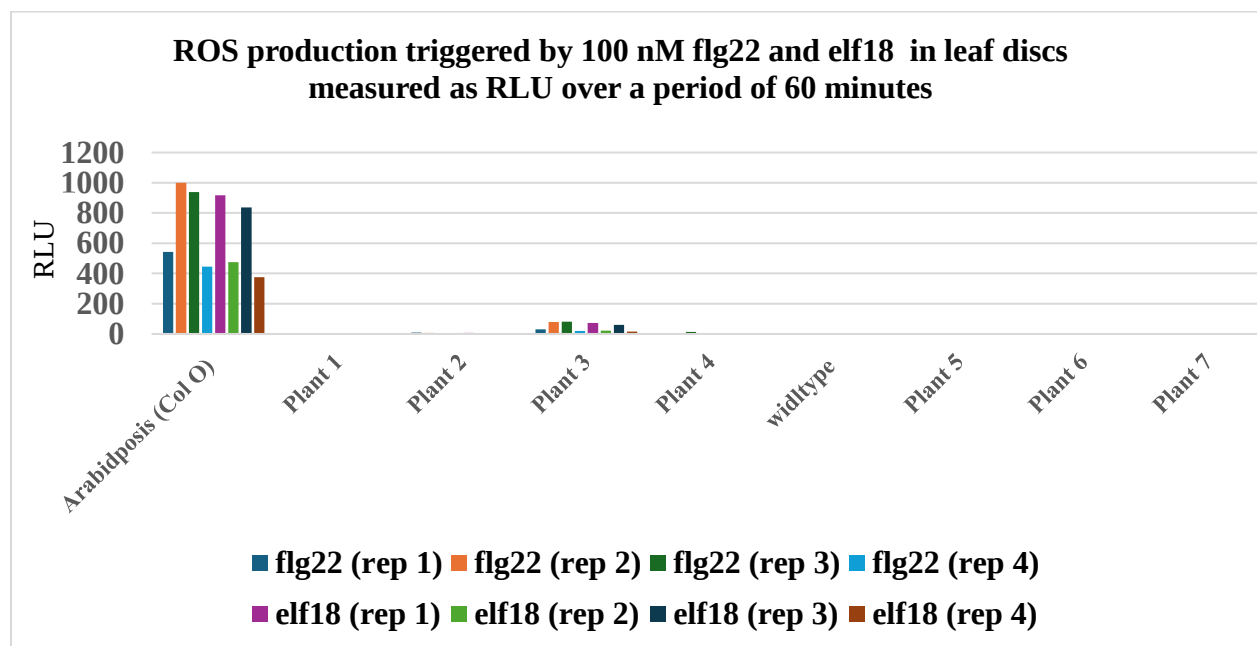


Figure 3.2: Relative luminescence units (RLU) over 60 minutes for 6 candidate EFR lines, wildtype and *Arabidopsis* Col 0 as positive control.

EFR Genotyping results

Out of the received T₀ EFR candidate seeds from our collaborators in University of North Texas representing four transformation events designated as plants 1, 2, 5, and 6, 36, 40, 26 and 34 samples were tested from plants 1, 2, 5 and 6 respectively. All 136 samples tested positive for the TFIIA γ gene,(conducted as positive control for the DNA template) however, none of the samples tested positive for the EFR transgene, the CaMV 35S promoter, or the kanamycin

resistance marker. In contrast, the positive control (extracted plasmid) tested positive for all three features.

The DNA samples extracted from these plants had acceptable quality and concentration with 260/280 ratios ranging from 1.7 to 2.1 and concentrations exceeding 95 µg/ml.

RNA Extraction and qPCR results

RNA extraction yielded low concentrations (less than 20 µg/ml) for all eight samples with successful extraction, including six EFR candidate plants, one wild-type plant, and one putative EFR-deleted negative control. Subsequently, cDNA synthesis resulted in low cDNA concentrations (19-16 µg/ml) for the eight samples. No amplification was observed in the 3 candidate EFR plants, one wild-type plant, and one putative EFR-deleted negative control while some amplification was observed in three candidate EFR plant 10, 12 and 2 with amplification efficiencies of 1.948, cq EFR values of 23.828, 21.859 and 26.079 respectively and ratios of 0.909, 0.963 and 0.953 respectively when normalized with cq *GhUBQ1*. (table figure 3.3).

Table 3.3: Gene expression of 3 EFR candidate plants 10, 12 and 2

	Plant 10	Plant 12	Plant 2
Eff. EFR	1.948	1.948	1.948
Cq. EFR	23.828	21.859	26.079
Cq. <i>GhUBQ1</i>	26.208	22.698	27.373
Ratio	0.909	0.963	0.953

DISCUSSION

Cotton, an important crop for the global textile industry faces significant challenges from biotic and abiotic agents. To improve cotton's resistant to these agents, most especially the biotic agents, classical/traditional breeding techniques has been used to develop new cotton varieties with desired traits such as disease resistance. However, this technique can be time-consuming sometimes taking up to 10 years thereby making the cotton plant to be disadvantaged in the evolutionary arms race with the pathogen. To accelerate this process, genetic engineering can be used to develop transgenic cotton varieties with enhanced resistance.

Our first strategy was to introduce a surface immune receptor gene (*EFR*) from *Arabidopsis* into cotton plants to enhances the recognition of not only Xcm but other bacterial pathogens as well. Here, 136 plants were planted from T₀ EFR candidate seeds that were received from our collaborators in University of North Texas representing four transformation events designated as plants 1, 2, 5, and 6. All were positive for the TFIIA γ gene (positive control) but none was positive for the EFR transgene, the CaMV 35S promoter, or the kanamycin resistance marker suggesting that the transgene may not have integrated into the cotton genome or may have been silenced. Our TFIIA γ amplification, good quality DNA of 260/280 ratios ranging from 1.7 to 2.1 and concentration exceeding 95 μ g/ml informed our decision to rule out the possibility of DNA template as the reason for the non-amplification of the EFR transgene.

Our collaborators at Clemson university have generated another set of EFR candidate lines and confirmed EFR on 30 differentiated plants from 10 independent transformation events but we were unable to transport these plants to Athens due to permit and regulatory issues so tissue samples for ROS and RNA extraction were taking at Clemson university and transported to Athens.

The production of reactive oxygen species (ROS) is one of the rapid immune responses triggered by plant in recognition of PAMPs (Couto & Zipfel, 2016). One well-studied PAMP is the 22-amino acid peptide flg22, derived from bacterial flagellin. Upon recognition by the FLS2 receptor, a signaling cascade is initiated, leading to the activation of Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases which is localized in the plasma membrane and subsequent ROS production in the apoplast (Kadota et al., 2014). Monitoring ROS production has been a valuable tool for investigating early immune responses and stress signaling pathways in plants. For instance, recognition of another PAMP EF-TU using EF-Tu derived peptide elf18 also lead to the production of ROS burst in leaves of apple (Piazza et al., 2021) and potato (Boschi et al., 2017). The most basic and widely used method for measurement of ROS production is luminol-based chemiluminescence in which H₂O₂ reacts with luminol in the presence of horseradish peroxidase, and produces an unstable intermediate that emits a photon of light.(Smith & Heese, 2014) and (Zhu et al., 2016). The photon emission is then measured by using a microplate reader.

In this study, Spectramax ID3 multi-mode plate reader was used to measure ROS where *Arabidopsis* Col-0 serve as a positive control for both flg22^{Pae} and elf18^{Eco} PAMP recognition as it possesses functional FLS2 and EFR receptors. Wild-type cotton on the other hand was used as a control for flg22^{Pae} recognition only as it lacks an EFR homolog.

As observed in figure 3.2 above, only one of the candidate EFR lines (plant 3) produced some response to flg22^{Pae} and elf18^{Eco} while other plants showed no response to flg22^{Pae} and elf18^{Eco}. As expected, *Arabidopsis* Col-0 exhibited a robust response to both flg22^{Pae} and elf18^{Eco}. However, surprisingly, the wild-type cotton Coker312 did not respond to flg22^{Pae}. This unexpected result may be attributed to the use of mature cotton plants, which might have a

reduced PAMP-triggered immunity response compared to younger plants because we observed response to flg22^{Pae} and elf18^{Eco} in our preliminary test with younger plants. Furthermore, previous studies have shown the suitability of 4-week-old healthy *Solanum tuberosum* (Boschi et al., 2017), *Arabidopsis thaliana* and *Nicotiana benthamiana*. (Sang & Macho, 2017) for ROS assays.

Some of leaves samples from candidate EFR lines collected at Clemson university were also used for RNA extraction but unfortunately, we encountered difficulties in isolating quality and concentrated RNA from the *EFR* candidate lines. Several factors may have contributed to this issue including collection of tissue samples from mature plant and the use of dry ice instead of liquid nitrogen for tissue preservation may have led to RNA degradation especially during transportation. Furthermore, fluctuations in freezer temperature where backup samples were kept made them unreliable for RNA extraction because the increase in temperature to 6°C may have likely compromised the RNA quality of the backup samples. However, the three RNA that were extracted and used for cDNA synthesis and qPCR indicates that plants 10, 12 and 2 are expressing the EFR transgene with cq EFR values of 23.828, 21.859 and 26.079 and ratios of 0.909, 0.963 and 0.953 respectively after normalizing with cq of the reference gene *GhUBQ1*. While this result suggests successful transformation and expression of the EFR transgene, lack of control plants for comparison altogether made these observations inconclusive.

Our second strategy is based on the disruption of interaction between Xcm TAL effectors and *GhTFIIA* to induce are recessive resistance since the successful interaction between γ subunit of TFIIA and TAL effectors using a can lead to induction of transcription factor binding motif and this strategy has been previous demonstrated to be successful in rice, tomato and citrus by (Huang et al., 2017)

In this study, cotton lines putatively expressing the designed transgene (*Ghxa5*) with natural TFIIA γ allele mutations were received from our collaborators in Clemson University, acclimatized in the greenhouse and genotyped for the presence of the transgene. Only 17 out of the 23 candidate lines were positive for the transgene and none of the control (Cas 9 only) lines was positive for the transgene. While this may suggest a successful integration of the transgene into cotton's genome, the less than 100% confirmation of *Ghxa5* can be due to a combination of factors including random integration site within the plant genome under non-selective conditions as reported by (Francis & Spiker, 2005; Kim et al., 2007; Shilo et al., 2017) and other factors like the transformation method used, the nature of the transgene construct itself, potential DNA damage during the process and epigenetic silencing mechanisms which can prevent proper expression of the inserted gene even if it integrates successfully.

Among this 17 *Ghxa5* lines, only two lines 22/E4P1 and 23/E4P2 had an high edit efficiency of 97.2% and 95.2% respectively and four base pair deletion at the expected cut site while other candidate lines have low edit efficiency between 0.2% in line 15/E2P1 to 33.2% in line 33/E11P2. The high edit efficiency of 88.9% observed in line 32/E11P1 does not represent the expected four base pair deletion but 3 base pair insertion and 1 base pair deletion. Several factors can be responsible for this variation in edit efficiency including expression levels of CRISPR Components like sgRNA and Cas9, the delivery method, features of target sequence and the potential of off-target effects as well.

Because these plants were produced from tissue culture, the variation in flowering and seed production in these lines, specifically the inability of lines 22/E4P1 and 23/E4P2 to produce flower and seed can be explained by somaclonal variation a termed coined by (Larkin & Scowcroft, 1981) for plant variants derived from any form of cell or tissue cultures. Though it can play a major role

in crop improvement through the creation of additional genetic variability we observed here some of the demerits of somaclonal variation. Other undesirable phenotypes that were observed while growing the transgenic plants included stunted growth, late flowering, no seed production in some plants that produced flowers, low seed production and inability of some plants to survive till adult stage. Despite all this variations, line 29/E10P2 with a lower editing efficiency of 29% was selected to further this study because of its ability to produce flower and highest seeds since seeds are the most efficient way to propagate plants and to ensure the stability and heritability of the transgene in subsequent generations which is essential for maintaining the newly incorporated trait.

The discrepancy in plant development between the high-efficiency lines and line 29/E10P2 highlights the complex nature of gene editing and its potential unintended consequences. While high editing efficiency is desirable, it is also essential to consider the possible unintended impact on plant phenotype and fitness. However, the lack of seed production in highly edited lines could not be entirely linked to the transformation events because control (Cas 9 only) lines 8, 9 and 10 also produced lower seeds when compared to *Ghxa5* candidate lines 17, 18 and 19.

We followed the *Agrobacterium*/two-vector approach described by (Uranga et al., 2023) and (Aragonés et al., 2022) to address this low editing efficiency, we successfully transformed *E. coli* MaHI cells with our vectors followed by electroporation to transform *Agrobacterium tumefaciens* EHA105 strain with TRV1 and TRV2 which contains an engineered TRV RNA2 sequence for editing *TFIIA γ* gene in cotton using cotton's PDS as control. This assembled recombinant viral replicons will be delivered into line 29/E10P2 a Cas9-expressing plant through agroinoculation to induce a systemic viral infection which will results in germline genome editing and recovery of edited progeny.

In our experiment, plant with a lower editing efficiency but a viable plant phenotype proved to be more useful for the advancement of this objective. Further investigation is needed to understand the underlying reasons for the observed phenotypes in plants with high edit efficiency. other alternatives could include optimizing this gene editing strategy and exploring other gene editing strategies.

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Table 3.4. Primers used in this study

Primer name	Sequence (5'-3')	Reference
<i>GhGAPDH</i>	F- CATTGTTGCCAATAGCTGGA R- GAAATTGCTGAAGCCGAAAG	(McGarry et al.,
<i>GhUBQ1</i>	F- CTGAATCTTCGCTTTCACGTTATC R- GGGATGCAAATCTTCGTGAAAAC	(K. L. Cox et al., 2017)
<i>EFR</i>	F-CTTGAATTTATTGGGGCTGTGGCG R- CCTGCAAGTTCAAAAGCTTCCCGA	(Piazza et al., 2021)
<i>EFR (qPCR)</i>	F- TTGTGGCTTCTCTGTGTTGG R- TTACCGAAATTGCCTGAACC	(Piazza et al., 2021)
<i>Ghxa5^{V39E}</i>	F- CGAGCAATAGCAAAGTGCAT	(Mijatovic et. al., 2022) (unpublished)
<i>transgene</i>	R- TGTTCTAAAACCTGAATAGC	
<i>guide</i>	F- GACTTGCCTTCCGCACAATA R- TTGTAAAACGACGGCCAGTG	
<i>cas9</i>	F- GGCTATCCTCTCTGCTAGGC R- AGGCAAGAGGATTTCTACCC	
<i>nptII (KanR)</i>	F- GAATCCAGAAAAGCGGCCAT R- ACAACAGACAATCGGCTGCT	

CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

Cotton bacterial blight (CBB) caused by *Xanthomonas citri* pv. *malvacearum* (Xcm) is an economically important pathogen of cotton as it can cause significant crop losses ranging from 5% to 35% annually (Delannoy et al., 2005). This disease is characterized by water-soaked lesions on leaves and stems, leading to tissue necrosis and plant death. (Phillips et al., 2017) showed that the re-emergence of CBB in the 21st century poses a major threat to the cotton industry. While (Mijatović et al., 2021) determined that Xcm can effectively colonize CBB-resistant cotton after seed inoculation, there is limited literature about the potential reservoir for Xcm inoculum that could have enhanced its resurgence and a need for new resistance to Xcm in order to prepare for when the current resistant genes would be ineffective against the pathogen. Work done here investigated seed-borne dissemination of the cotton bacterial blight pathogen under field conditions and strategies for translational resistance to Xcm.

In Chapter 2 we aim to test if resistant cotton cultivars can serve as inoculum source for surrounding susceptible cultivars. We conducted field trials over two growing seasons of 2023 and 2024 in a randomized complete block design of 24 plots which include 3 treatments and 8 replicates. To facilitate inoculum source tracking, the CBB-resistant and CBB-susceptible seed were each inoculated with Xcm strains carrying distinct spontaneous antibiotic resistance mutations. Harvested seed from resistant seed-inoculated plots were also tested for Xcm contamination.

This field study provides valuable insights into the complex dynamics between cotton and Xcm and its potential for spread through seed contamination and latent infections. We observed that seed inoculated resistant cultivar (PHY 411 W3FE) and susceptible cultivar (DP 2141NR

B3XF) can almost equally spread Xcm to uninoculated plants even in uninoculated plots. This finding highlights the importance of seed health in preventing the spread of the disease. Furthermore, our observation of very few symptoms in infected leave samples collected three times at approximately 40, 60 and 80 days after planting in each year suggest that latent infections where plants harbor the pathogen without showing symptoms can contribute to the spread of Xcm.

Understanding the factors influencing the development and progression of Xcm is crucial for effective disease management. Further research is needed to investigate the impact of environmental conditions, cotton genetics, movement and location of Xcm within the plant on disease incidence, severity and spread.

Providing durable resistant cultivars could augment current management strategies of this disease and reduce the cost of production. To avoid the risk of disease resistance breakdown, targeting highly conserved and stable pathogen components could be a promising strategy.

In Chapter 3, we explored two strategies for increased resistance to cotton bacterial blight, first, the introduction of the receptor gene EFR. The EFR receptor from *Arabidopsis thaliana* recognizes the bacterial PAMP, EF-Tu, and triggers immune responses. By introducing EFR into cotton, we aim to broaden its spectrum of disease resistance, as demonstrated by previous studies in tomato (Lacombe et al., 2010), apple (Piazza et al., 2021) wheat (Schoonbeek et al., 2015) and rice (Schwessinger et al., 2015). However, despite genotyping 136 candidate EFR plants that were received from our collaborators, we were unable to confirm the successful integration of the EFR transgene in any of the lines suggesting potential challenges in the transformation process such as low integration efficiency or gene silencing.

Tissue samples from candidate EFR lines generated by a different collaborator were also taken and transported to Athens before conducting ROS assays using flg22-Pae and elf18-Eco as

elicitors. While *Arabidopsis* Col-0, our positive control, exhibited a robust response to both PAMPs, only a single EFR transgenic lines showed a weak response. This unexpected result may be due to factors such as plant maturity. Additionally, attempts to analyze gene expression in the EFR lines were hindered by difficulties in RNA extraction. Low RNA quality and quantity limited our ability to draw definitive conclusions about EFR gene expression in the transgenic lines.

Our second strategy targets the *TFIIA γ* gene, a host susceptibility factor. A single amino acid change (V39E) in this gene has been reported by (Yuan et al., 2016) to confer recessive quantitative resistance to bacterial blight in rice. This resistance mechanism is dependent on the interaction between the bacterial TAL effectors and the *TFIIA γ* protein. By disrupting this interaction, we aim to induce recessive resistance in cotton. Similar strategies have been successfully implemented in other crops, such as rice, tomato, and citrus, resulting in increased resistance to their respective *Xanthomonas* pathogens.

While we successfully confirmed by genotyping that 17 transgenic cotton lines carried the desired transgene, challenges arose in terms of edit efficiency and fertility. The 2 lines with high edit efficiency (greater than 95%) were infertile while the fertile line line 29/E10P2 had low edit efficiency.

To address this low editing efficiency, we assembled viral replicons following agrobacterium/two-vector approach described by (Uranga et al., 2023) and (Aragonés et al., 2022) which will be delivered to the Cas9-expressing line 29/E10P2 via agroinfiltration to induce a systemic viral infection that will lead to germline genome editing and the recovery of edited progeny.

To reduce the impact of Xcm on boll yield and possible future resurgence of this pathogen, a comprehensive approach that includes the development of durable resistant cultivars, use of

appropriate cultural practices and the implementation of effective seed health management strategies is required. We would also need to address the challenges posed by latent infections by developing diagnostic methods/tools that can detect Xcm in asymptomatic phase. Further work is also needed to optimize cotton transformation protocols and improve its gene editing efficiency.

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