tale-Based Genetic Diversity of Chinese Isolates of the Citrus Canker Pathogen *Xanthomonas citri* subsp. *citri*

Gang Ye and Ni Hong, National Key Laboratory of Agromicrobiology, Huazhong Agricultural University, Wuhan, Hubei 430070, China; Li-Fang Zou, Hua-Song Zou, and Muhammad Zakria, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China; Guo-Ping Wang, National Key Laboratory of Agromicrobiology, Huazhong Agricultural University; and Gong-You Chen, School of Agriculture and Biology, Shanghai Jiao Tong University

Abstract

Ye, G., Hong, N., Zou, L.-F., Zou, H.-S., Zakria, M., Wang, G.-P., and Chen, G.-Y. 2013. *tale*-Based genetic diversity of Chinese isolates of the citrus canker pathogen *Xanthomonas citri* subsp. *citri*. Plant Dis. 97:1187-1194.

Pathotype A of *Xanthomonas citri* subsp. *citri*, the cause of citrus bacterial canker (CBC), is assumed to have originated in southern China. PthA, a type III secreted transcriptional activator-like effector (TALE), is a major pathogenicity determinant in *X. citri* subsp. *citri*. To investigate the diversity of *X. citri* subsp. *citri* in China, genomic and plasmid DNA of 105 *X. citri* subsp. *citri* isolates, collected from nine citrus-growing provinces of China, were digested by *Bam*HI and hybridized with an internal repeat region of *pthA*. Strains were classified into 14 different genotypes (designated A to N) based on the number and size of *pthA* homologues. Genotypes B and G represented 19 and

62% of the isolate collection, respectively. Genotypes J and L lacked *pthA* or a *pthA*-hybridizing fragment and were less virulent on grapefruit (*C. paradisi*) and sweet orange (*C. sinensis*) compared with strains containing *pthA* or a *pthA* homologue. The virulence of genotypes J and L was increased when the wild-type *pthA* was introduced. Genotype I, which was isolated from sweet orange in Jiangxi province, caused typical canker symptoms and may contain a novel *pthA*-like gene. To our knowledge, this is the first description of genetic diversity in Chinese CBC strains based on *tale* gene analysis.

Citrus bacterial canker (CBC) is one of the most economically important diseases of citrus in tropical and subtropical countries (9,17). Characteristic symptoms of CBC include the formation of necrotic, raised lesions on leaves, twigs, stems, and fruit. When the disease is severe, the pathogen can cause defoliation, twig dieback, decline, and premature fruit drop (18,37). The geographical origin of CBC is controversial. It is assumed that the disease may have originated in southern China with *Fortunella hindsii* as the wild host (24). In contrast, there are reports that CBC may have originated from India or Java (14,21,27). In geographical areas without CBC, the pathogen is considered a quarantine pest due to its potential threat to citrus production and food safety (18).

It is widely accepted that *Xanthomonas citri* subsp. *citri* (13,36) is one of the most devastating pathogens of commercial citrus. However, bacteria associated with CBC have been divided into different pathotypes based on host range and symptomology. These include pathotypes A (*X. axonopodis* pv. *citri*), B/C/D (*X. axonopodis* pv. *aurantifolii*), and E (*X. axonopodis* pv. *citrumelo*) (18,35,42). Three separate taxa—*X. smithii* subsp. *citri*, *X. fuscans* subsp. *aurantifolii*, and *X. alfalfae* subsp. *citrumelo*—are currently classified as *X. citri* (pathotype A), *X. fuscans* (B/C/D), and *X. alfalfae* (E) (39). Pathotype A, which is the Asiatic form of CBC, has a wide host range and is pathogenic on almost all citrus varieties. Pathotype A contains *pthA* or *pthA* homologues; *pthA* is a major virulence gene and encodes a type III secreted transcriptional activator-like effector (TALE) that promotes cellular hypertrophy (1,12,40,41).

TALEs are a structurally and functionally distinct class of proteins present in the Hrp II group of plant-pathogenic bacteria. TALE proteins are translocated into plant nuclei, bind to TALE-

specific DNA coding regions, and activate transcription of downstream genes for disease susceptibility or resistance (2,4,11,22,30–32). These statements are based on studies of AvrBs3, a TALE protein produced by the pepper and tomato pathogen *X. campestris* pv. *vesicatoria* (5). TALEs share several common features, including an N-terminal region required for secretion via the type III secretion system, a C-terminus containing nuclear localization signals, and an acidic activation domain typical of transcription factors. TALEs contain a central domain of tandem, nearly identical 33- to 34-amino-acid repeats that confer DNA-binding specificity (1,38,46). The number of repeats varies among TALEs, and the residues at the 12th and 13th positions are hypervariable and known as repeat-variable diresidues (RVDs). The RVDs bind to specific, single nucleotides and, consequently, determine specificity for disease susceptibility or resistance (2,4,31). Within the host cell, TALEs bind DNA at up-regulated by TALE boxes, a region that is not described for PthA (or PthA homologues) and citrus. The identification of new *pthA*-homologues in *X. citri* subsp. *citri* strains could advance efforts to understand this phenomenon.

There are many approaches to differentiate pathogens associated with CBC, including physiological and serological tests, phage typing, restriction enzyme analysis, total soluble protein profiles, and polymerase chain reaction (PCR)-based methods (15,16,19, 20,29,33,43,45). However, the differentiation of pathogen strains by these methods is not linked with virulence factors. To address this issue, a *pthA*-based PCR strategy to rapidly and sensitively detect CBC strains has been developed (34). However, the number of *pthA* homologues in different strains is not known and warrants further investigation. The genome sequence of a typical CBC strain (e.g., *X. citri* subsp. *citri* 306) revealed the presence of four plasmid-encoded *tale* genes, including *pthA* (10). *X. citri* subsp. *citri* strains from Korea were grouped into 13 genotypes based on *pthA* hybridization studies (25). Although the identification of *tale* genes in CBC strains may reveal potential interactions with citrus, the diversity of *tale* genes has not been studied in China, where CBC pathogens presumably originated (27).

The objective of the current study was to evaluate the distribution of *tale* genes in *X. citri* subsp. *citri* strains from China. This

Corresponding authors: G.-Y. Chen and G.-P. Wang,
E-mail: gyouchen@sjtu.edu.cn and gpwang@mail.hzau.edu.cn

Accepted for publication 16 March 2013.

was undertaken by collecting isolates from nine citrus-growing provinces and investigating the number and size of *tale* genes by hybridization analysis using *pthA* as a probe.

Materials and Methods

Isolation of bacterial strains. Samples of *Citrus sinensis*, *C. reticulata*, *C. paradisi*, *C. unshiu*, *Fortunella margarita*, and *Poncirus trifoliata* with CBC symptoms were collected from nine citrus-growing provinces in southern China from 2008 to 2011 (Table 1). Diseased tissues were surfaced sterilized and crushed in 1 ml of sterile distilled water (dH₂O) using a glass rod. Samples were plated in nutrient agar (NA) (28) and incubated at 28°C for 3 days. Yellow bacterial colonies were consistently isolated. Purified isolates were obtained from single colonies and analyzed by PCR using the *X. citri* subsp. *citri*-specific primers *J-pth1* and *J-pth2*

(8). Isolates were maintained at –80°C in fresh nutrient broth containing 25% glycerol.

Potential pathogenicity of isolates was screened in hypersensitive response (HR) assays. Bacterial suspensions 1×10^8 CFU/ml in dH₂O were infiltrated into tomato (*Lycopersicon esculentum* var. *esculentum*) and tobacco (*Nicotiana benthamiana*) leaves. Infiltration assays were repeated three times. Isolates producing an HR were deemed pathogenic and were later used for pathogenicity assay in citrus.

Southern hybridizations. Total genomic DNA was extracted from bacteria using the cetyltrimethylammonium bromide method, as described previously (3). DNA samples (3 µg) were digested with *Bam*HI at 37°C for 4 h, separated in 1.2% agarose gels, and then transferred to Hybond N⁺ nylon membranes. The probe was a digoxigenin-labeled 2.3-kb internal *Sph*I fragment of *pthA* (Gen-

Table 1. Genotype and virulence analysis of *Xanthomonas citri* subsp. *citri* isolates from China

Strains	<i>tale</i> Genes ^z	Genotype	Virulence ^y		Origin (cultivar, area)
			Grapefruit	Sweet orange	
1	5	G	++	++	<i>Citrus unshiu</i> , Jiangxi
2	5	G	++	++	<i>C. sinensis</i> , Jiangxi
3	5	A	++	++	<i>C. paradisi</i> , Guangdong
4	5	G	++	++	<i>C. sinensis</i> , ZheJiang
5	5	G	++	++	<i>C. sinensis</i> , ZheJiang
6	4	B	++	++	<i>C. sinensis</i> , Guangxi
7	5	G	++	++	<i>C. sinensis</i> , Guangxi
8	5	G	++	++	<i>C. sinensis</i> , Guangxi
9	4	B	++	++	<i>C. sinensis</i> , Guangxi
10	5	G	++	++	<i>C. sinensis</i> , Jiangxi
11	5	G	++	++	<i>C. sinensis</i> , Jiangxi
12	6	D	++	++	<i>C. sinensis</i> , Jiangxi
13	5	G	++	++	<i>C. sinensis</i> , Jiangxi
14	5	G	++	++	<i>C. sinensis</i> , Jiangxi
15	5	C	++	++	<i>C. sinensis</i> , Jiangxi
16	5	G	++	++	<i>C. sinensis</i> , Jiangxi
17	5	G	++	++	<i>C. sinensis</i> , Jiangxi
18	5	G	++	++	<i>C. sinensis</i> , Jiangxi
19	5	E	++	++	<i>C. sinensis</i> , Jiangxi
20	5	G	++	++	<i>C. sinensis</i> , Guangxi
21	4	F	++	++	<i>C. sinensis</i> , Jiangxi
22	5	G	++	++	<i>C. sinensis</i> , Jiangxi
23	5	G	++	++	<i>C. sinensis</i> , Jiangxi
24	5	G	++	++	<i>C. sinensis</i> , Jiangxi
25	5	G	++	++	<i>C. sinensis</i> , Jiangxi
26	5	G	++	++	<i>C. sinensis</i> , Jiangxi
27	4	H	++	++	<i>C. sinensis</i> , Jiangxi
28	4	H	++	++	<i>C. sinensis</i> , Jiangxi
029-1	5	I	++	++	<i>C. sinensis</i> , Jiangxi
029-2	5	J	+	+	<i>C. sinensis</i> , Jiangxi
30	5	G	++	++	<i>C. sinensis</i> , Jiangxi
31	5	G	++	++	<i>C. sinensis</i> , Jiangxi
32	5	I	++	++	<i>C. sinensis</i> , Jiangxi
33	5	G	++	++	<i>C. sinensis</i> , Jiangxi
34	5	I	++	++	<i>C. paradisi</i> , Jiangxi
35	5	I	++	++	<i>C. sinensis</i> , Jiangxi
36	5	G	++	++	<i>C. sinensis</i> , Jiangxi
37	5	G	++	++	<i>C. sinensis</i> , Jiangxi
38	5	G	++	++	<i>P. trifoliata</i> , Jiangxi
39	4	B	++	++	<i>C. sinensis</i> , ZheJiang
40	4	B	++	++	<i>C. sinensis</i> , ZheJiang
41	4	B	++	++	<i>C. sinensis</i> , ZheJiang
42	5	K	++	++	<i>C. paradisi</i> , ZheJiang
43	5	K	++	++	<i>C. paradisi</i> , ZheJiang
44	5	G	++	++	<i>C. paradisi</i> , ZheJiang
45	5	G	++	++	<i>C. paradisi</i> , ZheJiang
46	5	K	++	++	<i>C. sinensis</i> , ZheJiang
47	5	G	++	++	<i>C. sinensis</i> , ZheJiang
48	5	G	++	++	<i>C. sinensis</i> , ZheJiang
49	3	L	+	+	<i>C. sinensis</i> , ChongQing

(continued on next page)

^y Symbols: ++ indicates full virulence, where normal canker symptoms formed in the tested citrus cultivars; otherwise, + indicates reduced virulence.

^z Number of transcriptional activator-like effector (*tale*) genes in a strain was putatively figured out according to the hybridized bands displayed in Figure 2. It was assumed that there were two *tale* genes in a band which was more heavily hybridized than the upper and lower bands when the *Bam*HI-digested genomic DNA of a strain was hybridized with the internal *Sph*I fragment of *pthA* as the probe.

Bank accession number U28802.1) originating from *X. citri* subsp. *citri* strain 3213 (41). This 2.3-kb *SphI* fragment of *pthA* contains 17.5 tandem repeats of a 102-bp sequence and part of the leucine zipper coding region (Fig. 1) (38). The internal *SphI* fragment of the *pthA* gene, which was cloned in plasmid pZit45 (provided by Dr. Yong-Ping Duan) (1), was used as a probe to detect the number and size of *tale* genes in each strain. Labeling, hybridization, and detection procedures were performed as recommended by the manufacturer (Roche) and repeated twice.

Complementation studies with *pthA*. The sequences flanking the upstream and downstream of the *Bam*HI sites are almost identical in the *tale* genes of *X. oryzae* pv. *oryzae* strain KACC10331 (23) and *X. citri* subsp. *citri* strain 306 (10). In an attempt to complement Chinese *X. citri* subsp. *citri* strains lacking *pthA*, the 3.4-kb *Bam*HI fragment of *pthA* in pZit45 was subcloned into

pUAVPD, replacing the 2.4-kb *Bam*HI fragment of *avrXa3* (46) and generating the recombinant plasmid pPthA. After confirmation by sequence analysis, this plasmid was transferred into *X. citri* subsp. *citri* strains 029-2 and 049, which lack a 3.4-kb homologue of *pthA*. Recombinant *X. citri* subsp. *citri* strains containing pPthA were selected on NA with kanamycin. Putative recombinants were then verified by PCR amplification with the pPthA-specific primers *J-pth1* and *J-pth2* (8,48) and designated *X. citri* subsp. *citri* strains 029-2(pPthA) and 049(pPthA), respectively.

Pathogenicity assays and bacterial growth in planta. Citrus (*C. paradisi*, grapefruit and *C. sinensis*, sweet orange) plants were raised in pots under greenhouse conditions to establish the pathogenicity of *X. citri* subsp. *citri* strains (1,18,38,39,47). *X. citri* subsp. *citri* strains were grown for 2 days on NA at 28°C and then suspended in sterile distilled water. Bacterial suspensions (1×10^8

Table 1. (continued from preceding page)

Strains	<i>tale</i> Genes ^z	Genotype	Virulence ^y		Origin (cultivar, area)
			Grapefruit	Sweet orange	
50	4	B	++	++	<i>C. sinensis</i> , ChongQing
51	4	B	++	++	<i>C. sinensis</i> , ChongQing
52	5	G	++	++	<i>C. sinensis</i> , Jiangxi
53	5	G	++	++	<i>C. sinensis</i> , Jiangxi
54	5	G	++	++	<i>C. sinensis</i> , Jiangxi
55	5	G	++	++	<i>C. sinensis</i> , Jiangxi
56	5	G	++	++	<i>C. sinensis</i> , Jiangxi
57	5	G	++	++	<i>C. sinensis</i> , Jiangxi
58	5	G	++	++	<i>C. sinensis</i> , Jiangxi
59	5	G	++	++	<i>C. sinensis</i> , Jiangxi
60	5	G	++	++	<i>C. reticulata</i> , Guangdong
61	5	G	++	++	<i>C. reticulata</i> , Guangdong
62	4	B	++	++	<i>C. reticulata</i> , Guangdong
63	4	B	++	++	<i>C. reticulata</i> , Guangdong
64	4	B	++	++	<i>C. reticulata</i> , Guangdong
65	4	B	++	++	<i>C. reticulata</i> , Guangdong
66	4	B	++	++	<i>C. reticulata</i> , Guangdong
67	4	B	++	++	<i>C. reticulata</i> , Guangdong
68	4	B	++	++	<i>C. reticulata</i> , Guangdong
69	4	B	++	++	<i>C. reticulata</i> , Guangdong
70	4	B	++	++	<i>C. reticulata</i> , Guangdong
71	5	G	++	++	<i>C. reticulata</i> , Guangdong
72	4	B	++	++	<i>C. reticulata</i> , Guangdong
73	5	G	++	++	<i>C. reticulata</i> , Guangdong
74	4	B	++	++	<i>C. sinensis</i> , Zhejiang
75	5	G	++	++	<i>C. sinensis</i> , Zhejiang
76	5	G	++	++	<i>C. sinensis</i> , Zhejiang
77	4	B	++	++	<i>C. sinensis</i> , Zhejiang
78	5	G	++	++	<i>C. sinensis</i> , Zhejiang
79	5	G	++	++	<i>C. sinensis</i> , Zhejiang
80	5	G	++	++	<i>C. reticulata</i> , Fujian
81	5	G	++	++	<i>C. reticulata</i> , Fujian
82	5	M	++	++	<i>C. sinensis</i> , Hubei
83	5	M	++	++	<i>C. sinensis</i> , Hubei
84	5	M	++	++	<i>C. sinensis</i> , Hubei
85	5	G	++	++	<i>Fortunella margarita</i> , Guangxi
86	5	N	++	++	<i>F. margarita</i> , Guangxi
87	5	G	++	++	<i>C. sinensis</i> , Jiangxi
88	5	G	++	++	<i>C. sinensis</i> , Jiangxi
89	5	G	++	++	<i>C. unshiu</i> , Zhejiang
90	5	G	++	++	<i>C. unshiu</i> , Zhejiang
91	5	G	++	++	<i>C. unshiu</i> , Zhejiang
92	5	G	++	++	<i>C. sinensis</i> , Guizhou
93	5	G	++	++	<i>C. sinensis</i> , Guizhou
94	4	B	++	++	<i>C. sinensis</i> , Guizhou
95	5	G	++	++	<i>C. sinensis</i> , Guizhou
96	5	G	++	++	<i>C. sinensis</i> , Guizhou
97	5	G	++	++	<i>C. sinensis</i> , Guizhou
98	5	G	++	++	<i>C. sinensis</i> , Guizhou
99	5	G	++	++	<i>C. sinensis</i> , Guizhou
100	5	G	++	++	<i>C. sinensis</i> , Guangdong
101	5	G	++	++	<i>C. sinensis</i> , Guangdong
102	5	G	++	++	<i>C. sinensis</i> , Guangdong
103	5	G	++	++	<i>C. sinensis</i> , Hunan
104	5	G	++	++	<i>C. sinensis</i> , Hunan

CFU/ml) were injected into the intercellular spaces of fully expanded citrus leaves with needleless syringes. Inoculated plants were maintained in a greenhouse under a temperature regimen of 28°C (day) and 25°C (night). Twelve days after inoculation, the aggressiveness of each strain was evaluated by assessing the appearance of water-soaked margins. To measure bacterial growth in planta, leaf disks (0.8 cm in diameter) were excised with a cork borer and macerated in 1 ml of dH₂O. Macerated leaves were serially diluted, plated on NA, and incubated at 28°C for 3 days. The CFU per square centimeter of leaf tissue was calculated at 0, 2, 4, 6, 8, 10, and 12 days post inoculation (26). All of the tests above were repeated three times independently.

Results

Isolation of *X. citri* subsp. *citri*. Strains were identified as *X. citri* subsp. *citri* by PCR (Table 1) and confirmed to induce an HR

Fig. 1. Functional map of conserved regions in transcriptional activator-like effector (TALE) family members of *Xanthomonas citri* subsp. *citri* as represented by PthA. Abbreviations: B and S indicate *Bam*HI and *Sph*I sites in *pthA*, respectively. The horizontal dashed line between the two *Sph*I sites indicates the location of the probe, which is a 2.3-kb fragment containing 17.5 repeats of a 102-bp sequence involved in PthA specificity. T3S represents the secretion signal at the N-terminus of TALEs that is responsible for secretion via the type III secretion system; LZ = leucine zipper, NLS = nuclear localization signal, and AD = an acidic transcriptional activation domain.

in tomato and tobacco. The number of strains isolated from the different provinces was as follows: Jiangxi, 40; Zhejiang, 21; Guangdong, 19; Guizhou, 8; Guangxi, 7; Chongqing, 3; Hubei, 3; Hunan, 2; and Fujian, 2 (Table 2). The strains were isolated primarily from *C. sinensis* (71.43%), *C. reticulata* (16.19%), *C. paradisi* (5.71%), *C. unshiu* (3.81%), *F. margarita* (1.91%), and *P. trifoliata* (0.95%) (Table 3). Pathogenicity was assessed in grapefruit and sweet orange. *X. citri* subsp. *citri* strains 029-2 and 049, which were originally isolated from *C. sinensis*, showed reduced virulence on both grapefruit and sweet orange (Table 1), suggesting a potential genetic variation in virulence compared with the other 103 strains.

Chinese strains of *X. citri* subsp. *citri* are classified into 14 genotypes. All strains contained multiple fragments homologous to the *Sph*I fragment of *pthA* (Fig. 2). The largest and smallest hybridizing bands were approximately 4.6 and 2.0 kb, respectively (Figs. 2 and 3), suggesting that the potential *tal*e genes contain either more or fewer 102-bp repeat units than *pthA*. Some bands hybridized more intensely to the probe and may possibly contain at least two similar *tal*e genes, such as the 3.2-kb heavily hybridized band in *X. citri* subsp. *citri* 006 (Figs. 2 and 3). Thus, the putative *tal*e genes of each *X. citri* subsp. *citri* isolates (Table 1) were calculated one more than the hybridized bands if an intensively hybridized band existed correspondingly (Figs. 2 and 3). The Chinese strains generally contained four to six bands with homology to the probe, suggesting the presence of multiple *tal*e genes in all 105 strains. Because the *tal*e genes are highly conserved, the size variation of hybridizing bands presumably represents the number of 102-bp internal repeat units (Fig. 1). Based on the results in Figure 2, the strains were classified into 14 genotypes designated A to N (Table 1; Fig. 3). Notably, the strains isolated from *C. sinensis*

Table 2. Genotypes of isolates collected from different provinces of China

Location	Genotype ^w														Total ^x
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	
Jiangxi	...	...	1	1	1	1	29	2	4	1	...	...	...	...	40
Guangdong	1	10	...	...	...	...	8	...	...	...	...	...	...	...	19
Zhejiang	...	5	...	...	...	...	13	...	...	...	3	...	...	...	21
Guangxi	...	2	...	...	...	...	4	...	...	...	...	...	...	1	7
Guizhou	...	1	...	...	...	...	7	...	...	...	...	...	...	...	8
Chongqing	...	2	...	...	...	...	...	...	...	...	...	1	...	...	3
Hubei	...	...	...	...	...	...	...	...	...	...	...	...	3	...	3
Hunan	...	...	...	...	...	...	2	...	...	...	...	...	...	...	2
Fujian	...	...	...	...	...	...	2	...	...	...	...	...	...	...	2
Total ^y	1	20	1	1	1	1	65	2	4	1	3	1	3	1	105
Percent ^z	1.0	19.0	1.0	1.0	1.0	1.0	61.9	1.9	3.8	1.0	2.9	1.0	2.9	1.0	...

^w Number of isolates belonging to each genotype in each province.

^x Total number of the isolates from each province.

^y Total number of each genotype in 105 collected strains in China.

^z Genotypes in percentage of 105 collected strains in China.

Table 3. Numbers and genotypes of *Xanthomonas citri* subsp. *citri* strains in different citrus varieties

Genotype	Citrus varieties						Total
	<i>Citrus sinensis</i>	<i>C. reticulata</i>	<i>C. paradisi</i>	<i>C. unshiu</i>	<i>Fortunella margarita</i>	<i>Poncirus trifoliata</i>	
A	...	...	1	...	...	...	1
B	10	10	...	...	...	...	20
C	1	...	...	...	...	...	1
D	1	...	...	...	...	...	1
E	1	...	...	...	...	...	1
F	1	...	...	...	...	...	1
G	50	7	2	4	1	1	65
H	2	...	...	...	...	...	2
I	3	...	1	...	...	...	4
J	1	...	...	...	...	...	1
K	1	...	2	...	...	...	3
L	1	...	...	...	...	...	1
M	3	...	...	...	...	...	3
N	...	...	...	...	1	...	1
Total	75 (71.43%)	17 (16.19%)	6 (5.71%)	4 (3.81%)	2 (1.91%)	1 (0.95%)	105

belonged to 12 genotypes, excluding A and N (Table 3). The strains assigned to genotype G were distributed in seven provinces (excluding Hubei and Chongqing) and were associated with *C. sinensis*, *C. reticulata*, *C. paradisi*, *C. unshiu*, *F. margarita*, and *P. trifoliata*. These results suggest that genotype G, which represents 61.9% of the total strains used in this study, is the dominant genotype in China, followed by genotype B (Tables 2 and 3). Interestingly, the strains collected from Jiangxi exhibited more diversity than those collected from other provinces (Table 2).

Identification of Chinese strains potentially lacking *pthA*.

The *pthA* gene is essential for the pathogenicity of *X. citri* subsp. *citri* group A strains (1,41). Interestingly, when *X. citri* subsp. *citri* strains 029-1, 029-2, and 049 (genotypes I, J, and L, respectively) were hybridized with the *pthA* probe, there was no evidence of a 3.4-kb *pthA* homologue (Figs. 2 and 3), suggesting that these strains are novel. In contrast, 11 genotypes of *X. citri* subsp. *citri* strains contained a 3.4-kb *pthA* homologue (Figs. 2 and 3). Strain 029-1 (genotype I) and the other 102 strains caused typical symptoms of CBC (Table 1) when inoculated to grapefruit and sweet orange. However, strains 029-2 and 049, representing genotypes J and L, respectively, showed attenuated virulence and reduced bacterial growth in planta (Table 1; Fig. 4).

***pthA* restores full virulence to genotypes lacking a *pthA*-sized gene.** We speculated that the attenuated virulence of strains 029-2 and 049 might be due to the absence of a 3.4-kb *pthA* homologue. When pPthA was transferred into these strains, 029-2(pPthA) and 049(pPthA) caused symptoms of CBC comparable with strain 025 (Fig. 4A), which contains a *pthA* homologue. In these experiments, the wild-type *X. citri* subsp. *citri* strains 049 and 029-2 showed weak virulence and reduced water-soaking in both sweet orange and grapefruit (Fig. 4A). Bacterial growth in planta was evaluated by plating inoculated leaves to NA. At each time point, the population of 029-2(pPthA) and 049(pPthA) was equivalent to 025 and 029-1 in grapefruit and sweet orange (*data not shown*). However, the 029-2 and 049 population was significantly lower than 025 and 029-1 (Fig. 4B and C); the latter two strains either contain a 3.4-kb *pthA*-sized gene (strain 025) or a potential *pthA* homologue (strain 029-1). Thus, the results demonstrate that the introduction of *pthA* restored a full level of virulence to 029-2 and 049 when introduced *in trans*.

Discussion

In this report, we describe *tale*-based genetic diversity in bacterial strains causing CBC isolated from nine citrus-growing provinces in southern China. In all, 105 strains (Table 1) were classified into 14 genotypes (A to N) based on the size and number of *pthA* homologues. The majority of strains were assigned to genotype G (Tables 2 and 3). In general, four to six potential *tale* genes were present in Chinese strains based on Southern blot hybridizations using the internal *SphI* fragment of *pthA* as a probe (Table 1; Figs. 2 and 3). The importance of *pthA* in symptomology and bacterial growth has been previously established. Previously, *pthA* mutants of strains 3213 and KC21 exhibited decreased symptom severity and bacterial growth on citrus, which indicates that *pthA* plays a critical role in conferring virulence (38).

Southern blot analysis revealed that genotypes J and L lacked a hybridizing fragment representing the size of *pthA* (e.g. a 3.4-kb

Fig. 3. Southern blot analysis of the 14 genotypes *Xanthomonas citri* pv. *citri* strains identified in Figure 2. Genomic DNA of the 14 genotypes was digested with *Bam*HI and hybridized with a 2.3-kb internal *SphI* fragment containing the central repeat region of *pthA* (Fig. 1). Hybridized bands underlined by a dashed white line stand for transcriptional activator-like effector (*tale*) genes, possibly homologous to *pthA* in the same size. The band with an asterisk (*) marker indicates a *pthA* homologue which may be a 102-bp repeat unit larger than *pthA*. The assay was repeated twice and representative results are shown.

Fig. 2. Southern blot analysis of potential transcriptional activator-like effector (*tale*) genes in 105 strains of *Xanthomonas citri* pv. *citri*. Genomic DNA of the strains was digested with *Bam*HI and hybridized with an internal *SphI* fragment of *pthA* (Fig. 1). Numbers to the left of the lanes indicate the size of hybridizing bands (kb). Southern blot assays were repeated three times with similar results.

*Bam*HI fragment). The two strains in genotypes J and L (029-2 and 049, respectively) were attenuated in their ability to induce CBC symptoms and showed reduced growth in planta when compared with other genotypes. A high level of virulence (e.g., increased symptom severity and higher growth in planta) was conferred to strains 029-2 and 049 when the *pthA* gene was introduced *in trans*

(Fig. 4). These results further support the role of *pthA* as a major virulence effector in *X. citri* subsp. *citri*.

Pathotypes of *X. citri* pv. *citri* have been previously described with a very narrow host range. For example, the host range of pathotypes A^w from Florida and A^{*} from southwest Asia is restricted to Mexican lime (*C. aurantifolia*) (39,45). Grapefruit (*C.*

Fig. 4. Virulence analysis and bacterial growth of *Xanthomonas citri* pv. *citri* strains in grapefruit and sweet orange. **A**, Symptoms induced by *X. citri* pv. *citri* strains 025, 029-1, 029-2, 049, 029-2(pPthA), and 049(pPthA). Bacterial suspensions (approximately at 1×10^8 CFU/ml) were infiltrated into citrus leaves using a needleless syringe, and symptoms were recorded 12 days post inoculation. **B** and **C**, Bacterial growth in grapefruit and sweet orange. Numbers represent CFU per square centimeter in leaf tissue at 0, 2, 4, 6, 8, 10, and 12 days after inoculation with strains 025, 029-1, 029-2, 049, 029-2(pPthA), and 049(pPthA). Data represent the mean $\pm$ the standard deviation from three replicates, each containing three leaves. Columns labeled with different letters represent significant differences ($P = 0.01$, *t* test) between the tested strains.

paradisi) can serve as a differential host for A^w strains; this pathotype elicits a strong HR on grapefruit, whereas A^s strains show reduced symptoms or fail to elicit an HR on this host (6). In the present study, assays for the HR and pathogenicity indicated that the 105 CBC strains did not include A^w and A^s pathotypes. This conclusion is based on the absence of strains triggering an HR in grapefruit or showing attenuated symptoms on this host.

In the present study, we were not able to unequivocally confirm that the 3.4-kb hybridizing band in 102 of the strains was *pthA*. However, the results do suggest that all strains contain multiple *tale* genes, which likely contain different numbers of repeat units and RVDs. The largest (4.6-kb *Bam*HI fragment in genotype A) and smallest (2.0-kb *Bam*HI fragment in genotype E) *pthA* homologues could potentially contain 29 and 8 RVDs, respectively, based on the conserved, structural properties of TALEs (Fig. 1), although confirmation by sequence analysis is needed. Our results also suggest that some of the *tale* genes may be redundant; for example, the genotype H strain (*X. citri* subsp. *citri* 027) did not contain a 4.0-kb *pthA* homologue (Figs. 2 and 3) but showed full virulence in the tested host plants (Table 1). A new screening or assessment approach may be required to precisely define the roles of these different *tale* genes. In the related pathogen, *X. oryzae* pv. *oryzae*, TALEs with substantial effects on virulence were shown to activate members of the *OsSWEET* family of sucrose transporter genes (7) to promote disease. In a recent study, a TALE-free strain of *X. oryzae* was used to dissect the function of individual TALEs on rice cultivars (44), a promising approach that could also be used for *X. citri*.

Further study of the *tale* genes in *X. citri* subsp. *citri* may reveal an evolutionary interaction with citrus host plants. The *tale*-based diversity of Chinese strains may be more complex than CBC strains isolated from Korea (25), India (45), and others citrus-growing regions (27). Genomic sequencing of the Asian group A strain 306 (10) and our *X. citri* subsp. *citri* strain 029-1 (*unpublished data*) indicate that *tale* genes are plasmid borne in *X. citri* subsp. *citri*, indicating that horizontal gene transfer and homologous recombination could easily occur in *tale* loci and result in new *tale* genes that improve pathogen fitness (46). A *pthA*-homologue gene in 029-1, possibly a repeat unit larger than *pthA* (Figs. 2 and 3), may act similarly to *pthA* as a major virulence effector. We assume that the RVDs in this TALE may be different from that of *pthA*.

We acknowledge that the genetic diversity reported herein is not a comprehensive sampling of what might reside throughout the whole citrus-growing regions in China. Nonetheless, the present study indicates that the majority of CBC strains could be assigned to the G genotype (Tables 1–3) and four to six potential *tale* genes were present in strains (Table 1; Figs. 2 and 3). The strains and genotypes defined in this study will be useful in designing future studies aimed at defining genetic diversity in newly isolated strains of CBC.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31230059), the Special Fund for Agro-Scientific Research in the Public Interest (201003067-09), and the National Science and Technology Project (2007BAD61B05). We thank Y.-P. Duan at the United States Department of Agriculture for providing plasmid pZit45, C.-X. Chen at the Fruit Protection Station for CBC sample collection in Jiangxi province, and the Department of Agriculture of China for official permission to collect CBC samples from citrus-growing regions in China.

Literature Cited

- Al-Saadi, A., Reddy, J. D., Duan, Y. P., Brunings, A. M., Yuan, Q., and Gabriel, D. W. 2007. All five host-range variants of *Xanthomonas citri* carry one *pthA* homolog with 17.5 repeats that determines pathogenicity on citrus, but none determine host-range variation. *Mol. Plant-Microbe Interact.* 20:934-943.
- Antony, G., Zhou, J., Huang, S., Li, T., Liu, B., White, F., and Yang, B. 2011. Rice xa13 recessive resistance to bacterial blight is defeated by induction of the disease susceptibility gene Os-11N3. *Plant Cell* 22:3864-3876.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1995. *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.
- Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, A., and Bonas, U. 2009. Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 326:1509-1512.
- Bonas, U., Stall, R. E., and Staskawicz, B. 1989. Genetic and structural characterization of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria*. *Mol. Gen. Genet.* 218:127-136.
- Brunings, A. M., and Gabriel, D. W. 2003. *Xanthomonas citri*: breaking the surface. *Mol. Plant Pathol.* 4:141-157.
- Chen, L. Q., Hou, B. H., Lalonde, S., Takanaga, H., Hartung, M. L., Qu, X. Q., Guo, W. J., Kim, J. G., Underwood, W., Chaudhuri, B., Chermak, D., Antony, G., White, F. F., Somerville, S. C., Mudgett, M. B., and Frommer, W. B. 2010. Sugar transporters for intercellular exchange and nutrition of pathogens. *Nature* 468:527-532.
- Cubero, J., and Graham, J. H. 2002. Genetic relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR. *Appl. Environ. Microbiol.* 68:1257-1264.
- Das, A. K. 2003. Citrus canker—a review. *J. Appl. Hortic.* 5:52-60.
- Da Silva, A. C. R., Ferro, J. A., Reinach, F. C., Farah, C. S., Furlan, L. R., Quaggio, R. B., Monteiro-Vitorello, C. B., Van Sluys, M. A., Almeida, N. F., Alves, L. M. C., Amaral, A. M., Bertolini, M. C., Camargo, L. E. A., Camarotte, G., Cannavan, F., Cardoso, J., Chambergo, F., Ciapina, L. P., Cicarelli, R. M. B., Coutinho, L. L., Cursino-Santos, J. R., El-Dorry, H., Faria, J. B., Ferreira, A. J. S., Ferreira, R. C. C., Ferro, M. I. T., Formighieri, E. F., Franco, M. C., Greggio, C. C., Gruber, A., Katsuyama, A. M., Kishi, L. T., Leite, R. P., Lemos, E. G. M., Lemos, M. V. F., Locali, E. C., Machado, M. A., Madeira, A. M. B. N., Martinez-Rossi, N. M., Martins, E. C., Meidanis, J., Menck, C. F. M., Miyaki, C. Y., Moon, D. H., Moreira, L. M., Novo, M. T. M., Okura, V. K., Oliveira, M. C., Oliveira, V. R., Pereira, H. A., Rossi, A., Sena, J. A. D., Silva, C., De Souza, R. F., Spinola, L. A. F., Takita, M. A., Tamura, R. E., Teixeira, E. C., Tezza, R. I. D., 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.
- Deng, D., Yan, C., Pan, X., Mahfouz, M., Wang, J., Zhu, J. K., Shi, Y., and Yan, N. 2012. Structural basis for sequence-specific recognition of DNA by TAL effectors. *Science* 335:720-723.
- Duan, Y. P., Castaneda, A., Zhao, G., Erdos, G., and Gabriel, D. W. 1999. Expression of a single, host-specific, bacterial pathogenicity gene in plant cells elicits division, enlargement, and cell death. *Mol. Plant-Microbe Interact.* 12:556-560.
- Dye, D. W., Bradbury, J. F., Goto, M., Hayward, A. C., Lelliot, R. A., and Schroth, M. N. 1980. International standards for naming pathogens of phytopathogenic bacteria and a list of pathovar names and pathotype strains. *Rev. Plant Pathol.* 59:153-168.
- Fawcett, H. S., and Jenkins, A. E. 1933. Records of citrus canker from herbarium specimens of the genus *Citrus* in England and the United States. *Phytopathology* 23:820-824.
- Egel, D. S., Graham, J. H., and Stall, R. E. 1991. Genomic Relatedness of *Xanthomonas campestris* strains causing diseases of citrus. *Appl. Environ. Microbiol.* 57:2724-2730.
- Gabriel, D., Hunter, G., Kingsley, M., Miller, J., and Lazo, G. 1988. Clonal population structure of *Xanthomonas campestris* and genetic diversity among citrus canker strains. *Mol. Plant-Microbe Interact.* 1:59-65.
- Gottwald, T. R., Graham, J. H., and Schubert, T. S. 2002. Citrus canker: The pathogen and its impact. *Plant Health Prog.* Online publication. doi:10.1094/PHP-2002-0812-01-RV
- Graham, J. H., Gottwald, T. R., Cubero, J., and Achor, D. S. 2004. *Xanthomonas axonopodis* pv. *citri*: Factors affecting successful eradication of citrus canker. *Mol. Plant Pathol.* 5:1-15.
- Graham, J. H., Hartung, J. S., Stall, R. E., and Chase, A. R. 1990. Pathological, restriction-fragment length polymorphism, and fatty acid profile relationship between *Xanthomonas campestris* from citrus and noncitrus hosts. *Phytopathology* 80:829-836.
- Hartung, J. S. 1992. Plasmid-based hybridization probes for detection and identification of *Xanthomonas campestris* pv. *citri*. *Plant Dis.* 76:889-893.
- Hasse, C. H. 1915. *Pseudomonas citri*, the cause of citrus canker. *J. Agric. Res.* 4:97-100.
- Lahaye, T., and Bonas, U. 2001. Molecular secrets of bacterial type III effector proteins. *Trends Plant Sci.* 6:479-485.
- Lee, B. M., Park, Y. J., Park, D. S., Kang, H. W., Kim, J. G., Song, E. S., Park, I. C., Yoon, U. H., Hahn, J. H., Koo, B. S., Lee, G. B., Kim, H., Park, H. S., Yoon, K. O., Kim, J. H., Jung, C. H., Koh, N. H., Seo, J. S., and Go, S. J. 2005. The genome sequence of *Xanthomonas oryzae* pathovar *oryzae* KACC10331, the bacterial blight pathogen of rice. *Nucleic Acids Res.* 33:577-568.
- Lee, H. A. 1918. Further data on the susceptibility of rutaceous plants to citrus canker. *J. Agric. Res.* 15:661-666.
- Lee, S., Lee, J., Lee, D. H., and Lee, Y. H. 2008. Diversity of *pthA* Gene of *Xanthomonas* strains causing citrus bacterial canker and its relationship with virulence. *Plant Pathol. J.* 24:357-360.
- Li, J. Y., and Wang, N. 2012. The *gpsX* gene encoding a glycosyltransferase is important for polysaccharide production and required for full virulence in

- Xanthomonas citri* subsp. *citri*. BMC Microbiol. 12:31.
27. Li, W., Song, Q., Brlansky, R. H., and Hartung, J. S. 2007. Genetic diversity of citrus bacterial canker pathogens preserved in herbarium specimens. Proc. Natl. Acad. Sci. USA 104:18427-18432.
28. Li, Y. R., Che, Y. Z., Zou, H. S., Cui, Y. P., Guo, W., Zou, L. F., Biddle, E. M., Yang, C. H., and Chen, G. Y. 2011. Hpa2 is required by HrpF to translocate *Xanthomonas oryzae* TAL effectors into rice for pathogenicity. Appl. Environ. Microbiol. 77:3809-3818.
29. Louws, F. J., Fulbright, D. W., Stephens, C. T., and de Bruijn, F. J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strain generated with repetitive sequences and PCR. Appl. Environ. Microbiol. 60:2286-2295.
30. Mak, A. N., Bradley, P., Cernadas, R. A., Bogdanove, A. J., and Stoddard, B. L. 2012. The crystal structure of TAL effector *PthXo1* bound to its DNA target. Science 335:716-719.
31. Moscou, M. J., and Bogdanove, A. J. 2009. A simple cipher governs DNA recognition by TAL effectors. Science 326:1501.
32. Ponciano, G., Ishihara H., Tsuyumu, S., and Leach, J. E. 2003. Bacterial effectors in plant disease and defense: keys to durable resistance? Plant Dis. 87:1272-1282.
33. Pruvost, O., Hartung, J. S., Civerolo, E. L., Dubios, C., and Perrier, X. 1992. Plasmid DNA fingerprints distinguish pathotypes of *Xanthomonas campestris* pv. *citri*, the causal agent of citrus bacterial canker disease. Phytopathology 82:485-490.
34. Rigano, L. A., Marano, M. R., Castagnaro, A. P., Do Amaral, A. M., and Vojnov, A. A. 2010. Rapid and sensitive detection of citrus bacterial canker by loop-mediated isothermal amplification combined with simple visual evaluation methods. BMC Microbiol. 10:176-173.
35. Schaad, N. W., Postnikova, E., Lacy, G., Sechler, A., Agarkova, I., Stormberg, P. E., Stormberg, V. K., and Vidaver, A. K. 2005. Reclassification of *Xanthomonas campestris* pv. *citri* (ex Hasse 1915) Dye 1978 forms A, B/C/D, and E as *X. smithii* subsp. *citri* (ex Hasse) sp. nov. nom. rev. comb. nov., *X. fuscans* subsp. *aurantifolii* (ex Gabriel 1989) sp. nov. nom. rev. comb. nov., and *X. alfalfae* subsp. *citrumelo* (ex Riker and Jones) Gabriel et al., 1989 sp. nov. nom. rev. comb. nov.; *X. campestris* pv. *malvacearum* (ex Smith 1901) Dye 1978 as *X. smithii* subsp. *smithii* nov. comb. nov. nom. rev.; *X. campestris* pv. *alfalfae* (ex Riker and Jones, 1935) Dye 1978 as *X. alfalfae* subsp. *alfalfae* (ex Riker et al., 1935) sp. nov. nom. rev.; and "var. *fuscans*" of *X. campestris* pv. *phaseoli* (ex Smith, 1987) Dye 1978 as *X. fuscans* subsp. *fuscans* sp. nov. Syst. Appl. Microbiol. 28:494-518.
36. Schaad, N. W., Postnikova, E., Lacy, G., Sechler, A., Agarkova, I., Stormberg, P. E., Stormberg, V. K., and Vidaver, A. K. 2006. Emended classification of *xanthomonad* pathogens on citrus. Syst. Appl. Microbiol. 29:690-695.
37. Schubert, T. S., and Miller, J. W. 1996. Bacterial citrus canker. Pages 110-111 in: Plant Pathology Circular Volume 377. Florida Department of Agriculture and Consumer Services DoPI, Gainesville.
38. Shiotani, H., Fujikawa, T., Ishihara, H., Tsuyumu, S., and Ozaki, K. 2007. A *pthA* homolog from *Xanthomonas axonopodis* pv. *citri* responsible for host-specific suppression of virulence. J. Bacteriol. 189:3271-3279.
39. Sun, X. A., Stall, R. E., Jones, J. B., Cubero, J., Gottwald, T. R., Graham, J. H., Dixon, W. N., Schubert, T. S., Chaloux, P. H., Stromberg, V. K., Lacy, G. H., and Sutton, B. D. 2004. Detection and characterization of a new strain of citrus canker bacteria from key Mexican lime and Alemow in South Florida. Plant Dis. 88:1179-1188.
40. Swarup, S., De Feyter, R., Brlansky, R. H., and Gabriel, D. W. 1991. A pathogenicity locus from *Xanthomonas citri* enables strains from several pathovars of *Xanthomonas campestris* to elicit canker-like lesions on citrus. Phytopathology 81:802-809.
41. Swarup, S., Yang, Y., Kingsley, M. T., and Gabriel, D. W. 1992. An *Xanthomonas citri* pathogenicity gene, *pthA*, pleiotropically encodes gratuitous avirulence on nonhosts. Mol. Plant-Microbe Interact. 5:204-213.
42. Vauterin, L., Hoste, B., Kersters, K., and Swings, J. 1995. Reclassification of *Xanthomonas*. Int. J. Syst. Bacteriol. 45:472-489.
43. Vauterin, L., Yang, P., Hoste, B., Vancannet, M., Civerolo, E. L., Swings, J., and Kersters, K. 1991. Differentiation of *Xanthomonas campestris* pv. *citri* strains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins, fatty acid analysis, and DNA-DNA hybridization. Int. J. Syst. Bacteriol. 41:535-542.
44. Verdier, V., Triplett, L. R., Hummel, A. W., Corral, R., Cernadas, R. A., Schmidt, C. L., Bogdanove, A. J., and Leach, J. E. 2012. Transcription activator-like (TAL) effectors targeting *OsSWEET* genes enhance virulence on diverse rice (*Oryza sativa*) varieties when expressed individually in a TAL effector-deficient strain of *Xanthomonas oryzae*. New Phytol. 196:1197-1207.
45. Verniere, C., Hartung, J. S., Pruvost, O. P., Civerolo, E. L., Alvarez, A. M., Maestri, P., and Luisetti, J. 1998. Characterization of phenotypically distinct strains of *Xanthomonas axonopodis* pv. *citri* from Southwest Asia. Eur. J. Plant Pathol. 104:477-487.
46. Wu, X. M., Li, Y. R., Zou, L. F., and Chen, G. Y. 2007. Gene-for-gene relationships between rice and diverse *avrBs3/pthA* avirulence genes in *Xanthomonas oryzae* pv. *oryzae*. Plant Pathol. 56:26-34.
47. Yan, Q., and Wang, N. 2011. The ColR/ColS two-component system plays multiple roles in the pathogenicity of the citrus canker pathogen *Xanthomonas citri* subsp. *citri*. J. Bacteriol. 193:1590-1599.
48. Yang, Y. N., and Gabriel, D. W. 1995. *Xanthomonas* avirulence/pathogenicity gene family encodes functional plant nuclear targeting signals. Mol. Plant-Microbe Interact. 8:627-631.
49. Young, J. M., Dye, D. W., Bradbury, J. F., Panagopoulos, C. G., and Robbs, C. F. 1978. A proposed nomenclature and classification for plant pathogenic bacteria. N.Z. J. Agric. Res. 21:153-177.