



Identification of new genetic sources of resistance to bacterial blight race 18 in diploid Asiatic cotton and resistance transfer to tetraploid Upland cotton (*Gossypium hirsutum*)

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Received: 23 February 2024 / Accepted: 11 April 2024 / Published online: 9 May 2024
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Abstract Bacterial blight (BB) caused by *Xanthomonas citri* pv. *malvacearum* (Xcm), poses a significant threat to Upland cotton (*Gossypium hirsutum* L.) production worldwide, and Xcm race 18 is the most virulent and widespread and can cause serious yield loss. Understanding the genetic basis of resistance in diploid Asiatic cotton (*G. arboreum*) and successfully transferring the resistance to tetraploid Upland cotton are crucial for developing resistant cotton cultivars. This study aimed to identify chromosomal regions for BB resistance through a genome-wide association study (GWAS) using 245 *G. arboreum* accessions evaluated in two replicated greenhouse tests and to evaluate an introgression BC2F7 population derived from a tri-species hybrid (*G. arboreum*/*G. aridum*/*G. hirsutum*). In response

to Xcm race 18 infections after artificial inoculation, 80% of the accessions exhibited a high level of resistance, including 151 accessions showing immunity with no visible foliar water-soaked lesions. A GWAS based on 7009 polymorphic SNP markers detected 9 major BB resistance QTLs on chromosomes A01, A02, A05, A06, A10, A12 and A13 in the Asiatic cotton. The tri-species introgression population showed segregation in BB resistance with significantly lower disease incidence of BB than the susceptible check Acala 1517-18 GLS (30.2 vs. 100%), suggesting that the resistance in the diploid species has been successfully transferred into Upland cotton. The identification of Xcm race 18 resistant diploid Asiatic cotton germplasm and specific chromosomal regions and candidate genes delineated by SNPs for resistance for the first time provides strong evidence that the Asiatic cotton is a new genetic source of resistance to Xcm race 18. The results will facilitate further genetic and genomic studies toward the eventual identification of resistance genes in Asiatic cotton and their transfer into tetraploid cotton through marker-assisted selection.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10681-024-03342-1>.

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Keywords Diploid cotton · Bacterial blight · Race 18 · SNPs · GWAS

Introduction

Bacterial blight (BB), caused by *Xanthomonas citri* pv. *malvacearum* (Xcm), poses a significant threat to cotton (*Gossypium* spp.) production worldwide, causing various symptoms such as seedling blight, angular leaf spot, vein blight, black arm lesion, or boll blight (Smith 1920; Knight 1948; Brinkerhoff 1970; Verma 1986; Hillocks 1992). This bacterium infects plants through open stomata, wounds, and bolls (Bird and Smith 1961), particularly in windy environments with blowing rain and dust events during the growing season. There are 22 races of Xcm identified in cotton-producing regions globally over time (Hunter et al. 1968; Brinkerhoff 1970; Hillocks 1992; Delannoy et al. 2005; Jalloul et al. 2015; Phillips et al. 2017), of which race 18 is highly virulent and wide spread in the United States and worldwide (El-Zik and Thaxton 1995; Allen and West 1991; Hussain 1984; Thaxton et al. 2001; Verma and Singh 1975; Zhang et al. 2020).

BB can lead to substantial yield loss and fiber quality reduction (Knight and Hutchinson 1950; Wickens 1953). Annual yield reductions in Upland cotton caused by this disease ranged from 1 to 2% under normal conditions to 5–15% in severe outbreaks (Hillocks 1992), with potential losses exceeding 50% under rainy weather and high humidity (Knight 1950; Verma 1986). Effective control measures were established in the 1970s through the development of BB-resistant cultivars and the use of acid-delinted seeds (Zhang et al. 2020). However, BB's resurgence in the United States due to the adoption of BB-susceptible transgenic cultivars prompted renewed efforts to combat this disease through breeding (Bird 1986; Wheeler et al. 2007, 2022; Wheeler 2018). Xcm races evolve in response to the development of new cotton cultivars with resistance genes.

Historically, research efforts focused on cotton breeding for BB resistance, notably in Sudan from the 1930s to 1950s (Zhang et al. 2020). Pioneering work by Knight in transferring resistance genes from various cotton species to Egyptian cotton (*G. barbadense* L.) known as Sakel, which was cultivated in Sudan (Knight 1963) using traditional breeding methods, resulted in the identification of 10 major BB resistance genes. These genes, including B_1 – B_{10} , were successfully introduced into commercial Upland cotton cultivars in Sudan, significantly enhancing BB

resistance. Currently, 22 BB resistance genes have been reported in cotton, of which genes B_1 – B_{12} are major genes (Zhang et al. 2020). Genes B_1 , B_2 , B_3 , B_7 , B_{10} and B_{12} were identified in Upland cotton (Knight and Clouston 1939; Brinkerhoff et al. 1979; Knight 1944; Knight 1953a, b; Green and Brinkerhoff 1956; Innes and Brown 1969; Lagiere 1960; Innes 1965a–d; Knight 1963; Follin et al. 1988; Wallace and El-Zik 1989). Genes B_4 , B_6 , B_8 and B_{11} were identified in diploid cottons (Knight 1948, 1954; Knight 1953a, b; Innes 1966). Gene B_5 was identified in perennial *G. barbadense* (Knight 1950). All these genes except B_8 were transferred to Sakel and Upland cotton (Zhang et al. 2020).

In the United States, studying the genetic basis for BB resistance primarily focused on Upland cotton. Multiple major B genes, including B_7 , B_{12} , B_{In} , B_N and B_S , were identified, along with several polygene modifiers or complexes (Zhang et al. 2020). Wright et al. (1998) employed restricted fragment length polymorphism (RFLP) markers to map quantitative trait loci (QTLs) using four different F_2 populations generated from crosses between *G. barbadense* Pima S-7 and resistant Upland cotton lines. As such, B_{12} was first mapped to chromosome c14 (D02) and later confirmed by Rungis et al. (2002) using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers. Xiao et al. (2010) further fine-mapped B_{12} on D02 within a 3.4-cM region flanked by 4 SSR markers and 4 single nucleotide polymorphism (SNP) markers. In the QTL mapping study, Wright et al. (1998) mapped six major QTLs for resistance to races 2 and 4, including two QTLs on c20 (i.e., D10, corresponding to B_2 and B_3) and four QTLs (on c05/A05, c14/D02, c20/D10 and LGD02) corresponding to b_6 . Interestingly, QTLs corresponding to B_3 and b_6 explained 53–56% of the phenotypic variation in reaction to races 7 and 18. Therefore, BB resistance genes other than B_{12} may confer partial resistance to Xcm race 18. Ellassbli et al. (2021b) conducted a genome-wide association study (GWAS) involving over 330 Upland cotton germplasm accessions. The results showed that 55 SNPs were associated with resistance to BB race 18 on different chromosomes including A01, A05, D02, D08 and D10. Further segregation analysis was conducted in 95 F_2 populations between resistant and susceptible parents to study segregation ratios for BB resistance (Ellassbli et al. 2021c).

Results showed that all F_1 were resistant, and 65 F_2 populations exhibited the expected 3:1 resistant/susceptible ratio, indicating a dominant resistance gene conferring resistance to Xcm race 18. Further, all the resistant germplasm tested possessed the B_{12} gene based on DNA markers analysis, indicating that the B_{12} gene is widely distributed in U.S. Upland cotton (Elassbli et al. 2021c). In another most recent study, Gowda et al. (2022) reported a major resistance locus (BB-13) on D02 based on GWAS of the same set of Upland cotton accessions used by Elassbli et al. (2021b), followed by linkage mapping in a bi-parental recombinant inbred line (RIL) population. However, no major resistance gene has been identified in the A-subgenome.

In general, *G. barbadense* is often considered to be more susceptible to Xcm compared to *G. hirsutum* (Brown and Ware 1958; Brinkerhoff 1970; Elassbli et al. 2021a), while a broad spectrum of disease susceptibility exists among Upland cotton germplasm. The quantitative response of resistance to Xcm indicated that multiple genetic factors come into play, including additive, dominant, and epistatic gene interactions, all contributing to the overall resistance (Bird 1960; El-Zik and Bird 1967, 1970; Innes 1969, 1974, 1983; Wallace and El-Zik 1989; Zhang et al. 2020). Modifier genes play a crucial role in influencing how major resistance genes are expressed in different genetic backgrounds. The combination of several resistance genes resulted in a high level of resistance to Xcm (Brinkerhoff 1970; Bird 1982; Zhang et al. 2020). Thus, the cultivation of resistant cotton cultivars is an effective strategy for mitigating disease epidemics and managing BB. However, it is important to note that as environmental conditions change, the stability of resistant cultivars may also vary. The specific combination of resistance genes and their quantity influence how effectively cotton resists the disease under different environmental conditions (Knight and Hutchinson 1950).

The A-genome *Gossypium* taxa, a subgroup of cotton species, exhibits near-immunity to Xcm. This level of resistance was deemed valuable enough to prompt efforts to introduce those resistance traits into cultivated tetraploid cotton (Knight 1953a). The immunity observed in A-genome cottons aligns with the likelihood that Xcm originated in the Old World (Knight 1948), particularly Africa (Follin 1981, 1983). The A-genome species has been used

as a primary source of resistance (Zhang et al. 2020), providing a valuable pool of genetic traits that can be harnessed for the improvement of cultivated tetraploid cotton. However, the resistance of the cultivated diploid species to Xcm race 18 has not been explicitly evaluated or reported, and no genetic or QTL study has been reported on resistance to Xcm race 18 in the A-genome, although the race is currently the most prevalent. There is also a lack of development and reporting of introgressed Upland or *G. barbadense* lines from the diploid species that manifest race 18 resistance or immunity. This presents a compelling opportunity for genetic exploration and molecular investigations.

The hypothesized approach involves conducting controlled crosses between resistant and susceptible lines within diploid species, to elucidate the genetic basis of immunity. By employing advanced molecular markers, resistance genes can be transferred to Upland cotton through carefully designed interspecific hybridizations. Therefore, this study aimed to evaluate an association panel of 245 diploid accessions for resistance to Xcm race 18, identify QTLs for BB resistance, and transfer the BB resistance to tetraploid cotton.

Materials and methods

Plant materials, inoculations, and evaluations of BB resistance

The association mapping panel for the study of BB resistance in diploid cotton comprised 245 accessions of *G. arboreum* selected from the National Plant Germplasm System (NPGS) cotton collection (<https://npgsweb.ars-grin.gov>) (Wallace et al. 2009). The 245 accessions, detailed in Table S1, represented a diversity of landraces, conventional cultivars, and breeding lines released over a long period of breeding history (Li and Erpelding 2016; Li et al. 2018; Abdelraheem et al. 2024). To minimize within-accession variation, self-pollinated bolls from a single plant were chosen for each accession, and the increased seeds were utilized for both evaluating BB resistance and DNA extraction.

To assess BB resistance within this panel, two independent tests each with two replicates were conducted using a randomized complete block

design (RCBD) in a greenhouse at the Fabian Garcia Research Center, New Mexico State University, Las Cruces, NM, USA. Each test included 10 seedlings per genotype per replicate. Planting was done in 10-cm plastic pots, with 10 seeds per pot. Susceptible and resistant controls, Acala 1517-18 GLS (PI688431, Zhang et al. 2019) and FiberMax (FM) 2334GLT (BASF), respectively, were included. The pots were filled with Miracle-Gro Moisture Control Potting Mix 2 CF (Scotts Co., Marysville, OH, USA).

Inoculation was performed using a newly developed backpack sprayer method (Zhang et al. 2024). Briefly, a Xcm race 18 culture was maintained and cultured on the ATCC medium (carrot potato dextrose agar) at 30 °C for 3–5 days until a uniform culture covered the petri dishes. The culture was then transferred to a liquid ATCC medium until the bacterial concentration of 10^6 ml⁻¹ was reached. The organosilicone non-ionic wetting agent, Silwet L-77 (Loveland Industries, Greeley, CO, USA) was added to the inoculum at 0.25% (v/v) and mixed before spraying using a backpack sprayer (Model #190,479, Husqvarna 4-Gallon Plastic Backpack Sprayer, Charlotte, NC, USA). Seedlings at 3 weeks post planting were topically sprayed with the inoculum until water dripping on the edge of leaves became apparent. Following inoculation, seedlings were kept at 99% relative humidity at 28–35 °C for 4 days in a sealed container with water at the bottom. Seedlings were then watered daily to maintain soil moisture without insect control. The response of the cotton seedlings to Xcm infections was evaluated on an individual plant basis at 21-day post-inoculation (dpi), using a rating scale from 0 to 5 for foliar disease severity ratings (DSR) on a plant basis, where 0 for a healthy plant with no water-soaked lesions, 1 for a plant with a few water-soaked lesions, 2 for a plant with a low number of water-soaked lesions, 3 for a plant with a moderate number of water-soaked lesions, 4 for a plant with a high number of water-soaked lesions, and 5 for a plant with a very high number of water-soaked lesions (Zhang et al. 2023). The disease incidence (DI, percentage of infected plants with symptoms) and average DSR (the sum of the DSR divided by the number of plants) were calculated. Depending on mean DSR, an accession was subjectively rated as immune with no susceptible plants (DSR = 0.0), highly resistant (HR at $0 < \text{DSR} \leq 1$), moderately

resistant (MR at $1 < \text{DSR} \leq 2$), partially resistant (PR at $2 < \text{DSR} \leq 2.5$), partially susceptible (PS at $2.5 < \text{DSR} \leq 3$), and highly susceptible (HS, with $\text{DSR} > 3.5$).

The plot data were then subjected to a separate and a combined analysis of variance (ANOVA) for the two tests using SAS 9.3 (SAS Institute Inc., Cary, NC, USA). The PROC GLM statistical approach was employed to assess the statistical significance of various sources of variation. Broad-sense heritability (h^2) was calculated using variance components, as the following: $h^2 = \text{genotypic variance} / [(\text{genotypic variance} + (\text{genotype} \times \text{test variance} / n) + (\text{error variance} / nr))]$, where r = number of replicates and n = number of tests.

Genotyping of *G. arboreum* accessions

DNA extraction was performed on the entire set of 245 accessions, and this procedure followed the protocols specified in the DNeasy Plant Mini kit (Qiagen, Valencia, CA) manufacturer's instructions. To quantify the extracted DNA, a PicoGreen dsDNA Assay kit (Molecular Probes, Inc., Eugene, OR) was utilized. This involved loading 1 µL of DNA to a 1% agarose gel for electrophoresis using $1 \times$ TBE running buffer.

The genotyping of the panel was conducted through a genotyping-by-sequencing (GBS) approach at the Institute for Genomic Diversity, Cornell University, Ithaca, New York, utilizing the method originally proposed by Elshire et al. (2011). Following this, polymorphic SNPs were identified using the TASSEL-GBS pipeline (Glaubitz et al. 2014) within the TASSEL software, following the approach detailed by Li and Erpelding (2016).

In summary, the sequencing reads underwent alignment to the reference genome of *G. arboreum* (cultivar Shixiyal, 'SXY1' genome CRI-updated_V1) (Du et al. 2018)) using the Burrows–Wheeler Aligner (Li and Durbin 2009). Tags that uniquely aligned to positions in the genome were retained for subsequent SNP identification. SNPs were further filtered with criteria of minimum minor allele frequency of 0.05, a minimum taxa coverage of 0.8, a minimum site coverage of 0.1 and a maximum heterozygosity ratio of 0.2. As a result, 7009 SNPs were employed for the GWAS (Li et al. 2014; Li and Erpelding 2016).

Genome-wide association analysis and identification of candidate genes

For GWAS, TASSEL 5 software (www.maizegenetics.net/bioinformatics/tasselindex.htm) was utilized (Bradbury et al. 2007). A mixed linear model (MLM) association test was conducted by simultaneously accounting for multiple levels of population structure (Q-matrix) and K-matrix (MLM (Q+K)). To reduce the number of false positives, the principal component analysis MLM (PCA+K) mixed linear model correcting for both Q-matrix and K-matrix was used. A QTL was declared when two or more significant SNPs (at $P < 0.001$) were found within a 1 to 5 Mb region. A false discovery rate (FDR) adjusted P -value for DSR was obtained for a multiple comparison correction (Benjamini and Hochberg 1995). The Bonferroni threshold for SNP significance was set at $P < 1.43 \times 10^{-4}$ ($p = 1/n$, where n is the number of SNPs, $-\log_{10}(1/7009) \approx 3.85$) (Li et al. 2013; Yang et al. 2015; Liu et al. 2006). The R software package “Cmplot” was employed to generate Manhattan plots (Turner 2014).

To identify candidate genes for BB resistance, annotated genes that were within a QTL region from the GWAS were searched through the cotton functional genomic database (CottonFGD, <https://cottonfgd.org/>) (Zhu et al. 2017). The function of the identified genes was determined through gene annotation. Then, the annotated genes within the QTL region were filtered based on relevant gene ontology terms, including response to fungus or biotic stimulus. Genes that had expression levels greater than 1.0 FPKM (fragments per kilobase of million mapped reads) were considered as candidate genes for BB resistance.

Evaluation of a tri-species population involving *G. arboreum* for BB resistance

A tri-species introgression population developed from Upland cotton and two diploid species, *G. arboreum* and *G. aridum* was screened for BB resistance. The development of this population was previously described (Sacks and Robinson 2007). Briefly, a synthetic hexaploid from a cross of *G. hirsutum*/*G. aridum* was crossed with five Asiatic cotton accessions- A1-024 (PI408782), A2-019 (PI129723), A2-087 (PI417895), A2-113 (PI529740), and A2-190

(PI615699) to produce fertile tri-species tetraploid (*G. hirsutum*/*G. aridum*/*G. arboreum*, AADD). The synthetic tri-species hybrids were backcrossed twice with MD51ne (Meredith 1993). The resulted BC2F1 were advanced to BC2F7 through a single boll descent method in each generation, in which one boll from each plant was collected and bulked to advance to the next generation. The bulked seeds of BC2F7, together with the susceptible Upland check Acala 1517-18 GLS and the resistant Upland check FM 2334GLT, were used for evaluation of the BB resistance in a greenhouse at Las Cruces, New Mexico, using the procedure described above.

Results and discussion

Response of diploid astatic to BB Xcm race 18

The artificial inoculation was highly successful as all the plants from the resistant Upland cotton check FM 2334GLT displayed resistant responses with no water-soaked lesions at an average DSR and DI of 0 and 0%, respectively, while Acala 1517-18 GLS was highly susceptible with an average DSR and DI of 3.9 and 100%, respectively. The DSR among the diploid accessions for BB resistance ranged from 0 to 4 with an average of 0.47, while DI ranged from 0 to 100% with an average of 16.86% (Supplementary Table 1). Averaging across the two tests, many accessions had significantly and consistently lower DSR and a higher level of resistance including immunity and high resistance, similar to the resistant check (FM 2334GLT); and most of the susceptible accessions were more susceptible than the susceptible check (Acala1517-18 GLS) in both tests. We used the method described by Ellassbli et al. (2021a, b) to classify the 245 diploid accessions into different categories based on DSR evaluated across the two tests at 21 dpi, 152 accessions (62%) were immune (Immune, with DSR=0), with no symptomatic plants observed at 21 dpi (Supplementary Table 1). In addition, 34 accessions (14%) were highly resistant. Fourteen accessions (6%) were considered as MR. Eight accessions (3%) were PR, while 10 accessions were PS, and seven accessions (3%) were HS. This result indicates that a majority of the diploid accessions (>80%) exhibited varying degrees of resistance to Xcm race 18, with a significant portion being classified as immune or

HR. Therefore, a favorable genetic diversity for BB resistance within the diploid cotton germplasm was observed. This is consistent with Knight (1953a) that the A-genome *Gossypium* taxa show near-immunity to Xcm (with race designation unknown), which is deemed sufficiently valuable to impel introgression into the cultivated tetraploid cotton. The Xcm immunity of A-genome cotton supported the probable Old-World origin of the pathogen (Knight 1948; Knight and Hutchinson 1950; Follin 1981, 1983). The notion that A-genome diploids may have evolved resistance over an extended period of exposure to the pathogen before polyploid formation occurred adds an evolutionary perspective to the observed resistance (Wright et al. 1998). In addition, the spraying inoculation method gave consistent results between the two tests. Spraying bacterial suspension onto the lower surfaces of leaves was the most frequent method of inoculation used. With this method, the inoculum was forced through the open stomata into the substomatal cavity. Therefore, it was most effective when most of the stomata were open (Bird and Blank 1951). The existence of quantitative genetic variation in BB resistance among the 245 Asiatic diploid accessions suggests the potential for identifying and harnessing genetic factors contributing to BB resistance in the diploid panel.

Responses to BB infections in each plant were assessed based on DSR symptoms from 0 for no symptoms to 5 for very high levels of water-soaked lesions at 21 dpi. The combined analysis of variance is shown in Table 1 and indicates that DSR was significant (at $P < 0.001$) in test, genotype, and genotype \times test interaction. Therefore, a further analysis was conducted on an individual test basis. In both Tests 1 and 2, DSR showed a significant genotypic variation (at $P < 0.001$). Despite being conducted in the same greenhouse at different periods, utilizing distinct inoculum preparations and inoculations, along with other uncontrollable factors in the greenhouse, genotype \times test interactions were observed. However, it is noteworthy that the coefficient of variation (CV) remained relatively low and consistent, ranging from 11.88 to 13.15 in Test 1 and Test 2, respectively (Table 1). Broad-sense heritability estimates for DSR in the two tests were similar, ranging from 0.65 in Test 1 to 0.66 in Test 2, and the estimate was 0.57 in a combined ANOVA from

Table 1 Analysis of variance (mean square) of bacterial blight resistance in the diploid panel of 245 accessions evaluated in two replicated tests in the greenhouse, Las Cruces, NM, USA

Source of variance	DF	DSR
Combined		
Test	1	14.76***
Genotype	244	1.85***
Genotype \times Test	244	0.52***
Error	487	0.25
CV (%)	10.17	
h^2	0.570	
Test 1		
Rep	1	4.11***
Genotype	244	1.85***
Error	244	0.38
CV (%)	11.88	
h^2		0.659
Test 2		
Rep	1	0.84*
Genotype	244	0.75***
Error	244	0.15
CV (%)	13.15	
h^2		0.666

h^2 broad-sense heritability, DSR disease severity rating, CV coefficient of variation

*Significant at the < 0.05 level

**Significant at the < 0.01 level

***Significant at the < 0.001 level

the two tests (Table 1). Wright et al. (1998) reported moderate to high heritability estimates ranging from 0.58 to 0.90 in four mapping populations tested for resistance to race 18 based on qualitative screening. Even for high-heritability traits such as those, analysis of quantitative phenotype can improve the reliability of genetic mapping. Therefore, in this study, we treated the response to BB as a quantitative instead of a qualitative trait to narrow down the genomic regions, because of a qualitative scoring led to map inflation ranges for QTL detection and map to large gaps in linkage groups or at the telomere of the chromosomes and/or the linkage groups (Wright et al. 1998; Said et al. 2013; Abdelraheem et al. 2017). Moving forward, further investigation into specific genetic factors governing BB resistance is needed.

QTLs mapped for BB resistance in the diploid panel

Mean DSRs for each accession from Tests 1 and 2 and the average from the two tests were used for GWAS based on 7009 polymorphic SNP markers using the MLM model, which accounts for both population structure and kinship. Seventy-two significant

SNPs were detected for BB resistance based on DSR (Fig. 1; Table 2), leading to the identification of 9 major QTLs distributed on 7 chromosomes, each explaining 17–41% of the phenotypic variation (PVE) in the diploid panel (Table 2). Based on mean DSR across the two tests, 3 QTLs including one each on chromosomes A01, A05 and A12 were detected. In

Fig. 1 A Manhattan plot showing chromosomal regions of SNPs with significant association with bacterial blight resistance based on disease severity ratings in the 245 diploid cotton accessions in two tests including mean across the two tests (bottom), Test 1 (middle), and Test 2 (top)

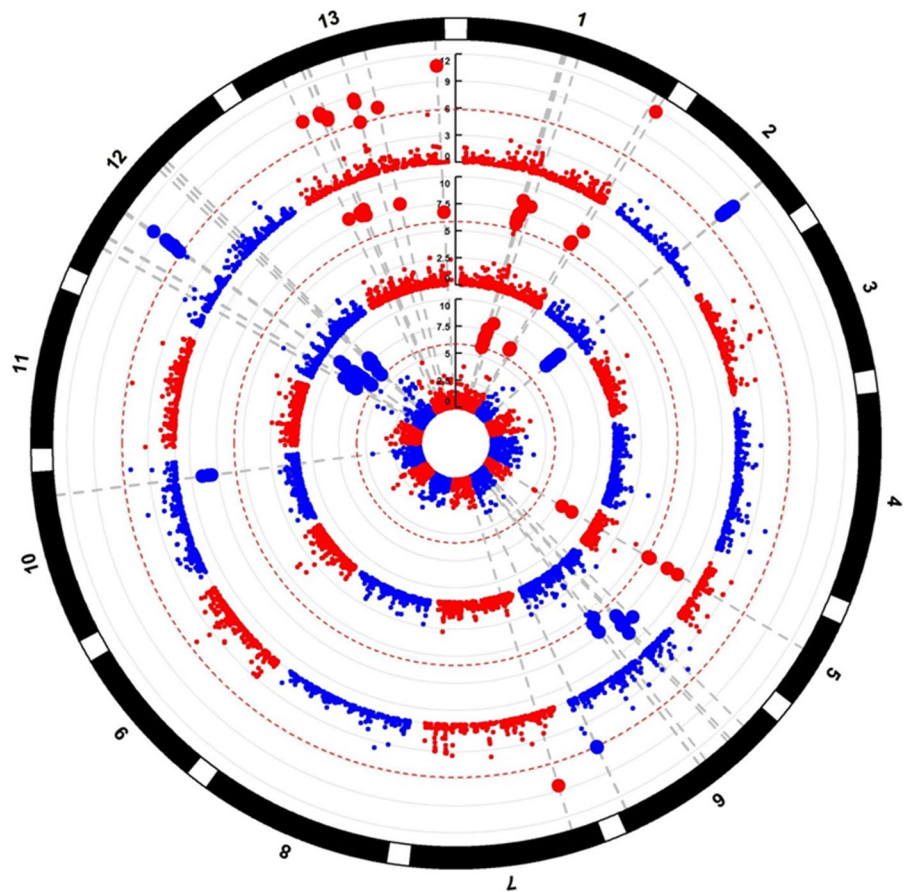


Table 2 QTL detected for bacterial blight resistance based on disease severity rating in 245 diploid accessions with artificial inoculations in the greenhouse, Las Cruces, NM, USA

QTL name	Test	Chr	No. of SNPs	Start (bp)	End (bp)	PVE%
qBB-A2-A01-1	Mean	A01	4	65,897,579	68,509,352	18.54–32.15
qBB-A2-A02-1	Test 2	A02	16	69,815,651	70,535,537	17.51–41.54
qBB-A2-A05-1	Mean	A05	4	25,876,469	25,897,507	20.00–35.00
qBB-A2-A05-2	Test 1	A05	9	29,626,300	34,202,551	17.52–29.54
qBB-A2-A06-1	Test 2	A06	8	50,578,474	56,936,664	17.40–35.01
qBB-A2-A10-1	Test 1	A10	4	96,586,824	96,797,988	22.15–25.54
qBB-A2-A12-1	Test 2	A12	15	48,156,415	49,842,423	17.14–38.91
qBB-A2-A12-2	Mean	A12	5	90,156,651	94,238,463	17.53–33.15
qBB-A2-A13-1	Test 1	A13	7	43,348,365	49,194,823	21.93–39.54

Chr. Chromosome, PVE phenotypic variance explained by SNPs

Test 1, 3 QTLs were detected, including one each on A05, A10 and A13; and in Test 2, 3 QTLs with one each on A02, A06 and A12 were also detected. One common QTL on A01 (*qBB-A2-A05-1*) was detected between Test 1 and the overall means across the two tests, while two QTLs (*qBB-A2-A12-1* and *qBB-A2-A12-2*) were detected on chromosome A12 based on results in Test 2 and the overall means across the two tests.

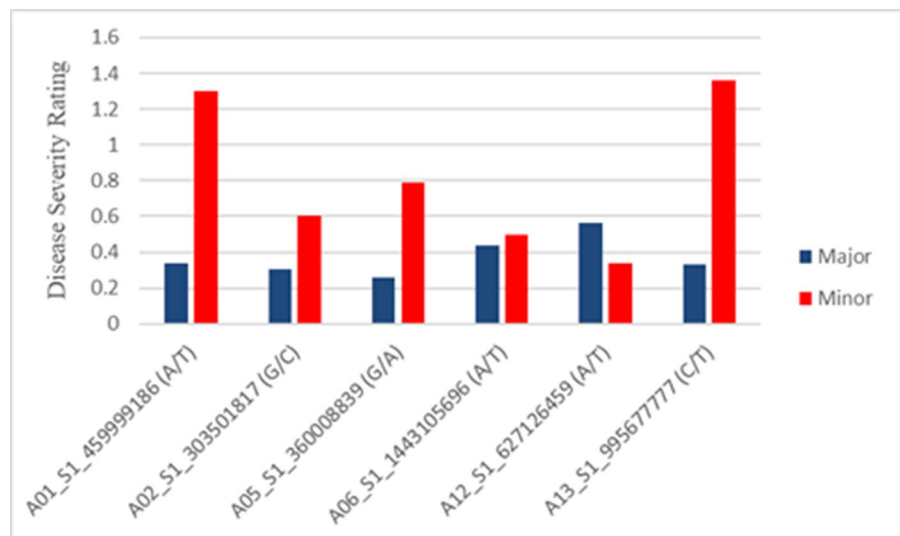
Haplotype analysis was performed on a chromosome basis for several QTLs to explore the importance of the QTLs for resistance to BB. On chromosome A01, 142 blocks were detected. Among those regions, LD block 42 has four SNPs (*SI_459999186*, *SI_459999197*, *SI_460021209*, and *SI_460021239*) within 22 kb, forming three linkage haplotype blocks. Results showed that >80% of the accessions (197) carrying the major haplotype (ACGT) had a DSR of 0.34 which significantly reduced BB infections compared to the accessions (49) carrying the haplotypes (TTTG or ACTG) with DSR values two and four-folds higher, respectively (Fig. 2). Within the major haplotype, out of 197 accessions, 127 considered immune with 0% of DI, while 12 accessions considered MR and HS to BB resistance (Table S1).

On chromosome A02, five SNPs (*SI_303501817*, *SI_303505134*, *SI_303507674*, *SI_303507711*, and *SI_303507839*) were clustered within 6 kb with three haplotypes. Notably, 147 accessions with the haplotype (GCCAT) had a DSR of 0.61, with 114 accessions classified as immune and HR, and 33

accessions considered MR and HS. Seventy accessions carrying the haplotype (CTTGG) had significantly lower DSR, with 64 accessions classified as immune and HR, and five accessions were MR and HS (Fig. 2).

In addition, on chromosome A12, four SNPs (*SI_627126363*, *SI_627126459*, *SI_627134046*, and *SI_627275529*) were clustered within 149 kb, forming a major (TAAC) and a minor (GTTA) haplotype. The 134 accessions carrying the major haplotype exhibited a DSR of 0.56, with 85 accessions classified as immune, 33 as HR, and 16 as MR and HS (Fig. 2). The result indicates the complexity of the genetic basis for resistance to Xcm race 18 and suggests the existence of major and minor alleles/haplotypes in both resistant and susceptible accessions with multiple genetic variants contributing to the observed phenotypic differences. Further, it highlights the importance of considering the interactions between alleles on different genetic backgrounds. In addition, BB resistance in the diploid may be controlled by multiple genes, each with different alleles. These genes could interact in complex ways to influence the phenotype, and other genetic or environmental factors could also play a role in BB resistance. Wright et al. (1998) indicated that the complexity of the *b6* resistance, involving 4 genes (*Qb6a*, *Qb6b*, *Qb6c*, *Qb6d*) with very different dosage effects and derived from different parents was an unexpected discovery that demonstrates a horizontal component of bacterial blight resistance. In addition, selection pressure

Fig. 2 The mean phenotype values of the 245 diploid accessions with major and minor alleles/haplotypes in significant loci on chromosomes A01, A02, A05, A06, A12 and A13 for the bacterial blight resistance in diploid cotton based on disease severity rating



acting on the diploid species has maintained diversity in the genetic variants associated with the trait.

In this study, 9 major QTLs were identified in the A genome for the first time, all considered novel due to the lack of gene mapping studies for validation in the diploid species. Notably, QTLs on chromosomes A01 and A05 were consistent with findings by Ellassbli et al. (2021b) and Wright et al. (1998). Wright et al. (1998) mapped six major QTLs for resistance to races 2 and 4, including two QTLs on c20 (i.e., D10, corresponding to B_2 and B_3) and four QTLs (on c05/A05, c14/D02, c20/D10, LGD02) corresponding to b_6 . Interestingly, QTL corresponding to B_3 and B_6 explained 53–56% of the phenotypic variation in reaction to races 7 and 18. Therefore, BB resistance genes other than B_{12} may confer partial resistance to Xcm race 18. In addition, it is worthwhile to study the homoeologous relationship between the QTLs identified on A02 and A10 in this study and these on D02 and D10 reported by Wright et al. (1998) and Ellassbli et al. (2021b)

Despite varying degrees of resistance observed in D-genome diploid cotton taxa against Xcm, complete immunity is not exhibited, challenging their role as primary sources of resistance genes (Knight 1948). However, Wright et al. (1998) noted that among seven resistance genes from tetraploid cotton, six genes (86%) were mapped to D-subgenome chromosomes. Wright et al. (1998) further explained that this may be due to the differences in evolutionary rates between subgenomes, gene conversion or other intergenomic exchanges that escaped detection by genetic mapping, or other factors. Most BB resistance genes were traditionally identified through Mendelian genetic studies using biparental crosses, and these B genes exhibit resistance to different Xcm races (Zhang et al. 2020). In addition to the well-documented B_{12} in S295, conferring resistance to all US Xcm races (Wallace and El-Zik 1989), our study suggests that resistance to race 18 may be conferred by other major B genes. However, the specific B genes possessed by the identified resistant accessions remain unknown, including whether they have a homoeologous gene to B_{12} in addition to b_6 and B_4 . Thus, conducting segregation analysis and gene mapping emerges as a potent strategy to map and validate those genes, providing a comprehensive understanding of the genetic architecture within the diploid species for BB resistance (Ellassbli et al. 2021c).

Identification of candidate genes for BB resistance QTL

In this study, a total of 150 genes were identified within eight QTL regions, of which 32 were found to be related to disease resistance and considered as candidate genes (Supplementary Table 2). The QTL $qBB-A2-A01$ at 68–70 Mb co-localized with a gene encoding U-box domain-containing protein 4 which had a role in disease defense. This QTL region was close to the reniform nematode (RN) resistant gene encoding wound-induced protein 1-like (*Cotton_A_00283* at 70 Mb) (Li et al. 2018). Therefore, it appeared that the BB resistance QTL and the reniform nematode (RN) resistance QTL locus were closely linked (ca. 0.39 Mb apart). One major QTL $qBB-A2-A05$ at 25.8–25.9 Mb region contained a candidate gene (*Ga05G2600*) encoding chalcone synthase 2 (CHS2) and is highly expressed in leaves and stems. CHS2 is a key enzyme in the biosynthesis of diverse flavonoids involved in disease resistance, nodulation, and pigmentation in different crops that respond to challenges by pathogens. Flavonoids are secondary metabolites involved in plant development and defense. Recently, Zu et al. (2023) conducted a transcriptome analysis of F_6 RILs and the parent lines following *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) infection. The findings highlighted the significant role of *CHI* genes in cotton in response to FOV infection. This gene has also been reported for BB resistance in common bean (*Phaseolus vulgaris* L.) (Cox et al. 2021). Two QTLs ($qBB-A2-A06$ and $qBB-A2-A12$) were associated with one gene each within the leucine-rich repeat (LRR) protein kinase family. Within this family, one prominent group consists of genes encoding LRR domain-containing receptor-like kinases (LRR-RLKs), which are known as resistance (R) genes and play a crucial role in plant immune responses (Jones and Dangl 2006). These R genes, upon detecting invading pathogens, can initiate a cascade of defense-related responses, ultimately leading to enhanced plant resistance. In cotton, previous studies have identified LRR domain containing genes that confer resistance in interactions with *Fusarium* wilt, *Verticillium* wilt, and plant-parasite nematodes (Li et al. 2015a, b; Abdelraheem et al. 2020). The findings presented in this study align with the hypothesis that R genes could be pivotal regulators in cotton's resistance to BB. However, the mechanisms remain

largely unknown and there are no R genes cloned in cotton against any pathogens. Cotton presents challenges to cloning R genes due to a long growth cycle, a large genome size with an allopolyploid nature, and a relatively long and technically challenging crop transformation process (DeFeyter et al. 1993; Cox et al. 2019). Thus, further functional molecular studies are necessary to validate and confirm this hypothesis, providing a deeper understanding of the specific mechanisms through which R genes contribute to resistance in cotton against BB.

Interestingly, on chromosome A13, at the major QTL *qBB-A2-A13*, 10 genes were detected within 2 Mb, eight of which (*Ga13G1095*, *Ga13G1096*, *Ga13G1097*, *Ga13G1098*, *Ga13G1099*, *Ga13G1100*, *Ga13G1101*, and *Ga13G1104*) responded to auxin, which plays an important role in plant–microbe interactions, including interactions between plant hosts and pathogenic microorganisms that cause disease. It is now well-established that indole-3-acetic acid (IAA), the most well-studied form of auxin, promotes disease in many plant–pathogen interactions (Kunkel and Johnson 2021). Recently, studies have shown that IAA can act both as a plant hormone that modulates host signaling and physiology to increase host susceptibility and as a microbial signal that directly impacts the pathogen to promote virulence. To make things even more complex, this signaling network can be further modulated by several other hormones, including other auxin, abscisic acid (ABA), and gibberellins (GAs). Not surprisingly, plant pathogens have evolved mechanisms for taking advantage of this regulatory crosstalk as a strategy for promoting pathogenesis, for example, by producing hormones or other virulence factors that modulate hormone signaling (Spoel and Dong 2008; Kazan and Manners 2009; Robert-Seillanantz et al. 2011; Kazan and Lyons 2014; Ma and Ma 2016).

The BB resistance in a tri-species population involving *G. arboreum* and *G. aridum*

Of the five diploid Asiatic accessions used in producing the tri-specific hybrid, two accessions (A2-019 and A2-190) were included in the GWAS panel for evaluation for Xcm race 18 resistance, while the other three accessions were not included due to seed availability. Results showed that both accessions tested were HR and MR, respectively, to Xcm race 18.

A total of 731 seedlings of the tri-species introgression population were evaluated for BB resistance, and 221 plants (32.2%) displayed typical water-soaked lesions. As a comparison, the resistant control FM 2334GLT and the susceptible control Acala 1517-18 GLS incurred 1 and 100% DI, respectively. The differences among the three groups were significant (Fig. 3), indicating that there existed BB resistance in the introgression population. The BB resistance was not detected in the Upland cotton parents used to develop the tri-specific population. *G. aridum* was identified to possess a resistance gene *Ren^{ari}* transferred to Upland cotton for reniform nematode (*Rotylenchulus reniformis*) resistance (Romano et al. 2009). Therefore, the resistance detected in the introgression population is likely transferred from the diploid Asiatic cotton, although resistance from *G. aridum* could not be ruled out because its response to Xcm race 18 has not been reported. Recombinant inbred lines from the bulk introgression population have been developed and are being used for segregation analysis and QTL/gene mapping for BB resistance.

Conclusions

This study utilized a panel of 245 accessions of *G. arboreum* to study the genetic basis of BB resistance in a diploid species. Many accessions were immune to BB race 18; thus, genetic resources possessing different BB resistance genes should be utilized for developing

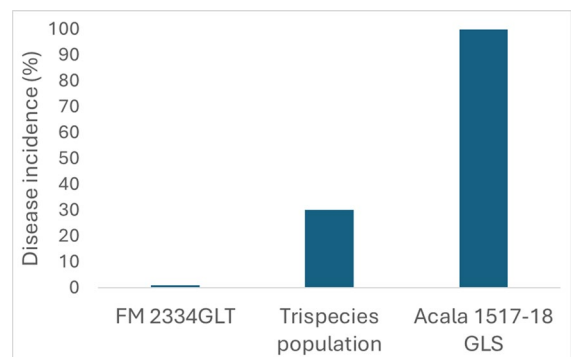


Fig. 3 Evaluation of resistance to bacterial blight race 18 in an introgressed trispecific population derived from *Gossypium hirsutum*/*G. aridum*/*G. arboreum*, as compared to resistant Upland check FM 2334GLT and susceptible Upland cotton Acala 1517-18 GLS, based on disease incidence (DI)

resistant cultivars to BB. The identification of QTLs on chromosomes A01, A02, A05, A06, A10, A12 and A13 suggest multiple genetic loci conferring resistance to Xcm race 18. The homologous or homoeologous relationships between the QTLs on A01, A02, A05 and A10 identified in the diploid A genome from this study and these reported in tetraploid cotton should be investigated. Because susceptible *G. arboreum* lines exist, genetic and molecular studies can be performed to identify *B* gene(s) that confer immunity to BB in resistant by susceptible crosses within the diploid species. Through careful selection, breeding, and genotyping, researchers can ultimately identify and isolate specific resistance genes within the diploid *Gossypium* species. Markers can then be used to trace their transfer to Upland cotton through interspecific hybridizations. Therefore, segregation analysis and gene mapping could be a powerful tool for mapping and validating resistance genes to give a comprehensive understanding of the genetic architecture for BB resistance within the diploid species. The development of portable markers closely linked to those resistance genes would further enhance their utility in marker-assisted selection for BB resistance.

Acknowledgements This research was supported in part by the U.S. Department of Agriculture, Agricultural Research Service. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Author contributions AA conducted the study, analyzed the results, and drafted the manuscript. YZ inoculated the plants and collected data for the study. LZ conceived the study and provided the tri-species population. SS and CD provided the seeds for the diversity panel. TW shared expertise in the pathogen and screening techniques. JZ conceived and directed the study. All authors reviewed and edited the manuscript.

Funding The authors have not disclosed any funding.

Declarations

Conflict of interest The authors declare no competing interests.

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