

Identification of a virulence tal gene in the cotton pathogen, *Xanthomonas citri* pv. *malvacearum* strain Xss-V2-18

Fazal Haq

Shanghai Jiao Tong University

Shiwang Xie

Shanghai Jiao Tong University

Kunxuan Huang

Shanghai Jiao Tong University

Syed Mashab Ali Shah

Shanghai Jiao Tong University

Wenxiu Ma

Shanghai Jiao Tong University

LuLu Cai

Shanghai Jiao Tong University

Xiameng Xu

Shanghai Jiao Tong University

Zhengyin Xu

Shanghai Jiao Tong University

Sai Wang

Shanghai Jiao Tong University

Lifang Zou

Shanghai Jiao Tong University

Bo Zhu

Shanghai Jiao Tong University

Gongyou Chen (✉ gyouchen@sjtu.edu.cn)

Shanghai Jiao Tong University

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Abstract

Background: Bacterial blight of cotton (BBC), which is caused by the bacterium *Xanthomonas citri* pv. *malvacearum* (Xcm), is a destructive disease in cotton. Transcription activator-like effectors (TALEs), encoded by tal-genes, play critical roles in the pathogenesis of xanthomonads. Characterized strains of cotton pathogenic Xcm harbor 8-12 different tal genes and only one of them is functionally decoded. Further identification of novel tal genes in Xcm strains with virulence contributions are prerequisite to decipher the Xcm-cotton interactions. Results: In this study, we identified six tal genes in Xss-V2-18, a highly-virulent strain of Xcm from China, and assessed their role in BBC. RFLP-based Southern hybridization assays indicated that Xss-V2-18 harbors the six tal genes on a plasmid. The plasmid-encoded tal genes were isolated by cloning BamHI fragments and screening clones by colony hybridization. The tal genes were sequenced by inserting a Tn5 transposon in the DNA encoding the central repeat region (CRR) of each tal gene. Xcm TALome evolutionary relationship based on TALEs CRR revealed relatedness of Xss-V2-18 to MSCT1 and MS14003 from the United States. However, Tal2 of Xss-V2-18 differs at two repeat variable diresidues (RVDs) from Tal6 and Tal26 in MSCT1 and MS14003, respectively, inferred functional dissimilarity. The suicide vector pKMS1 was then used to construct tal deletion mutants in Xcm Xss-V2-18. The mutants were evaluated for pathogenicity in cotton based on symptomology and growth in planta. Four mutants showed attenuated virulence and all contained mutations in tal2. One tal2 mutant designated M2 was further investigated in complementation assays. When tal2 was introduced into Xcm M2 and expressed in trans, the mutant was complemented for both symptoms and growth in planta, thus indicating that tal2 functions as a virulence factor in Xcm Xss-V2-18. Conclusions: Overall, the results demonstrated that Tal2 is a major pathogenicity factor in Xcm strain Xss-V2-18 that contributes significantly in BBC. This study provides a foundation for future efforts aimed at identifying susceptibility genes in cotton that are targeted by Tal2.

Background

Cotton (*Gossypium* spp.) is an economically-important crop worldwide and is a significant source of fiber, feed, oil and biofuel [1]. The primary cotton production areas are located in the southern United States (USA), Central America, western Africa, and central and eastern Asia. According to the 2017/18 world ranking, China leads the world in cotton production followed by India, the USA and Pakistan [2]. *Gossypium* spp. contains over 50 species, including *G. arboreum*, *G. herbaceum*, *G. hirsutum* and *G. barbadense*. *G. arboreum* and *G. herbaceum* are diploid ($2n = 26$), whereas *G. hirsutum* and *G. barbadense* are tetraploid ($4n = 52$) [3, 4]. *G. hirsutum* is the predominant species and produces with 90% of the world's cotton fiber production [5]. This species is impacted by a devastating bacterial disease known as bacterial blight of cotton (BBC), which is caused by *Xanthomonas citri* pv. *malvacearum*. The first detailed description of BBC was reported in the USA [6]. However, this disease currently occurs in all cotton production areas and causes significant yield losses (5-35%) either by injury to the plant or direct damage to the boll [7].

Xcm is able to infect all above-ground parts of cotton at any developmental stage starting with seedlings [8]. Typical BBC symptoms include cotyledon/seedling blight, angular leaf spots, water-soaked lesions, black arm of petioles and stems, boll rot and boll shedding [8, 9]. The main virulence factors that contribute to the pathogenicity and adaptation of bacterial pathogens include exopolysaccharides, lipopolysaccharides, adhesins, protein secretion systems, siderophores, quorum sensing, biofilms, chemotactic sensors and degradative enzymes [10-13]. Particularly, type III secreted effector (T3SE) proteins play an important role in bacterial pathogenicity [10-12, 14] and have been identified in *Xanthomonas* spp. [14-20]. One of the most studied groups of T3SEs are the transcriptional-activator like (*tal*) effector (TALE) proteins [21-28].

TALE proteins, functionally resemble eukaryotic transcription factors, are localized to the host plant nucleus where they bind to specific promoter sequences known as effector-binding elements (EBEs), thus regulating host gene expression [29-31]. TALEs belong to the *avrBs3/pthA* gene family [26], which is highly conserved among different *Xanthomonas* spp. TALEs contain an N-terminal T3S signal domain, a central repeat region (CRR), C-terminal nuclear localization signals (NLS), and an acid activation domain (AD) [30, 31]. 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 [30-32]. TALE-mediated activation of EBEs can induce host susceptibility (*S*) or resistance (*R*) genes [29, 30]. For example, the TALEs PthXo1 and PthXo2 from *X. oryzae* pv. *oryzae* (*Xoo*) were shown to enhance the expression of rice genes *OsSWEET11* and *OsSWEET13*, which are required for susceptibility to bacterial leaf blight [33, 34]. However, rice cultivars were resistant to *Xoo* when they contained *OsSWEET11* and *OsSWEET13* alleles lacking PthXo1 and PthXo2 EBEs [35-37]. Recently, a new rice *S* gene (*OsERF#123*) was shown to be targeted by TalB in African strains of *Xoo* [38]. Other examples of TALEs include AvrBs3 that targets the pepper resistance gene *Bs3* and AvrXa10, AvrXa23 and AvrXa27 that interact with rice *R* genes *Xa10*, *Xa23* and *Xa27*, respectively [29, 39-41]. Recently, Cai et al. [21] reported that Tal7 from *Xoo* binds and activates the expression of the rice gene *Os09g29100*, an interaction that suppresses *avrXa7-Xa7*-mediated resistance in rice. A number of truncated TALEs (truncTALEs) and interfering TALEs (iTALEs) have also been reported in *Xoo* that function as suppressors of *Xa1*-mediated defense in rice [42, 43].

Resistance to *Xcm* has been identified primarily in *G. hirsutum*. The genetic nature of resistance to BBC was first revealed in 1939, and efforts to breed cotton plants for resistance ensued shortly thereafter [44]. About 20 major *R* genes or polygene complexes (*B* genes) participate in resistance to BBC in cotton [7, 8]. Based on their virulence phenotype in differential cotton hosts, *Xcm* strains have been classified into 22 races that are named 1-22 [7]. Race 18 is the most common variant and was first isolated in 1973 [45, 46]. In some cases, the outcome of interactions between *Xcm* strains and differential cotton varieties is dependent on the *avrBs3/pthA* gene family in *Xcm*, which indicates that *Xcm*-cotton interactions follow the gene-for-gene model for host plant resistance [7, 10, 47, 48].

The number and diversity of *tal* effector genes varies among different species, pathovars and strains of *Xanthomonas*. For example, *Xoo* strains harbor 8-26 TALEs [49-53], *Xoc* strains contain 19-28 [49, 54, 55],

Xtt strains contains 5-12 [56], *Xtu* strains contains 7-8 [56-58] and *Xcm* strains harbor 8-12 genes encoding *tal* effectors [27, 46, 59]. Some *Xanthomonas* spp. lack *tal* effector genes, such as *X. citri* pv. *raphani* strain 756C [54]. To date, at least 20 TALEs have been cloned and characterized from *Xcm* strains [25, 26, 28, 48]. Among these, Avrb6 was the first *Xcm* TALE shown to be important for virulence [25]. Cox et al. [27] demonstrated that Avrb6 induced the expression of the cotton *S* gene, *GhSWEET10*, thus enhancing bacterial virulence and adaptation to the host.

The aim of the current study is to identify a novel virulent *tal*-gene encoding TALE protein in a highly virulent cotton pathogen, *Xcm* strain Xss-V₂-18 (from China).

Results

TALEs of Xss-V₂-18

Restriction fragment length polymorphism (RFLP) analysis was conducted to estimate the number and size of *tal* genes in *Xcm* Xss-V₂-18. Since most *tal* genes retain two *Bam*HI sites, *Xcm* Xss-V₂-18 plasmid and genomic DNAs were digested with *Bam*HI and analyzed by Southern blotting as described above. Six bands hybridized to the probe in *Bam*HI-digested genomic and plasmid DNA, indicating that Xss-V₂-18 contained six plasmid-encoded *tal* genes (Fig. 1A).

The six *tal* genes were cloned in pBluescript as *Bam*HI fragments, giving rise to pB-*tal1*, pB-*tal2*, pB-*tal3*, pB-*tal4*, pB-*tal5* and pB-*tal6* (Fig. 1B) and confirmed by colony hybridization and sequence analysis. To obtain the complete DNA sequence of each *tal* gene, we inserted the Tn5 transposon into the CRR region and used primer sets tal-F/RP and FP/tal-R to obtain the sequences (Fig. 1C). The *tal* gene sequences have been deposited in GenBank under the following accession numbers: MK654746 (*tal1*), MK654747 (*tal2*), MK654748 (*tal3*), MK654749 (*tal4*), MK654750 (*tal5*) and MK654751 (*tal6*). Each *tal* gene encodes various numbers of RVDs, which are tandemly arranged and encoded within 102-bp direct repeats. There were 27.5, 102-bp repeat units in *tal1*, 25.5 in *tal2*, 21.5 in *tal3*, 18.5 in *tal4*, 15.5 in *tal5* and 13.5 in *tal6* (Fig. 2A).

To better understand the features of Xss-V₂-18 TALEs, we compared them with TALEs in *Xcm* strains MSCT1, H1005, N1003, MS14003 and AR81009 [27, 46, 59]. Phylogenetic tree of TALEs from *Xcm* strains were constructed by aligning TALE-CRR with DisTAL v1.1. All 53 TALEs (Xss-V₂-18=6, MSCT1=8, H1005=12, N1003=9, MS14003=8 and AR81009=12) were classified into 6 major groups and 33 sub-groups. Tal2 of Xss-V₂-18, TAL6 of MCST and Tal26 of MS14003 fall in same group (Fig. 2B).

Nearly identical RVD sequences were observed for the six TALEs in Xss-V₂-18, MSCT1, H1005, MS14003 and AR81009 (Fig. 2A). Differences of two RVDs between Tal2 of Xss-V₂-18 and TAL6 of MSCT1, Tal26 of MS14003 indicate that they are functionally different from each other and may target a different EBE. The predicted theoretical EBE box for Tal2, Tal6 and Tal26 of Xss-V₂-18, MSCT1 and MS14003,

respectively, are mentioned in Figure S1. RVDs in *Xcm* strains included NI, NG, NS, HD and NN; the latter RVD was absent in Tal1, Tal2, Tal3 and Tal4.

Xss-V₂-18 *tal* deletion mutants

To assess the role of *tal* genes in the virulence of Xss-V₂-18, we generated *tal* deletion mutants by homologous recombination using the suicide vector pKMS1 [60]. Fragments *a* (580 bp) and *b* (350 bp) were amplified on the left and right sides of DNA encoding the CRR, respectively, and cloned as a fused fragment in pKMSA1 (Fig. 3A, B). Construct pKMSA1 was introduced into *Xcm* Xss-V₂-18; after homologous recombination, 41 putative mutants were selected for PCR amplification using primers pKMSA1-5F/pKMSA1-3R (Table S1). Four putative mutants designated M1, M2, M3 and M4 contained a 930-bp PCR product, which is consistent with the size of the insert in pKMSA1 (Table 1, Fig. 3C). Southern hybridization indicated that one or more *tal* genes were deleted in the four mutants (Fig. 3D). M1 and M2 were lacking *tal3* and *tal2*, respectively, M3 was missing *tal2* and *tal4*, and M4 lacked *tal2*, *tal4*, *tal5*, and *tal6*. These results indicated that four *tal* loci underwent homologous exchange via pKMSA1, and copies of the plasmid pKMSA1 functioned to delete multiple *tal* genes simultaneously in M3 and M4.

A second round of deletion mutagenesis was conducted with plasmid pKMSA2, which contains a fusion of fragments *c* (150 bp) and *d* (300 bp) on the left and right sides of the DNA encoding the CRR, respectively (Fig. 3A). Construct pKMSA2 was used to generate new deletions in the M4 mutant, and potential new mutants were analyzed by PCR with primer pairs pKMSA2-5F/pKMSA2-3R (Table S1). Two mutants designated M5 and M6 contained a 450-bp PCR product that is consistent with the size of the insert in pKMSA2 (Fig. 3E). In addition to *tal2*, *tal4*, *tal5*, and *tal6*, Southern hybridization indicated that mutant M5 contained a deletion in *tal3*. M6 was lacking both *tal1* and *tal3* (Fig. 3F); thus, M6 lacked all six *tal* genes and can be considered a *tal*-free mutant of Xss-V₂-18.

Virulence assays

Xss-V₂-18 and mutants M1-M6 were inoculated into cotton leaves and phenotypes were observed 3-5 days post-inoculation (Fig. 4A). Xss-V₂-18, M1, and M4 produced substantial water-soaked lesions in the inoculation sites; however, water-soaking was reduced in leaves inoculated with M2, M3, and M5 (Fig. 4A). In contrast, the region inoculated with the *tal*-free mutant M6 showed cell death and necrosis (Fig. 4A) signifying that the loss of *tal* genes affect the virulence of Xss-V₂-18. On the second day post-inoculation, the populations of the M2 and M6 mutants were significantly lower than Xss-V₂-18, M1, M3, M4 and M5 (Fig. 4B). On days 4 and 6 post-inoculation, the growth of Xss-V₂-18 was significantly higher than mutants M1-M6 with no significant difference among the mutants. These results indicated that some of the *tal* genes are involved in Xss-V₂-18 virulence, and the absence of selected *tal* genes impacted growth of the pathogen *in planta*.

Mutant M2, which lacks *tal2*, exhibited reduced symptomology and bacterial growth when compared to wild-type Xss-V₂-18 (Fig. 4A, B). Based on these observations, we speculated that *tal2* might be involved

in virulence; this was addressed by constructing pHZW-*tal2* (Table 1) for complementation analysis. The pHZW-*tal2* construct was introduced into *Xcm* M2, and the empty vector (ev, pHM1) was used as a negative control. Western blot analysis indicated that the Tal2 protein was produced in *Xcm* M2 (Fig. 4C). The wild-type Xss-V₂-18, mutant M2, M2(ev), and M2(*tal2*) were inoculated into cotton leaves; phenotypes were observed at 5-7 days post-inoculation (Fig. 4D), and bacterial growth was measured at 0, 2, 4, and 6 days post-inoculation (Fig. 4E). Both water-soaking and bacterial growth *in planta* were restored to wild-type levels in *Xcm* M2 containing pHZW-*tal2* (Fig. 4D, E). Based on results shown in Fig. 4, we conclude that Tal2 is major virulence factor in Xss-V₂-18.

Discussion

Until recently, BBC has been effectively controlled using classical *R* genes [61-63]; however, in 2011 the disease re-emerged with a vengeance [46]. A known virulence factors, transcription activator-like effectors (TALEs), in *Xcm* are important for BBC. In previous studies, 8-12 *tal* genes were reported in *Xcm* [26-28, 48, 59]. Some *Xcm tal* genes, notably *avrB101*, *avrB102* and *avrBln*, are known to cause an hypersensitive response (HR) on cotton [28], whereas *avrb6* elicits water-soaking [48]. In this study, RFLP-based Southern hybridizations indicated that the highly-virulent *Xcm* strain Xss-V₂-18, which was originally isolated from Hainan, China, harbors six plasmid-borne *tal* genes (Fig. 1). In the genus *Xanthomonas*, the location and number of *tal* genes varies among species, pathovars and strains [55, 64]. For example, strains of *X. oryzae* pv. *oryzicola* (*Xoc*) encode over 250 chromosomally-borne *tal* genes [55]; however, plasmid-encoded *tal* genes are common in other *Xanthomonas* spp. Examples include the *tal* genes in *X. citri* pv. *citri*, *X. citri* pv. *aurantifolii* and *X. axonopodis* pv. *manihotis*, which were identified on plasmids pXAC66, pXcB and pXam46, respectively [65-67]. Feyter and Gabriel [28] and Showmaker et al. [59] reported the existence of plasmid-borne *tal* genes in *Xcm* strains XcmH and MSCT1, respectively. A draft genome sequence of the *Xanthomonas translucens* pv. *cerealis* strain CFBP 2541 also indicate a plasmid borne *tal*-gene [68].

The presence of highly repetitive sequences in *tal* genes complicates efforts to obtain their nucleotide sequence; therefore, we used a Tn5 insertion method as a sequencing strategy. This sequencing strategy for *tal*-genes was also used by others previously [21, 69]. Normally the number of repeats in *tal* genes varies between 1.5 and 33.5, and each repeat encodes 33-34 amino acids that vary only at positions 12 and 13 (RVDs) [30]. In *Xcm* Xss-V₂-18, we identified 27.5, 25.5, 21.5, 18.5, 15.5 and 13.5 tandemly arranged 102-bp direct repeats (encoding 34 amino acids) in *tal1*, *tal2*, *tal3*, *tal4*, *tal5* and *tal6*, respectively. In order to understand how *Xcm* TALome differ from each other within and between strains, DisTAL and AnnoTALE were used to characterized [50, 70]. *Xcm* encodes very diverse TALE effectors that were classified exclusively into 6 major groups and 33 sub-groups. TALE phylogenetic tree of *Xcm* strains showed that Tal2 of Xss-V₂-18, TAL6 of MCST and Tal26 (M26) of MS14003 fall in same group. Furthermore, RVDs based analysis showed that the six TALEs in Xss-V₂-18 were identical or nearly identical to plasmid-borne TALEs in *Xcm* MSCT1, MS14003, H1005 and AR81009 which suggests that these genes may have been horizontally transferred [65, 71, 72]. The number and location of *tal* genes

varied in the six *Xcm* strains; MSCT1 possess eight (seven plasmid-borne) [59], XcmH1005 has 12 (six plasmid- and six chromosomally-encoded) [27], XcmN1003 has nine (four plasmid-encoded) [27], MS14003 has 8 (7 plasmid-encoded) [46], AR81009 has 12 (six plasmid-encode) [46] and Xss-V₂-18 has six plasmid-encoded *tal* genes (Fig. 1, 2). The variation in number, location and RVD sequence in *Xcm* TALEs could be important for maintaining virulence in cotton cultivars grown in different geographical regions.

To assess the role of *tal* genes in Xss-V₂-18, we generated deletions in Xss-V₂-18 by homologous recombination with pKMS1 [60], which was previously used to generate deletion mutants in the rice pathogen, *Xoc* [73-75]. This is the first report where pKMS1 was used to generate *tal* deletion mutants in *Xcm*, and the basic strategy was to replace the CRR (encoded by 102-bp repeat units) with up- and downstream fragments flanking the *tal* genes. Using construct pKMSA1, we obtained four mutants; M1 and M2 lacked *tal3* and *tal2*, M3 had deletions in *tal2* and *tal4*, and M4 lacked *tal2*, *tal4*, *tal5* and *tal6*. We speculate that *tal5* and *tal6* might be located in the same gene cluster. The second knockout was obtained using pKMSA2 where up- and downstream flanking fragments (homology arms) were located closer to the CRR. Mutant M4 was used as a parental strain for the deletions generated with pKMSA2, and we recovered two new mutants designated M5 and M6. In addition to *tal2*, *tal4*, *tal5* and *tal6*, mutant M5 also lacks *tal3*, whereas M6 contains deletions in all six *tal* genes (Fig. 3). The symptoms induced by M2, M3, M5 and M6 were significantly reduced relative to the wild-type, thus indicating that one or more *tal* genes contribute to symptom development in Xss-V₂-18. Mutants M2, M3, M5 and M6 all lack the *tal2* gene; thus the potential contribution of *tal2* to symptom development was further investigated. Expression of *tal2 in trans* restored symptoms and growth *in planta* to the M2 mutant, thus confirming that Tal2 is a virulence factor (Fig. 4). Although the TALE repertoire of *Xcm* Xss-V₂-18, MSCT1, MS14003, H1005 and AR81009 is somewhat identical, Tal2 of Xss-V₂-18 differs at two repeat variable diresidues (RVDs) from Tal6 in MSCT1 and Tal26 in MS14003, inferred functional dissimilarity.

TALEs functionally resemble eukaryotic transcription factors that target and regulate the expression of host genes by binding to their promoter sequences. TALE-triggered susceptibility has been well-studied, and the contribution of TALEs to virulence has been evaluated in many *Xanthomonas* spp. [21-23, 27, 57, 76-79]. For example, the TALEs PthXo1 and PthXo2 from *Xoo* were shown to enhance the expression of rice genes *OsSWEET11* and *OsSWEET13*, which are required for susceptibility to bacterial leaf blight in rice [33, 34]. However, rice cultivars were resistant to *Xoo* when they contained *OsSWEET11* and *OsSWEET13* alleles lacking PthXo1 and PthXo2 EBEs [35-37]. A recent study by Peng et al. [80] reported that Tal8 from *Xtu* target and induce the expression of host gene *Ta-NCED-5BS*, encode enzyme required for rate-limiting step in ABA biosynthesis, to promote disease susceptibility. In another new study, Wu et al. [81] shown that TAL-effector Brg11 from *Ralstonia solanacearum* enhance the expression of 5-truncated *ADC* (*arginine decarboxylase*) transcripts that subvert translational control and thereby inhibit competing pathogens. In *Xcm*, Avrb6 was the first TALE shown to be important for virulence [25]. Recently, the *Xcm* effector Avrb6 was shown to target and induce the expression of the cotton *S* gene, *GhSWEET10*, thus enhancing virulence and promoting disease [27]. The present study provides an

important foundation for identifying potential *S* genes that interact with Tal2, which will ultimately help us develop better control strategies for BBC.

Conclusions

In this study, we identified genes encoding TALEs in the highly-virulent *Xcm* strain, Xss-V₂-18 (from China), and assessed TALE roles in BBC. We found that Xss-V₂-18 encodes six plasmid-borne *tal* genes. Knockout mutagenesis of Xss-V₂-18 *tal* genes and complementation analysis demonstrated that Tal2 is required for full virulence of Xss-V₂-18 on cotton. The identification of the Tal2 target in cotton will ultimately provide new avenues for developing BBC-resistant varieties.

Methods

Bacterial strains, growth conditions, and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (5g yeast extract, 10g NaCl, 10g tryptone/L) or LB with agar at 37°C. *Xcm* strains were grown in nutrient broth (NB) (1 g yeast extract, 3 g beef extract, 5 g polypeptone and 10 g sucrose/L) or NB with agar at 28°C. *Xcm* transformants containing the first crossover event were grown on NAN (nutrient agar without sucrose) or NBN (NAN without agar) medium. For the second crossover event, transformants were plated on NAS agar (NAN with 10% sucrose) [60]. When appropriate, antibiotics were added at the following concentrations (µg/mL): ampicillin, 100; kanamycin, 20; spectinomycin, 25; and rifampicin, 50. The pH of both solid and liquid media was adjusted to 7.0-7.2.

DNA preparation

Total genomic DNA of Xss-V₂-18 was isolated using the Bacterial Genomic DNA Extraction Kit (TaKaRa, China). The isolated gDNA pellet was re-suspended in double-distilled water. Bacterial plasmid DNA was isolated using the Plasmid Miniprep Kit (Omega, USA). The quality and quantity of genomic DNA and plasmid DNA were checked with NanoDrop spectrophotometer (Eppendorf). Routine plasmids isolation from *E. coli* was carried out by using the plasmid DNA Mini Kit (GBS Biotechnology, China).

Isolating, cloning and sequencing of Xss-V₂-18 *tal* genes

The isolation and cloning of *tal* genes from *Xcm* strain Xcc-V₂-18 followed a previously described procedure [21, 69, 77, 82] with minor modifications. Plasmid DNA and genomic DNA (50 µg) were isolated from *Xcm*, digested with *Bam*HI, and separated on 1.2% agarose gels. Specific *tal* DNA fragments were then gel-purified and ligated into pBluescriptII SK(-) that was digested with *Bam*HI and treated with calf intestinal phosphatase (CIP). The ligated products were introduced into competent *E. coli* cells by the heat shock method according to the manufacturer's protocol (Bio-Rad, USA). The successful cloning of *tal* genes in pBluescript II was validated by restriction digestion, colony hybridization and sequence analysis.

The repeat units in *tal* genes complicate abilities to sequence the genes using conventional approaches. Thus, after cloning into pBluescript II SK(-), we used the EZ-Tn5TM<KAN-2>Tnp TransposomeTM Kit to insert Tn5 into each *tal* gene as recommended by the manufacturer (Epicentre, Madison, WI). Clones with Tn5 insertions in the middle of the repeat region were selected by *SphI* digestion and sequenced using primers pair tal-F/RP and FP/tal-R (Table S1).

TALEs phylogenetic tree construction and RVDs comparison

For TALEs phylogeny, available genome sequences of *Xcm* strains MSCT1, H1005, N1003, MS14003 and AR81009 were obtained from the NCBI. *TALE* genes were predicted and analyzed in each genome using AnnoTALE v1.4.1 [50]. DisTAL v1.1 were used to align and classify TALEs based on their central repeat region [70].

For the TALE RVDs analysis, we used AnnoTALE version 1.4.1. The TALEs are grouped into classes based on the RVDs that shows possible functional and evolutionary relationship [50, 83].

Construction of Xss-V₂-18 *tal* deletion mutants

The *tal* genes in *Xcm* Xss-V₂-18 were deleted by homologous recombination using the suicide vector pKMS1 [60]. The 5' and 3' fragments that flank the CRR repeat in *tal* genes are conserved [64] and were used as sites for homologous recombination. The left- and right-flanking fragments of each *tal* gene were PCR-amplified using genomic DNA of *Xcc* strain Xcc049 (Table 1) as the template, and ligated into the MCS of pKMS1 [4], resulting in constructs pKMSA1 and pKMSA2, respectively. The new constructs were verified by restriction digestion and sequence analysis (TaKaRa, China). Constructs pKMSA1 and pKMSA2 were introduced into *Xcm* strain Xss-V₂-18 by electroporation; cells were then plated on NAN medium supplemented with kanamycin and incubated at 28°C for 4 days. Single colonies were then cultured in NBN broth at 28°C to OD₆₀₀ ≤ 0.2 (~3×10⁸ cells/mL), inoculated to NAS agar medium, and incubated for two days at 28°C. Single colonies that grew on NAS were then transferred to NA and NA containing kanamycin. Colonies that grew on NA, but not on NA^{Km}, were selected as potential deletion mutants. The mutants were then analyzed by Southern blot hybridization and PCR with primer pairs pKMSA1-5F/pKMSA1-3R and pKMSA2-5F/pKMSA2-3R (Table S1).

Southern hybridizations

Xcm plasmid and genomic DNA were extracted as described above. After *Bam*HI digestion, DNA was separated on 1.2% agarose gels and then transferred onto Hybond N⁺ nylon membranes (Roche, Germany). The 2898-bp internal *SphI* fragment of *pthXo1* (GenBank accession no: AY495676) from *Xoo* [84] was labeled with digoxigenin (DIG) and used as a hybridization probe to detect the *tal* genes. Probe labeling and Southern blotting were performed using the DIG Probe Synthesis Kit as recommended by the manufacturer's instructions (Roche, Sweden).

Virulence assays

Cotton cultivar TM-1 (*G. hirsutum*) was used in this study. Plants were grown in a greenhouse at 23°C with a 12-h light/dark photoperiod and ~80% RH. Two-week-old plants were used in virulence assays. Single colonies of *Xcm* were inoculated to 4 mL NB and cultured overnight at 28°C. Bacterial cells were harvested by centrifugation (5000 rpm, 3 min); pellets were washed twice in sterile 10 mM MgCl₂ and then re-suspended in 10 mM MgCl₂ buffer to OD₆₀₀=0.1 (~2×10⁸ cells/mL). The suspensions were inoculated to the abaxial surface of leaves by infiltration with a sterile needleless syringe. Inoculation with simply 10mM MgCl₂ buffer served as a mock. Leaf phenotypes were examined 4-5 days after inoculation. Three independent plants were used, and the experiments were repeated three times with similar results. For the quantification of bacterial growth in cotton, triplicate leaf samples (1cm² in diameter) were collected for each inoculated strain and washed with 70% ethanol and double-distilled water (ddw). Samples were macerated in 1mL ddw and incubated for 30 minutes at room temperature. Serial dilutions were then plated on NB agar medium with appropriate antibiotics for colony counts. The experiment was repeated three times, and the significant differences were determined by using student's *t*-test.

Expression of *tal2* gene in *Xcm* M2

The plasmid pZWavrXa7 (supplied by Dr. Bing Yang) was used to construct the plasmid for expression of *tal2* in Xss-V₂-18 strain. Plasmid pZWavrXa7 contains a FLAG-tag epitope immediately downstream of the second *SphI* site in the C-terminus of AvrXa7. The central *SphI* fragment of *avrXa7* was replaced with the *SphI* fragment of Xss-V₂-18 *tal2* gene to generate pZW-*tal2* (Table 1). The recombinant plasmid was then fused with broad-host-range vector pHM1 at the *HindIII* site giving rise to pHZW-*tal2*. The constructs were transformed into *Xcm* M2 (Δ *tal2* strain, see below) by electroporation (2.5 kv, 4 ms).

The expression of *tal2* in M2 was confirmed by western blotting with flag-labelled antisera. Briefly, the M2 strain containing pHZW-*tal2* was cultured in NB to the logarithmic phase and harvested by centrifugation. The pellets were washed twice, and re-suspended in 1X PBS buffer to OD₆₀₀ = 1.0 (~3×10⁹ cells/mL). SDS loading buffer (5X) was added to the bacterial suspensions and boiled in a water bath for 10 min. Proteins were separated on 8% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes for immunoblotting using anti-FLAG (TransGene, Beijing, China) as the primary antibody. Primary antibodies were detected using goat anti-mouse IgG (H + L) (TransGen) and visualized with the EasySee Western Kit (TransGen). *E. coli* RNA polymerase subunit α (RNAP) was used as a loading control.

Declaration

Authors' contributions

GC contributed in conceiving and designing the experiments. FH and HX conducted the experiments and analyzed the data with assistance from SMAS, SW, WM, LC, XX, ZX, SW. LZ, and BZ. FH and GC wrote the paper; and all authors read, commented on, and approved the manuscript.

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Availability of data and materials

All the dataset generated or analyzed during this study are included in this published article. The nucleotide sequences have been deposited in GenBank under the following accession numbers (MK654746-MK654751). The plasmids are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not Applicable.

Consent for publication

Not Applicable.

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1. List of strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source
<i>Escherichia coli</i>		
DH5 α	<i>F</i> ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17(rk-, mk+)</i> <i>phoA supE44</i> λ - <i>thi-1 gyrA96 relA1</i>	Clontech
<i>X. citri</i> pv. <i>malvacearum</i>		
MSCT1	Wild-type, causes bacterial blight of cotton	[59]
XcmH1005	Spontaneous Rif ^r derivative of XcmN	[27]
XcmH1003	Sp ^r , Rif ^r derivative of XcmN	[27]
Xcc049	Wild-type <i>Xcc</i> strain, used for construction of pKMSA1/A2	This lab
Xss-V ₂ -18	Wild-type, causes bacterial blight of cotton	Hainan University, China
M1	<i>tal3</i> deletion mutant of Xss-V ₂ -18	This study
M2	<i>tal2</i> deletion mutant of Xss-V ₂ -18	This study
M3	<i>tal2 tal4</i> deletion mutant of Xss-V ₂ -18	This study
M4	<i>tal2 tal4 tal5 tal6</i> deletion mutant of Xss-V ₂ -18	This study
M5	<i>tal2 tal3 tal4 tal5 tal6</i> deletion mutant of Xss-V ₂ -18	This study
M6	<i>tal</i> -free mutant of Xss-V ₂ -18	This study
Plasmids		
pBluescript II SK(-)	Ap ^r , phagemid, pUC derivative	Lab collection
pMD18-T	Ap ^r , pUC18 derivative, TA cloning vector, 2692 bp	TaKaRa
pKMS1	Km ^r , <i>sacB mob lacZ oriV</i> , 6475 bp	[60]
pHM1	Broad-spectrum cosmid vector, <i>cos, parA, IncW</i> , Sp ^r	[85]
pKMSA1	pKMS1 containing a 930-bp <i>Xba</i> I/ <i>Sma</i> I fragment; insert contains a fusion of <i>a</i> and <i>b</i> fragments that encode the N- and C-terminal sides of the <i>tal</i> CRR; Km ^r	This study
pKMSA2	pKMS1 containing a 450-bp <i>Xba</i> I/ <i>Sma</i> I fragment; insert contains a fusion of the <i>c</i> and <i>d</i> fragments that encode the N- and C-terminal sides of the <i>tal</i> CRR; Km ^r	This study
pB- <i>tal1</i>	pBluescript II SK(-) containing <i>tal1</i> of Xss-V ₂ -18	This study
pB- <i>tal2</i>	pBluescript II SK(-) containing <i>tal2</i> of Xss-V ₂ -18	This study
pB- <i>tal3</i>	pBluescript II SK(-) containing <i>tal3</i> of Xss-V ₂ -18	This study
pB- <i>tal4</i>	pBluescript II SK(-) containing <i>tal4</i> of Xss-V ₂ -18	This study

pB- <i>tal5</i>	pBluescript II SK(-) containing <i>tal5</i> of Xss-V ₂ -18	This study
pB- <i>tal6</i>	pBluescript II SK(-) containing <i>tal6</i> of Xss-V ₂ -18	This study
pZWavrXa7	<i>avrXa7</i> in pBluescript II KS+, contains FLAG epitope immediately downstream of the second <i>SphI</i> site in the C-terminus of AvrXa7, Ap ^r	[86]
pZW- <i>tal2</i>	<i>SphI</i> fragment of <i>tal2</i> in pZWavrXa7, Ap ^r	This study
pHZW- <i>tal2</i>	pHM1 fused with pZW- <i>tal2</i> at <i>HindIII</i> , <i>lacZ</i> promoter upstream of <i>tal2</i> , Ap ^r , Sp ^r	This study

Figures

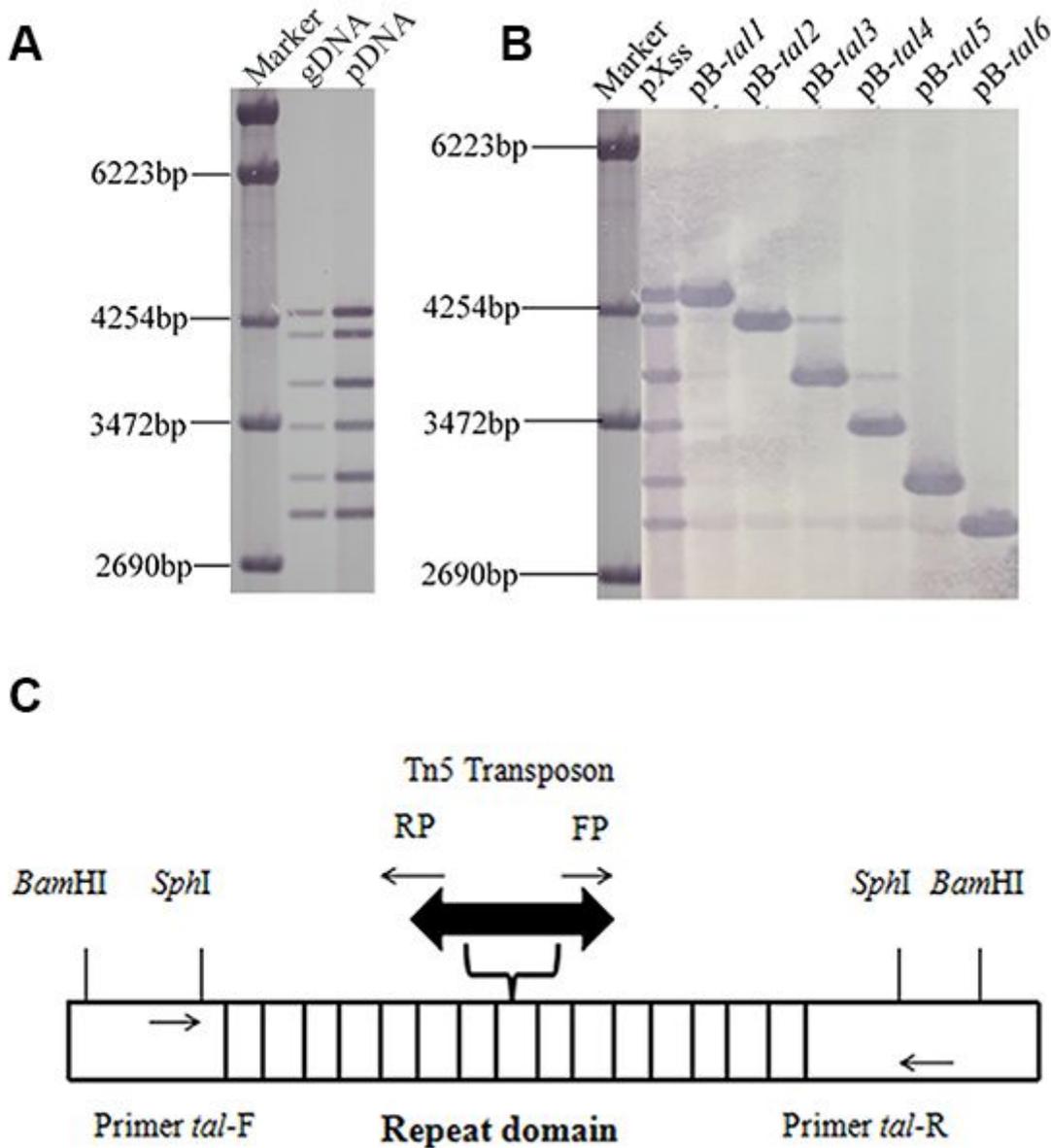


Figure 1

Southern blotting, and Isolation and sequencing of Xss-V2-18 tal-genes. (A) Southern blot analysis of BamHI-digested genomic (gDNA) and plasmid DNA (pDNA) of Xcm strain Xss-V2-18. A 2.9-kb SphI fragment of pthXo1 (from Xoo) was labeled with digoxigenin (DIG) and used as a probe to detect tal genes in Xcm Xss-V2-18. (B) Plasmid DNA of Xss-V2-18 was digested with BamHI, and fragments were gel-purified and ligated into BamHI-digested and CIP-treated pBluescript II SK(-). Southern blot analysis was performed by the using internal SphI fragment of pthXo1 as a probe to confirm each clone (pB-tal1 – pB-tal6). (C) Schematic diagram of strategy used to sequence tal genes. After cloning into pBluescript II SK(-), the EZ-Tn5™<KAN-2>Tnp Transposome™ Kit was used to insert Tn5 into each tal gene. Clones with Tn5 insertions in the middle of the CRR were selected by SphI digestion and sequenced using primer pairs tal-F/RP and FP/tal-R.

A

TALEs	Repeat Variable Diresidues (RVDs)																											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
MSCT1 Tal8	NI	NG	NI	NI	NI	NG	HD	HD	NS	NI	HD	NI	HD	HD	NI	NS	NG	HD	NS	NS	NS	NG	NS	NG	NG	NG	NG	NG
Xss-V ₂ -18 Tal1	NI	NG	NI	NI	NI	NG	HD	HD	NS	NI	HD	NI	HD	HD	NI	NS	NG	HD	NS	NS	NS	NG	NS	NG	NG	NG	NG	NG
MS14003 Tal28b	NI	NG	NI	NI	HD	NG	HD	HD	NS	NI	HD	NI	HD	HD	NI	NS	NG	HD	NS	NS	NS	NG	NS	NG	NG	NG	NG	NG
M14003 Tal26	NI	NG	NI	NI	NI	NG	NG	NS	NG	NS	NS	NG	NS	NG	HD	NS	HD	HD	NG	NS	NG							
MSCT1 Tal6	NI	NG	NI	NI	NI	NG	NG	NS	NG	NS	NS	NG	NS	NG	HD	NS	HD	HD	NG	NS	NG							
Xss-V ₂ -18 Tal2	NI	NG	NI	NI	NI	NG	NG	NS	NG	NS	NS	NG	NS	NG	HD	NS	HD	NS	HD	NS	NS	NS	NG	NG	NG	NG	NG	NG
MS14003 Tal22	HD	NI	NG	NI	NI	NS	NG	NG	NI	NG	NS	HD	NS	HD	NS	NG	NS	NG	HD	NG								
MSCT1 Tal1	HD	NI	NG	NI	NI	NS	NG	NG	NI	NG	NS	HD	NS	HD	NS	NG	NS	NG	HD	NG								
Xss-V ₂ -18 Tal3	HD	NI	NG	NI	NI	NS	NG	NG	NI	NG	NS	HD	NS	HD	NS	NG	NS	NG	HD	NG								
MS14003 Tal19	HD	NI	NG	NI	NI	NI	HD	HD	NS	NS	HD	HD	NS	NS	NG	NS	NS	NG	NS	NG								
MSCT1 Tal7	HD	NI	NG	NI	NI	NI	HD	HD	NS	NS	HD	HD	NS	NS	NG	NS	NS	NG	NS	NG								
Xss-V ₂ -18 Tal4	HD	NI	NG	NI	NI	NI	HD	HD	NS	NS	HD	HD	NS	NS	NG	NS	NS	NG	NS	NG								
H1005 AvrB7	HD	NI	NG	NI	NI	NI	NS	HD	HD	NS	HD	HD	NS	NS	NG	NS	NS	NG	NS	NG								
AR81009 Tal19b	HD	NI	NG	NI	NI	NI	NS	HD	HD	NS	HD	HD	NS	NS	NG	NS	NS	NG	NS	NG								
MS14003 Tal16	NI	NI	NI	NN	NI	NS	HD	NG	NN	NS	NN	NN	HD	NG	N*	NN												
MSCT1 Tal5	NI	NI	NI	NN	NI	NS	HD	NG	NN	NS	NN	NN	HD	NG	N*	NN												
Xss-V ₂ -18 Tal5	NI	NI	NI	NN	NI	NS	HD	NG	NN	NS	NN	NN	HD	NG	N*	NN												
MS14003 Tal14b	NI	NI	NI	NN	NI	NS	HD	NG	HD	NS	NG	HD	HD	NG														
MSCT1 Tal2	NI	NI	NI	NN	NI	NS	HD	NG	HD	NS	NG	HD	HD	NG														
Xss-V ₂ -18 Tal6	NI	NI	NI	NN	NI	NS	HD	NG	HD	NS	NG	HD	HD	NG														

B

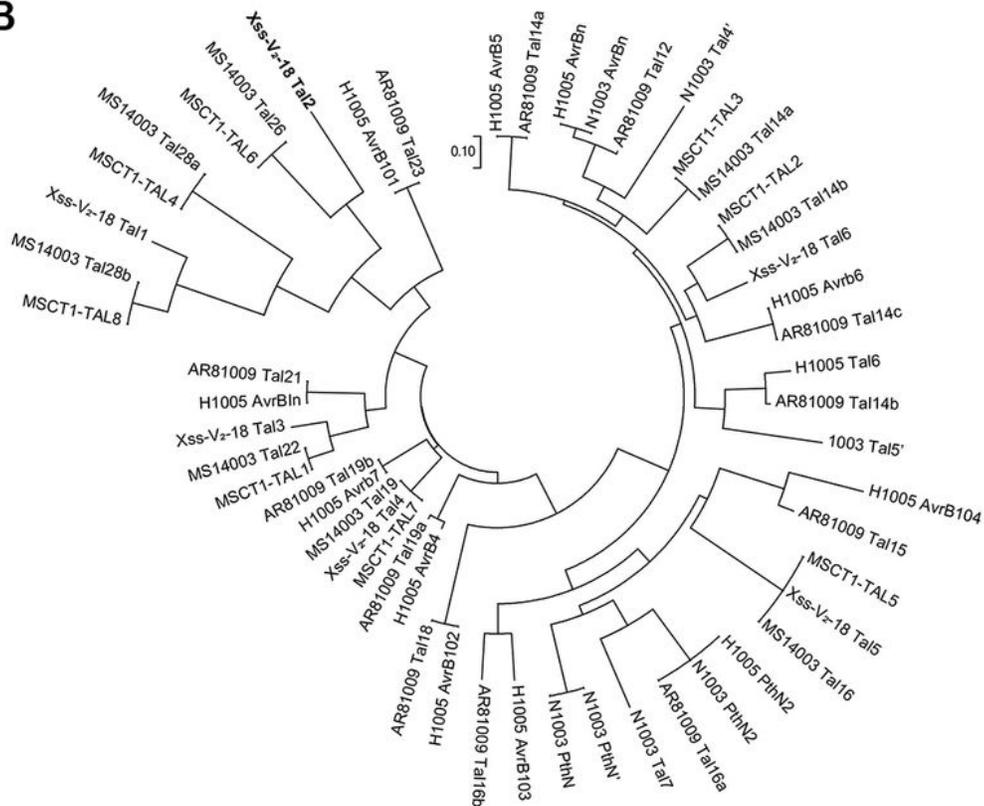


Figure 2

TALEs Phylogeny and Alignment of TALE RVDs. (A) Alignment of TALE RVDs from Xcm strains Xss-V₂-18, MSCT1, H1005, MS14003 and AR81009 with AnnoTALE (version 1.4.1). Letters in red font indicate RVDs that differ between the two strains. The asterisk represents a missing amino acid residue (B) Construction of phylogenetic tree based on central repeat amino acid sequences of TALEs. A set of 53 TAL effector sequences from 6 different Xcm strains were used to construct tree with DisTAL program

using default parameters. TALEs were classified into 6 major groups and 33 sub-groups showing the relationship of Xcm Xss-V2-18 to other Xcm strains published previously. Tal2 of Xss-V2-18, TAL6 of MCST and Tal26 (M26) of MS14003 fall in same group. Scale is shown below the tree.

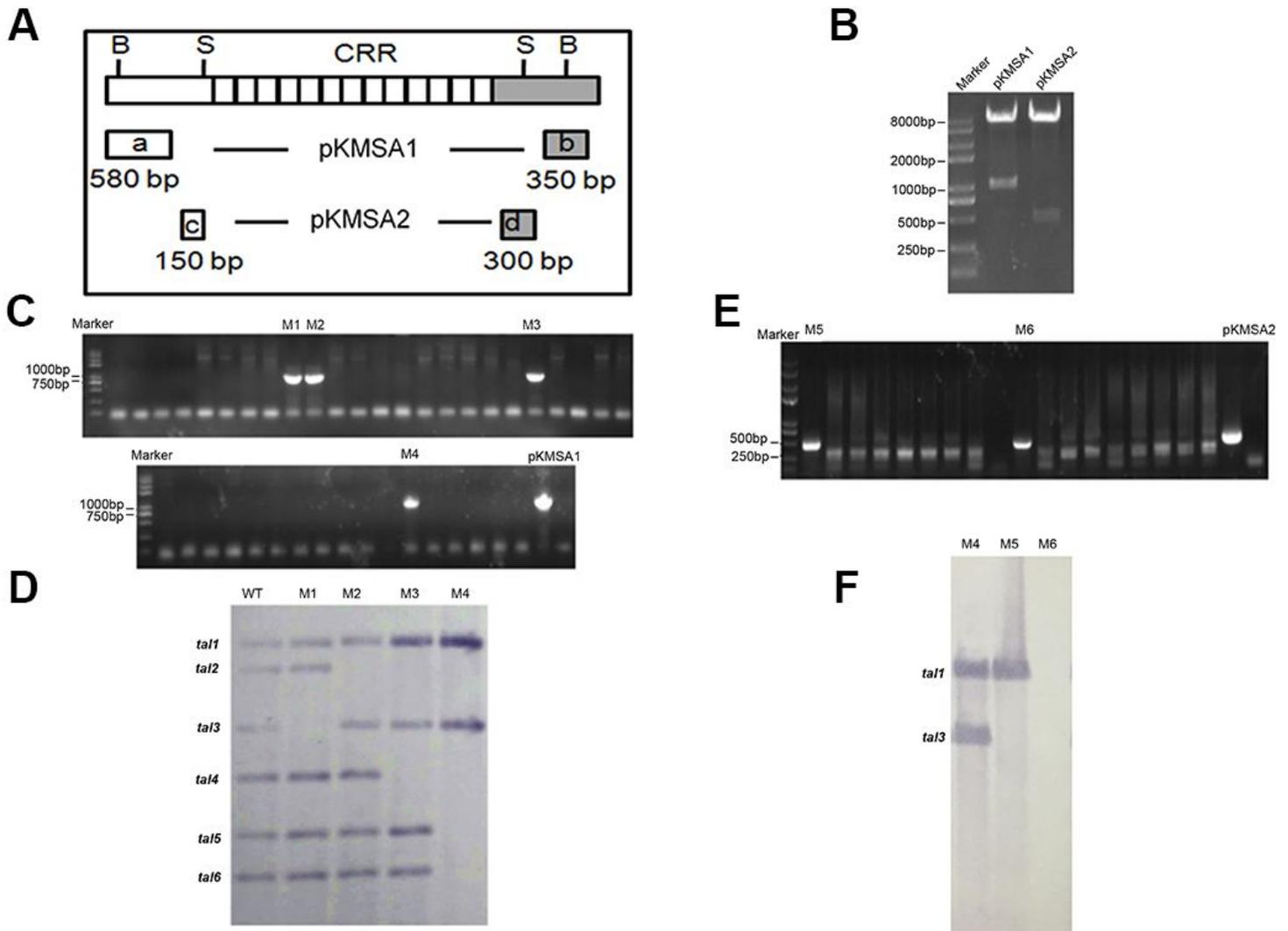


Figure 3

Deletion mutagenesis of Xss-V2-18 tal genes. (A) Schematic diagram of suicide plasmids pKMSA1 and pKMSA2. Fragments a (580 bp) and b (350 bp) were amplified on the left and right sides of the CRR, respectively, and cloned as a fused fragment in pKMSA1. Fragments c (580 bp) and d (150 bp) were amplified on the left and right sides of the CRR, respectively, and cloned as a fused fragment in pKMSA2. Constructs pKMSA1 and pKMSA2 were introduced into Xcm strain Xss-V2-18 by electroporation, and deletion of the CRR region was conducted as described in Methods. (B) Confirmation of 930- and 450-bp inserts in pKMSA1 and pKMSA2, respectively, by digestion with XbaI and SmaI. (C) PCR analysis of 41 putative mutants with primers pKMSA1-5F and pKMSA1-3R. A 930-bp fragment was amplified in M1, M2, M3, and M4, indicating that these four mutants underwent a homologous recombination and potential deletion of the CRR region. pKMSA1 was included as a control. (D) Southern hybridization analysis of Xss-V2-18 and mutant strains M1-M4. Plasmid DNA of WT Xss-V2-18 and mutants were isolated and

digested with BamHI. The internal SphI fragment of pthXo1 (from Xoo) was used as a hybridization probe to detect tal genes. (E) PCR screening for putative mutants using primers pKMSA2-5F and pKMSA2-3R. pKMSA2 was included and used as a positive control. (F) Southern hybridization analysis of mutant M4 (used for second round of mutagenesis), M5 and M6. Plasmid DNA of M4, M5 and M6 were isolated and digested with BamHI, and the internal SphI fragment of pthXo1 was used a hybridization probe to detect tal genes.

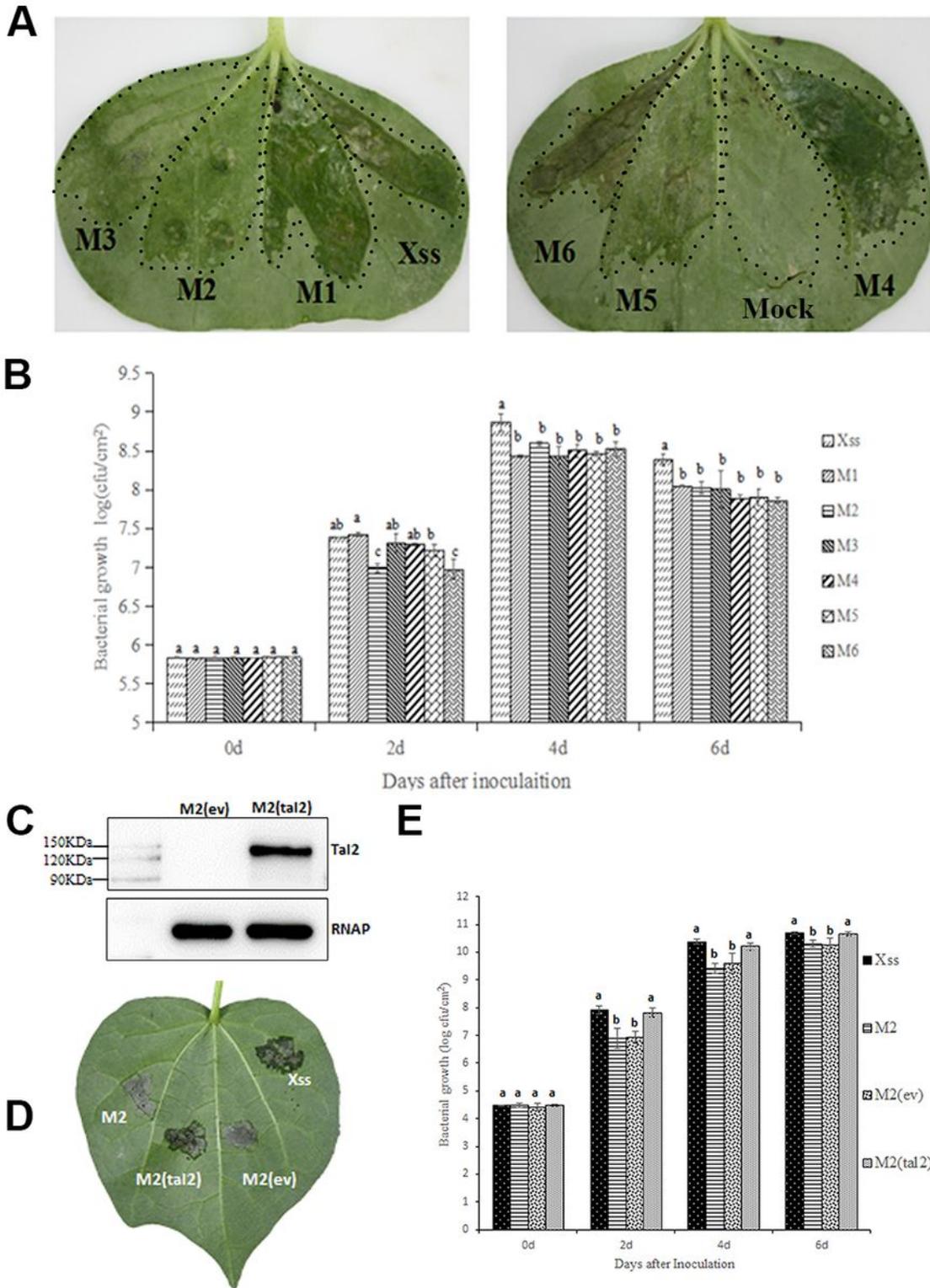


Figure 4

Tal2 contribution to virulence of Xss-V2-18 on cotton variety TM-1. (A) Phenotypes of the mutant strains relative to wild-type Xss-V2-18. Wild-type (WT) and mutant strains were inoculated to the lower surface of cotton leaves (two-week-old plants) using a needleless syringe. Infiltration with simply 10mM MgCl₂ served as a mock. Phenotypes were observed 3-5 days post-inoculation. (B) In planta growth of WT Xss-V2-18 and mutants. Growth was measured at 0, 2, 4, and 6 days post-inoculation. Error bars represent means and standard deviations (means \pm SD), and columns labeled with different letters represent significant differences ($P < 0.05$). (C) Western blot analysis of TALE production in Xcm M2. Plasmid pHZW-tal2 was transferred into Xcm M2 by electroporation. Production of TALE was analyzed by western blotting using an anti-FLAG primary antibody (see Methods). RNA polymerase subunit alpha (RNAP) from *E. coli*, was used as a loading control. (D) Symptoms in cotton leaves inoculated with Xss-V2-18, mutant M2, M2 containing empty vector and M2 containing tal2 in trans. Bacterial strains were inoculated to cotton leaves using a needleless syringe, and phenotypes were observed within 5-7 days post-inoculation. (E) In planta growth of the WT Xss-V2-18, mutant M2 and complemented strain. Growth was measured at 0, 2, 4, and 6 days post-inoculation. Error bars represent means and standard deviations (means \pm SD), and columns labeled with different letters represent significant differences ($P < 0.05$).

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