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Short communication

Genome segments encoding capsid protein-like variants of Pyrus pyrifolia cryptic virus

Hideki Osaki[a,*], Atsuko Sasaki[a], Eiko Nakazono-Nagaoka[b], Nobuyoshi Ota[a], Ryoji Nakaune[a]

[a] NARO Institute of Fruit Tree and Tea Science, 2-1 Fujimoto, Tsukuba, Ibaraki 305-8605, Japan
[b] JIRCAS (Japan International Research Center for Agricultural Sciences), 1-1 Ohwashi, Tsukuba, Ibaraki 305-8686, Japan

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ABSTRACT

According to previous studies, three double-stranded (ds) RNA molecules (dsRNA1, 2, and 3) detected in Japanese pear are transmitted to the next generation with high frequency through both ovules and pollen. Nucleotide sequence analysis of dsRNA1-encoding RNA-dependent RNA polymerase (RdRp) has suggested that these dsRNAs are related to a cryptovirus named Pyrus pyrifolia cryptic virus (PpCV). In this study, purified dsRNA prepared from a PpCV-infected Japanese pear cultivar was subjected to next-generation deep sequencing. This sequencing generated two *de novo* assembled contigs corresponding to dsRNA2 and 3, with BLAST analysis of the predicted amino acid sequences indicating homology to capsid proteins (CPs) of the cryptoviruses persimmon cryptic virus and Sinapis alba cryptic virus 1, respectively. Relationships between the two contigs and dsRNA2 and 3 were confirmed by northern blot hybridization with probes generated using primers designed from the assembled contigs. Rapid amplification of cDNA ends analyses of 5′- and 3′-terminal sequences of dsRNA2 and 3 revealed that these two dsRNAs consist of 1523 and 1481 bp, respectively. The 5′-terminal sequences (AGAAUUUC) of dsRNA1, 2 and 3 were found to be conserved. Phylogenetic analysis of deduced amino acid sequences of the two CP-like variants indicated that PpCV belongs to *Deltapartitivirus* (*Partitiviridae*). Our results imply that PpCV is tri-segmented.

Plant-infecting partitiviruses (so called cryptoviruses), which are widespread among plants (both angiosperms and gymnosperms), feature isometric particles, *ca.* 30 nm in diameter, and genomes generally consisting of two monocistronic double-stranded (ds) RNA segments. Cryptoviruses have no known natural vectors. These viruses are transmitted to the seed embryo with high frequency only by ovules and pollen, with no graft transmission and apparently no cell-to-cell transport. Cryptoviruses possess no cell-to-cell movement proteins from other viruses, but instead spread throughout their host along with the host's cell division (Ghabrial et al., 2008).

Cryptoviruses belong to *Partitiviridae*, a family with an exceedingly broad host range spanning three kingdoms: plants, fungi, and protists (King et al., 2011). The family *Partitiviridae* has recently been taxonomically reorganized (Nibert et al., 2014). According to the results of a phylogenetic analysis, former genera *Partitivirus*, *Alphacryptovirus*, and *Betacryptovirus* were replaced by the new genera *Alphapartitivirus*, *Betapartitivirus*, *Gammapartitivirus*, and *Deltapartitivirus*. *Cryspovirus* was found to be phylogenetically distinct and was therefore maintained as a genus. In partitiviruses, which are bipartite dsRNA viruses, the segment encoding the RNA-dependent RNA polymerase (RdRp) protein is designated as dsRNA1, while the region encoding the capsid protein (CP) is termed dsRNA2. The two segments are separately encapsidated. Additional dsRNA molecules have been reported to be associated with some of these viruses and are suggested to be satellite molecules or additional genomic segments (Chen et al., 2006; Tzanetakis et al., 2008; Salem et al., 2008; Sabanadzovic and Abou Ghanem-Sabanadzovic, 2008).

Three dsRNA molecules (dsRNA1, 2, and 3) have been reported to be cultivar-specific in Japanese pear (*Pyrus pyrifolia*; Osaki et al., 1998a). According to the previous study, these dsRNA molecules are transmitted with high frequency to the next generation through both ovules and pollen, not by grafting (Osaki et al., 1998a). Although attempts to purify virus-like particles associated with these dsRNAs were unsuccessful, nucleotide sequence analysis of dsRNA1-encoding RdRp (Osaki et al., 1998b) suggested that these dsRNAs are related to a cryptovirus named Pyrus pyrifolia cryptic virus (PpCV).

In the present study, we analyzed dsRNA2 and 3 by conventional and next-generation sequencing. Sequence comparisons and phylogenetic analysis of the putative amino acid sequences revealed that PpCV has two CP-like variants and is thus tri-segmented.

* Corresponding author.
E-mail address: hideki@affrc.go.jp (H. Osaki).

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dsRNA was extracted from bark tissue of Japanese pear cultivar Niitaka as described by Osaki et al. (1998a) The resulting dsRNA samples were electrophoretically fractionated on 5% polyacrylamide gels in TAE buffer (40 mM Tris, 40 mM acetate, and 1 mM Na_2EDTA). After staining of gels with ethidium bromide, the dsRNAs were visualized on a UV transilluminator. For use with the Illumina MiSeq sequencing system, the dsRNA samples were further purified with a Plant Viral dsRNA Enrichment kit (Medical and Biological Laboratories Co., Japan).

Sequencing on the Illumina MiSeq system and *de novo* assembly were performed as previously described (Osaki et al., 2016). Deduced amino acid sequences were predicted using CLC Main Workbench 6 software (CLC bio, Cambridge, MA, USA). Sequence comparisons with nucleic acid and protein databases were performed using the BLAST program (Altschul et al., 1990). Multiple sequence alignment, neighbor-joining phylogenetic tree construction (Saitou and Nei, 1987), bootstrap analysis (Felsenstein, 1985), and prediction of secondary structures of 5′-terminal regions were also carried out in CLC Main Workbench 6.

Deep sequencing revealed two contig sequences (nos. 154 and 39) showing homology to CPs of cryptoviruses. To confirm a relationship between the two contigs and dsRNA2 and 3, northern blot hybridization was carried out using probes generated by RT-PCR from primers based on sequences of the two contigs. The mobilities of dsRNA2 and 3 on polyacrylamide gel electrophoresis (PAGE) gels were quite similar (Fig. 1A a). Consequently, a probe against dsRNA1 was used to distinguish between dsRNA2 and 3, with relative distances then estimated between the dsRNA1 hybridization signal and hybridization signals of dsRNA2 or dsRNA3. The following primer pairs were used to amplify portions of dsRNA1 (*ca.* 610 bp), contig no. 154 (*ca.* 700 bp), and contig no. 39 (*ca.* 700 bp), respectively: PpRdr/PpRd1 (5′-GATCCGTTGGATAGCGTACC-3′ and 5′-GGCTCGATATGCAGAAATCC-3′), PCF1/PCF2 (5′-ACAGGAGAAGAAACGGCTCC-3′ and 5′-CAATCTAGGAGCCAGCATGG-3′), and SaCF1/SaCF2 (5′-CTAGTATGCCTTTGACCTCC-3′ and 5′-CTTCTACCCAATCGAAGAGC-3′). Amplified cDNA products were cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Digoxigenin (DIG)-labeled probes were produced by PCR amplification of the plasmid carrying the product using each primer pair. DIG-labeling and detection were carried out according to the protocol of the DIG DNA Labeling and Detection kit (Sigma-Aldrich, St. Louis, MO, USA). Northern blot hybridization of these RNAs on 5% polyacrylamide gels was performed as previously described (Valverde et al., 1990).

For RACE analysis, the 3′ ends of the plus and minus strands of the dsRNAs were polyadenylated and then used as templates for initial reverse transcriptions using an oligo-dT-containing adapter primer. Both terminal regions were amplified using a 3′ RACE System for Rapid Amplification of cDNA Ends kit (Thermo Fisher Scientific), with primers 5PC1 (5′-ACTGACTCACCTATTATGCG-3′) and 3PC1 (5′-GTTCGGCTATGTAATCATGG-3′) used for amplification of 5′- and 3′-terminal regions of dsRNA 2, respectively. To amplify 5′- and 3′-terminal dsRNA 3 regions, primers 5SC1 (5′-GTGCTTAAGGTCAACATACC-3′) and 3SC1 (5′-ACGCCCGATAATCAGAACCG-3′) were respectively used. Amplified cDNA products were cloned using a TOPO TA cloning kit (Invitrogen) and sequenced on an automated DNA sequencer (ABI PRISM 3130xl Genetic Analyzer, Applied Biosystems).

An Illumina MiSeq system was used to analyze the dsRNA virome of the Japanese pear cultivar Niitaka. A total of 3,202,561 reads were obtained. These reads were assembled into 2474 larger contigs using Trinity software and then subjected to BLAST analysis. The BLAST analysis uncovered two contig sequences (nos. 154 and 39) showing homology to CPs of cryptoviruses Persimmon cryptic virus (PerCV) (Morelli et al., 2015) and Sinapis alba cryptic virus 1 (SaCV1) (Li et al., 2016), respectively (Fig. 1B). Except a contig corresponding to dsRNA1 (contig no.12), no contigs with predicted amino acid sequences showing homology to RdRps of cryptoviruses were obtained. Sequence of the contig no.12 was same to the reported sequence of dsRNA1 (Osaki et al., 1998b). Moreover, based on BLAST results, other RNA viruses or virus-like agents were not found in the starting material.

To confirm the relationship between the two contigs and dsRNA2 and 3, a northern blot hybridization analysis was conducted. The distance between the northern blot hybridization signal for dsRNA1 and that of contig no. 39 was apparently longer than the distance between dsRNA 1 and contig no. 154 hybridization signals (Fig. 1A b-1 and 3), indicating that contig nos. 154 and 39 corresponded precisely to dsRNA2 and dsRNA3, respectively.

In a previous study (Osaki et al., 1998b), dsRNA2 and dsRNA3 were hybridized with a probe against dsRNA1 in a dot-blot hybridization experiment. Because dsRNA1, dsRNA2, and dsRNA3 bands excised and eluted from PAGE gels had significantly similar mobilities, the dsRNA2 and dsRNA3 samples were thought to have been contaminated with dsRNA1.

According to RACE analyses, dsRNA2 and 3 consist of 1523 and 1481 bp, respectively (Fig. 1B). The full-length cDNA sequences of PpCV dsRNA2 and dsRNA3 were deposited in GenBank under accession nos. LC221824 and LC221825, respectively. The 5′-terminal sequences (AGAAUUUC) of dsRNA1, 2, and 3 were found to be conserved. Similar 5′-terminal sequences are harbored by other cryptoviruses in the genus *Deltapartitivirus*, including Beet cryptic virus 3 (AGAAUUU on both dsRNA segments) (Xie et al., 1993), PerCV (GGAAUUAC on dsRNA1 and AGAAUUUC on dsRNA2) and SaCV1 (AGAAUUUU on both segments). This similarity supports the inclusion of PpCV in the genus. The conserved terminal sequence of PpCV (AGAAUUUC) is also consistent with the *Deltapartitivirus* 5′-terminal consensus sequence (GAWWWU) reported by Nibert et al. (2014).

The 5′-untranslated regions of dsRNA1, 2 and 3 were predicted to fold into stem-loop structures (Fig. 1C). Similar stem-loop structures have also been observed in other partitiviruses (Lesker et al., 2013; Guo et al., 2017). These structures are considered as likely having an important role in dsRNA replication and virion assembly.

Segments dsRNA2 and 3 were found to contain single open reading frames (ORFs) with the potential to encode putative proteins of 419 and 411 amino acids (aa), respectively, similar to cryptoviral CPs (Fig. 1B). PpCV dsRNA1, determined to comprise 1592 bp and containing a single ORF, was predicted to encode a protein of 477 aa with conserved motifs characteristic of RdRp (Osaki et al., 1998b; Fig. 1B). Size-range differences in genome-segment and/or protein lengths among partitiviruses have been observed to parallel phylogenetic findings (Nibert et al., 2014). According to this criterion, PpCV can be considered to belong to *Deltapartitivirus*. The CP-like variants predicted from dsRNA2 and 3 nucleotide sequences were designated as PpCV CP1 and CP2, respectively.

In a previous study (Osaki et al., 1998b), as noted above, dsRNA2 and 3 were hybridized with a probe against dsRNA1, encoding RdRp, in a dot-blot hybridization experiment by probable contaminations. Because segments for CP genes had not been found, the dsRNAs were not defined to be of viral origin. In this study, however, deep sequencing analysis shows contigs corresponding to dsRNA2 and 3 are related to cryptoviral CPs. Thus, it is concluded that the dsRNAs are genome of PpCV.

Nibert et al. (2014) showed that PpCV belonged to *Deltapartitivirus*, based on phylogenetic analysis on RdRps of partitiviruses. Meanwhile, PpCV CP1 and CP2 were also placed in two separate clades within the *Deltapartitivirus* cluster including CPs of PerCV and SaCV1 in a generated phylogenetic tree based on CPs and CP-like variants (Fig. 2). The results of the phylogenetic analyses thus also support assignment of PpCV to this genus. In addition, as already described by Nibert et al. (2014), these results suggest a hypothesis: when a Japanese pear tree is co-infected with two different cryptoviruses, the more efficient of the two RdRps will be selected along with the two surviving CP genes. On the other hand, CP-like variants of other tri-segmented deltapartitiviruses, Fragaria chiloensis cryptic virus (FcCV; Tzanetakis et al., 2008), rose cryptic virus 1 (RCV1; Sabanadzovic and Abou Ghanem-