MPMI Vol. 22, No. 11, 2009, pp. 1401–1411. doi:10.1094/MPMI-22-11-1401. *e*-Xtra*

Identification of Six Type III Effector Genes with the PIP Box in *Xanthomonas campestris* pv. *campestris* and Five of Them Contribute Individually to Full Pathogenicity

Wei Jiang,[1] Bo-Le Jiang,[1] Rong-Qi Xu,[2] Jun-Ding Huang,[1] Hong-Yu Wei,[1] Guo-Feng Jiang,[1] Wei-Jian Cen,[1] Jiao Liu,[1] Ying-Ying Ge,[1] Guang-Hua Li,[1] Li-Li Su,[1] Xiao-Hong Hang,[1] Dong-Jie Tang,[1] Guang-Tao Lu,[1] Jia-Xun Feng,[1] Yong-Qiang He,[1] and Ji-Liang Tang[1]

[1]Guangxi Key Laboratory of Subtropical Bioresources Conservation and Utilization, The Key Laboratory of Ministry of Education for Microbial and Plant Genetic Engineering, and College of Life Science and Technology, Guangxi University, 100 Daxue Road, Nanning, Guangxi 530004, China; [2]Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China

Submitted 3 April 2009. Accepted 7 June 2009.

***Xanthomonas campestris* pv. *campestris* is the pathogen of black rot of cruciferous plants. The pathogenicity of the pathogen depends on the type III secretion system (T3SS) that translocates directly effector proteins into plant cells, where they play important roles in the molecular interaction between the pathogen and its hosts. The T3SS of *Xanthomonas* spp. is encoded by a cluster of hypersensitive response and pathogenicity (*hrp*) genes. It has been demonstrated that the expression of *hrp* genes and some type III secreted (T3S)-effector genes is coactivated by the key *hrp* regulatory protein HrpX. The regulation by HrpX can be mediated by the binding of HrpX protein to a *cis*-regulatory element named the plant-inducible promoter (PIP) box present in the promoter region of HrpX-regulated genes. A genome screen revealed that *X. campestris* pv. *campestris* 8004 possesses 56 predicted genes with the PIP box. Nine of these genes have been shown to encode T3S effectors, Hrp, and Hrp-associated proteins. In this study, we employed an established T3S effector translocation assay with the hypersensitive-reaction-inducing domain of *X. campestris* pv. *campestris* AvrBs1 as a reporter to characterize the remaining 47 genes with the PIP box and showed that 6 of them, designated as XopXccE1, XopXccP, XopXccQ, XopXccR1, XopXccLR, and AvrXccB, harbor a functional translocation signal in their N-terminal regions, indicating that they are T3S effectors of *X. campestris* pv. *campestris*. We provided evidence to demonstrate that all these effectors are expressed in an HrpX-dependent manner and their translocation into plant cells relies on the translocon protein HrpF and the chaperone HpaB. Mutational analyses demonstrated that all these effectors, except AvrXccB, are individually required for full virulence and growth of *X. campestris* pv. *campestris* in the host plant Chinese radish.**

Numerous gram-negative phytopathogenic bacteria translocate so-called effector proteins directly into plant cells via a type III secretion system (T3SS). The type III secreted (T3S) effectors inside the plant cells interact with plant substrates to activate or suppress plant defense signal transduction, resulting in elicitation of disease resistance or promotion of disease symptoms (Greenberg and Vinatzer 2003; Alfano and Collmer 2004; Mudgett 2005; Büttner and Bonas 2006; Grant et al. 2006). A given bacterial pathogen species or pathovar may develop its unique type of T3S effectors during the evolution of its pathogenicity, although homologues of some identified T3S effectors have been observed among different bacteria. As a first step toward understanding the molecular mechanisms by which T3S effectors function in the interaction between pathogens and plants, many research groups have been making efforts to identify T3S effectors from different important plant pathogens. However, the identification was limited for a long period of time to those effectors, termed avirulence (Avr) proteins, which can individually stimulate strong defense responses often associated with the hypersensitive reaction (HR) on some resistant host or nonhost plants. HR is a visible disease resistance phenomenon with a type of rapid, localized, and programmed cell death in the pathogen-infection tissues of plants (Alfano and Collmer 2004; Jones and Dangl 2006). This reaction is easily detected and has been used to identify a large number of Avr effector proteins from various pathogens. For most T3S effectors, loss of a single one did not affect pathogen virulence, or else its influence in pathogenicity could not be detected due to the absence of a sensitive detection method (Gürlebeck et al. 2006). Recently developed efficient identification methods and the availability of the genome sequences of major bacterial phytopathogens have facilitated the comprehensive identification of T3S effectors. Despite the fact that the authentic functional roles in pathogenicity of most T3S effectors remain to be further determined, large-scale identification of T3S effectors has been achieved in several pathogens such as *Pseudomonas syringae* pv. *maculicola* (Guttman et al. 2002), *P. syringae* pv. *phaseolicola* (Chang et al. 2005; Vencato et al. 2006), *P. syringae* pv. *tomato* (Petnicki-Ocwieja et al. 2002; Chang et al. 2005; Vinatzer et al. 2005; Schechter et al. 2006), *Ralstonia solanacearum* (Cunnac et al. 2004b), *Xanthomonas campestris* pv. *vesicatoria* (Roden et al. 2004), and *X. oryzae* pv. *oryzae* (Furutani et al. 2009). One of the strategies used to identify T3S effectors is the application of the HR-inducing domains of Avr proteins. In general, the C-terminal domain of an Avr protein is sufficient to elicit an HR in a resis-

Wei Jiang and Bo-Le Jiang contributed equally to this work.

Corresponding author: Ji-Liang Tang; telephone: +86-771-3239566; Fax: +86-771-3239413; E-mail: jltang@gxu.edu.cn

*The *e*-Xtra logo stands for "electronic extra" and indicates that three supplemental tables are published online.

tance host or nonhost plant that possesses a cognate resistance (*R*) gene; T3S effectors have a modular structure, and their N-terminal 50 to 100 amino acids harbor a functional signal to target proteins into plant cells (Mudgett et al. 2000; Guttman and Greenberg 2001; Greenberg and Vinatzer 2003; Schechter et al 2004). A number of T3S effectors have been successfully identified from *P. syringae* pv. *maculicola* and *X. campestris* pv. *vesicatoria* by using the HR-inducing C-terminal domain of AvrRpt2, AvrBs2, or AvrBs3 as a reporter (Guttman et al. 2002; Noël et al. 2003; Roden et al. 2004).

It is well known that the expression of T3SS-encoding genes and most T3S-effector genes is repressed in rich media and induced in plant tissues or in minimal media. In *Xanthomonas* spp., the T3SS is encoded by a cluster of hypersensitive response and pathogenicity (*hrp*) genes consisting of at least six *hrp* operons (Arlat et al. 1991; Bonas et al. 1991). The expression of the *hrp* operons is activated by the key *hrp* regulatory proteins HrpG and HrpX. HrpG is a member of the OmpR family of two-component signal transduction response regulators and HrpX is an AraC-type transcriptional regulator. HrpG and HrpX form a regulatory cascade, in which HrpG regulates the expression of *hrpX* and HrpX then regulates the expression of downstream *hrp* operons and some T3S-effector genes (Wengelnik and Bonas 1996; Wengelnik et al. 1996; Huang et al. 2009). It has been demonstrated that the regulation by HrpX in *Xanthomonas* spp. could be mediated by the binding of HrpX protein to a *cis*-regulatory element named the plant-inducible promoter (PIP) box (discussed below), present in the promoter region of HrpX-regulated genes (Fenselau and Bonas 1995; Tsuge et al. 2005; Koebnik et al. 2006). Such a regulation mode also exists in *R. solanacearum* (Cunnac et al. 2004a).

X. campestris pv. *campestris* is the causal agent of black rot disease of cruciferous plants. The pathogen infects almost all members of the crucifer family (*Brassicaceae*), including important vegetables such as broccoli, Brussels sprouts, cabbage, cauliflower, kale, mustard, and radish; the major oil crop rape; as well as the model plant *Arabidopsis thaliana* (Alvarez 2000). After determination of the whole-genome sequence of the *X. campestris* pv. *campestris* 8004 (Qian et al. 2005), we have made an effort to identify T3S effectors from the pathogen and have recently reported two such effectors discovered by translocation assay using the HR-inducing domain of the Avr protein AvrBs1 from *X. campestris* pv. *campestris* as a reporter (Jiang et al. 2008; Xu et al. 2008). Bioinformatics analysis revealed that the genome of the *X. campestris* pv. *campestris* 8004 possesses 56 predicted genes with the PIP box. In this work, we employed the previously established translocation assay to characterize these genes and showed that six of them are T3S effectors of *X. campestris* pv. *campestris*. We also demonstrated that five of the T3S effectors are individually required for full virulence and growth of the pathogen in the host plant Chinese radish.

RESULTS

Fifty-six predicted genes with the PIP box present in the genome of the *X. campestris* pv. *campestris* 8004.

A survey of the genomic sequence of the *X. campestris* pv. *campestris* 8004 (Qian et al. 2005) for the presence of the typical motif of the PIP box, TTCGB-N_{15}-TTCGB (B refers to the base C, G, or T but not A) (Cunnac et al. 2004a; Koebnik et al. 2006), revealed that 56 predicted genes contain such a motif within the upstream sequence of their putative translational start codons. Among these genes, six are *hrp*, *hrp*-conserved (*hrc*), or *hrp*-associated (*hpa*) genes; that is, *XC_3001* (*hpa2*), *XC_3002* (*hpa1*), *XC_3011* (*hrpB1*), *XC_3012* (*hrcU*), *XC_3015* (*hrcQ*), and *XC_3019* (*hrpD5*). These *hrp*/*hrc*/*hpa* genes do not encode T3S effectors and their function has been studied in some detail. Thus, they were excluded for further study in this work. In addition, three of the PIP-box-containing genes have already been demonstrated to encode T3S effectors acting as Avr proteins; that is, *XC_0052* (*avrBs2*) (Ignatov et al. 2002), *XC_2004* (*avrXccC*) (Castañeda et al. 2005), and *XC_2602* (*avrXccE1*) (He et al. 2007). The remaining 47 genes with the PIP box were taken as candidates for identification of novel T3S effectors of *X. campestris* pv. *campestris* in this study. One of the candidates, named *XC_3802*, was annotated to encode an Avr protein (AvrXccB) (da Silva et al. 2002; Qian et al. 2005); however, no experimental evidence has been reported to show that it is an Avr protein or T3S-effector.

Identification of six T3S-effector genes from the PIP box-containing genes by translocation assay.

Our previous work has demonstrated that the Avr protein AvrBs1 of *X. campestris* pv. *campestris* 8004 is responsible for the pathogen to induce an HR on pepper cv. ECW-10R in a T3SS-dependent manner (Xu et al. 2008). The *avrBs1*-deletion mutant harboring $avrBs1_{59\text{-}445}$, an *avrBs1*-derivative lacking the first 58 codons that encode amino acids for a functional translocation signal, cannot elicit an HR. However, a fragment coding for a functional translocation signal from another T3S effector fused to the 5′ end of $avrBs1_{59\text{-}445}$ in an in-frame manner can target the fusion protein into plant cells and elicit an HR in the pepper ECW-10R (Xu et al. 2008). To investigate whether any products of the above 47 candidate open reading frames (ORF) encode T3S effectors, we determined the functionality to target proteins into plant cells of their N-terminal domains by using the HR-inducing domain $AvrBs1_{59\text{-}445}$ as a reporter. To facilitate the determination, a reporter plasmid, named pJAG, was constructed, which contains the P*lac* promoter upstream of the multiple cloning sites and $avrBs1_{59\text{-}445}$ fused with the 5′ end of 3× FLAG (details provided below). A DNA fragment harboring the putative promoter and T3S signal-encoding sequence from each of the 47 candidate genes was cloned into the plasmid pJAG, giving an in-frame fusion between the N-terminal amino acids of a candidate and the $AvrBs1_{59\text{-}445}$.

All of the obtained 47 recombinant plasmids were introduced into the *avrBs1*-deletion mutant strain 8004ΔavrBs1 (Xu et al. 2008) by triparental conjugation. The resulting transconjugant strains were then tested for HR induction on the leaves of pepper cv. ECW-10R. The results showed that, 24 h after inoculation, the mutant strain 8004ΔavrBs1 carrying the recombinant plasmid pJAG2994, pJAG2995, pJAG3160, pJAG3177, pJAG3802, or pJAG4273, which carried an in-frame fusion between $AvrBs1_{59\text{-}445}$ and the N-terminal amino acids of the ORF *XC_2994*, *XC_2995*, *XC_3160*, *XC_3177*, *XC_3802*, or *XC_4273*, could stimulate a typical HR on pepper ECW-10R (Fig. 1A), whereas 8004ΔavrBs1 harboring any of the other 41 recombinant plasmids could not (data not shown). These results revealed that the N-terminal regions of the proteins encoded by the genes *XC_2994*, *XC_2995*, *XC_3160*, *XC_3177*, *XC_3802*, and *XC_4273* possess a translocation signal with the functionality to target proteins into plant cells, suggesting that these proteins are T3S effectors.

Translocation of the six T3S effectors relies on the translocon protein HrpF and the chaperone HpaB.

The HrpF of *X. campestris* is a putative T3SS translocon protein required for the translocation of T3S effectors across the plant plasma membrane (Büttner et al. 2002; Meyer et al. 2006), whereas HpaB is a T3SS chaperone crucial for the efficient translocation of T3S-effector proteins (Büttner et al., 2004). To investigate whether HrpF and HpaB are involved in

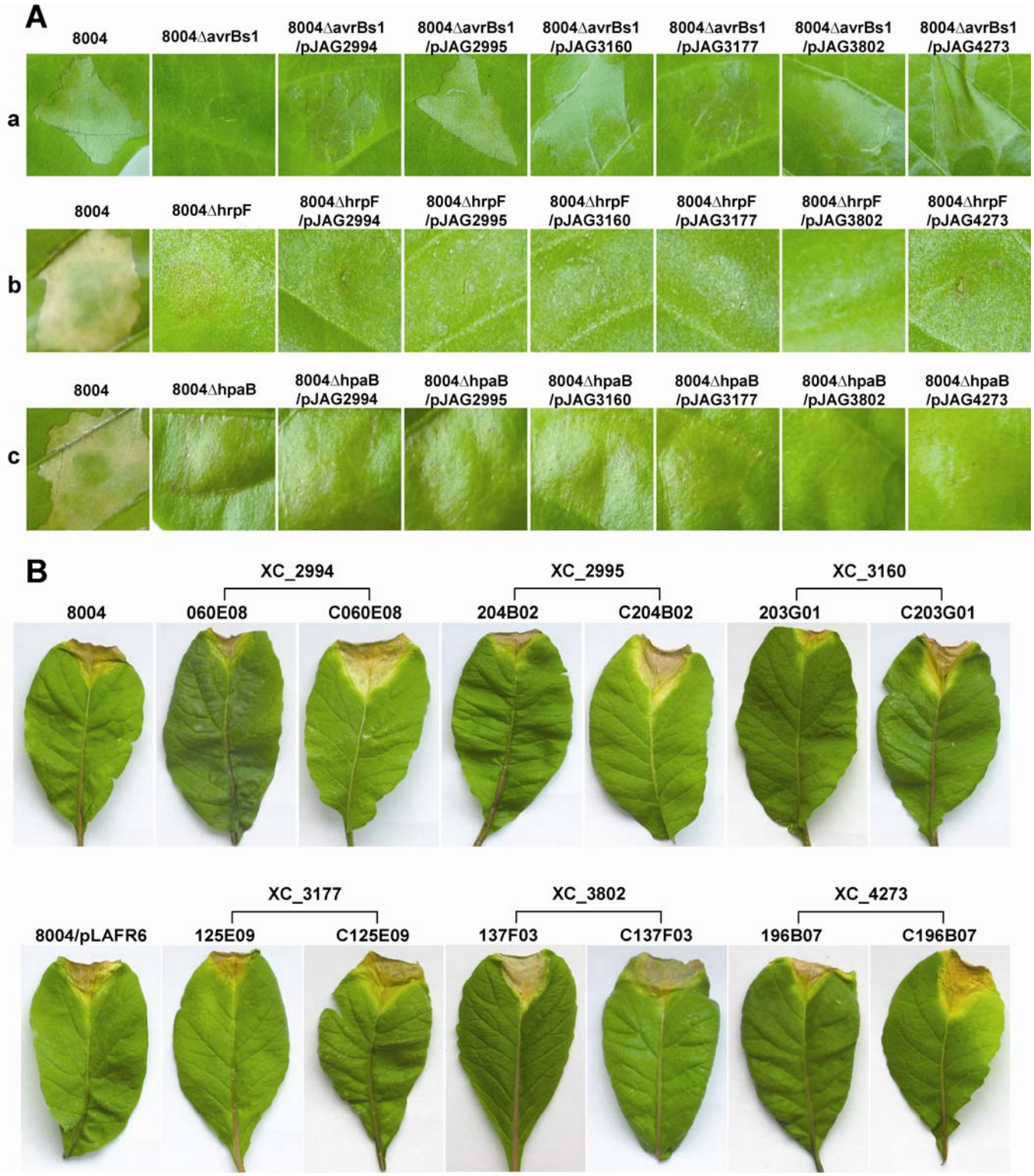

Fig. 1. Analyses of hypersensitive reaction (HR) induction and virulence of *Xanthomonas campestris* pv. *campestris* strains. **A,** HR-induction analysis on the leaves of pepper ECW-10R (*Capsicum annuum* cv. ECW-10R) carrying the resistance gene *Bs1*. The wild-type 8004 induced HR symptoms but the *avrBs1*-deletion mutant strain 8004ΔavrBs1, the hypersensitive response and pathogenicity (*hrp*)*F*-deletion mutant strain 8004ΔhrpF, and the *hpaB*-integration mutant strain 8004ΔhpaB did not (a, b, and c). The mutant 8004ΔavrBs1 harboring the six recombinant plasmids pJAG2994, pJAG2995, pJAG3160, pJAG3177, pJAG3802, and pJAG4273, which carried an in-frame fusion between $AvrBs1_{59\text{-}445}$ and the N-terminal amino acids of the open reading frames (ORF) XC_2994, XC_2995, XC_3160, XC_3177, XC_3802, or XC_4273, respectively, could stimulate typical HR symptoms (a); however, the mutants 8004ΔhrpF and 8004ΔhpaB harboring the same plasmids could not (b and c), suggesting that the ORF XC_2994, XC_2995, XC_3160, XC_3177, XC_3802, and XC_4273 encode type III secreted (T3S)-effector proteins and the translocation of which into plant cells relies on the T3S translocon HrpF and the T3S chaperone HpaB. The photographs were taken 24 h (a) and 72 h (b and c) postinoculation. **B,** Virulence analysis on the leaves of Chinese radish (*Raphanus sativus* var. *radiculus*) cv. Manshenhong. The mutants 060E08, 204B02, 203G01, 125E09, and 196B07 but not the mutant 137F03 showed reduced black rot disease symptoms compared with the wild-type 8004, and the complemented strains (C060E08, C204B02, C203G01, C125E09, C137F03, and C196B07) and the wild type harboring the vector plasmid pLAFR6 (8004/pLAFR6) showed wild-type disease symptoms. The photographs were taken on day 9 postinoculation.

the secretion of the identified six T3S effectors, the recombinant plasmids pJAG2994, pJAG2995, pJAG3160, pJAG3177, pJAG3802, and pJAG4273 were also introduced by triparental conjugation into the *hrpF*-deletion mutant strain 8004ΔhrpF and the *hpaB*-integration mutant strain 8004ΔhpaB. Transconjugant strains for each of the six recombinant plasmids were obtained and tested for HR production on pepper ECW-10R. At 72 h after inoculation, no visible HR was elicited by any of the transconjugant strains (Fig. 1A). These results suggest that the translocation of all six T3S effectors into plant cells relies on the T3SS translocon protein HrpF and the chaperone HpaB.

Expression of the six T3S-effector genes is regulated by HrpG and HrpX.

Although it has been demonstrated that the PIP box serves as a regulatory element and HrpX activates the expression of genes with the PIP box within their promoters via binding to the PIP box (discussed below), HrpX-independent PIP-box-containing promoters have been observed (Gürlebeck et al. 2006). To determine whether the expression of the six identified T3S-effector genes is regulated by HrpX and HrpG, a promoter-reporting plasmid carrying the promoter region of each of the six effector genes 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 (details provided below). The GUS activities of the obtained reporter strains cultured in the minimal medium MMX (Daniels et al. 1984), in which expression of *hrp* genes is strongly induced (Wei et al. 2007b), and the rich medium NYG (Daniels et al. 1984) were then measured and compared. The results are presented in Table 1. When grown in MMX medium, each and every reporter of the six effector genes in the *hrpX* or *hrpG* mutation background produced significantly lower GUS activity than in the wild-type background ($P = 0.01$ by *t* test). The GUS activity values produced by the reporters in the wild-type background are 4- to 72-fold higher than those in the *hrpX* or *hrpG* mutation background (Table 1). All the reporter strains produced very feeble GUS activity when grown in NYG rich medium compared with those grown in MMX minimal medium (Table 1). These results demonstrate that the expression of *XC_2994*, *XC_2995*, *XC_3160*, *XC_3177*, *XC_3802*, and *XC_4273* is induced in MMX minimal medium and positively regulated by the *hrp* regulators HrpX and HrpG.

Five of the six identified T3S effectors are individually essential for full pathogenicity of *X. campestris* pv. *campestris*.

In order to investigate the role of the identified T3S effectors in pathogenicity of *X. campestris* pv. *campestris*, we screened the mutant library of the *X. campestris* pv. *campestris* 8004, which was constructed by transposon Tn*5gusA5* insertion mutagenesis in our laboratory (details provided below), for mutants of the six T3S-effector-coding genes. Mutants named 060E08, 204B02, 203G01, 125E09, 137F03, and 196B07, in which a Tn*5gusA5* was inserted in each of the six T3S-effector-coding genes *XC_2994*, *XC_2995*, *XC_3160*, *XC_3177*, *XC_3802*, and *XC_4273*, respectively, were obtained. All of the mutants grew identically to the wild-type strain 8004 in NYG rich medium as well as MMX minimal medium (data not shown). The virulence of the mutants was tested on the host plant Chinese radish (*Raphanus sativus* var. *radiculus*) cv. Manshenhong by the leaf-clipping method (Dow et al. 2003). The results showed that five mutants (i.e., 060E08, 204B02, 203G01, 125E09, and 196B07) exhibited a significant reduction in virulence compared with the wild-type strain ($P = 0.01$ by *t*-test), while the mutant 137F03 displayed the same virulence as the wild type (Fig. 1B). Ten days after inoculation, the mean lesion length of the disease symptoms caused by the mutants 060E08, 204B02, 203G01, 125E09, and 196B07 was only 8.2, 8.2, 4.5, 9.4, and 8.7 mm, respectively, whereas the wild type caused a mean lesion length of 13.2 mm (Fig. 2A).

To validate whether the reduced virulence phenotype of the mutants was indeed caused by the transposon Tn*5gusA5* insertion disruption of the five corresponding T3S-effector-coding genes, a complemented strain for each of the mutants was constructed in this study by transferring a recombinant plasmid, in which an entire corresponding gene was cloned into the plasmid pLAFR6 (Huynh et al. 1989), into the corresponding mutant (details provided below). The virulence of the resulting complemented mutant strains was then examined. The results displayed that the mean lesion lengths caused by the complemented strains, the wild-type strain 8004, and the strain 8004 harboring the vector pLAFR6 alone were not significantly different ($P = 0.05$ by *t* test) (Fig. 2A), suggesting that the virulence reduction of the five mutants was indeed initiated by the Tn*5gusA5* insertion disruption of the corresponding T3S-effector-coding genes. Taken together, these results demonstrate that *XC_2994*, *XC_2995*, *XC_3160*, *XC_3177*, and *XC_4273* are essential for full virulence of *X. campestris* pv. *campestris*.

To evaluate the role of the T3S-effector genes in the growth of *X. campestris* pv. *campestris* in host plants, the bacterial populations of the mutants of the T3S-effector genes, the wild-type strain, and the complemented strains in the infected radish leaves were compared. The results showed that, besides the mutant strain 137F03 (the mutant of *XC_3802*), the bacterial numbers of the other five mutants recovered from the infected leaves were significantly fewer ($P = 0.01$ by *t* test) than that of the wild-type strain 4 days postinoculation and onward (Fig. 2B). There was no significant difference among the growth

Table 1. β-Glucuronidase (GUS) activities produced by the type III secreted (T3S)-effector promoter-*gusA* reporters under different genetic backgrounds in different growth conditions[z]

	GUS activities (mg/ml/min/OD_{600})					
Strain	**pLGUS2994**	**pLGUS2995**	**pLGUS3160**	**pLGUS3177**	**pLGUS3802**	**pLGUS4273**
8004 (N)	0.07 ± 0.04 a	0.08 ± 0.00 a	0.02 ± 0.01 a	0.03 ± 0.00 a	1.11 ± 0.14 a	0.07 ± 0.01 a
8004 (M)	1.07 ± 0.12 b	4.79 ± 0.32 b	0.90 ± 0.09 b	0.72 ± 0.17 b	10.27 ± 0.83 b	0.23 ± 0.01 b
8004ΔhrpG (M)	0.08 ± 0.02 a	0.67 ± 0.18 a	0.03 ± 0.01 a	0.01 ± 0.01 a	0.62 ± 0.03 a	0.05 ± 0.01 a
8004ΔhrpX (M)	0.05 ± 0.03 a	0.69 ± 0.08 a	0.04 ± 0.01 a	0.04 ± 0.03 a	0.65 ± 0.04 a	0.04 ± 0.01 a

[z] The T3S-effector promoter-*gusA* reporters pLGUS2994, pLGUS2995, pLGUS3160, pLGUS3177, pLGUS3802, and pLGUS4273 were constructed by cloning the DNA fragments containing the promoter regions of the T3S-effector genes *XC_2994*, *XC_2995*, *XC_3160*, *XC_3177*, *XC_3802*, and *XC_4273*, respectively, into the reporter plasmid pLGUS which harbors the promoterless *gusA* gene. The T3S-effector promoter-*gusA* reporters were introduced into the wild-type 8004, the hypersensitive response and pathogenicity (*hrp*)*G* mutant 8004ΔhrpG, and the *hrpX* mutant 8004ΔhrpX by triparental conjugation, and their GUS activities were detected by measurement of the optical density at 415 nm (OD_{415}) using ρ-nitrophenyl-β-D-glucuronide as substrate after being grown in NYG (N) (Daniels et al. 1984) for 16 h or MMX (M) (Daniels et al. 1984) for 24 h. The different letters in each data column indicate significant differences at $P = 0.01$ by *t* test. Values given are the means and standard deviations of triplicate measurements. Data presented were from a representative experiment and similar results were obtained in two other independent experiments.

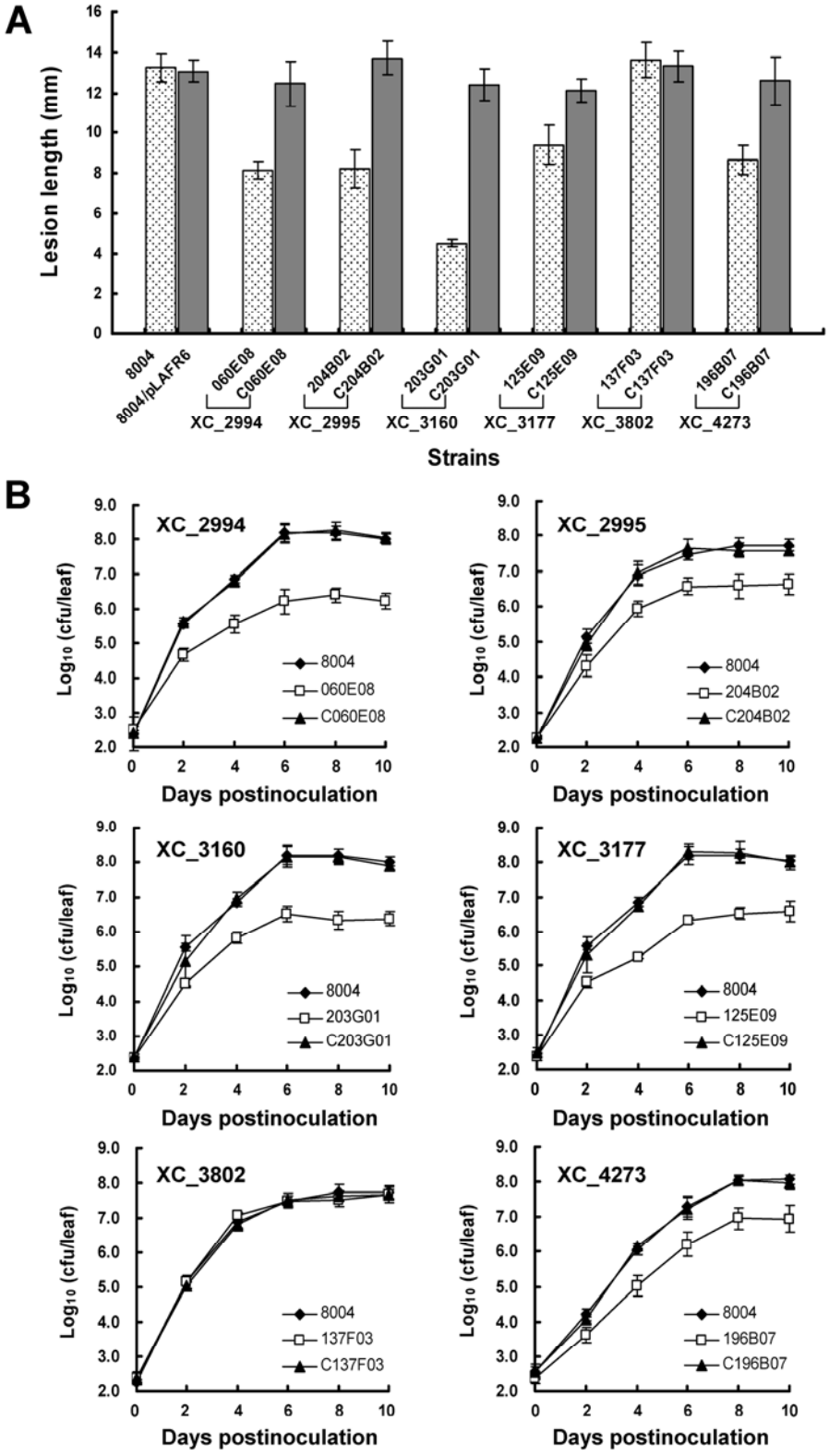

Fig. 2. Type III secreted (T3S) effectors XC_2994, XC_2995, XC_3160, XC_3177, and XC_4273 but not XC_3802 contribute individually to the full pathogenicity of *Xanthomonas campestris* pv. *campestris*. **A,** Virulence of *X. campestris* pv. *campestris* strains. The average lesion lengths caused by the mutants (060E08, 204B02, 203G01, 125E09, and 196B07) of the T3S-effector genes *XC_2994, XC_2995, XC_3160, XC_3177*, and *XC_4273* are significantly shorter than that caused by the wild-type 8004 ($P = 0.01$ by t test). The average lesion lengths caused by the mutant (137F03) of the T3S-effector gene *XC_3802*, the complemented strains (C060E08, C204B02, C203G01, C125E09, C137F03, and C196B07), the wild type, and the wild type harboring the vector plasmid pLAFR6 (8004/pLAFR6) are similar. Values are the mean ± standard deviation (SD) from three repeats, each with 60 leaves. The lesion lengths were measured 10 days postinoculation. **B,** The in planta growth of *X. campestris* pv. *campestris* strains. The bacterial numbers (CFU) of the mutants (060E08, 204B02, 203G01, 125E09, and 196B07) of the T3S-effector genes *XC_2994, XC_2995, XC_3160, XC_3177,* and *XC_4273*, recovered from the infected leaves, are significantly fewer ($P = 0.01$ by t test) than that of the wild-type strain 8004 4 days postinoculation and onward. There is no significant difference between the bacterial numbers of the wild type and the complemented strains C060E08, C204B02, C203G01, C125E09, and C196B07. The bacterial numbers of the mutant 137F03 of the effector gene *XC_3802*, the complemented strain C137F03, and the wild type are almost identical. Data are the mean ± SD from three repeats.

rates of the mutant 137F03, the wild type, and all of the complemented strains (Fig. 2B). This suggests that the growth capacity of the five virulence-reduced mutants in planta could be restored by in trans complementation. These data reveal that the T3S-effector genes *XC_2994*, *XC_2995*, *XC_3160*, *XC_3177*, and *XC_4273* are required for *X. campestris* pv. *campestris* to proliferate well in host plants.

DISCUSSION

As described above, T3S effectors play important roles in molecular interactions between a phytopathogen and its hosts. The translocation of the effectors into plant cells relies on the effector targeting signal that generally resides in the N-terminal domain (Mudgett et al. 2000; Schechter et al. 2004). In this work, we have identified six T3S effectors of *X. campestris* pv. *campestris* by employing the HR-inducing domain of the Avr protein AvrBs1 as a reporter to analyze the translocation capability of the N-termini of proteins encoded by the genes with a PIP motif. By *gus*-reporter analyses, we showed that the expression of all of these effectors is positively regulated by the *hrp* regulators HrpX and HrpG, and their translocation into plant cells relies on the translocon protein HrpF and the chaperone HpaB. Koebnik and associates (2006) have demonstrated by DNA affinity enrichment that the HrpX of *X. campestris* pv. *vesicatoria* specifically binds to the PIP boxes present in the promoters of *hrp* operons, suggesting that HrpX directly activates the expression of HrpX-regulated genes via binding to corresponding PIP boxes. In *X. oryzae* pv. *oryzae*, the PIP boxes also have been shown to be essential for HrpX-dependent gene activation (Furutani et al. 2006). Because the *hrp* cluster and the *hrp* key regulators *hrpX* and *hrpG* are highly conserved among different *Xanthomonas* spp. and pathovars, it has been considered that the regulation of the *hrp* genes may be similar among different *Xanthomonas* bacteria. It is possible that the expression of the T3S-effector-coding genes with the PIP box identified in this work is directly initiated by HrpX. However, an alternative regulatory model has been proposed by Koebnik and associates (2006). They suggested that HrpX may activate genes independent of a PIP box, one of which encodes a regulator which recognizes the PIP box and activates the corresponding genes. Therefore, in *X. campestris* pv. *campestris*, whether HrpX directly activates the expression of the T3S effectors needs to be validated by further experimental evidence.

In addition to strain 8004, the genome sequences of two other strains (ATCC 33913 and B100) of *X. campestris* pv. *campestris* have been also determined (da Silva et al. 2002; Vorhölter et al. 2008). A database sequence comparison displayed that all of the effectors identified in this work from strain 8004, with the exception of XC_3802, are present in strains ATCC 33913 and B100, and the homologs for each of them are almost identical (Table 2). XC_3802 is present in strain ATCC 33913 but not B100 (Table 2). This observation fits with the results of our previous array-based comparative genome hybridization (aCGH) analyses of 18 virulent *X. campestris* pv. *campestris* strains, isolated from different host plants and various geographical regions over a wide range of latitudes across China, which showed that the ORF XC_3802 homologs are only present in 8 of the strains, whereas orthologs of the other five effector-coding ORF (i.e., *XC_2994*, *XC_2995*, *XC_3160*, *XC_3177* and *XC_4273*) are present in all of the 18 strains (He et al. 2007). These may suggest that the effectors, except XC_3802, are highly conserved among *X. campestris* pv. *campestris* strains. These facts may also imply that, in *X. campestris* pv. *campestris*, the T3S effectors contributing significantly to pathogenicity, no matter whether they were acquired or inherent, may be eternal; otherwise, they will be lost during evolution.

A further protein BLAST analysis revealed that homologs or analogs of XC_2994, XC_3177, and XC_4273 are present in all sequenced *Xanthomonas* spp. (i.e., *X. campestris* pv. *vesicatoria*, *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *X. axonopodis* pv. *citri*) whereas XC_2995 homologs or analogs are present only in *X. campestris* pv. *vesicatoria* and *X. axonopodis* pv. *citri*, and the XC_3802 homolog or analog is present only in *X. campestris* pv. *vesicatoria* (Table 2). In addition, homologs or analogs of all the effectors are also present in *Ralstonia solanacearum* and three of them are present in *P. syringae* pv. *tomato* (Table 2).

Table 2. Distribution of the six type III secreted (T3S) effectors among other phytopathogenic bacteria[u]

Xanthomonas campestris* pv. *campestris				***X. oryzae* pv. *oryzae***						
8004	**ATCC33913**	**B100**	**85-10[v]**	**KACC10331**	**PXO99A**	**MAFF311018**	**BLS256[w]**	**306[x]**	**GMI1000[y]**	**DC3000[z]**
XopXccP XC_2994	XCC1247 (99/100)	xccb100_305 7 (99/99)	XopP XCV1236 (38/56)	XOO3425 (39/58)	PXO_021 07 (39/57)	XOO_3222 (39/57)	Xoryp_06110 (42/61)	XAC120 8 (39/56)	hlk3 RSp0160 (35/54)	N
XopXccE1 XC_2995	XCC1246 (100/100)	xccb100_305 8 (100/100)	XopE1 XCV0294 (26/40)	N	N	N	N	XAC028 6 (24/38)	AvrPphE RSc3369 (27/45)	HopX1 PSPTOA0 012 (25/40)
XopXccR1 XC_3160	XCC1089 (100/100)	xccb100_325 6 (99/100)	N	N	N	N	N	N	RSp1281 (26/39)	HopR1 PSPTO08 83 (49/64)
XopXccQ XC_3177	XCC1072 (99/99)	XopQ xccb100_327 4 (99/100)	XopQ XCV4438 (63/74)	XOO4466 (64/75)	PXO_039 01 (64/75)	XOO4208 (61/72)	Xoryp_00510 (63/74)	XAC433 3 (63/74)	RSc0245 (47/62)	HopQ1-1 PSPTO08 77 (61/72)
AvrXccB XC_3802	XCC3731 (99/100)	N	XopJ XCV2156 (24/41)	N	N	N	N	N	PopP2 RSc0321 (24/37)	N
XopXccLR XC_4273	XCC4186 (100/100)	xccb100_440 0 (99/100)	XCV3220 (43/60)	XOO1762 (41/58)	PXO_016 20 (42/59)	XOO1662 (41/58)	ZP_0224412 7 (40/58)	XAC309 0 (42/59)	RSp0842 (29/45)	N

[u] Numbers in parentheses represent percentages of amino-acid identity or similarity; N denotes no homologous (or similar) gene was found.
[v] *X. campestris* pv. *vesicatoria.*
[w] *X. oryzae* pv. *oryzicola.*
[x] *X. axonopodis* pv. *citri.*
[y] *Ralstonia solanacearum.*
[z] *Pseudomonas syringae* pv. *tomato.*

The ORF coding for the effectors XC_2994 and XC_2995 lie side by side on the chromosomes of all sequenced *X. campestris* pv. *campestris* strains, whereas the loci of their homologs or analogs are disconnected in *X. campestris* pv. *vesicatoria*, *X. axonopodis* pv. *citri*, and *R. solanacearum*. Interestingly, the three ORF next to the left and right flanks of *XC_2994* and *XC_2995* encode putative mobile elements; that is, a putative IS1479 transposase (XC_2993) and two putative IS1404 transposases (XC_2996 and XC_2997) (Fig. 3) (Qian et al. 2005). Furthermore, the GC contents of *XC_2994* and *XC_2995* are 57 and 49%, respectively, which are much lower than the 65%, in average, of the *X. campestris* pv. *campestris* genome. These suggest that the effectors XC_2994 and XC_2995 of *X. campestris* pv. *campestris* are horizontally acquired. More interestingly, the predicted ORF *XC_2992*, *XC_2999*, and *XC_3000* of *X. campestris* pv. *campestris* share more than 70% DNA sequence identity with the 5′ terminal, middle, and 3′ terminal sequences of the ORF *XCV0442* of *X. campestris* pv. *vesicatoria*, respectively (Fig. 3), hinting that the allele of *XCV0442* in *X. campestris* pv. *campestris* was truncated in the ancient bacterium. An analysis of the determined genome sequences showed that *XCV0442* and its alleles in other *Xanthomonas* spp. share high sequence identity and are tightly flanked by the *hrp* gene cluster (Fig. 3). The *hrp* cluster is conserved in *Xanthomonas* spp. and is a typical pathogenicity island acquired by horizontal gene transfer (Dobrindt et al. 2004; Gürlebeck et al. 2006). *XC_2998* and its alleles in the other two *X. campestris* pv. *campestris* strains are identical and have been annotated to encode a hypothetical protein. Our previous aCGH analyses revealed that, like *XC_2994* and *XC_2995*, *XC_2998* is highly conserved in all 18 different *X. campestris* pv. *campestris* strains tested (He et al. 2007). However, no homolog or analog of *XC_2998* is present in other sequenced *Xanthomonas* spp. Taken together, all of these facts may indicate that the effectors XC_2994 and XC_2995 were horizontally acquired after the acquisition of the *hrp* cluster in *X. campestris* pv. *campestris*.

The effector XC_2994 shows 39% (58%) amino-acid identity (similarity) to the effector XopP, which was identified from *X. campestris* pv. *vesicatoria* (Table 2), and its function is unknown (Roden et al. 2004). A BLAST search against the *X. campestris* pv. *campestris* 8004 genome with the effector XopP of *X. campestris* pv. *vesicatoria* revealed no other sequence-similar deduced protein except XC_2994. Therefore, we renamed XC_2994 as XopXccP. The effector XC_2995 shares 26% (40%) amino-acid identity (similarity) with the *X. campestris* pv. *vesicatoria* effector XopE1 (Table 2). XopE1 belongs to the HopX (AvrPphE) family of putative transglutaminases, with a cysteine-based catalytic triad essential for function (Nimchuk et al. 2007; Thieme et al. 2007). An alignment analysis showed that XC_2995 possesses a catalytic triad (122 cysteine, 152 histidine, and 174 aspartic acid) and a conserved N-terminal domain (data not shown), suggesting that XC_2995 may belong to the HopX (AvrPphE) family. Therefore, we renamed XC_2995 as XopXccE1.

The effector XC_3160 shares 49% (64%) amino-acid identity (similarity) with the effector HopR1 identified from *P. syringae* (Chang et al. 2005; Schechter et al. 2006), although it exists only in *X. campestris* pv. *campestris* among the sequenced *Xanthomonas* spp. and pathovars (Table 2). Based on the amino-acid sequence similarity, we renamed XC_3160 as XopXccR1. The function of HopR1 in the interaction between the pathogen and hosts is unknown because no mutant phenotype in planta for HopR1 has been observed (Chang et al. 2005; Schechter et al. 2006). Similar to HopR1, the predicted XopXccR1, consisting of 2,032 amino acids, is one of the few very large proteins deduced in *X. campestris* pv. *campestris* (Qian et al. 2005). To predict the secondary structure of XopXccR1, we performed a three-dimensional position-specific scoring matrix (3D-PSSM)

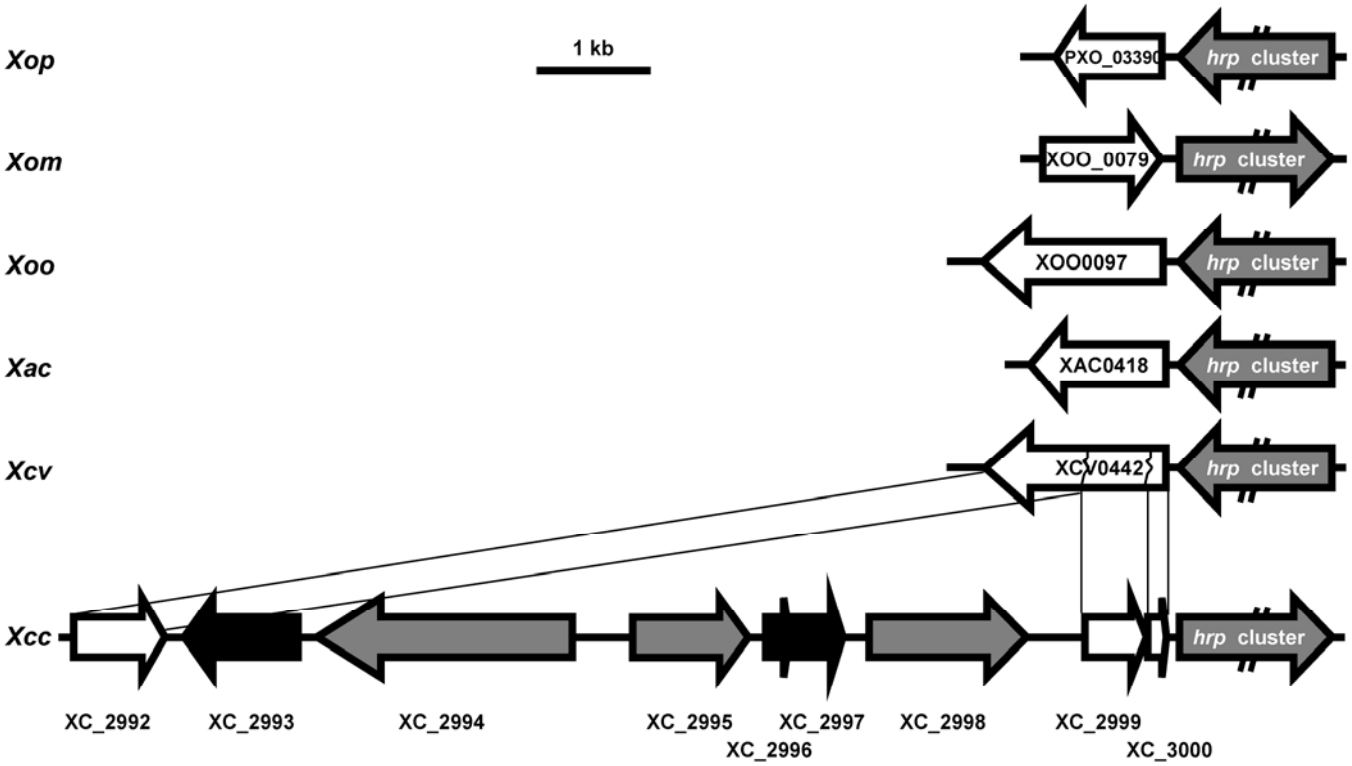

Fig. 3. *XC_2994* and *XC_2995* were horizontally acquired after the acquisition of the hypersensitive response and pathogenicity (*hrp*) cluster in *Xanthomonas campestris* pv. *campestris*. The open reading frames (ORF) *XC_2992*, *XC_2999*, and *XC_3000* of the *X. campestris* pv. *campestris* 8004 are homologous to the 5′ terminal, middle, and 3′ terminal sequences, respectively, of the ORF *XCV0442* of *X. campestris* pv. *vesicatoria*. The ORF *XC_2993*, *XC_2996*, and *XC_2997* encode putative transposases.

analysis (www.sbg.bio.ic.ac.uk/~3dpssm) (Kelley et al. 2000). Interestingly, the result showed that the N-terminal domain of XopXccR1 shares structural homology with the extracellular domain of mycobacterium tuberculosis serine/threonine protein kinase PknD, while the middle region shares structural homology with a superfamily of proteins containing tandemly repeated α-helices, known as ARM/HEAT repeats, which are commonly present in eukaryotes but are rare in bacteria (Andrade et al. 2001; Roden et al. 2004), and the C-terminal region is homologous to the constant regulatory domain of the protein phosphatase 2A. Protein phosphatase 2A is a highly regulated family of eukaryotic serine/threonine phosphatases implicated in cell growth and signaling (Janssens and Goris 2001). As a T3SS-effector protein, XopXccR1, with such eukaryotic structural motifs, may be significant for the pathogen to manipulate its host plants.

XC_3177 shows more than 60% amino-acid sequence identity to the *X. campestris* pv. *vesicatoria* effector XopQ (Roden et al. 2004) and the *P. syringae* pv. *tomato* effector HopQ1-1 (Wei et al. 2007a) (Table 2). Based on the sequence similarity, we renamed XC_3177 as XopXccQ. Although the *xopQ* mutant of *X. campestris* pv. *vesicatoria* exhibited no significant growth defect in susceptible host plants pepper and tomato (Roden et al. 2004), HopQ1-1 of *P. syringae* pv. *tomato* has been demonstrated to be an Avr protein (Wei et al. 2007a). BLAST analysis showed that XopXccQ contains a nuc_hydro_2 (NH_2) domain, a subgroup of nucleoside hydrolases, with an E value of $1e^{-60}$. These nucleoside hydrolases have been found in eukaryotes and prokaryotes. They cleave the N-glycosidic bond in nucleosides generating ribose and the respective base and vary in their substrate specificity.

XC_3802 shares 24% (41%) amino-acid identity (similarity) with the effector XopJ of *X. campestris* pv. *vesicatoria* (Noël et al. 2003). XopJ probably belongs to the YopJ/AvrRxv family of C55 cysteine proteases (Kay and Bonas 2009). Analysis of the predicted amino-acid sequence of XC_3802 showed that XC_3802 possesses the conserved characteristics of cysteine proteases (data not shown), suggesting that the effector XC_3802 may also be a member of the YopJ/AvrRxv family. As mentioned above, XC_3802 was annotated as the Avr protein AvrXccB; therefore, we retained XC_3802 as AvrXccB although its authentic avirulence function remains to be further evaluated. XC_3802 is almost identical to the XCC3731 of the *X. campestris* pv. *campestris* ATCC33913 (528^T) (Table 2). Although whether XCC3731 in strain ATCC33913 (528^T) is a T3S effector has not been experimentally validated, it has been demonstrated that mutation of *XCC3731* did not affect the pathogenicity of strain ATCC33913 (528^T) on a number of host species (Castañeda et al. 2005). This study also showed that mutation of *XC_3802* did not affect the virulence and growth of strain 8004 either. As described above, *XC_3802* is expendable among virulent strains. Being secreted into host cells, whether the effector AvrXccB plays any role in the interaction between *X. campestris* pv. *campestris* and plants needs further investigations.

XC_4273 and *XC_1553* in *X. campestris* pv. *campestris* 8004 were predicted to encode proteins containing the leucine-rich repeat (LRR) motif (Qian et al. 2005). The LRR motif is a typical protein motif commonly observed in eukaryotic proteins and appears to be implicated in the mediation of protein–protein interactions (Kobe and Kajava 2001). LRR-containing proteins have been shown to be involved in the host defense systems of both plants and mammals, and many plant *R* genes identified to date encode proteins possessing the LRR motif (Kobe and Kajava 2001; Jones and Dangl 2006). LRR motif-containing proteins have also been found in diverse groups of animal and plant bacterial pathogens and some of them have been shown to be T3S effectors; for instance, PopC and GALAs from *R. solanacearum* (Gueneron et al. 2000; Angot et al. 2006). Recently, we have demonstrated that XC_1553 of *X. campestris* pv. *campestris*, which was renamed as $AvrAC_{Xcc8004}$, is a T3S effector and functions as an Avr protein, which seems to be recognized in vascular tissues of *A. thaliana* ecotype Col-0 (Xu et al. 2008). Although homologs or analogs of XC_4273 are present in a number of other pathogens (Table 2), whether they are T3S effectors is unknown. We renamed the effector XC_4273 as XopXccLR (LR = leucine-rich repeat).

We would like to point out that the technique using AvrBs1 as a reporter and pepper for the translocation assay to identify T3S effectors in this study may have limitations, because pepper is not the host of *X. campestris* pv. *campestris* and AvrBs1 may have a high threshold level for HR induction. The effectors secreted in a relatively low amount might not be identified by this method. Therefore, in addition to the above six genes, whether any of the other genes with the PIP box listed in Supplementary Table S1 encode T3S effectors needs further investigations by other methods such as the cya assay (Casper-Lindley et al. 2002). Furthermore, a number of T3S effectors identified from different *Xanthomonas* pathogens are encoded by genes without a PIP box. It is not doubted that *X. campestris* pv. *campestris* may also possess T3S-effector genes without a PIP box. The deduced proteins encoded by the non-PIP-box-containing genes *XC_0052*, *XC_0241*, and *XC_2210* in the *X. campestris* pv. *campestris* 8004 share more than 80% amino-acid identity with the T3S effectors identified from *X. oryzae* pv. *oryzae* (Furutani et al. 2009), implying that they may be T3S effectors. Further identification of the T3S effectors encoded by non-PIP-box-containing genes in *X. campestris* pv. *campestris* has been recently initiated in our laboratory.

Many studies have shown that a block in the T3SS of a pathogenic bacterium results in a severe reduction in virulence and in planta growth of the pathogen, indicating that the T3S effectors in a pathogen contribute collectively to pathogenicity. However, apart from *avr* genes, for the overwhelming majority of T3S-effector genes identified from different pathogens so far, mutations affecting individual genes in a given pathogen gave no significant phenotypes. This may be due to apparent redundancy of the effectors in a pathogen (Gürlebeck et al. 2006; Schechter et al. 2006; Wei et al. 2007a). Interestingly, among the six T3S effectors of *X. campestris* pv. *campestris* identified in this work, five showed individually a significant contribution to the full virulence and growth of the pathogen in host plants. Does this mean that *X. campestris* pv. *campestris* harbors fewer T3S effectors with functional redundancy than other pathogens? How do these effectors contribute to disease development? These will be topics that merit further investigations.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in this work are listed in Supplementary Table S2. All of the *X. campestris* pv. *campestris* strains were grown at 28°C in the rich medium NYG (Daniels et al. 1984) or the minimal medium MMX (Daniels et al. 1984). *Escherichia coli* strains were grown in LB medium (Miller 1972) at 37°C. Antibiotics were used at the following final concentrations as required: gentamycin (Gm), 5 μg/ml; kanamycin (Km), 25 μg/ml; rifampicin (Rif), 50 μg/ml; spectinomycin (Spc), 50 μg/ml; and tetracycline (Tc), 15 μg/ml for *E. coli* and 5 μg/ml for *X. campestris* pv. *campestris*.

DNA manipulations.

Standard DNA manipulations were performed following the procedures described by Sambrook and associates (1989). The

conjugation between the *X. campestris* pv. *campestris* and *E. coli* strains was performed as described by Turner and associates (1985). Restriction enzymes and DNA ligase were used in accordance with the manufacturer's instructions (Promega, Shanghai, China).

Mutant construction and complementation.

The Tn5*gusA5* insertional mutants of the six identified T3S-effector-coding genes (*XC_2994*, *XC_2995*, *XC_3160*, *XC_3177*, *XC_3802*, and *XC_4273*), named 060E08, 204B02, 203G01, 125E09, 137F03, and 196B07, were from the insertional mutant library of the *X. campestris* pv. *campestris* 8004 in the authors' laboratory. The mutants were selected by mating the *X. campestris* pv. *campestris* 8004/pLAFR1::Tn5*gusA5* with 8005/pPH1JI (Turner et al. 1985) (the plasmids pPH1JI [Gm- and Spc-resistant] [Hirsch and Beringer 1984] and pLAFR1 [Tc-resistant] are incompatible) and by plating them on the NYG selection medium containing Rif (for 8004), Km (for Tn5*gusA5*), Gm, and Spc (for pPH1JI). The Rif-Km-Gm-Spc-resistant but Tc-sensitive individual transconjugants were chosen as candidate mutants. The genomic positions of the transposon Tn5*gusA5* in the mutants were determined by thermal asymmetric interlaced polymerase chain reaction (PCR) (Liu and Huang 1998) and subsequent sequencing for comparison with the whole genome sequence of strain 8004 (Qian et al. 2005). The insertional site of Tn5*gusA5* was further confirmed by PCR using the primers on transposon and on the gene upstream or downstream of the gene disrupted.

For complementation of the mutants, the DNA fragments containing the entire individual effector genes were amplified by PCR using the total DNA of the *X. campestris* pv. *campestris* 8004 as the template and the primer sets listed in Supplementary Table S3. After confirmation by sequencing, the amplified DNA fragments were cloned into pLAFR6. The obtained recombinant plasmids and the control plasmid pLAFR6 were transferred into the corresponding Tn5*gusA5* insertion mutants by triparental conjugation. The transconjugants were screened on NYG plates with appropriate antibiotics.

An *hpaB* mutant was constructed by homologous suicide plasmid integration as described by Windgassen and associates (2000). A 356-bp internal fragment of the *hpaB* gene (*XC_3022*, AAY50070) was amplified using the total DNA of the *X. campestris.* pv. *campestris* 8004 as template and the primer set XC3022F/XC3022R. After confirmation by sequencing, the amplified DNA fragment was cloned into pK18mob (Schafer et al. 1994) to create the recombinant plasmid pK3022. The pK3022 was introduced from *E. coli* JM109 (Yanisch-Perron et al. 1985) into the *X. campestris.* pv. *campestris* 8004 by triparental conjugation. Transconjugants were screened on NYG plates supplemented with Rif and Km, and the obtained transconjugants with a mutation in the *hpaB* gene were confirmed by PCR. Confirmation PCR was performed using the total DNA of the obtained transconjugants as templates and the primers P18conF (located in pK18mob) and XC3023R (located downstream of the *hpaB* gene). The expected PCR products were further confirmed by sequencing. A negative control was performed using the same primers and the total DNA of the *X. campestris.* pv. *campestris* 8004 as the template. One of the confirmed transconjugants was randomly selected for further study and named 8004ΔhpaB. For complementation of 8004ΔhpaB, the 1,461-bp fragment containing 483-bp *hpaB* ORF and its 542-bp upstream and 436-bp downstream flanking sequences was amplified by PCR using the total DNA of the *X. campestris* pv. *campestris* 8004 as the template and the primer set C3022-F/C3022-R, and cloned into pLAFR6 to generate the plasmid pLhpaB. The plasmid pLhpaB was introduced into the mutant 8004ΔhpaB by triparental conjugation, generating the complemented strain 8004ΔhpaB/pLhpaB. The *hrpF*-deletion mutant strain 8004ΔhrpF was constructed previously (Jiang et al. 2008).

Translocation assay.

To facilitate the identification of T3S effectors of *X. campestris* pv. *campestris*, the reporter plasmid pJAG was constructed. The artificial synthesized fragment of 3× FLAG was cloned into the *Pst*I and *Hin*dIII sites of pLAFRJ, a derivate of pLAFR3, which contains the multiple cloning sites of pUC19, generating the plasmid pJXG. The 1,174-bp DNA fragment of the 3′ end of *avrBs1* was amplified using the total DNA of the *X. campestris* pv. *campestris* 8004 as the template and the primer set 59aa-F/445aa-R (Xu et al. 2008). After confirmation by sequencing, the fragment was cloned into the *Xba*I and *Pst*I sites of pJXG, yielding the recombinant plasmid pJAG. The validities of pJAG in secretion and translocation assay were confirmed with the known T3S effector XopXccN (data not shown). The DNA fragments containing the putative promoter and T3S signal sequence of candidate genes were amplified using the total DNA of *X. campestris* pv. *campestris* 8004 as the template and the primer sets. After confirmation by sequencing, the fragments were cloned into pJAG to generate a series of recombinant plasmids. The obtained recombinant plasmids were introduced into the *avrBs1*-deletion mutant (8004ΔavrBs1), *hrpF*-deletion mutant (8004ΔhrpF), and *hpaB*-integration mutant (8004ΔhpaB) by triparental conjugation.

The ability of the obtained strains to elicit HR was tested on pepper ECW-10R (*Capsicum annuum* cv. ECW-10R), a non-host plant commonly used to test the HR of *X. campestris* pv. *campestris* (Castañeda et al. 2005; Xu et al. 2008). Bacterial cells suspended in 10 mM sodium phosphate buffer (5.8 mM Na_2HPO_4 and 4.2 mM NaH_2PO_4, pH 7.0) at an optical density 600 (OD_{600}) of 0.3 were hand infiltrated into the leaf tissues using a needleless syringe. The inoculated plants were maintained in a greenhouse with a day and night cycle of 16 and 8 h, respectively, with illumination by fluorescent lamps and a constant temperature of 28°C at 80% relative humidity. At least three plants were inoculated in each experiment and each experiment was repeated at least three times. HR symptoms were photographed 24 h postinoculation in the 8004ΔavrBs1 background or 72 h postinoculation in the 8004ΔhrpF or 8004ΔhpaB background.

Transcription assay.

The reporter plasmid pLGUS, harboring the promoterless *gusA* gene in the *Bam*HI/*Sph*I sites of pLAFR6 (Jiang et al. 2008), was employed to determine the transcription level of the identified T3S-effector genes. The DNA fragments containing the putative promoters of the identified T3S-effector genes were cloned into pLGUS. The attained recombinant plasmids were introduced into the wild-type 8004, the *hrpG*-deletion mutant 8004ΔhrpG, and the *hrpX*-deletion mutant 8004ΔhrpX by triparental conjugation. The transconjugants were screened on NYG plates with appropriate antibiotics and assayed for GUS activity. The transconjugant strains were grown in NYG for 16 h or MMX for 24 h and their GUS activities were detected by measurement of the OD_{415} using ρ-nitrophenyl-β-D-glucuronide as substrate, as described by Jefferson and associates (1986).

Virulence assay and determination of bacterial load in planta.

The virulence of *X. campestris* pv. *campestris* strains was tested on potted Chinese radish (*Raphanus sativus* var. *radiculus*) cv. Manshenhong grown in a greenhouse with a day and night cycle of 12 and 12 h, respectively, with illumination by

fluorescent lamp at temperatures of 25 to 28°C as described previously (Tang et al. 2005). Seedlings with four fully expanded leaves were used for inoculation. Bacterial cells were grown in NYG medium at 28°C with shaking at 200 rpm for 15 h. The cell concentration was adjusted to OD_{600} = 0.001. Two leaves per plant were inoculated by the leaf-clipping method (Dow et al. 2003). Sixty leaves were inoculated for each strain in each independent experiment. After being maintained at 100% humidity for 24 h, the inoculated plants were maintained in the growth conditions described above. Lesion length was measured 10 days postinoculation. Experiments were repeated independently three times.

To determine the bacterial growth of *X. campestris* pv. *campestris* strains in planta, Chinese radish leaves were inoculated by the same method used for the virulence test as described above, and five inoculated leaves for each sampling were homogenized in 9 ml of sterile water. Diluted homogenates were plated on NYG plates supplemented with appropriate antibiotics. Bacterial CFU were counted after incubation at 28°C for 3 days.

ACKNOWLEDGMENTS

This work was supported by the '973' Program of the Ministry of Science and Technology of China (2006CB101902) and the National Science Foundation of China (30870071 and 30770075).

LITERATURE CITED

Alfano, J. R., and Collmer, A. 2004. Type III secretion system effector proteins: Double agents in bacterial disease and plant defense. Annu. Rev. Phytopathol. 42:385-414.

Alvarez, A. M. 2000. Black rot of crucifers. Pages 21-52 in: Mechanisms of Resistance to Plant Diseases. A. J. Slusarenko, R. S. S. Fraser, and L. C. van Loon, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Andrade, M. A., Petosa, C., O'Donoghue, S. I., Muller, C. W., and Bork, P. 2001. Comparison of ARM and HEAT protein repeats. J. Mol. Biol. 309:1-18.

Angot, A., Peeters, N., Lechner, E., Vailleau, F., Baud, C., Gentzbittel, L., Sartorel, E., Genschik, P., Boucher, C., and Genin, S. 2006. *Ralstonia solanacearum* requires F-box-like domain-containing type III effectors to promote disease on several host plants. Proc. Natl. Acad. Sci. U.S.A. 103:14620-14625.

Arlat, M., Gough, C. L., Barber, C. E., Boucher, C., and Daniels, M. J. 1991. *Xanthomonas campestris* contains a cluster of *hrp* genes related to the larger *hrp* cluster of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. 4:593-601.

Bonas, U., Schulte, R., Fenselau, S., Minsavage, G. V., Staskawicz, B. J., and Stall, R. E. 1991. Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. Mol. Plant-Microbe Interact. 4:81-88.

Büttner, D., and Bonas, U. 2006. Who comes first? How plant pathogenic bacteria orchestrate type III secretion. Curr. Opin. Microbiol. 9:193-200.

Büttner, D., Nennstiel, D., Klüsener, B., and Bonas, U. 2002. Functional analysis of HrpF, a putative type III translocon protein from *Xanthomonas campestris* pv. *vesicatoria*. J. Bacteriol. 184:2389-2398.

Büttner, D., Gürlebeck, D., Noël, L. D., and Bonas, U. 2004. HpaB from *Xanthomonas campestris* pv. *vesicatoria* acts as an exit control protein in type III-dependent protein secretion. Mol. Microbiol. 54:755-768.

Casper-Lindley, C., Dahlbeck, D., Clark, E. T., and Staskawicz, B. J. 2002. Direct biochemical evidence for type III secretion-dependent translocation of the AvrBs2 effector protein into plant cells. Proc. Natl. Acad. Sci. U.S.A. 99:8336-8341.

Castañeda, A., Reddy, J. D., El-Yacoubi, B., and Gabriel, D. W. 2005. Mutagenesis of all eight *avr* genes in *Xanthomonas campestris* pv. *campestris* had no detected effect on pathogenicity, but one *avr* gene affected race specificity. Mol. Plant-Microbe Interact. 18:1306-1317.

Chang, J. H., Urbach, J. M., Law, T. F., Arnold, L. W., Hu, A., Gombar, S., Grant, S. R., Ausubel, F. M., and Dangl, J. L. 2005. A high-throughput, near-saturating screen for type III effector genes from *Pseudomonas syringae*. Proc. Natl. Acad. Sci. U.S.A. 102:2549-2554.

Cunnac, S., Boucher, C., and Genin, S. 2004a. Characterization of the cis-acting regulatory element controlling HrpB-mediated activation of the type III secretion system and effector genes in *Ralstonia solanacearum*. J. Bacteriol. 186:2309-2318.

Cunnac, S., Occhialini, A., Barberis, P., Boucher, C., and Genin, S. 2004b. Inventory and functional analysis of the large Hrp regulon in *Ralstonia solanacearum*: Identification of novel effector proteins translocated to plant host cells through the type III secretion system. Mol. Microbiol. 53:115-128.

Daniels, M. J., Barber, C. E., Turner, P. C., Sawczyc, M. K., Byrde, R. J. W., and Fielding, A. H. 1984. Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad host range cosmid pLAFR1. EMBO (Eur. Mol. Biol. Organ.) J. 3:3323-3328.

da Silva, A. C., Ferro, J. A., Reinach, F. C., Farah, C. S., Furlan, L. R., Quaggio, R. B., Monteiro-Vitorello, C. B., Sluys, M. A., Almeida, N. F., Alves, L. M., Do Amaral, A. M., Bertolini, M. C., Camargo, L. E., Camarotte, G., Cannavan, F., Cardozo, J., Chambergo, F., Ciapina, L. P., Cicarelli, R. M., Coutinho, L. L., Cursino-Santos, J. R., El-Dorry, H., Faria, J. B., Ferreira, A. J., Ferreira, R. C., Ferro, M. I., Formighieri, E. F., Franco, M. C., Greggio, C. C., Gruber, A., Katsuyama, A. M., Kishi, L. T., Leite, R. P., Lemos, E. G., Lemos, M. V., Locali, E. C., Machado, M. A., Madeira, A. M., Martinez-Rossi, N. M., Martins, E. C., Meidanis, J., Menck, C. F., Miyaki, C. Y., Moon, D. H., Moreira, L. M., Novo, M. T., Okura, V. K., Oliveira, M. C., Oliveira, V. R., Pereira, H. A., Rossi, A., Sena, J. A., Silva, C., De Souza, R. F., Spinola, L. A., Takita, M. A., Tamura, R. E., Teixeira, E. C., Tezza, R. I., Trindade Dos Santos, M., Truffi, D., Tsai, S. M., White, F. F., Setubal, J. C., and Kitajima, J. P. 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. Nature 417:459-463.

Dobrindt, U., Hochhut, B., Hentschel, U., and Hacker, J. 2004. Genomic islands in pathogenic and environmental microorganisms. Nat. Rev. Microbiol. 2:414-424.

Dow, J. M., Crossman, L., Findlay, K., He, Y. Q., Feng, J. X., and Tang, J. L. 2003. Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. Proc. Natl. Acad. Sci. U.S.A. 100:10995-11000.

Fenselau, S., and Bonas, U. 1995. Sequence and expression analysis of the *hrpB* pathogenicity operon of *Xanthomonas campestris* pv. *vesicatoria* which encodes eight proteins with similarity to components of the Hrp, Ysc, Spa, and Fli secretion systems. Mol. Plant-Microbe Interact. 8:845-854.

Furutani, A., Nakayama, T., Ochiai, H., Kaku, H., Kubo, Y., and Tsuge, S. 2006. Identification of novel HrpXo regulons preceded by two *cis*-acting elements, a plant-inducible promoter box and a -10 box-like sequence, from the genome database of *Xanthomonas oryzae* pv. *oryzae*. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 259:133-141.

Furutani, A., Takaoka, M., Sanada, H., Noguchi, Y., Oku, T., Tsuno, K., Ochiai, H., and Tsuge, S. 2009. Identification of novel type III secretion effectors in *Xanthomonas oryzae* pv. *oryzae*. Mol. Plant-Microbe Interact. 22:96-106.

Grant, S. R., Fisher, E. J., Chang, J. H., Mole, B. M., and Dangl, J. L. 2006. Subterfuge and manipulation: Type III effector proteins of phytopathogenic bacteria. Annu. Rev. Microbiol. 60:425-449.

Greenberg, J. T., and Vinatzer, B. A. 2003. Identifying type III effectors of plant pathogens and analyzing their interaction with plant cells. Curr. Opin. Microbiol. 6:20-28.

Gueneron, M., Timmers, A. C., Boucher, C., and Arlat, M. 2000. Two novel proteins, PopB, which has functional nuclear localization signals, and PopC, which has a large leucine-rich repeat domain, are secreted through the *hrp*-secretion apparatus of *Ralstonia solanacearum*. Mol. Microbiol. 36:261-277.

Gürlebeck, D., Thieme, F., and Bonas, U. 2006. Type III effector proteins from the plant pathogen *Xanthomonas* and their role in the interaction with the host plant. J. Plant Physiol. 163:233-255.

Guttman, D. S., and Greenberg, J. T. 2001. Functional analysis of the type III effectors AvrRpt2 and AvrRpm1 of *Pseudomonas syringae* with the use of a single-copy genomic integration system. Mol. Plant-Microbe Interact. 14:145-155.

Guttman, D. S., Vinatzer, B. A., Sarkar, S. F., Ranall, M. V., Kettler, G., and Greenberg, J. T. 2002. A functional screen for the type III (Hrp) secretome of the plant pathogen Pseudomonas syringae. Science 295:1722-1726.

He, Y. Q., Zhang, L., Jiang, B. L., Zhang, Z. C., Xu, R. Q., Tang, D. J., Qin, J., Jiang, W., Zhang, X., Liao, J., Cao, J. R., Zhang, S. S., Wei, M. L., Liang, X. X., Lu, G. T., Feng, J. X., Chen, B., Cheng, J., and Tang, J. L. 2007. Comparative and functional genomics reveals genetic diversity and determinants of host specificity among reference strains and a large collection of Chinese isolates of the phytopathogen *Xanthomonas campestris* pv. *campestris*. Genome Biol. 8:R218.

Hirsch, P. R., and Beringer, J. E. 1984. A physical map of pPH1JI and pJB4JI. Plasmid 12:139-141.

Huang, D, L., Tang, D. J., Liao, Q., Li, X. Q., He, Y. Q., Feng, J. X., Jiang, B. L., Lu, G. T., and Tang, J. L. 2009. The Zur of *Xanthomonas cam-*

pestris is involved in hypersensitive response and positively regulates the expression of the *hrp* cluster via *hrpX* but not *hrpG*. Mol. Plant-Microbe Interact. 22:321-329.
Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. Staskawicz. 1989. Bacterial blight of soybean: Regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377.
Ignatov, A. N., Monakhos, G. F., Dzhalilov, F. S., and Pozmogova, G. V. 2002. Avirulence gene from *Xanthomonas campestris* pv. *campestris* homologous to the *avrBs2* locus is recognized in race-specific reaction by two different resistance genes in *Brassica* plant species. Genetika 38:1656-1662.
Janssens, V., and Goris, J. 2001. Protein phosphatase 2A: A highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem. J. 353:417-439.
Jefferson, R. A., Burges, S. M., and Hirsh, D. 1986. β-glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc. Natl. Acad. Sci. U.S.A. 83:8447-8451.
Jiang, B. L., He, Y. Q., Cen, W. J., Wei, H. Y., Jiang, G. F., Jiang, W., Hang, X. H., Feng, J. X., Lu, G. T., Tang, D. J., and Tang, J. L. 2008. The type III secretion effector XopXccN of *Xanthomonas campestris* pv. *campestris* is required for full virulence. Res. Microbiol. 159:216-220.
Jones, J. D., and Dangl, J. L. 2006. The plant immune system. Nature 444:323-329.
Kay, S., and Bonas, U. 2009. How *Xanthomonas* type III effectors manipulate the host plant. Curr. Opin. Microbiol. 12:37-43.
Kelley, L. A., MacCallum, R. M., and Sternberg, M. J. E. 2000. Enhanced genome annotation using structural profiles in the program 3D-PSSM. J. Mol. Biol. 299:499-520.
Kobe, B., and Kajava, A. V. 2001. The leucine-rich repeat as a protein recognition motif. Curr. Opin. Struct. Biol. 11:725-732.
Koebnik, R., Kruger, A., Thieme, F., Urban, A., and Bonas, U. 2006. Specific binding of the *Xanthomonas campestris* pv. *vesicatoria* AraC-type transcriptional activator HrpX to plant-inducible promoter boxes. J. Bacteriol. 188:7652-7660.
Liu, Y. G., and Huang, N. 1998. Efficient amplification of insert end sequences from bacterial artificial chromosome clones by thermal asymmetric interlaced PCR. Plant Mol. Biol. Rep. 16:175-181.
Meyer, D., Cunnac, S., Gueneron, M., Declercq, C., Van Gijsegem, F., Lauber, E., Boucher, C., and Arlat, M. 2006. PopF1 and PopF2, two proteins secreted by the type III protein secretion system of *Ralstonia solanacearum*, are translocators belonging to the HrpF/NopX family. J. Bacteriol. 188:4903-4917.
Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
Mudgett, M. B. 2005. New insights to the function of phytopathogenic bacterial type III effectors in plants. Annu. Rev. Plant Biol. 56:509-531.
Mudgett, M. B., Chesnokova, O., Dahlbeck, D., Clark, E. T., Rossier, O., Bonas, U., and Staskawicz. B. J. 2000. Molecular signals required for type III secretion and translocation of the *Xanthomonas campestris* AvrBs2 protein to pepper plants. Proc. Natl. Acad. Sci. U.S.A. 97:13324-13329.
Nimchuk, Z. L., Fisher, E. J., Desveaux, D., Chang, J. H., and Dangl, J. L. 2007. The HopX (AvrPphE) family of *Pseudomonas syringae* type III effectors require a catalytic triad and a novel N-terminal domain for function. Mol. Plant-Microbe Interact. 20:346-357.
Noël, L., Thieme, F., Gäbler, J., Büttner, D., and Bonas, U. 2003. XopC and XopJ, two novel type III effector proteins from *Xanthomonas campestris* pv. *vesicatoria*. J. Bacteriol. 185:7092-7102.
Petnicki-Ocwieja, T., Schneider, D. J., Tam, V. C., Chancey, S. T., Shan, L., Jamir, Y., Schechter, L. M., Buell, C. R., Tang, X., Collmer, A., and Alfano, J. R. 2002. Genomewide identification of proteins secreted by the Hrp type III protein secretion system of *Pseudomonas syringae* pv. *tomato* DC3000. Proc. Natl. Acad. Sci. U.S.A. 99:7652-7657.
Qian, W., Jia, Y., Ren, S. X., He, Y. Q., Feng, J. X., Lu, L. F., Sun, Q., Ying, G., Tang, D. J., Tang, H., Wu, W., Hao, P., Wang, L., Jiang, B. L., Zeng, S., Gu, W. Y., Lu, G, Rong, L., Tian, Y., Yao, Z., Fu, G., Chen, B., Fang, R., Qiang, B., Chen, Z., Zhao, G. P., Tang, J. L., and He, C. 2005. Comparative and functional genomic analyses of the pathogenicity of phytopathogen *Xanthomonas campestris* pv. *campestris*. Genome Res. 15:757-767.
Roden, J. A., Belt, B., Ross, J. B., Tachibana, T., Vargas, J., and Mudgett, M. B. 2004. A genetic screen to isolate type III effectors translocated into pepper cells during *Xanthomonas* infection. Proc. Natl. Acad. Sci. U.S.A. 101:16624-16629.
Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.
Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G., and Puhler, A. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: Selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145:69-73.
Schechter, L. M., Roberts, K. A., Jamir, Y., Alfano, J. R., and Collmer, A. 2004. *Pseudomonas syringae* type III secretion system targeting signals and novel effectors studied with a Cya translocation reporter. J. Bacteriol. 186:543-555.
Schechter, L. M., Vencato, M., Jordan, K. L., Schneider, S. E., Schneider, D. J., and Collmer, A. 2006. Multiple approaches to a complete inventory of *Pseudomonas syringae* pv. *tomato* DC3000 type III secretion system effector proteins. Mol. Plant-Microbe Interact. 19:1180-1192.
Tang, D. J., He, Y. Q., Feng, J. X., He, B. R., Jiang, B. L., Lu, G. T., Chen, B., and Tang, J. L. 2005. *Xanthomonas campestris* pv. *campestris* possesses a single gluconeogenic pathway that is required for virulence. J. Bacteriol. 187:6231-6237.
Thieme, F., Szczesny, R., Urban, A., Kirchner, O., Hause, G., and Bonas, U. 2007. New type III effectors from *Xanthomonas campestris* pv. *vesicatoria* trigger plant reactions dependent on a conserved N-myristoylation motif. Mol. Plant-Microbe Interact. 20:1250-1261.
Tsuge, S., Terashima, S., Furutani, A., Ochiai, H., Oku, T., Tsuno, K., Kaku, H., and Kubo, Y. 2005. Effects on promoter activity of base substitutions in the cis-acting regulatory element of HrpXo regulons in *Xanthomonas oryzae* pv. *oryzae*. J. Bacteriol. 187:2308-2314.
Turner, P., Barber, C. E., and Daniels, M. J. 1985. Evidence for clustered pathogenicity genes in *Xanthomonas campestris* pv. *campestris*. Mol. Gen. Genet. 199:338-343.
Vencato, M., Tian, T., Alfano, J. R., Buell, C. R., Cartinhour, S., DeClerk, J., Guttman, D. S., Joardar, V., Lindeberg, M., Bronstein, P. A., Mansfield, J., Myers, C. R., Collmer, A., and Schneider, D. J. 2006. Bioinformatics-enabled identification of the HrpL regulon and type III secretion system effector proteins of *Pseudomonas syringae* pv. *phaseolicola* 1448A. Mol. Plant-Microbe Interact. 19:1193-1206.
Vinatzer, B. A., Jelenska, J., and Greenberg, J. T. 2005. Bioinformatics correctly identifies many type III secretion substrates in the plant pathogen *Pseudomonas syringae* and the biocontrol isolate *P. fluorescens* SBW25. Mol. Plant-Microbe Interact. 18:877-888.
Vorhölter F. J., Schneiker, S., Goesmann, A., Krause, L., Bekel, T., Kaiser, O., Linke, B., Patschkowski, T., Rückert, C., Schmid, J., Sidhu, V. K., Sieber, V., Tauch, A., Watt, S. A., Weisshaar, B., Becker, A., Niehaus, K., and Pühler, A. 2008. The genome of *Xanthomonas campestris* pv. *campestris* B100 and its use for the reconstruction of metabolic pathways involved in xanthan biosynthesis. J. Biotechnol. 134:33-45.
Wei, C. F., Kvitko, B. H., Shimizu, R., Crabill, E., Alfano, J. R., Lin, N. C., Martin, G. B., Huang, H. C., and Collmer, A. 2007a. A *Pseudomonas syringae* pv. *tomato* DC3000 mutant lacking the type III effector HopQ1-1 is able to cause disease in the model plant *Nicotiana benthamiana*. Plant J. 51:32-46.
Wei, K., Tang, D. J., He, Y. Q., Feng, J. X., Jiang, B. L., Lu, G. T., Chen, B., and Tang, J. L. 2007b. *hpaR*, a putative *marR* family transcriptional regulator, is positively controlled by HrpG and HrpX and involved in the pathogenesis, hypersensitive response, and extracellular protease production of *Xanthomonas campestris* pathovar *campestris*. J. Bacteriol. 189:2055-2062.
Wengelnik, K., and Bonas, U. 1996. HrpXv, an AraC-type regulator, activates expression of five of the six loci in the *hrp* cluster of *Xanthomonas campestris* pv. *vesicatoria*. J. Bacteriol. 178:3462-3469.
Wengelnik, K., Van den Ackerveken, G., and Bonas, U. 1996. HrpG, a key *hrp* regulatory protein of *Xanthomonas campestris* pv. *vesicatoria* is homologous to two-component response regulators. Mol. Plant-Microbe Interact. 9:704-712.
Windgassen, M., Urban, A., and Jaeger, K. E. 2000. Rapid gene inactivation in *Pseudomonas aeruginosa*. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 193:201-205.
Xu, R. Q., Blanvillain, S., Feng, J. X., Jiang, B. L., Li, X. Z., Wei, H. Y., Kroj, T., Lauber, E., Roby, D., Chen, B., He, Y. Q., Lu, G. T., Tang, D. J., Vasse, J., Arlat, M., and Tang, J. L. 2008. $AvrAC_{Xcc8004}$, a type III effector with a leucine rich repeat domain from *Xanthomonas campestris* pathovar *campestris* confers avirulence in vascular tissues of the *Arabidopsis thaliana* ecotype Col-0. J. Bacteriol. 190:343-355.
Yanisch-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103-119.

AUTHOR-RECOMMENDED INTERNET RESOURCES

National Center for Biotechnology Information BLAST server:, www.ncbi.nlm.nih.gov/
3D-PSSM website: www.sbg.bio.ic.ac.uk/~3dpssm