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Research Article 研究报告

Identification of a novel type III secreted effector in *Xanthomonas campestris* pv. *campestris*

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Abstract: [Objective] To identify a novel type III secreted effector (T3SE) gene in the genome of *Xanthomonas campestris* pv. *campestris* (*Xcc*) strain 8004. **[Methods]** A Tn5 transposon system integrated with AvrBs1$_{59\text{-}445}$ was constructed for library screening. The mutant library was generated based on *avrBs1*-deleted mutant and then screened on *Capsicum annuum* cv. ECW-10R. **[Results]** Seven clones with visible hypersensitive response (HR) were screened out via large-scale HR assay. In addition to 3 mutants inserted into known T3SE genes, a new locus was identified, which was located between *XC_0438* and *XC_0439* and un-annotated in *Xcc* 8004 by plasmid rescue and sequencing. We annotated the new gene designated as *XC_0438a* according to the bioinformatics analysis results. The translocation assay confirmed that the signal region of XC_0438a could guide the secretion and translocation of the reporter protein AvrBs1 and elicit the HR of ECW-10R. The results of *β*-glucuronidase (GUS) activity assay demonstrated that the expression of *XC_0438a* was induced in nutrition sterile medium and activated by the key regulatory proteins HrpG and HrpX. However, in our tested conditions, *XC_0438a* had no significant contribution to the pathogenesis of *Xcc*. **[Conclusion]** In summary, we identified a novel effector gene *XC_0438a* dependent on the type III secretion system.

Keywords: *Xanthomonas campestris* pv. *campestris*; type III secreted effector (T3SE); Tn5 transposon; hypersensitive response (HR); *XC_0438a*

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十字花科黑腐病菌一个新的 III 型效应物的鉴定

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摘　要：【目的】在全基因组范围内鉴定十字花科黑腐病菌 *Xcc* 8004 中新的III型效应物(type III secreted effector，T3SE)基因。【方法】通过构建与 AvrBs1$_{59\text{-}445}$ 整合的 Tn5 转座子系统，进行文库筛选。在 *avrBs1* 缺失突变体背景下生成突变文库，在辣椒 ECW-10R 上进一步筛选。【结果】大规模 HR 测定筛选出 7 个具有明显过敏反应(hypersensitive response，HR)的克隆。通过质粒拯救和测序发现除了插入已知 T3SE 基因的 3 个突变体外，还鉴定了一个位于 *XC_0438* 和 *XC_0439* 之间且在 *Xcc* 8004 中未注释的新基因。结合生物信息学，我们将其命名为一个新的基因 *XC_0438a*。易位实验证实 XC_0438a 信号区可引导报告蛋白 AvrBs1 的分泌和易位，并诱导辣椒 ECW-10R 产生 HR 反应。*β*-葡萄糖醛酸酶(*β*-glucuronidase，GUS)活性测定，*XC_0438a* 在基本培养基中诱导表达，并由关键调控蛋白 HrpG 和 HrpX 激活。然而，在我们的实验条件下，*XC_0438a* 对 *Xcc* 的致病力没有显著的贡献。【结论】我们鉴定了一种新的依赖于III型分泌系统的效应基因 *XC_0438a*。

关键词：十字花科黑腐病菌；III型效应物；Tn5 转座子；过敏反应；*XC_0438a*

Most Gram-negative plant pathogenic bacteria employ type III secretion system (T3SS) which directly translocates type III secreted effectors (T3SE) proteins into plant cells[1]. T3SS and T3SE are closely related to the pathogenicity of plant pathogens[2]. *Xanthomonas campestris* pv. *campestris* (*Xcc*) infects many important crops such as cabbage, radish, kale, cauliflower, and the model plant *Arabidopsis thaliana*[3]. In *Xcc*, the T3SS device is encoded by 25 *hrp* (hypersensitive response and pathogenicity) genes, which are mainly regulated by three key regulatory factors, HpaS[4], HrpG (an OmpR family regulatory factor), and HrpX (an AraC-type transcriptional activator)[5]. HrpG activates the expression of HrpX, which in turn controls the expression of other *hrp* genes and effector genes[6–7].

T3SS, a complex membrane-spanning nanomachines, contain an extracellular pilus-like appendage which provides a transport channel for secreting T3SE proteins to the plant pathogen interface[8–9]. The interaction between different types of effectors and intracellular targets of host

determine bacterial pathogenicity and host specificity[10]. According to the function and sub-localization of type III effectors, in a broad sense, we can divide them into two categories: TAL (transcription activator-like) effectors and non-TAL effectors. TAL effectors are usually translocated into the plant cell nucleus, functioning as transcription factors and regulating expression of plant genes[11]. Non-TAL effectors are generally composed of secretory signal regions and functional regions. Many non-TAL effects were identified by fusing the signal sequences of candidate genes and the hypersensitive response (HR) induction regions of known effectors, such as AvrBs2[12] and AvrBs3[13].

In general, a bacterium was predicated to contain 30–50 type III effectors[14]. However, in *Xcc* 8004, only 13 type III effectors have been reported so far[15–20]. In this study, we established a modified Tn5 transposon integrated with an avirulence protein lack of N-terminal signal and utilized the chimeric transposon to construct a mutant library for large-scale HR phenotype screening. And one novel type III secreted effector (*XC_0438a*) was identified.

1 Materials and methods

1.1 Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant genotype or characteristics*	Reference or source
Plasmids		
pRK2073	Helper plasmid, Spc^r	Lab collection
pK18mob	Suicide plasmid in *Xanthomonas campestris* pv. *campestris*, Kan^r	Lab collection
pK18mobsacB	Sucrose-sensitive suicide plasmid, Kan^r	Lab collection
pK0438a	the flanking fragments of the *XC_0438a* were ligated into pK18mobsacB, Kan^r	This study
pLGUS	pLAFR6 containing a promoterless *gus* gene, Tc^r	Lab collection
pLGUS0438a	pLGUS containing the promoter and signal region of *XC_0438a*, Tc^r	This study
pUC19	Cloning plasmid, Amp^r	Lab collection
pJAG	pJXG containing the $avrBs1_{59\text{-}445}$, Tc^r	Lab collection
pJAG0438a	pJAG containing the promoter and signal region of *XC_0438a*, Tc^r	This study
Strains		
E. coli		
DH5α	Transforming receptor	Lab collection
ED8767	Helper strain, containing plasmid pRK2073, Spc^r	Lab collection
Xcc		
Xcc 8004	Wild type strain, Rif^r	Lab collection
8004ΔavrBs1	*avrBs*1 delete mutant of *Xcc* 8004, Rif^r	Lab collection
8004ΔhrcV	*hrcV* delete mutant of *Xcc* 8004, Rif^r	Lab collection
8004ΔhrpX	*hrpX* delete mutant of *Xcc* 8004, Rif^r	Lab collection
8004ΔhrpG	*hrpG* delete mutant of *Xcc* 8004, Rif^r	Lab collection
8004/pLGUS0438a	*Xcc* 8004 harboring pLGUS0438a, Rif^r, Tc^r	This study
8004ΔhrpX/pLGUS0438a	*Xcc* 8004ΔhrpX harboring pLGUS0438a, Rif^r, Tc^r	This study
8004ΔhrpG/pLGUS0438a	*Xcc* 8004ΔhrpG harboring pLGUS0438a, Rif^r, Tc^r	This study
8004ΔhrcV/pJAG0438a	*Xcc* 8004ΔhrcV harboring pJAG0438a, Rif^r, Tc^r	This study
8004ΔavrBs1/pJAG0438a	*Xcc* 8004ΔavrBs1 harboring pJAG0438a, Rif^r, Tc^r	This study
D0438a	*XC_0438a* delete mutant of *Xcc* 8004, Rif^r	This study

*: Amp: ampicillin; Rif: rifampicin; Kan: kanamycin; Spc: spectinomycin; Tc: tetracycline.

were grown at 37 °C in Luria-Bertani (LB) medium. All *Xcc* strains were cultured at 28 °C in the rich medium NYG, or the minimal medium MMX or XCM. Antibiotics were used in the following final concentrations: ampicillin (Amp), 100 μg/mL; kanamycin (Kan), 25 μg/mL; rifampicin (Rif), 50 μg/mL; spectinomycin (Spc), 100 μg/mL; tetracycline (Tc), 15 μg/mL for *E. coli*, 5 μg/mL for *Xanthomonas*.

1.2 Construction of Tn5 mutant library of *Xanthomonas campestris* pv. *campestris*

AvrBs1$_{59\text{-}445}$ is sufficient to induce hypersensitivity (HR) on pepper ECW-10R (*Capsicum annuum* cv. ECW-10R)[16] which contains the cognate resistance gene *Bs1*[21]. In order to facilitate large-scale HR phenotype screening on ECW-10R, a modified Tn5-transposon was firstly reconstructed and the functional region of AvrBs1 was inserted between left IS and Kan resistance fragments. Briefly, the DNA fragment 1 containing the IS sequence in the 5′ end and *avrBs1*$_{59\text{-}445}$ was amplified using the total DNA of *Xcc* 8004 as template with the primers sets P1/P2 (CTGTCTCTTATACACATCTGCTTTGCACAC CTCATCGTTAG/*TCGATGATGGTTG*TTACGC TTCTCCTGCATTTGTAAC) (The underlined in P1 is the IS sequence, and the italic part in P2 is complementary to the italic part in P3). Simultaneously, the fragment 2 containing Kan resistance fragment and the 3′-end IS sequence was amplified using the total DNA of D06B03, a Tn5 mutant of *Xcc* 8004, as template with the primer sets P3/P4 (*CAACCATCATCGAT*G AATTGTGTCTC/CTGTCTCTTATACACATCT CAACCCTGAAG) (The underlined in P4 is the IS sequence). The fragment 1 and fragment 2 were fused together by using fusion-PCR, generating the modified Tn5-transposon.

Using the modified Tn5-transposon, according to the manufacturer's instructions for EZ-Tn5<KAN-2>Transposon Insertion Kit (Epicentre, USA), the Tn5 mutant library of *Xcc* 8004ΔavrBs1 was constructed as described by Zou and associates[22]. Briefly, the competent cells of *Xcc* 8004ΔavrBs1 were prepared by water-washing method. The modified Tn5-transposon with sufficient DNA concentration was transferred to the competent cells of *Xcc* 8004ΔavrBs1 by electro-transformation. The transformants were diluted 10-fold and screened on Kan+Rif plates (more than 200 plates).

1.3 Large-scale HR screening

The large-scale HR phenotype screening were performed on pepper ECW-10R as described by Xu and associates[16]. The test bacteria (8 strains selected as a group) were cultured together in NYG for 16 h and harvested, adjusting the cell densities to OD_{600}=0.5 with sterile water, and the wild-type *Xcc* 8004 and mutant 8004ΔavrBs1 were used as positive control and negative control, respectively. At least two fully expanded pepper leaves were infiltrated. After infiltration, the inoculated peppers were grown at 28 °C, 16 h light/8 h dark photoperiod per day, and 80% relative humidity. Photographs are taken 1–3 d after infiltration. Once a set of mutants could induce visible HR, each of the corresponding 8 mutants was further analyzed for HR induction.

1.4 Plasmids rescue assays

Plasmid rescue strategy was employed to determine the insertion site of transposon in each mutant. Taking a-k-1 as an example, the *Eco*R I/*Kpn* I digested fragments of a-k-1 genomic DNA were cloned into the *Eco*R I/*Kpn* I digested pUC19. The recombinant colonies were screened on Kan+Amp plates. The recombinant plasmid was then extracted and verified with PCR using the primer sets P1/P4. The obtained positive clone was sent for sequencing (BGI, Shanghai) with the primer PS (TTATCTTAGCCAACC CTCTC). The sequences were analyzed in the genome of *Xcc* 8004 (taxid: 314565) by Nucleotide BLAST on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

1.5 Translocation assay

The promoter and signal region of *XC_0438a* were amplified using primer sets P-F/P-R (CCCG AATTCCAATACGTTCTAACTGCGCG/CCCGG

ATCCTCTGTAACTAAGTGCAGTGT) with the total DNA of *Xcc* 8004 as the template and cloned into *Eco*R I/*Bam*H I sites of pK18mob[23], generated pK18mob0438a. After confirmation by sequencing, the verified fragment was cloned into the *Eco*R I/*Bam*H I sites of the reporter plasmid pJAG, a derivative of pLAFRJ[17] in which contains *avrBs1*$_{59\text{-}445}$ fused with 3×FLAG, obtained the recombinant plasmid pJAG0438a. Through triparental conjugation, the recombinant plasmid pJAG0438a was transferred into 8004ΔavrBs1 and 8004ΔhrcV, respectively. The obtained positive conjugants were tested for HR assay on pepper ECW-10R.

1.6 Construction of deletion mutant

To generate a deletion mutant of *XC_0438a*, the flank fragments of *XC_0438a* were amplified by PCR using the primer sets D-FF/D-FR (GGCGAATTCCAATGTTCAGACCTTCCCTTA/CACGGATCC GAATCGGACTTGTCATGTAA) and D-RF/D-RR (GGCGGATCCGGTTAGAAA TTAAAGATAACC/AAGAAGCTTAACACTCA TTCACGCCGATG) and successively cloned into the *Eco*R I/*Bam*H I and *Bam*H I/*Hin*d III sites of pK18mobsacB[23], a sucrose-sensitive suicide plasmid in *Xcc*, generating the recombinant plasmid pK0438a. After confirmation by sequencing, the plasmid pK0438a was introduced from *E. coli* into the strain 8004 by triparental conjugation. The *XC_0438a* deletion mutant, named as D0438a, was selected on plates containing Rif and 5% sucrose. The mutant was confirmed by PCR with the primer sets D-FF/D-RR.

1.7 Virulence assays

Virulence assay of *Xcc* strains was preformed utilizing the leaf clipping method on the four-leaf stage Chinese radish (*Raphanus sativus* var. *radiculus* cv. Manshenhong) grown in greenhouse as described previously[24–25]. Overnight cultured bacteria were adjusted to OD_{600}=0.001 with sterile NYG, and the plants were cut with sterilized scissors. Lesion length was measured 9 d post inoculation. At least two repeated independent experiments were performed.

1.8 Glucuronidase activity assay

Determination of *β*-glucuronidase (GUS) activities was performed by using *ρ*-nitrophenyl-*β*-D-glucuronide (PNPG) as substrates as described by Henderson and associates[26]. Briefly, the promoter region of *XC_0438a* was amplified with the primer sets P-F/P-R and cloned into the *Eco*R I/*Bam*H I sites of pLGUS which contains a promoterless *gus* gene[17], generated the reporter plasmid pLGUS0438a. This obtained plasmid was then introduced into the wild type strain 8004, 8004ΔhrpX, and 8004ΔhrpG, by triparental conjugation, respectively. Wild-type and mutants harboring pLGUS0438a were cultured at 28 °C in MMX for 24 h or NYG for 16 h. The enzyme activity unit [(U, mg/(mL·min·OD_{600})] was defined as yielding of *p*-nitrophenol (mg) when hydrolyzing PNPG per minute in 1 mL bacterial cells with an OD_{600} of 1.

2 Results

2.1 Seven mutants with hypersensitive response were screened out from the EZ::Tn5 mutants library of *Xcc* 8004ΔavrBs1

It has been reported that the bacterial *avrBs1* gene can be recognized by the *Bs1* gene of pepper ECW-10R (*Capsicum annuum* cv. ECW-10R), eliciting hypersensitive response (HR)[21,27]. Generally, a bacterium might harbor 30–50 type III secreted effectors[14]. However, only 13 type III secreted effectors are identified and reported in *Xcc* 8004[15–20]. Therefore, it is necessary to identify novel type III secreted effectors through the Tn5 capture strategy.

To facilitate this strategy, a modified transposon DNA was reconstructed basing EZ::Tn5 (Epicentre) which contains the inverted repeat IS sequence at both ends and the sequence of *avrBs1*$_{59\text{-}445}$ fused with the Kan resistance fragments (Figure 1A). Using this modified Tn5 with a DNA concentration of 259 ng/μL, a highly saturated mutant library was established in the background of *Xcc* 8004ΔavrBs1 according to the manufacturer's instructions for EZ-Tn5 <KAN-2>Transposon Insertion Kit (Epicentre) (see details in Material and Methods), obtained

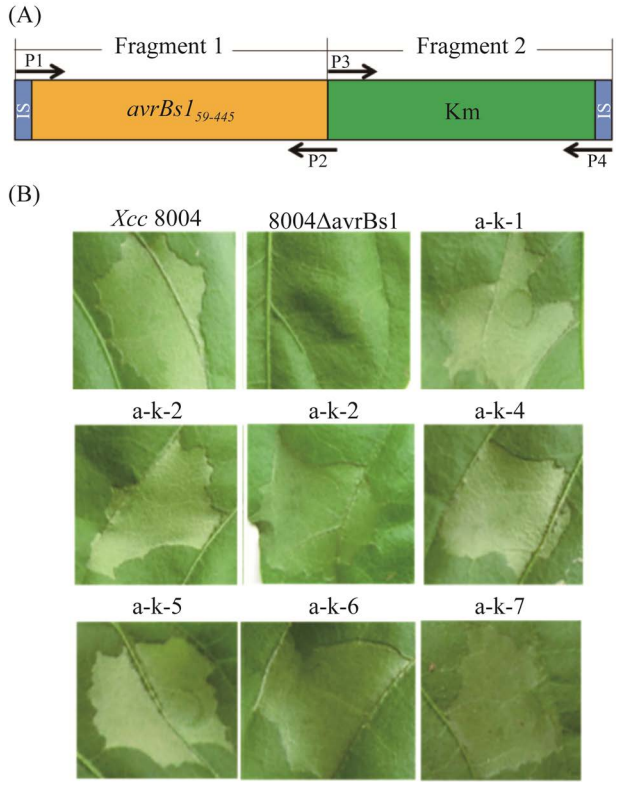

Figure 1 Construction of modified Tn5 transposon and Tn5 library screen. A: schematic diagram of Tn5 transposon DNA. B: hypersensitive response (HR) screening of seven candidate Tn5 insertion mutants (a-k-1–7) on pepper ECW-10R (*Capsicum annuum* cv. ECW-10R). *Xcc* 8004, the positive control; 8004ΔavrBs1, the negative control. The photographs were taken 24 h post infiltration. Experiments were repeated independently three times.

approximately 45 000 mutants. Sixteen mutants were randomly selected and verified by PCR with the primer sets P1/P4. The verification results showed that all the selected mutants harbor the chimeric *avrBs1*::Kan fusion fragments (data not shown). These indicated that the established Tn5 library is robust and credible with high saturation and good randomness.

After a large-scale HR phenotype detection on ECW-10R (see details in Material and Methods), seven mutants named a-k-1–7 could elicit visible HR singly were obtained (Figure 1B). The insertion sites of these mutants were determined by plasmid rescue strategy. The nucleotide BLAST (BLASTn) results indicated that the insertion sites of a-k-1, a-k-3, a-k-5, and a-k-7 are located in the intergenic region between *XC_0438* and *XC_0439* (The insertion sites were 522 417, 522 546, 522 612, and 522 630, respectively) (Figure 2). Although this fragment consists of 1 572 base pairs (bps), no open reading frame (ORF) is annotated in *Xcc* 8004[28]. Herein, using the website software ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) with the default parameter settings, we annotated a novel ORF *XC_0438a* which encodes a type III secreted effector protein and is started from 522 275 and stopped at 522 673, encoding 132 amino acid rescues (Figure 2). The putative domains of XC_0438a were analyzed using SMART (http://smart.embl-heidelberg.de/). The results showed that no distinguished domain was present in XC_0438a, and XC_0438a harbors no transmembrane helixes and no signal peptides (data not shown).

As for the other 3 mutants, the blastn results demonstrated that Tn5 of a-k-2 and a-k-4 were in-frame inserted in *xopXccLR* (*XC_4273*), and Tn5 of a-k-6 was in-frame inserted in *xopXccN* (*XC_0241*) (The insertion sites were 5 966 845, 5 066 752, and 291 132, respectively). These two genes are known type III secreted effector genes[16–17].

2.2 XC_0438a is a type III secreted effector

To further verify that whether XC_0438a is a new T3SE, the promoter region and the signal region of the *XC_0438a* were cloned in-frame into the reporter plasmid pJAG[17] to obtain a recombinant reporter plasmid pJAG0438a. The recombinant reporter plasmid pJAG0438a was then introduced into 8004/ΔavrBs1 and 8004ΔhrcV, yielding 8004ΔavrBs1/pJAG0438a and 8004ΔhrcV/pJAG0438a. Strains *Xcc* 8004, 8004ΔavrBs1, 8004ΔavrBs1/pJAG0438a, and 8004ΔhrcV/pJAG0438a were cultured overnight, adjusted to OD_{600}=0.05, and assayed for hypersensitive response on pepper ECW-10R. After 24 h post infiltration, similar with the wild type *Xcc* 8004, 8004ΔavrBs1/pJAG0438a could elicit a typical HR, while 8004ΔhrcV/pJAG0438a and 8004ΔavrBs1 could not cause HR (Figure 3).

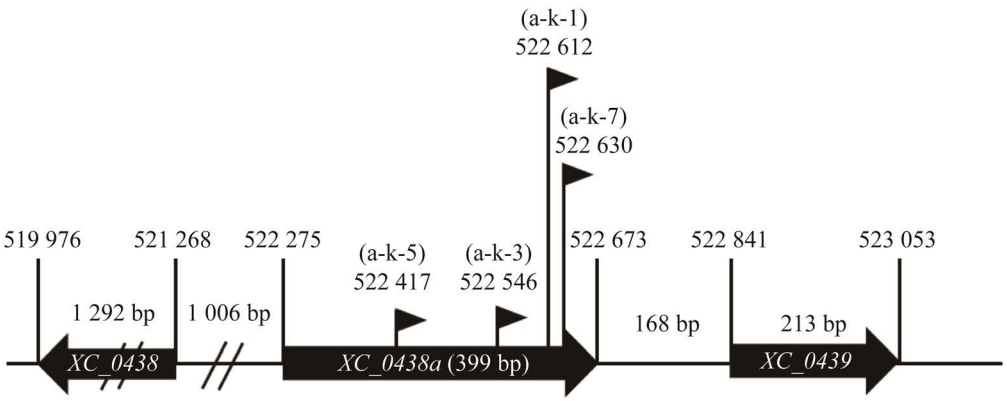

Figure 2 Genetic organization of locus of *XC_0438a*. The black flags denote the Tn5 insertion mutants and the insertion sites of corresponding Tn5 mutant (labeled in parentheses) are represented above the flags.

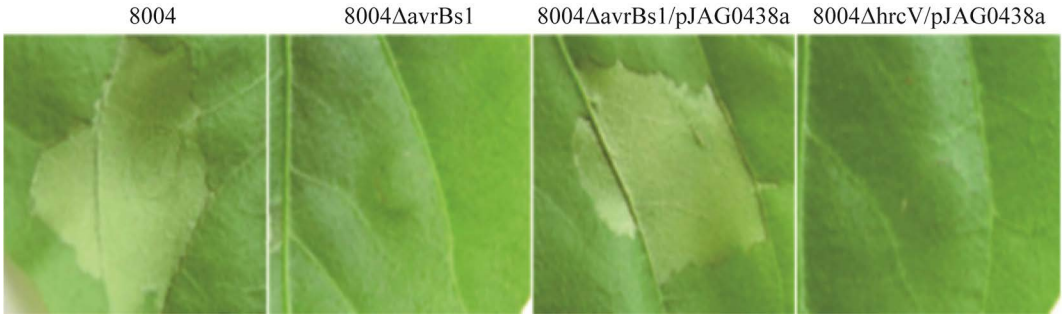

Figure 3 HR assay on pepper ECW-10R (*Capsicum annuum* cv. ECW-10R). The wild-type 8004 induced typical HR symptoms as a positive control, while the *avrBs1*-deletion mutant 8004ΔavrBs1 could not elicit any HR just as the negative control. The photographs were taken 24 h post infiltration. Experiments were repeated independently three times.

These results indicated that XC_0438a is indeed a T3SS dependent effector.

2.3 Expression of *XC_0438a* is positively regulated by HrpG and HrpX

To determine whether the expression of the *XC_0438a* is induced in minimal medium and activated by the key regulators HrpX and HrpG[29–32], a promoter-reporting plasmid carrying the promoter region of the *XC_0438a* fused with the promoterless *β*-glucuronidase (*gus*) gene was constructed and introduced into the wild-type strain 8004, the *hrpX*-deletion mutant 8004ΔhrpX, and the *hrpG*-deletion mutant 8004ΔhrpG by triparental conjugation, yielding the reporter strains 8004/pLGUS0438a, 8004ΔhrpX/pLGUS0438a, and 8004ΔhrpG/pLGUS0438a, respectively. The results showed that that strain 8004/pLGUS0438a produced very weak GUS activity (0.233 U) in the rich medium NYG, but about 13-fold stronger (3.019 U) in the minimal medium MMX (Figure 4A). Meanwhile, 8004ΔhrpX/pLGUS0438a and 8004ΔhrpG/pLGUS0438a produced imperceptible GUS activities, even in the minimal medium MMX (Figure 4B). These results indicated that the expression of *XC_0438a* is significantly induced in the minimal medium MMX (P<0.01, t-test) (Figure 4A), and significantly activated by the two key regulators, HrpG and HrpX (P<0.01, t-test) (Figure 4B).

2.4 *XC_0438a* has no significant contribution to pathogenicity of *Xcc* 8004 in Chinese radish

To investigate whether the new type III effector gene *XC_0438a* is involved in the pathogenesis of *Xcc* 8004, a deletion mutant of

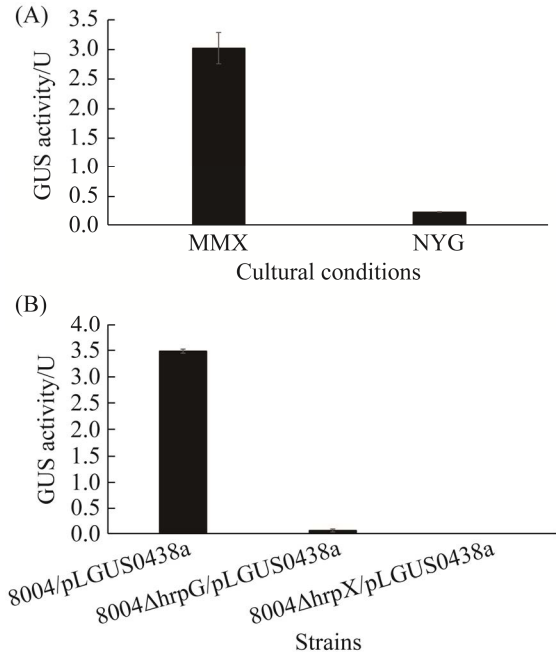

Figure 4 The expression of *XC_0438a* in the *Xanthomonas campestris* pv. *campestris* strains was induced in the minimal medium MMX (A) and positively regulated by *hrpG* and *hrpX* (B). A: strain 8004/pLGUS0438a was cultured in the rich medium NYG for 16 h or in the minimal medium MMX for 24 h; B: strains 8004/pLGUS0438a, 8004ΔhrpG/pLGUS0438a, and 8004ΔhrpX/pLGUS0438a were expressed in the minimal medium MMX for 24 h. The GUS activities were performed by using *ρ*-nitrophenyl-*β*-D-glucuronide (PNPG) as substrates. Values are the mean±standard deviation (SD) of triplicate measurements. Experiments were repeated independently three times.

D0438a was constructed. Strains D0438a and *Xcc* 8004 were cultured overnight and harvested, adjusting the cell densities to OD_{600}=0.001. The virulence assay of *Xcc* strains was performed on the Chinese radish by the leaf clipping inoculation method. The results showed that D0438a caused a disease symptom with a mean lesion length of 10.25 mm, which is not significantly different from that caused by the wild-type strain 8004 (P>0.05, t-test), indicating that *XC_0438a* did not contribute significantly to pathogenicity of *Xcc* in the tested condition (Figure 5).

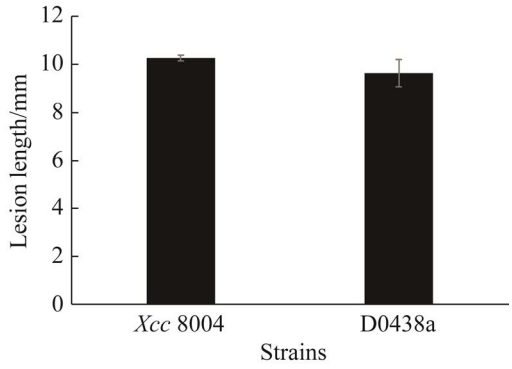

Figure 5 Pathogenicity analysis of *Xanthomonas campestris* pv. *campestris* (*Xcc*) wild type strain 8004 and the *XC_0438a* deletion mutant D0438a on Chinese radish (*Raphanus sativus* var. *radiculus*) cv. Manshenhong. The average lesion length caused by the D0438a mutant was not significantly different from that caused by *Xcc* 8004 (P>0.05 by t-test).

3 Discussion

In this study, we established a modified Tn5 transposon integrated with an avirulence protein lack of N-terminal signal and utilized the chimeric transposon to construct a mutant library for large-scale HR phenotype screening. Although a high saturated Tn5 mutant library was obtained, only one novel type III secreted effector was identified, except for two known type III secreted effectors XopXccN and XopXccLR. Therefore, although this system can be used to quickly identify novel type III secreted effectors, the efficiency seems to be not high enough. The reasons lie in at least two factors. Firstly, only the in-frame insertion mutants could elicit HR. Those unknown type III secreted effectors could not be identified though they were frame-shift inserted by the chimeric transposon. So, the capacity of the mutant library should be large enough. However, it would cause more workload. In fact, in order reducing workload, we select 8 mutants as a group to determine HR induction. This strategy might yield the second shortage. Due to the low secretion and translocation efficiency of most type III

secreted effectors, the amount of the bacteria should be high enough, otherwise the qualitative weak HR might not be detected. Therefore, there might be more than 7 candidate type III secreted effector genes interrupted by the Tn5 transposon in our constructed mutant library, but they failed be screened out eventually and need to be identified by other more sensitive detection methods.

The newly identified *XC_0438a* possesses the typical characteristics of type III secreted effector genes. Except that it is induced by nutrition sterile medium and activated by the key *hrp* regulators HrpG and HrpX, bioinformatics analysis results showed that an imperfect plant-inducible promoter (PIP) box (TTCGC-N15-TTCCT) is present 144–120 bp upstream of the start codon of *XC_0438a*. In addition, the flanking genes of *XC_0438a*, *XC_0439* and *XC_0440*, are all the truncated IS1479 transposase-like genes[28], indicating that *XC_0438a* also harbors horizontal gene transfer (HGT) characteristics which is a typical feature of many type III secreted effector genes[33].

Another important factor is that the nucleotide sequence of *XC_0438a* is only present in 13 *Xanthomonas* spp., i.e., 9 *Xcc* strains including MAFF302021 (upublished, GenBank accession number (No.) AP019684.1), 3811 (unpublished, GenBank accession No. CP025750.1), 17[34], ICMP 4013[35], ATCC 33913[36], B100[37], CN14, CN15, CN16[38], *Xanthomonas campestris* pv. *raphani* 756C[39], *Xanthomonas perforans* strains 91-118[40] and LH3 (unpublished, GenBank accession No. CP018475.1), and *Xanthomonas axonopodis* pv. *commiphoreae* strain LMG26789 (unpublished, GenBank accession No. CP031059.1). Remarkably, *XCR_4085a*, the homologue of *XC_0438a* in *Xanthomonas campestris* pv. *raphani* 756C, is annotated as type III effector candidate XopAR. We also noticed that at least one transposase gene is present in the flanking of the corresponding homologue of *XC_0438a* in other *Xanthomonas* spp.

We failed to detect the contribution of *XC_0438a* to pathogenesis of *Xcc* 8004. This might be due to the redundancy, a common feature of many type III secreted effectors[33]. More interestingly, *XC_0438a* is the smallest gene of the 14 identified type III secreted effector genes in *Xcc* 8004[15–20]. The factor that the mutant a-k-5 is inserted 143-bps downstream of the start codon of *XC_0438a* indicated that the secretion and translocation signal of protein XC_0438a are present in the N-terminal 47 amino acid rescues, and the functional region harbors the C-terminal 85 amino acid rescues (Table 2). However, the detailed functionality of this mini type III secreted effector should be investigated in future.

Table 2 Distribution of *XC_0438a* among other phytopathogenic bacteria

Gene	Strain	Length/bp	Identities/%	Accession No.
XC_0438a	*Xcc* 8004	399		
xcc-b100_0460	*Xcc* B100[37]	399	100.00	AM920689.1
/	*Xcc* AT33913[36]	399	100.00	AE008922.1
/	*Xcc* ICMP4013[35]	399	100.00	CP012146.1
/	*Xcc* 17[34]	399	100.00	CP011946.1
/	*Xcc* MAFF302021	399	100.00	AP019684.1
/	*Xcc* 3811	399	100.00	CP025750.1
/	*Xcc* CN14[38]	399	99.75	CP017317.1
/	*Xcc* CN15[38]	399	99.75	CP017323.1
/	*Xcc* CN16[38]	399	99.75	CP017389.1
XCR_4085a	*Xcr* 756C[39]	264	99.50	CP002789.1
XPE_10325	*Xp* 91-118[40]	399	98.75	CP019725.1
BJD13_14670	*Xp* LH3	399	98.75	CP018475.1
Xcom_19835	*Xac* LMG26789	399	98.50	CP031059.1

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