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Genetic diversity of *Prunus necrotic ringspot virus* infecting stone fruit trees grown at seven regions in China and differentiation of three phylogroups by multiplex RT-PCR

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ABSTRACT

Prunus necrotic ringspot virus (PNRSV) is an important pathogen of stone fruit trees (*Prunus* sp.) worldwide. In this study, 35 out of 166 (21.1%) samples of *Prunus* sp. randomly collected from seven regions in China were tested to be positive for PNRSV by reverse transcription-polymerase chain reaction (RT-PCR). The coat protein (CP) gene of 28 isolates and movement protein (MP) gene of 15 isolates shared 87.1–100% and 82.9–99.9% nucleotide sequence identity, respectively. Phylogenetic analyses of CP and MP gene sequences revealed three well-defined phylogroups represented by isolates PV96, PV32 and PE5, with distribution frequencies of 37.5%, 56.3% and 6.3%, respectively. Sequence variations around a hexanucleotide insertion at sites 124–129 in the CP gene in six PV32-type isolates from *Prunus avium* resulted in an alteration of secondary structure. A multiplex RT-PCR assay based on CP gene sequences was developed to differentiate isolates in three phylogroups.

1. Introduction

Prunus necrotic ringspot virus (PNRSV) causes economically important diseases in most *Prunus* species grown worldwide (Aparicio et al., 1999; Cui et al., 2012a; Myrta et al., 2001; Oliver et al., 2009; Uyemoto and Scott, 1992). The virus can be transmitted by pollen, seed and vegetative propagation materials (Greber et al., 1991; Vasková et al., 2000). PNRSV is a member of the genus *Ilarvirus* in the family *Bromoviridae*, possessing a genome consisting of three single-stranded, positive-sense RNAs. RNA 1 and RNA 2 encode proteins P1 and P2 involved in viral replication; the bicistronic RNA 3 encodes a 5′ proximal movement protein (MP) gene, and a 3′ proximal coat protein (CP) gene expressed via subgenomic RNA (Aparicio et al., 2010; Bujarski et al., 2012).

Serological and phenotypic diversities of PNRSV have been documented. In sweet cherry (*Prunus avium* L.), PNRSV induces a wide range of phenotypic responses from none (asymptomatic) to severe rugose mosaic symptoms on leaves (Crosslin and Mink, 1992; Howell and Mink, 1988). PNRSV isolates from naturally infected cherry trees have been divided into three serotypes (designated CH3, CH9, and CH30) with the CH9 serotype containing the greatest number of isolates (Crosslin and Mink, 1992; Mink et al., 1987).

Numerous PNRSV isolates have been characterized by comparison of MP and CP sequences. Three phylogroups, named PV32–I, PV96-II and PE5-III, have been identified (Aparicio and Pallás, 2002; Aparicio et al., 1999; Cui et al., 2012b; Fiore et al., 2008; Hammond, 2003; Sanchez-Navarro and Pallás, 1997; Spiegel et al., 1999; Vasková et al., 2000), and an additional phylogroup CH30, grouped previously in the PE5 group, has been proposed based on CP sequences (Glasa et al., 2002). Comparisons of MP and CP sequence diversity among PNRSV isolates demonstrate a correlation between serotype and pathotype (Hammond and Crosslin, 1998). Despite such variation, overall deduced amino acid (aa) sequences of the CP, regardless of geographic origins and host species, are highly conserved among PNRSV isolates (Scott et al., 1998). The available

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results suggest that there is no clear correlation between PNRSV phylogroups and host or geographic origins since PNRSV isolates from different host species and locations distributed into the three phylogroups (Aparicio and Pallás, 2002; Aparicio et al., 1999; Fiore et al., 2008; Glasa and Sub, 2002; Hammond, 2003; Vasková et al., 2000). However, it was observed that pathogenicity of some PNRSV isolates was related to molecular characteristics of their MP or CP gene. In phylogenetic trees based on MP or CP sequences, PNRSV isolates of the CH30 serotype (CH30 and CH71) clustered in the PE5 group, the mild isolates of the CH9 serotype (CH61 and CH39) clustered into the PV96 group, and the severe isolates (CH9, CH38 and CH57) causing rugose symptoms clustered into a subgroup within the PV32 group (Aparicio et al., 1999; Cui et al., 2012b; Hammond and Crosslin, 1998). Generally, isolates in PV32 group were more virulent than those in the PV96 group (Hammond, 2003). Recently, our results also showed PV32-type PNRSV isolate Pch12 was more virulent than PV96-type isolate Chr3 in experimental host cucumber cv. 'Straight Eight' or natural host cherry cv. 'Bing' (Cui et al., 2012b, 2013). Therefore, establishment of rapid and sensitive protocols for detection and grouping of PNRSV isolates may provide an important tool to manage diseases caused by PNRSV.

Although the presence of PNRSV in China has been documented (Hou et al., 2002; Li et al., 2009; Yu et al., 2013; Zhou et al., 1996), limited molecular information for PNRSV isolates infecting stone fruit trees is available. In this study, incidence of PNRSV in *Prunus* sp. grown in China was investigated by RT-PCR, and molecular diversity of Chinese PNRSV isolates was characterized based on sequences of CP and MP genes. This study provides useful information which enhances understanding of the molecular evolution of PNRSV.

2. Materials and methods

2.1. Materials

Leaf or young shoot samples were randomly collected from 166 *Prunus* trees grown in seven regions of China (Fig. S1), including 129 and 5 samples from Hubei and Jiangxi provinces in central China, 19 and 5 from Shandong and Henan provinces in northern China, 3 and 1 from Jiangsu and Zhejiang provinces in eastern China, and 1 from Shanxi in western China. Out of 166 samples, 110 samples from peach (*Prunus persica* L. Batsch), 4 from flowering peach (*Prunus mume* L.), 6 from nectarine (*P. persica* L. *var. nucipersica* Schneider), 28 from sweet cherry, 5 from flowering cherry (*Prunus serrulata* L.), 11 from plum (*Prunus domestica* L.), and 2 from apricot (*Prunus armeniaca* L.) (Table 2). All sampled trees were over five years old. Except for 13 sweet cherry samples collected from a germplasm collection, other samples were collected from commercial orchards or ornamental trees. Most of samples were collected in the spring and without regard to presence of symptoms. For the sampling of peach from Hubei provinces, samples were taken from three to five trees at each orchard. For other species, one to three trees at each location or one tree of each variety in the germplasm collection were sampled. All samples were analyzed for presence of PNRSV by reverse transcription-polymerase chain reaction (RT-PCR). Leaves or shoots from the same plant were pooled as one sample.

Table 2
Sources and species of host plants and the PNRSV detection results by RT-PCR.

Origin	Host species	Infected No./Tested no.		Positive no.
		CP	MP	
Hubei	Peach (*Prunus persica*)	11/102	6/80	13
	Sweet cherry (*P. avium*)	0/9	0/9	0
	Plum (*P. domestica*)	2/11	0/11	2
	Apricot (*P. armeniaca*)	0/1	0/1	0
	Flowering peach (*Prunus mume*)	0/3	0/3	0
	Nectarine (*P. persica* var. nucipersica)	3/6	–	3
Shandong	Sweet Cherry (*P. avium*)	10/15	8/13	10
	Flowering cherry (*P. serrulata*)	4/4	1/1	4
Henan	Sweet Cherry (*P. avium*)	1/5	–	1
Jiangxi	Peach (*P. persica*)	0/5	0/5	0
Jiangsu	Peach (*P. persica*)	0/3	–	0
Zhejiang	Flowering peach (*P. mume*)	1/1	–	1
Shanxi	Apricot (*P. armeniaca*)	1/1	1/1	1

Ten samples positive for PNRSV, of which FchrYT3, FchrYT4 and ChrYT26 belonged to PV96 group, PchHN1, PchHN2, PchHN9, ChrYT2, ChrYT5, and ChrYT6 belonged to PV32 group and Pch-b belonged to PE5 group, were used for multiplex RT-PCR analysis. Virus-free peach GF305 seedlings, maintained in a greenhouse, were used as negative controls in all RT-PCR tests.

2.2. Total nucleic acid extraction

Total nucleic acids were extracted from 100 mg leaf samples using a cetyltrimethyl ammonium bromide (CTAB)-based method essentially as described by Li et al. (2008). Total nucleic acid samples were dissolved in 400 μL DEPC-treated H_2O and mixed with an equal volume of 4.0 M LiCl. RNA was allowed to precipitate at −20 °C for 4 h (or overnight at 4 °C), followed by centrifugation at 4 °C for 15 min at 12,000 rpm. Pellets were washed with 75% ethanol, centrifuged at 12,000 rpm for 5 min, air-dried and dissolved in 40 μL DEPC-treated water. All RNA samples were stored

Table 1
Primers used for the RT-PCR and multiplex RT-PCR detection of PNRSV isolates.

Primer	Sequence (5′–3′)[a]	Target gene and/region[b]	Position (nt)	Product size (bp)	Reference
MG2	ATGGTTTGCCGAATTTGCAAT	CP	1–21	~680	Glasa et al., 2000
MG1	ACTCTAGATCTCAAGCAGGTC	CP	664–681		
MP-F	GATTGTTGGTTGTCTATTC	5′-UTR	–	~1000	this study
MP-R	GATTGCAAATTCGGCAAAC	ITS	–		
MP-F1	AGTGGAGTGTTCTATGGACGAA	MP	45–66	~910	
MP-R1	TATGATTGCAAATTCGGCAAAC	ITS	–		
PNR449	ACGCGCAAAAGTGTCGAAATCTAAA	CP	507–531	–	
PV96-CP	TCCCCGAATTCCTAAGGGG	CP	222–240	–	
PV32-CP	GAATAACCCGAATARGADTAGG	CP	108–129	–	
PE5-CP	TGACTCTTTTAACKRTTATCAA	CP	341–362	–	
PV96-MP	GGCCTACACATCTGTAAATC	MP	246–264	–	
PV32-MP	GATTTTCACCATTCGAACCTAA	MP	781–805	–	
PE5-MP	CGACTCTACCAGGGAWCGA	MP	414–432	–	

[a] D, R and K = A/G/T, A/G, and G/T, respectively.
[b] MP, movement protein; CP, coat protein; 5′-UTR, 5′-terminal untranslated region of RNA3; ITS, intergenic space between MP and CP genes.