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Letter to the Editor

Establishment of *in vitro* transcription system based on RNA-dependent RNA polymerase (RdRp) of Little cherry virus 1

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Little cherry virus 1 (LChV-1), belonging to the genus *Closterovirus*, family *Closteroviridae*, causes little cherry disease (LCD) with varying symptoms. LChV-1 has a positive single strand RNA genome with 17,000 nt length and no poly(A) tail or tRNA-like structure at the 3'-terminal region (*1-3*). At present, studies on LChV-1 have primarily focused on virus detection, genomic cloning and molecular evolution (*4*), but no research investigating the mechanism of LChV-1 genome replication was performed. Viral replication includes synthesis of nucleic acids and viral protein translation. Currently, virus-infected cultured cells and heterologous expression systems are used in the research of viral replication (*5*). Heterologous expression systems have been used to study the mechanism of genome replication of several plant RNA viruses (*6,7*). Replicases purified from virus-infected cells usually have properties resulting from post-translational modifications that can hamper protein purification. However, replicases obtained from heterologous sources (i.e. *Escherichia coli)* have no such properties, and are, therefore, favorable for high-purity isolation (*8*). In this study, we developed an *in vitro* transcription system based on prokaryotically expressed LChV-1 RdRp.

In order to facilitate the study on replication of LChV-1, RNA-dependent RNA polymerase (RdRp) of LChV-1 was expressed and purified. To amplify LChV-1 RdRp, the plasmid pMAL-LChV/RdRp was constructed by inserting the coding sequence of LChV-1 RdRp (1428 nt) into the vector pMAL-C2X. The coding sequence of RdRp was amplified using primers LCh1-BamHI-7070-F (5'-AAGGATC CATGGACTTGACTTTCAATGG-3') and LCh1-SalI-8497-R (5'-TTGTCGACCTATTACAACTGAATACTAATATCGT-3'), and was ligated to pMAL-C2X by double digestion with *Bam*HI and *Sal*I. The correct insertion (LChV1 RdRp) was confirmed by DNA sequencing. The expression of MBP-LChV/RdRp, which has the molecular weight approximately equal to the sum of MBP tag (40 kDa) and RdRp (50 kDa), was induced in *E. coli* using 0.4 mmol/l IPTG and cultured at 18°C. Bacterial cells were disrupted by sonication and the supernatant was subjected to affinity chromatography using amylose resin (NEB) as a matrix. In addition, to prepare RNA template for *in vitro* replication reaction, LChV-1 genomic fragment containing T7 promoter was prepared by PCR (primers for 3'UTR: LChV-1-T7-3F: 5'-ATtaatacgactcactataggGTTTATAATAA GTTTCTATATTATAAATATATTATCAA-3'; LChV-1-3R:

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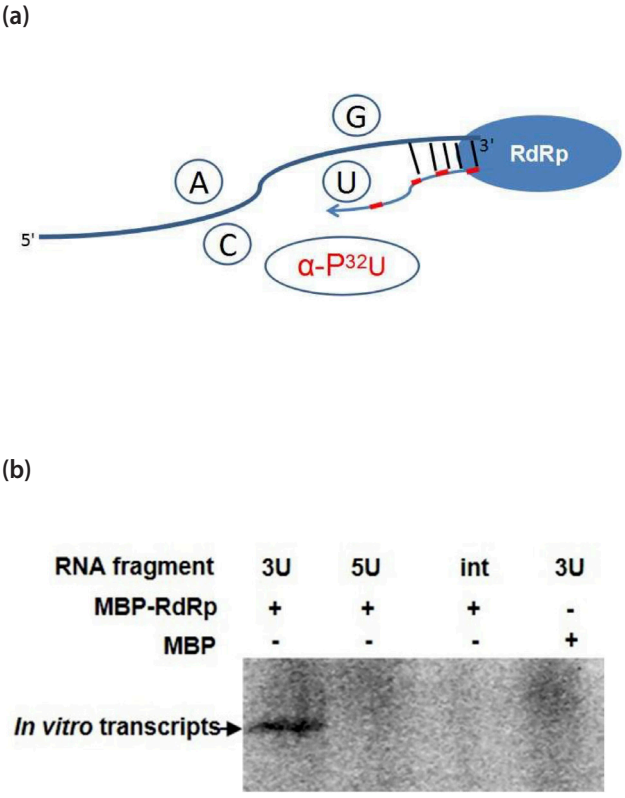
Abbreviations: LCD = little cherry disease; LChV-1 = Little cherry virus 1; RdRp = RNA-dependent RNA polymerase



***In vitro* transcription using RdRp of Little cherry virus 1**
(a) A schematic diagram of *in vitro* replication; **(b)** *In vitro* transcription, 5U indicates 5' terminal 75 nt of LChV-1 genome, int indicates 268 nt from position 271 to position 538 in LChV-1 genome, 3U indicates 3' terminal 207 nt of LChV-1 genome.

5'GCACCTTTTATTTTTTATATATGCA-3'. Primers for 5'UTR: LChV-1-T7-5F: 5'-ATtaatacgactcactataggCGTTTTTATCTCCCAGCTTT-3'; LChV-1-5R: 5'-TACTGAAAGGAAAGTTGCG-3'. Primers for the internal fragment LChV-1-T7-int271F: 5'-ATtaatacgactcactataggCCCGCAGCAATGAAGACATTCTC-3'; LChV-1-int538R 5'-TACAGCAACATCAGCACTTGAAA-3') on a large scale and purified by gel extraction kit (TaKaRa). Then RNA was transcribed *in vitro* with the bacteriophage T7 RNA polymerase (Promega) and RNA integrity and concentration were verified by 1.0% agarose gel electrophoresis.

To analyze the RNA polymerase activity and template specificity of MBP-LChV/RdRp fusion protein, three different RNA fragments corresponding to the LChV-1 genome (5U: 5'UTR 75 nt; int: internal fragment 276 nt; 3U: 3'UTR 207 nt) were used as templates for *in vitro* replication reaction. MBP-LChV/RdRp specifically recognized the 3' end (3U) of LChV-1 RNA genome and synthesized its complementary strand, whereas MBP-LChV/RdRp was not able to synthesize the complementary strands from the 5' end or internal fragment of LChV-1 genome (Fig. 1). In a control experiment, MBP protein alone did not synthesize the complementary strand of the 3' end (Fig. 1). This result indicates that MBP-LChV/RdRp fusion protein has an RNA polymerase activity and 3'-terminal region contains a specific regulatory sequence or structure that is important for LChV-1 replication.

In this study, we used a prokaryotic expression system to produce LChV-1 RdRp that has RNA polymerase activity for transcription of 3'-terminal region of LChV-1 genome RNA. LChV-1 is the main causative pathogen of LCD that affects cherry production worldwide. LChV-1 infection causes serious damage in cherry production (4,9). Due to the importance of the disease, current research focuses on LChV-1 detection and genome diversity. Considering the prevalence of complex infection of virus diseases in fruit trees, further detailed studies are needed to investigate whether the replication of different viruses infecting small fruit is compatible among each other, and whether LChV-1 RdRp can facilitate the replication of other fruit tree viruses. The *in vitro* transcription system that we have established in this study can be used as a research tool and material for the future studies on the mechanism of replication of LChV-1 and other RNA viruses.

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