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Complete genome sequence of an isolate of papaya leaf distortion mosaic virus from commercialized PRSV-resistant transgenic papaya in China

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Summary. – Papaya leaf distortion mosaic virus is highly destructive to commercial papaya production. Here, the complete genome sequence was determined for an isolate of papaya leaf distortion mosaic virus, designated PLDMV-DF, infecting the commercialized papaya ringspot virus (PRSV)-resistant transgenic papaya from China. Excluding the 3'-poly (A) tail, the sequence shares high sequence identity to several PLDMV isolates from Taiwan and Japan and is phylogenetically most closely related to the isolate from Japan. Infection of PLDMV-DF in transgenic PRSV-resistant papaya may indicate emergence of this disease in genetically engineered plants. The reported sequence for this isolate may help generate bi-transgenic papaya resistant to PRSV and PLDMV.

Keywords: papaya leaf distortion mosaic virus; papaya ringspot virus; complete genome; transgenic papaya

Papaya (*Carica papaya* L.), found in tropical and subtropical regions, is important to the diet and agriculture of many countries. Commercial papaya production can be devastated by the potyvirus papaya ringspot virus (PRSV), believed to be the most widespread and destructive disease affecting this crop (Manshardt, 1992; Ventura *et al*., 2004; Tripathi *et al*., 2008). However, a related virus identified in 1954, papaya leaf distortion mosaic virus (PLDMV), is similarly destructive and has limited the production of papaya in Okinawa, Japan (Kawano and Yonaha, 1992).

PLDMV infection is characterized by mosaic and distortion on leaves, water soaking streaks on petioles, and ring spots on papaya fruits (Kawano and Yonaha, 1992; Maoka *et al*., 1996). Because these symptoms overlap with those of PRSV, as do host range, non-persistently aphid-transmitted mode, and physical characteristics of the virus, the disease was initially thought to be PRSV (Maoka *et al*., 1996; Chen *et al*., 2002). However, serological diagnosis and complete genome sequencing of the virus revealed that PLDMV is a distinct species in the genus *Potyvirus* (Maoka and Hataya, 2005; Bau *et al*., 2008;). Several PLDMV isolates from Taiwan have also been detected (Bau *et al*., 2008). Complete genome sequences have been reported for the PLDMV isolate from Japan (Japan-J56p, Acc. No. AB088221) and two isolates from Taiwan (Taiwan-CZ and KS, Acc. Nos. JX416282 and EU233272). Another isolate from Taiwan, designated P-TW-WF, was found in diseased papaya that had been transgenically made resistant to PRSV by introduction of the coat protein (CP) gene (Bau *et al*., 2008). Up to date, the complete genome sequence of the PLDMV P-TW-WF isolate has not been reported.

PRSV-resistant transgenic papayas have been used to successfully control the disease caused by PRSV in some countries. Indeed, transgenic papayas have been planted commercially in Hawaii, United States since 1998 (Gonsalves, 1998; Tecson Mendoza *et al*., 2008). In 2006, China approved one new PRSV-resistant transgenic papaya, designated Huanong No. 1, containing the PRSV replicase (NIb) gene, for commercialization (Tecson Mendoza *et al*., 2008;

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Abbreviations: pipo = pretty interesting potyviridae ORF; PLDMV = papaya leaf distortion mosaic virus; PRSV = papaya ringspot virus

Guo *et al.*, 2009). Last year, we observed that some Huanong No. 1 lines exhibited symptoms similar to those caused by PRSV in Hainan Island, China. To determine the origin of this disease, we isolated and sequenced the viral genome from the PRSV-resistant plants.

We report here the complete genome sequence of PLDMV-DF isolated from commercialized PRSV-resistant transgenic papaya in China and describe molecular evidence that PLDMV-DF represents a unique and novel PLDMV isolate. Sequencing was initiated using two specific primer pairs (PLDMV-F: CCCTCCTTgCTTAgTCTgAAgTTC CAT, PLDMV-R: CgATATCCAATA AACTCACAAT gCCAAAAgT; PRSV-F: GACATATCTGGTGTCTGGGT TATGAT; PRSV-R: CTCTCATTCCAAGAGGCTCGAATAG) designed based on the conserved region of the 3'-untranslated region (UTR) of previously identified PLDMV and PRSV isolates. RT-PCR and sequence analyses revealed that symptoms in Huanong No. 1 papaya lines resulted from PLDMV infection, which is not prevented in plants resistant to PRSV. To our knowledge, this is the first report of PLDMV in papaya in Hainan Island and mainland of China. This evidence suggests that development and wide application of PRSV-resistant transgenic papaya may lead to the emergence of PLDMV.

Fresh samples of Huanong No. 1 PRSV-resistant papaya leaves showing severe distorted and mosaic symptoms were collected in August 2012 from an experimental plot in Dongfang, Hainan, China, and stored at -80°C until use. Total RNA was extracted from the diseased leaves using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Seven primer pairs were designed to produce overlapping amplicons spanning the PLDMV-DF draft genome sequence according to the complete nucleotide sequences of PLDMV- Japan-J56P from Okinawa and PLDMV-Taiwan-KS from Taiwan using the Takara RNA PCR Kit (AMV) Ver. 3.0 (Takara Bio, Japan) (Table 1). These seven overlapping DNA fragments covering the genome sequence of PLDMV-DF and ranging in sizes from 800 nt to 2,100 nt were amplified and cloned into pMD 18-T vector (Takara Bio, Japan), and at least three independent clones of each fragment were sequenced. Subsequently, the SMARTer RACE cDNA Amplification Kit (Clontech Bio, USA) was used to determine the 5'-terminal sequences including the 5'- UTR and a part of the P1-coding sequence based on two genome-specific nested reverse primers (Table 1). Amplicons were cloned and sequenced in both directions. Thus, sequences of these fragments were assembled and analyzed using Vector NTI Advance 11.0 software (Invitrogen, USA), and the complete genome nucleotide sequence of PLDMV-DF was deposited in the GenBank database (Acc. No. JX974555).

Table 1. Primers used for RT-PCR and RACE

Primer designation[a]	Position[b] (nt)	Primer sequence (5′→3′)
First-strand cDNA synthesis		
Oligo dT-Adapter Primer	provided by RNA PCR kit	GTTTTCCCAGTCACGAC$(T)_n$
PCR		
pldmv1F(+)	1-30	AAAAATATAAAAACTCAACAAAACTTATgC
pldmv1R(-)	1759-1791	CAAGTTCCTCTTCTTGTGACAACATTTCAAGCA
pldmv2F(+)	1628-1657	TCGAGATAGACCTAAGAACGCTCATGAGTG
pldmv2R(-)	3103-3137	CCAGTACTTTAAAGCTAATTCTAAATGCTGACTAT
pldmv3F(+)	2935-2967	ATTATTTAGTAGGTGGAGACCTCCATAGCAAGC
pldmv3R(-)	5039-5068	ATgTTggTTgCTACAATgAAATgTTTCTTC
pldmv4F(+)	4858-4886	GAACTGGGTCTGCACGAGATGTAATCAAT
pldmv4R(-)	6608-6640	AGAGGATTGTGTGGTTGCAAATCTACTTTCATG
pldmv5F(+)	6426-6455	TACATAGATCCCATAACTGGAGCAACGCGT
pldmv5R(-)	8136-8165	TGTCCATGGGCATTTCATATTAAGATCATA
pldmv6F(+)	7954-7985	TGCTACAGCTTAGTTGTCTCCGATTATTCAAG
pldmv6R(-)	10127-10153	CCCTCCTTgCTTAgTCTgAAgTTCCAT
3′RACE		
pld9286F(+)	9286-9316	CgATATCCAATAAACTCACAATgCCAAAAgT
M13 Primer M4(-)	provided by RNA PCR kit	GTTTTCCCAGTCACGAC
5′RACE		
UPM(Long) (+)	provided by RACE kit	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
UPM(Short) (+)	provided by RACE kit	CTAATACGACTCACTATAGGGC
pld302R(-)	273-302	CGCGCATCTATCAGTACAACCAAGAGTTAC
pld290R(-)	261-290	AGTACAACCAAGAGTTACTTCTTGGTAGTG

[a] (+) matching or (-) reverse complementary to virus sequence; [b] the primer positions refer to the PLDMV-DF sequence.

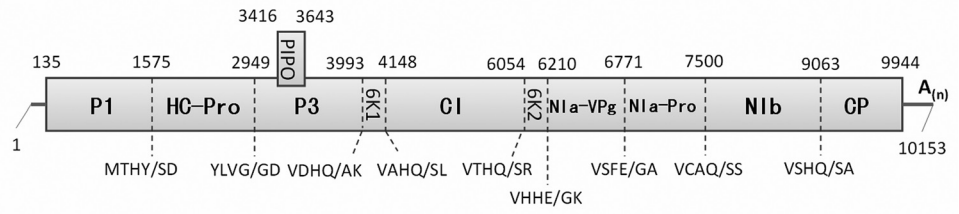

Fig. 1
Schematic representation of the genomic structure of PLDMV

The numbers above the ideogram indicate the start position (nt) of each protein-coding gene. The 5'- and 3'-untranslated regions (UTR) are represented by lines, and the ORFs are depicted by open box. The numbers below the ideogram represent the starting and terminating nucleotide position predicted for each gene. The putative proteinase cleavage sites in the polyprotein are presented below the ideogram.

Sequence analysis showed that the genome organization of PLDMV-DF is typical of *Potyvirus*. The full-length sequence comprises 10,153 nucleotides (nt), excluding the 3'-poly-A tail, and includes a 134-nt 5'-UTR and a 210-nt 3'-UTR (Fig. 1). A single, long open reading frame (ORF) and a short, overlapping coding sequence were predicted. The long ORF starts at nt position 135 with an AUG codon and terminates at nt 9,943 with a UAG stop codon. The sequence encodes a 373.68-kDa polyprotein that is processed into 10 putative mature proteins (P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb, and CP) in nine putative protease cleavage sites, identified by comparison with other potyviruses (Fig. 1) (Silvio *et al*., 2001). The other ORF, named *pipo* (Pretty Interesting *Potyviridae* ORF), is 225 nt long and was identified using the gene-finding software MLOGD (Firth and Brown, 2006; Chung *et al*., 2008) (Fig. 1). The PLDMV-DF PIPO protein is embedded within the P3 cistron of the polyprotein and contains a G_2A_6 motif, which is highly conserved in *Potyvirus* (Chung *et al*., 2008).

Pairwise comparisons were performed using MEGA 5 (Tamura *et al*., 2011) among PLDMV-DF, Japan-J56p, and Taiwan-CZ and KS, all of which are PLDMV isolates having complete genome sequences in GenBank. PLDMV-DF sequence shares 94.3–94.9% nucleotide identity with the other isolates. The 3'-UTR region is highly conserved (96.1–97.6% identical); the AT-rich, 5'-UTR region is considerably more divergent (88.1–91.1% identical). At the amino acid level, the polyprotein shares 95.9–96.3% identity among isolates. The 6K1 and

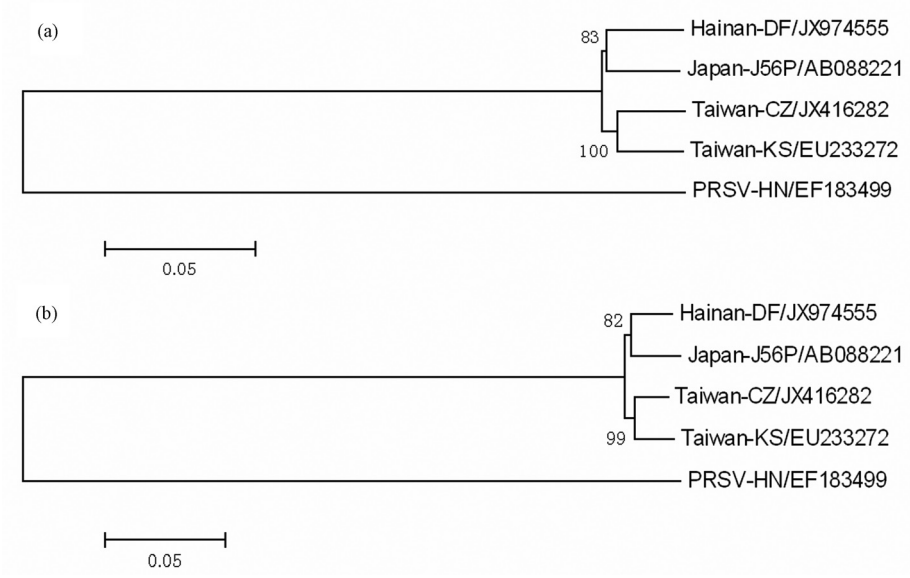

Fig. 2

Neighbor-joining tree analysis of the complete cDNA sequences (a) and the amino acid sequences (b) of the polyprotein of papaya leaf distortion mosaic virus (PLDMV, Hainan-DF) from China and other fully sequenced PLDMV isolates (virus isolate name/Acc. No.)

The complete genome of an isolate of papaya ringspot virus from Hainan Island (PRSV-HN, EF183499) was used as an outgroup. Numbers associated with branches represent percentages of non-parametric bootstrap support (1000 replicates) for those branches. GenBank Acc. Nos. are shown in the trees.

NIa-Pro proteins of PLDMV-DF display the highest sequence identity (98.1–100%) with the other PLDMV isolates; the P1 protein displays the lowest sequence identity (88.1–90.2%).

To further understand the relationships between PLDMV-DF and PLDMV isolates from Japan and Taiwan, phylogenetic trees were constructed using a multiple alignment of the complete nucleotide sequences of PLDMV isolates and the amino acid sequences of their polyproteins. The tree was constructed using the neighbor-joining method with MEGA 5, with a closely-related PRSV isolate (HN, accession no. EF183499) as an outgroup (Lu *et al*., 2008). Phylogenetic analysis identified two distinct clades (Fig. 2): one includes PLDMV-DF and Japan-J56p isolates, the other comprises two isolates from Taiwan (Taiwan-CZ and KS). PLDMV isolates from Taiwan are clustered as a single genetic group segregating from the Japanese isolate, consistent with a previous report (Bau *et al*., 2008). Notably, the PLDMV-DF isolate shares the most recent common ancestor of the Japan-J56p isolate rather than the Taiwan isolates, though the geographic distance between Hainan and Taiwan is shorter than that between Hainan and Japan. In addition, the genetic distance between PLDMV-DF and Japan-J56p (0.075) is slightly lower than that between PLDMV-DF and Taiwan isolates (0.080). Therefore, PLDMV-DF is a distinct isolate found in diseased transgenic papaya in China. It is possible that PLDMV-DF has been infecting papaya in China for years, but was mistakenly recognized as PRSV due to their similar disease symptoms. PLDMV-DF was recognized because of its appearance in commercialized PRSV-resistant transgenic papaya plants, which have become widely planted in China. Importantly, the emergence of PLDMV-DF in commercialized PRSV-resistant papaya indicates a potential threat to papaya in China. Bi-transgenic papaya lines resistant to both PRSV-YK and PLDMV P-TW-WF have been generated in Taiwan to reduce the threat of the disease (Kung *et al*., 2009). Therefore, our future studies will focus on the distribution of PLDMV-DF in China and how to appropriately control it.

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