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Virulence determination and molecular features of peach latent mosaic viroid isolates derived from phenotypically different peach leaves: A nucleotide polymorphism in L11 contributes to symptom alteration

LiPing Wang[a,b,c,d], Yan He[a,b,c,d], YanPing Kang[a,b,c,d], Ni Hong[a,b,c,d], Abu Bakr Umer Farooq[a,b,c,d], GuoPing Wang[a,b,c,d,**], WenXing Xu[a,b,c,d,*]

[a] State Key Laboratory of Agricultural Microbiology, Wuhan, Hubei 430070, PR China
[b] College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, PR China
[c] National Indoor Conservation Center of Virus-free Germplasms of Fruit Crops, Wuhan, Hubei 430070, PR China
[d] Lab of Key Lab of Plant Pathology of Hubei Province, Wuhan, Hubei 430070, PR China

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ABSTRACT

Symptoms of chlorosis along leaf edges (chlorosis-edge), along leaf veins (chlorosis-vein) and yellowing on peach leaves have been observed for a long history in the field, while the pathological factor(s) responsible for these symptoms remained unknown. Peach latent mosaic viroid (PLMVd) was detected in the leaves collected from three unique phenotypic peach trees showing above mentioned symptoms. The obtained PLMVd isolates were subjected to population structure analyses and biological assays to evaluate their pathogenicity on peach seedlings in an effort to elucidate the relationship between the PLMVd and the symptoms observed on peach trees in China. In addition, molecular features of PLMVd isolates were analyzed to obtain some insight into the structure–function relationships of this viroid. The results revealed that the symptoms of chlorosis-edge and yellowing were indeed incited by PLMVd, and a direct link between the nucleotide polymorphisms and the symptoms of yellowing and chlorosis-edge was established, i.e. residue U_{338} responsible for the yellowish symptom and C_{338} responsible for the chlorosis-edge symptom. This study provides an additional proof to endorse a previous proposal that PLMVd pathogenicity determinants reside in L11. The illustrative etiology of the disease, visualization of the symptoms progression and identification of the unique single nucleotide polymorphism possibly involved in the symptom induction will significantly increase understanding of the pathogenic mechanisms of PLMVd and will help in designing control strategies for the resulting disease.

1. Introduction

With a humble genome size (250–400 nt) and simple structure, viroids are giants in terms of functional versatility in the world of RNA, infecting higher plants and cause devastating plant diseases (Ding, 2009). Viroid RNAs do not code for proteins and thus depend on RNA sequence/structural motifs for interacting with host proteins to induce disease in host (Flores et al., 2012). Nucleotide polymorphisms in certain regions of some viroid variants are directly related to specific diseases or to dramatic alterations in symptom severity (for a review see Flores et al., 2005). Identification of the specific viroid sequence/structural elements is one of the most important and exciting challenges for future studies on viroid–host interactions (Ding, 2009).

Peach latent mosaic viroid (PLMVd) is approximately 336–351 nt in length, and is a typical species of the genus *Pelamoviroid* in the family *Avsunviroidae*. It does not include a central conserved region (CCR), replicates and accumulates in the chloroplast through asymmetric rolling-circle mechanism, and possesses self-cleaving properties by forming hammerhead ribozyme structures (Hernández and Flores, 1992). PLMVd possesses a branched secondary structure including stems P1–P11, Loops 1–11, and always forms two pseudoknots including P8 and the one formed by the interaction between nucleotides of Loops 1 and 11 (Bussière et al., 2000; Pelchat et al., 2000; Dubé et al., 2010).

PLMVd occurs in most commercial peach varieties worldwide, damages peach trees, affects fruit quality, reduces tree longevity,

* Corresponding author at: College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, PR China. Tel.: +86 27 87282498; fax: +86 27 87397735.
** Co-corresponding author.
E-mail addresses: gpwang@mail.hzau.edu.cn (G. Wang), xuwenxing@mail.hzau.edu.cn (W. Xu).

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and increases susceptibility to other biotic and abiotic stresses (for a review see Flores et al., 2006). The most conspicuous symptoms under field conditions are fruit deformation, bud necrosis, rapid aging of the trees, delay in foliation, flowering and ripening, and in some cases yellow mosaic, blotch or albinism (peach calico, PC) on the infected leaves (Ambrós et al., 1998). Symptoms of chlorosis along leaf edges (chlorosis-edge), chlorosis along leaf veins (chlorosis-vein) and yellowing have been observed for a long history, while the pathological factor(s) and mechanisms responsible for these symptoms remained unknown. PLMVd was already suspected as an etiological agent as it induces a wide foliar symptoms and occurs with a high incidence of 46.6–73.0% in China. Until now, Koch's postulates were only fulfilled on building the correlation between PLMVd and the symptoms of mosaic and PC by inoculating index seedlings with purified viroid RNAs, infectious cDNA clones or RNAs constructed in vitro (Flores et al., 1990). Additional leaf symptoms related to PLMVd requires further study.

Numerous reports demonstrated that viroids propagate in their hosts as a population of similar sequences (quasi-species) and display high plasticity in their genomes (for a review see Góra-Sochacka, 2004). The evolutionary rate of PLMVd in its natural host observed by inoculating single infectious cDNAs on the peach indicator GF-305 suggested the rapid accumulation of sequence heterogeneity after infection. Most progeny variants had unique sequences, revealing extremely heterogeneous character of PLMVd natural isolates and the very dynamic nature of the viroid populations (Ambrós et al., 1998, 1999; Fekih et al., 2007; Pelchat et al., 2001). Furthermore, most of the naturally occurring infections are asymptomatic on leaves, symptoms are unstable and frequently disappear with time, or the prolonged time, at least 2 years is required for the onset of symptoms under field conditions (for a review see Flores et al., 2006). The intrinsic ability of PLMVd to accumulate changes, together with the unstable symptomology has impeded the establishment of a correlation between a phenotype and any individual genotype (Ambrós et al., 1998, 1999). The only successful reported case was of PC symptom, which was shown to be incited by PLMVd variants containing a 12–13 nt insertion having a characteristic "UUUU" core in L11 between position 1 and 337 in the predicted secondary structure of PLMVd RNA (Malfitano et al., 2003; Rodio et al., 2006). Effort to map the pathogenicity determinant(s) of characteristic mosaic was also made by gaining insight into the molecular basis of the different phenotypic effects following PLMVd infection, but failed due to high variability observed in the progeny genomic sequences (Ambrós et al., 1998). More motifs responsible for other symptoms remained unidentified.

In this work, PLMVd isolates from different phenotypic peach trees were identified and subjected to population structure analysis and biological assay to evaluate their pathogenicity on its natural host in an effort to clarify the relationship between PLMVd and the symptoms found on peach trees in China. In addition, molecular features of PLMVd isolates were analyzed to obtain some insight into the structure–function relationships. Here we present the results to provide evidence suggesting that the symptoms of chlorosis-edge and yellowing are indeed incited by PLMVd, and a single nucleotide polymorphism located in L11 contributes to the symptoms alteration.

2. Materials and methods

2.1. Plant material

Young leaves of peach trees possessing unique symptoms were collected from different orchards in spring of 2009, in Wuhan, Hubei Province, PR China. Three samples of leaves from three trees were collected, in which dxh2.1, dxh12.1 and wh5.3 exhibited symptoms of chlorosis-vein, chlorosis-edge and yellowing, respectively.

2.2. Nucleic acid extraction, RT-PCR, and population structure analysis

Total RNA was extracted from the three or four peach leaves showing obvious disease symptoms were subjected to RNA extraction according to a previous method (Xu et al., 2008). The extracted RNAs were subjected directly to reverse transcription. The first-strand cDNA was synthesized using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega Corp., Madison, WI, USA) using reported primer Pr1 (5′-ATCACACCCCCCTCGGAACCAA-3′) (Hernández and Flores, 1992), which was complementary to position 205–184 on the PLMVd reference sequence (Fig. 2). Double-strand DNA was amplified with *Taq* DNA polymerase (TaKaRa Biotechnology Corp., Dalian, China) with primer Pr1 and Pr 2 (5′-CCAGGTACCGCCGTAGAAACTG-3′), which is identical to position 206–227 on the PLMVd reference sequence. PCR amplification was carried out using a thermal cycler (PTC-200; MJ Research Inc., Watertown, MA, USA). The reaction conditions were adjusted for 35 cycles as follow: 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, after predenaturation for 3 min at 94 °C, followed by a final extension of 7 min at 72 °C. PCR products were subjected to electrophoresis in 1% agarose gels, purified using the agarose gel DNA purification kit and ligated into vector pMD18-T (TaKaRa Biotechnology Corp., Dalian, China), and followed by transformation into *Escherichia coli* DH5α.

The constructed plasmids were used as template to recover the full-length cDNA by PCR amplification using the above protocol, which were then subjected to population structure analyses using single-strand conformation polymorphism (SSCP) described before (Xu et al., 2006). Aliquots of PCR products were mixed with denaturing loading buffer (95% formamide, 10 mmol l^{-1} NaOH, 0.05% bromphenol blue, 0.05% xylene cyanol), heated for 8 min at 100 °C and chilled on ice for 3 min. The samples were then loaded into 8% non-denaturing polyacrylamide (acrylamide:bisacrylamide, 49:1) gels and run at 200 V in a refrigerator at 4 °C in 1× TBE buffer (89 mmol l^{-1} Tris, 89 mmol l^{-1} boric acid, 2.5 mmol l^{-1} EDTA, pH 8.3) for 14 h. The gel was then stained with silver nitrate solution.

2.3. Construction of dimeric RNA for infectivity assay

The clones of predominant variants were PCR amplified using the phosphorylated primer Pr1 and non-phosphorylated primer Pr2 in the presence of *Pfu* polymerase (Bio Basic Canada Inc., Markham, Ontario, Canada). Only primer Pr1 was phosphorylated to reduce the possibility of unwanted dimerized products (i.e. non-head-to-tail copies) in the subsequent ligation reaction. The PCR products were polymerized with T4 DNA ligase in the presence of 1× T4 DNA ligase buffer (NEB, Ipswich, MA, USA), and the dimeric cDNAs were purified using the agrose gel DNA purification kit (Daopson Corp., Beijing, China). The dimeric cDNAs were then ligated into the pGEM-T vector after the addition of an A nucleotide to its 3′ termini in the presence of *Taq*, 0.5 μl of 10 mM dATP and 1× *Taq* buffer (TaKaRa Biotechnology Corp., Dalian, China) in a 50 μl reaction volume at 72 °C for 25 min. The vectors containing the PLMVd dimeric cDNAs were transformed into *E. coli* DH5α. The constructed dimeric cDNAs were sequenced to confirm that they harbored two head-to-tail copies of their original version.

The plasmids containing the dimeric full-length cDNAs were extracted, linearized by digestion with *Sal* I (TaKaRa Biotechnology Corp., Dalian, China), and subjected to in vitro transcription in the presence of T7 RNA polymerase (Thermo Fisher Scientific Inc., Shanghai, China) as described previously (Xu et al., 2012).