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Reverse transcription loop-mediated isothermal amplification assay for rapid detection of *Papaya ringspot virus*

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A B S T R A C T

Papaya ringspot virus (PRSV) and *Papaya leaf distortion mosaic virus* (PLDMV), which causes disease symptoms similar to PRSV, threaten commercial production of both non-transgenic-papaya and PRSV-resistant transgenic papaya in China. A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay to detect PLDMV was developed previously. In this study, the development of another RT-LAMP assay to distinguish among transgenic, PRSV-infected and PLDMV-infected papaya by detection of PRSV is reported. A set of four RT-LAMP primers was designed based on the highly conserved region of the *P3* gene of PRSV. The RT-LAMP method was specific and sensitive in detecting PRSV, with a detection limit of 1.15×10^{-6} μg of total RNA per reaction. Indeed, the reaction was 10 times more sensitive than one-step RT-PCR. Field application of the RT-LAMP assay demonstrated that samples positive for PRSV were detected only in non-transgenic papaya, whereas samples positive for PLDMV were detected only in commercialized PRSV-resistant transgenic papaya. This suggests that PRSV remains the major limiting factor for non-transgenic-papaya production, and the emergence of PLDMV threatens the commercial transgenic cultivar in China. However, this study, combined with the earlier development of an RT-LAMP assay for PLDMV, will provide a rapid, sensitive and cost-effective diagnostic power to distinguish virus infections in papaya.

1. Introduction

The pathogen *Papaya ringspot virus* (PRSV; family *Potyviridae*, genus *Potyvirus*) infects papaya (*Carica papaya*) and cucurbit plants (Purcifull et al., 1984; Tripathi et al., 2008). Based on its host specificity, PRSV is grouped into two types, one of which infects plants in the families *Caricaceae*, *Cucurbitaceae*, and *Chenopodiaceae* (type P) while the other that infects plants in the families *Cucurbitaceae* and *Chenopodiaceae* but not *Caricaceae* (type W). Both P and W type viruses are transmitted non-persistently via aphids and are serologically indistinguishable (Purcifull et al., 1984; Yeh et al., 1984; Chen et al., 2008). PRSV-P occurs in most tropical and subtropical areas where papaya is grown and devastates commercial production. In papaya, PRSV-P infection causes mosaic, yellow mottling and distortion of the leaves, vein clearing, classic "ringspot" and streaking on fruits, and water-soaked streaks on stems and petioles (Gonsalves et al., 2008; Tripathi et al., 2008). There are four main control strategies for PRSV: quarantine and geographic displacement, roguing and netting, cross-protection, and transgenic resistance (Tripathi et al., 2008). Transgenic resistance conferred by the viral gene via homology-dependent post-transcriptional gene silencing (PTGS) is, by far, the most effective way to protect plants from viral infections including PRSV (Ratcliff et al., 1999; Wani and Sanghera, 2010).

PRSV-resistant transgenic papayas engineered with the coat protein (CP) gene have been commercialized in Hawaii since 1998, representing the first commercialized transgenic fruit crop (Gonsalves, 1998). This virus-resistant transgenic papaya has revived Hawaii's papaya industry. Another transgenic papaya from China, designated Huanong No. 1, contains the PRSV replicase (*NIb*) gene. It was licensed for commercial production in Guangdong province by the Chinese government in 2006 (Ye and Li, 2010). However, the PRSV-resistant transgenic plants can be overcome by PRSV with geographically-distant homology to the transgene, or by PRSV strains with HC-Pro (a suppressor protein of RNA silencing) that can suppress the silencing mechanism of transgenic papaya (Bau et al., 2003; Tripathi et al., 2008). In addition, another potyvirus, *Papaya leaf distortion mosaic virus*

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Table 1
The four primers used for RT-LAMP assay for detection of PRSV.

Primer name	Length (nt)	Sequence (5′ → 3′)
F3	19	GTTGAAAAAACCTACGCCG
B3	19	GACCTCGTGTACACAGTAC
FIP (F1c + F2)	43	GCAAAGTACCTTAAGTGCAACTTT-CTCTCTTGCAGGAAGAGTG
BIP (B1c + B2)	44	TCAAATCACAACCATCTTTTTCCG-GGTGAGAAGTCGTACACAAT

(PLDMV), infects PRSV-resistant transgenic papaya on Taiwan and Hainan Island (China); it is difficult to distinguish between PLDMV and PRSV due to their similar disease symptoms (Tuo et al., 2013).

The popularity of papaya has been increasing in China due to its nutritional, digestive, and medicinal properties. China cultivated 6424 ha of papaya and produced 181,183 tons of fruits in 2011 based on statistical data from the Food and Agriculture Organization of the United Nations (FAO). However, the emergence of new PRSV isolates and PLDMV threatens commercial papaya production in China, especially when considering the increased international trade of papaya fruits and seeds (Bau et al., 2008; Tuo et al., 2013; Shen et al., 2014). Thus, the accurate diagnosis of PRSV is essential for forecasting transmission as it will help to reduce not only the economic loss on the papaya industry but also to reduce risk of introduction of other PRSV isolates from abroad.

Several diagnostic methods, such as enzyme-linked immunosorbent assay (ELISA), Western blotting, reverse transcription-polymerase chain reaction (RT-PCR), and real time RT-PCR have been introduced for the detection of PRSV (Ling et al., 1991; Chiang et al., 2001; Noa-Carrazana et al., 2006; Cruz et al., 2009; Shen et al., 2010; Usharani et al., 2013). Commercial rapid-ELISA detection kits, such as the Agdi DAS ELISA kit (Elkhart, IN, USA) have been used for the diagnosis of PRSV, but the kits are expensive and less sensitive than molecular diagnostic methods. However, RT-PCR and real time RT-PCR require a sophisticated thermocycler instrument and complicated detection protocol. In recent years, we and others have applied reverse transcription loop-mediated isothermal amplification (RT-LAMP) as a novel diagnostic tool for rapid detection of various plant RNA virus including PLDMV (Varga and James, 2006; Lee et al., 2011; Zhou et al., 2012; Almasi et al., 2013; Shen et al., 2014). This method is a one-step, single-tube, reverse transcription and amplification reaction that detects a target RNA sequence with high sensitivity and specificity under a constant temperature of 60–65 °C within 60 min (Notomi et al., 2000). RT-LAMP is more cost-effective than RT-PCR and real-time RT-PCR and can be performed in a

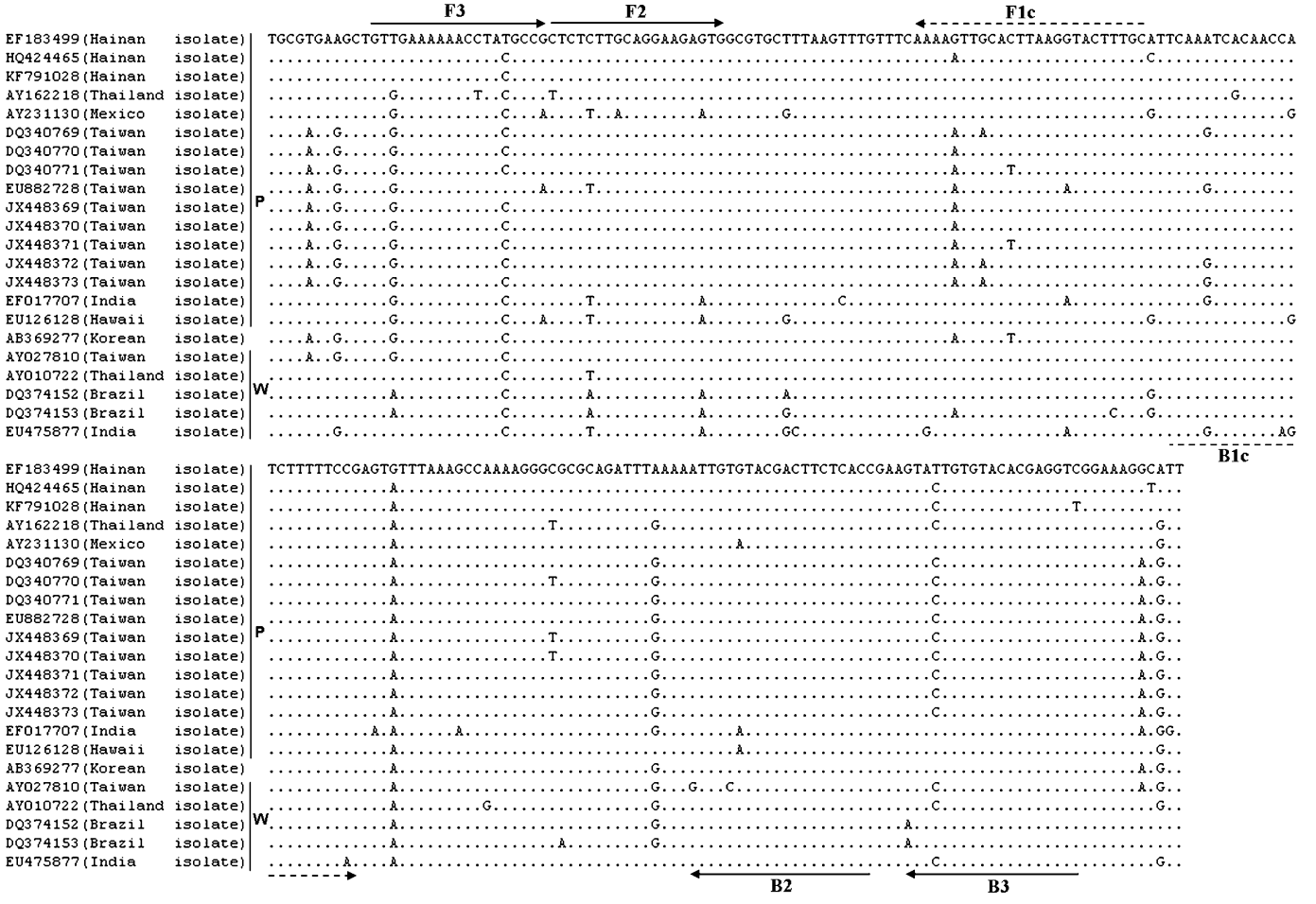

Fig. 1. Sequence alignment of the conserved region of the *P3* gene for PRSV isolates from different geographic origins. The geographic origins and their GenBank accession numbers of 22 PRSV isolates are shown to the right of the sequence of P3 genes. The nucleotide sequence of the *P3* gene of the PLDMV isolates from Hainan, China (GenBank accession numbers: EF183499, HQ424465, and KF791028) was used for the design of the RT-LAMP primers. The positions of primers (F3, F2, F1, B1, B2, and B3) are indicated by arrows. P and W in bold represent the PRSV-P and PRSV-W isolates, respectively.