Plant Pathol. J. 24(3) : 357-360 (2008)

The Plant Pathology Journal

Note

Diversity of *PthA* Gene of *Xanthomonas* Strains Causing Citrus Bacterial Canker and its Relationship with Virulence

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(Received on July 1, 2008; Accepted on August 20, 2008)

Several pathotypes have been recognized in citrus bacterial canker, which causing serious damage in citrus cultivation area. To control the disease, it is important to understand the pathological diversity and reason of difference in virulence of the causal pathogen. We analyzed 124 strains of *Xanthomonas* causing citrus bacterial canker by southern hybridization with an internal 3.4-kb *Bam*HI fragment from *pthA* gene. Assuming each band represented an intact gene, each strain of *Xanthomonas* was estimated to have approximately 1 to 4 copies of *pthA* gene. *X. a.* pv. *citri* A type had more than 3 copies of *pthA* gene, and the number of *pthA* gene in *X. a.* pv. *citri* A^*, A^w, and *X. a.* pv. *aurantifolii* B, C were different from 1 to 3 according to the strains. When the *pthA* gene profile was classified into 13 groups according to the number and size of hybridization bands, most of the A types belong to the 3A group, and 4A and 4B type was dominant when they had 4 bands. However, there was no general pattern of difference between the virulence and *pthA* gene group in this test.

Keywords **:** *avrBs3*, citrus bacterial canker, *Xanthomons axonopodis*. pv. *citri*

Citrus bacterial canker is an economically important disease in many tropical and subtropical countries, and quarantine restrictions have been enforced to prevent the spread of the pathogen to new areas (Graham et al., 2004). However, these efforts were not enough to contain its spread. In view of the importance of the disease, it is important to understand the pathological diversity and difference of virulence of the causal pathogen.

The plant-pathogen interactions are generally controlled by gene-for-gene complementarity (Daniels and Leach, 1993; Flor, 1955). In gene-for-gene interactions between plants and the pathogens, host plant resistance results from the genetic recognition of resistance genes in the plant and play a role in bacterial virulence in susceptible host plants (Yang et al., 1994). A pathogenicity gene, *pthA*, which is found in many *Xanthomonas* strains, is essential for the pathogen to cause hyperplastic canker symptoms on citrus (Swarup et al., 1991). Southern hybridization, restriction analysis, and partial DNA sequencing have shown that *pthA* belongs to a major *avrBs3/pthA* gene family (Swarup et al., 1992). When transferred to other xanthomonads, *pthA* confers the ability to induce hyperplastic cankers on citrus and a hypersensitive response on other hosts (Swarup et al., 1992). Therefore, *pthA* exhibits pleiotropic pathogenicity and avirulence functions.

In view of the great contribution of the *pthA* gene in citrus and the causal pathogen interaction, it is important to know the diversity of the gene. The objective of this research was to study the diversity of *avrBs3/pthA* genes of *X. a. pv. citri* strains collected from Korea. Moreover the difference of virulence on citrus plants was also compared between the strains of *X. a.* pv. *citri* A types based on the difference of *pthA* genes.

Bacterial strains were isolated and cultured as described in Lee et al. (2008). The citrus bacterial canker strains of which the pathotype was already known were obtained as reference strain from several institutes as described in Table 1. Total genomic DNA and plasmid DNA of 124 isolates was extracted following the procedure of Lee et al. (2008). The DNA from each strain were digested with *Bam*HI and separated in 0.8% agarose gels and southern blot hybridization was performed as described by Sambrook et al. (1989). The membranes were probed with ^{32}P-labled 3.4 kb internal *Bam*HI fragment of *AvrBs3/pthA* gene in pLAFR3 plasmid. The tested *Xanthomonas* strains revealed at least one band that hybridized to an internal 3.4-kb *Bam*HI fragment from *pthA*. At least one other hybridizing band of different size was present in each strain excepting *X. a.* pv. *citri* A^* strain IR04 and *X. a.* pv. *aurantifolii* B strain CFBP 2868 (Fig. 1A). Assuming each band represented an intact gene, each strain of *Xanthomonas* was estimated to have approximately 1 to 4 copies of *pthA* genes. DNA sequence analyses revealed an unusually high level of sequence conservation among *pth* gene members of Xanthomonads (Yang et al., 1994). Due to the very high level of sequence conservation among members of the *AvrBs3/pthA* gene family, the slight size differences of these bands, which

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Table 1. *Xanthomonas* isolates used in this study

Type	Name	Origin	Source[a]
X. a. pv. *citri* A	CFBP2859	Brazil	CIRAD
	JH410-1	China	CIRAD
	C43, CFBP1814	France	CIRAD
	CFBP2900	Japan	CIRAD
	JJ238-3, JK4-3	Korea	CIRAD
	CFBP2525	New Zealand	CIRAD
	JK148-2	Philippines	CIRAD
	M9, A-5246, M5, A-5208	USA	DPI
	SL-0870, 0874, 4021, 4024, 4026, 4028, 4029, 4036, 4040, 4041, 4043, 4044, 4046, 4047, 4050, 4054, 4056, 4057, 4062, 4063, 4064, 4066, 4068, 4070, 4071, 4072, 4073, 4090, 4093, 4095, 4096, 4098, 4099, 4100, 4468, 4469, 4474, 4477, 4478, 4483, 4484, 4492, 4493, 4500, 4501, 4510, 4511 4516, 4517, 4518, 4520, 4525, 4526, 4528, 4529, 4530, 4537, 4538, 4539, 4547, 4548, 4553, 4554, 4556, 4558, 4560, 4562, 4564, 4566, 4567, 4568, 4915, 4916, 4917, 4918, 4920, 4922, 4925, 4926, 4928, 4931, 4932, 4933, 4934, 4935, 4937, 4943, 4945, 4946, 4947, 4949, 4950, 4952, 4953, 4954, 4955, 4956, 4958, 4990	Korea	PPD
	KACC10443	Korea	KACC
X. a. pv. *citri* A*	IR01, IR02, IR03, IR04	Iran	PPDSI
	JF90-2	Oman	CIRAD
	JK2-10	Saudi Arabia	CIRAD
	A-1609	USA	DPI
X. a. pv. *citri* A^{w}	A-2032	USA	DPI
X. a. pv. *aurantifolii* B	CFBP2868, CFBP2903	Argentina	CIRAD
X. a. pv. *aurantifolii* C	CFBP2866	Brazil	CIRAD

[a]CIRAD, The Agricultural Research Centre for International Development; DPI, Division of Plant Industry, Florida, USA; PPD, Plant Pathology Division, NIAST, Korea; PPDSI, Plant Pests and Diseases Research Institute, Iran; KACC, Korean Agricultural Culture Collection.

were presumed to represent the internal repeat fragments of *avrBs3/pthA* genes, were likely due to different numbers of repeat units within the *BamH1* fragment. *X. a.* pv. *citri* A strain 306 has four members of the *pthA* family of proteins, named *pthA1*, *pthA2*, *pthA3* and *pthA4* (da Silva et al. 2002). Only *pthA4* is the same size as *pthA*, and *pthA2* and *pthA*3 gene have the same repeat regions, which is two repeats less than *pthA*. This sequence homology results indicate that the same size of *pthA* genes could be shown as one band, suggesting more than one copy of *pthA* gene could be present in the tested strains.

The profile of bands was classified into 13 groups according to the number and size of hybridization bands (Fig. 1B). Interestingly, the number of *pthA* gene was more than 3 in *X. a.* pv. *citri* A type (Table 2). The number of *pthA* genes in *X. a.* pv. *citri* A*, A^{w} and *X. a.* pv. *aurantifolii* B, C were different from 1 to 3 according to the strains. In case of *X. a.* pv. *citri* A type, 89 from 113 strains showed 3A band type, and 4A and 4B type was dominant in the group having 4 bands.

Pahtogenicity of Xanthomonads is not solely expressed by *pthA* gene (Kingsley et al., 1993). However the *pthA* genes of *X. a.* pv. *citri* are required for the full virulence to the citrus plants (Swarp et al., 1991), and the number and organization of the 102 bp repeats are key factors determining the interaction with plant resistance genes (Bonas et al., 1993; Herbers et al., 1992; Leach and White, 1996; Yang et al., 1995). Bacterial effectors encoded by *avrXa7*, *avrXa10*, and *apl1* might suppress hypersensitive reaction and associated phenotypes (Fujikawa et al., 2006) Wu et al. (2007) argued that the reasons why so many *pthA* genes are present in the pathogens and how new *pthA* genes arise remains to be determined. Recently, Schornack et al. (2008) reported that recognition efficiency of *AvrHah1* gene, a novel avrBs3-like gene, is predominantly determined by solvent-exposed residues, rather than by overall homology or repeat unit length.

To check the relationship between the number of *pthA* gene and virulence, six strains of *X. a.* pv. *citri* A type were tested for their virulence. The aggressiveness was evaluated after inoculation into leaves of different citrus host plants, such as *C. sinensis* (sweetorange), *C. paradisi* (grapefruit), *C. limon* (lemon and rough lemon), and *C. unshiu* (mandarin; Banpeiyu and Heungjinjosang). Bacterial strains were grown on peptone sucrose medium with 1.5% agar at 28°C for 1 day. A sharp insect pin was dipped in a bacterial

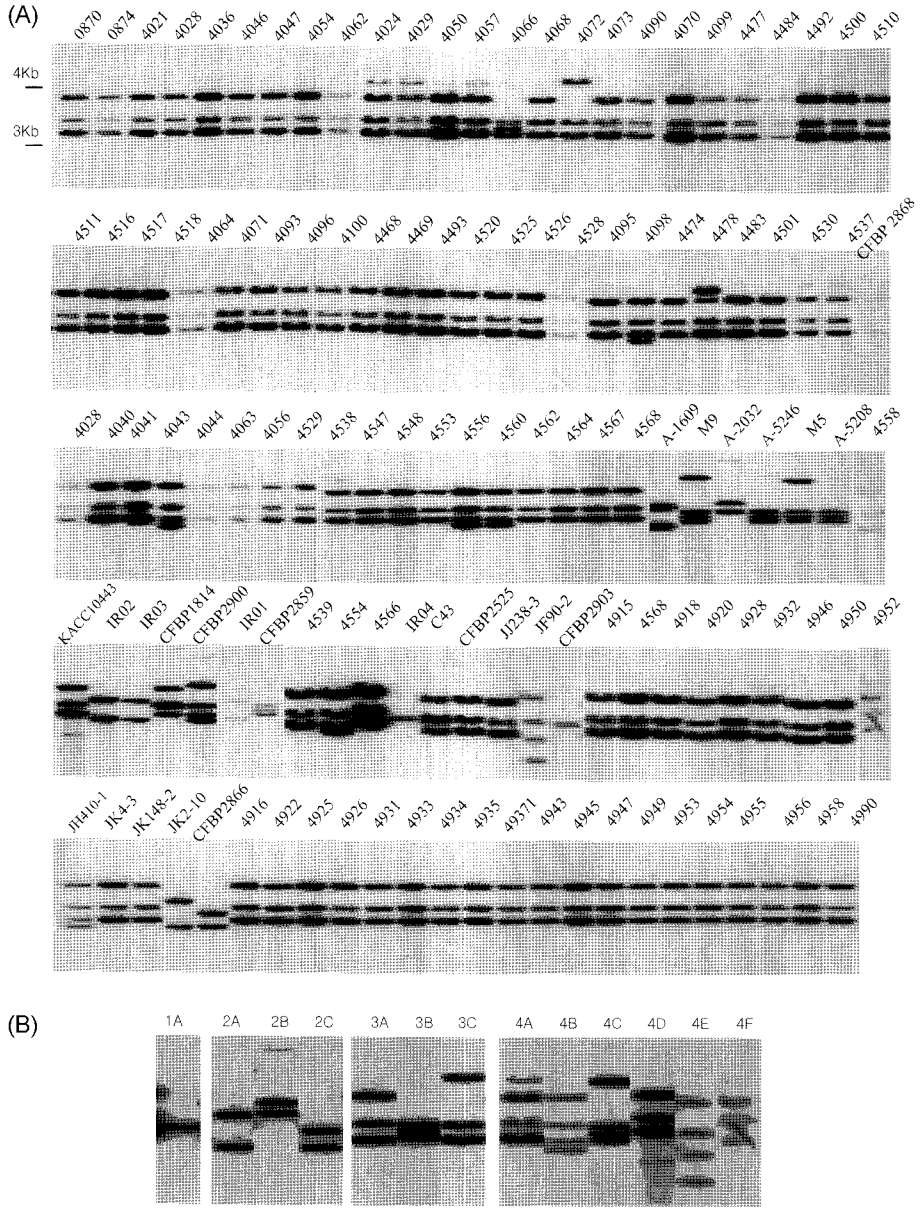

Fig. 1. Southern hybridization patterns of *AvrBs3/pthA* genes and representative group of *pthA* gene of *Xanthomonas* strains. (A) Total genomic DNA was digested with *BamHI* and probed with the internal 3.4-kb *BamHI* fragment of *avrBs3*. (B) The group was generated by the number and size of the *avrBs3/pthA* gene.

suspension about 10^8 cfu/ml and the pin was used to prick the epidermis. Four leaves of each citrus plant were used for each strain, and the assays were repeated three times. The inoculated plants were grown in a greenhouse in which the temperature was about 28°C. The diameters of the circular lesions, including cork tissue and yellow margin, were measured 8 weeks after inoculation and the inoculated plants were destroyed. There was diverse response of pathogenicity depending on the strains and citrus varieties. Grapefruit, sweet orange and Banpeiyu was more susceptible than the other host plants. However, there was no general pattern between difference of the pathogenicity and *pthA* gene group, which needs more investigation. For example, the strain 4096 and 4553 which belong to the same *pthA* group 3A had a wide difference of virulence in most of the tested host plants.

Overall the results of this test indicate that *X. a.* pv. *citri* A type has more copy of *pthA* gene than the other pathotypes, such as *X. a.* pv. *aurantifolii* B and C type. There weren't any general relationships between the number of the *pthA* genes and the geographical origin of the strains. Moreover, the number of the strain in each group was not directly related with the number of *PthA* gene. In our knowledge, this is the first report for analysis of *pthA* gene diversity

Table 2. Diversity of *pthA* gene depending on the pathotypes of *Xanthomonas*

pthA Group[a]	No. of strains[b]				
	A	A*	Aw	B	C
1A		1		1	
2A		5			
2B			1		
2C					1
3A	89			1	
3B	4				
3C	1				
4A	8				
4B	7				
4C	2				
4D	1				
4E		1			
4F	1				
Total No. of strains	113	7	1	2	1

[a]The pattern of *pthA* gene as described in Fig. 1A.
[b]A, *X. axonopodis* pv. *citri* A; A*, *X. a.* pv. *citri* A*; Aw, *X. a.* pv. *citri* Aw; B, *X. a.* pv. *aurantifolii* strains; and C, *X. a.* pv. *citrumelo*.

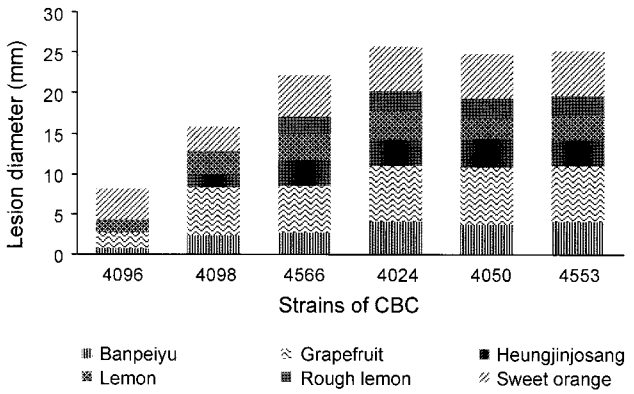

Fig. 2. Difference of lesion diameter 8 weeks after inoculation. A sharp insect pin was dipped in a bacterial suspension about 10^8 cfu/ml and the pin is used to prick the epidermis of citrus plants. Four leaves of each citrus plant were used for each strain, and the assays were repeated three times. The diameters of the circular lesions, including cork tissue and water-soaked margin, were measured 8 weeks after inoculation and the inoculated plants were destroyed.

with wide variety of bacterial collections causing citrus bacterial canker. However, the meaning of the difference in the number of *pthA* genes and the relationships with host plants still remains to be elucidated.

Acknowledgement

This research was supported by Agriculture R & D Promotion Center, Ministry of Agriculture and Forestry, Korea, 2005 project (105058-03-1-HD120).

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