Complete genome sequence of vallota mosaic virus detected in a narcissus bulb imported from the United States to Japan

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Received: 25 November 2021 / Accepted: 24 January 2022 / Published online: 5 March 2022
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Abstract
Narcissus (Narcissus albidus) imported from the United States exhibited leaf chlorosis during post-entry quarantine. We employed next-generation sequencing (NGS) on symptomatic leaf samples and detected vallota mosaic virus (ValMV), belonging to the genus Potyvirus, family Potyviridae, as the viral agent. Sanger sequencing of PCR products and rapid amplification of cDNA ends based on NGS contigs revealed that ValMV is 9,451 nucleotides (nt) in length, excluding the poly(A) tail. Nucleotide and amino acid (aa) sequences of the coat protein region had over 98% identity to previously reported ValMV isolates. In each of the 10 regions encoding mature proteins, however, the sequence identity to other potyviruses was 49.5–71.9% nt and 18.3–78.9% aa, values that are below the species demarcation thresholds for the family Potyviridae. Phylogenetic analysis revealed that our ValMV isolate is most closely related to known ValMV isolates and is grouped with other potyviruses. Taken together, our results indicate that the newly isolated ValMV belongs to a distinct species in the genus Potyvirus. This study provides the first report of the complete ValMV genome sequence and the first record of this virus in narcissus.

Narcissus (Narcissus L. spp.) is a bulbous plant of the family Amaryllidaceae, comprising numerous species. Although originating from the Mediterranean region [1], Narcissus plants are now grown worldwide. In Japan, narcissus bulbs imported from abroad are inspected during post-entry quarantine. Over 20 plant viruses are known to infect narcissus [1], including several members of the genus Potyvirus: cyrtanthus elatus virus A, lily mottle virus, narcissus degeneration virus, narcissus late season yellows virus (NLSYV), narcissus yellow stripe virus, and ornithogalum mosaic virus (OrMV) [1–4].

The potyvirus vallota mosaic virus (ValMV) [5] was originally identified in Vallota speciosa in the Netherlands, with infection causing flower colour breaking and chlorotic leaf lesions [6]. This virus can be transmitted by Myzus persicae, and its experimental host range includes Chenopodium amaranticolor, C. quinoa, Gomphrena globosa, Hyoscyamus niger, Nicotiana clevelandii, Spinacia oleracea, and Tetragonia expansa [6]. Three distinct ValMV isolates have been reported from Cyrtanthus elatus (synonym for V. speciosa [7]) in the United States (accession no. EF441726.1: direct submission), Nerine sarniensis in the United Kingdom (accession no. EF507688.1 [8]), and Nerine sp. in New Zealand (accession no. FJ618540.1 [9]). However, no study has analysed their sequences from the 5′ terminus to the Nib region, nor has the complete genome of ValMV been sequenced. This study now provides the complete ValMV genome sequence.

Narcissus bulbs imported from the United States were cultivated for inspection at one of Japan’s post-entry quarantine stations. One plant (N. albidus) exhibited leaf chlorosis in February 2020. To confirm viral infection, next-generation sequencing (NGS) of the symptomatic leaf sample was performed. Total RNA was extracted using a Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA). Reverse transcription of extracted RNA and cDNA amplification were performed using a TransPlex® Complete Whole Transcriptome Amplification (WTA) Kit (Sigma-Aldrich) and ExTaq DNA polymerase (Takara Bio, Shiga, Japan) as described by Yanagisawa et al. [10]. An Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific, Tokyo,
Japan) was used for NGS library preparation from WTA products. The library was sequenced on an Ion Personal Genome Machine (PGM) system (Thermo Fisher Scientific). Sequence data analysis and de novo assembly were performed using CLC Genomics Workbench version 20.0 (QIAGEN, Hilden, Germany) as described [10].

To confirm the assembled contig sequences from NGS and determine the complete ValMV genome sequence, we performed Sanger sequencing of products from RT-PCR and rapid amplification of cDNA ends (RACE). New primers were designed based on one contig showing high sequence similarity to known ValMV isolates, determined using BLASTn (Fig. 1 and Supplementary Table S1). Reverse transcription of extracted RNA was performed using PrimeScript™ Reverse Transcriptase (Takara Bio), using the random primer (N)6 and an oligo(dT)15 primer (Takara Bio). The novel primers and KOD-Plus-Neo (TOYOBO, Osaka, Japan) were used for PCR. The 5′- and the 3′-terminal ends of ValMV were amplified using a SMARTer® RACE 5′/3′ Kit (Takara Bio) and a 3′-Full RACE Core Set (Takara Bio), respectively. Amplicons were sequenced directly using SeqStudio Genetic Analyzer (Thermo Fisher Scientific). Acquired paired-end sequence data were assembled in MEGA X [11].

The open reading frame (ORF) of ValMV was identified using ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). To predict putative cleavage sites in the viral polyprotein, we compared the amino acid (aa) sequences of ValMV and other known potyviruses [5]. The PIPO protein was identified based on the highly conserved G1-2A6-7 motif [12].

We investigated the phylogenetic relationship of ValMV to other potyviruses using aa sequences of the coat protein (CP) and the polyprotein. The analysis was performed in MEGA X, using the maximum-likelihood method with the Jones-Taylor-Thornton (JTT) model and 1,000 bootstrap replications. Using BLAST, we performed pairwise sequence comparisons of the complete genome, the viral polyprotein, and the 10 putative mature proteins between ValMV and potyviruses in the same cluster of the CP-based phylogenetic tree (Fig. 2A).

Fifty contigs longer than 500 nucleotides (nt) were assembled from 659,956 NGS reads. The BLAST analysis identified a 9,339 nt contig with high sequence similarity (99.0, 98.9, and 98.8% identity) to the nt sequences of three known ValMV isolates (accession no. FJ618540.1, EF441726.1, and EF507688.1). The novel contig consisted of 5,273 NGS reads (mean coverage, 70.15). Five other contigs (749, 912, 1,058, 1,181, and 2,152 nt) showed high sequence similarity (94.8, 92.9, 89.9, 93.7, and 94.6% identity) to the NLSYV nt sequence (accession no. JQ326210.1). Furthermore, 370,856 NGS reads mapped to the genome sequence (accession no. JQ326210.1) of NLSYV (mean coverage, 4,586.71). Another 44 contigs (518-2,394 nt) were not related to plant virus sequences.

The complete genome of ValMV (isolate ValMV-Nar) has 9,451 nt (ORF: nt 135–9,236 and 3,033 aa) (accession no. LC658681), excluding the 3′ poly(A) tail (Fig. 1). Its genome structure is typical of potyviruses, and we identified PIPO via the GA7 motif at nt positions 2,808–2,815 (Fig. 1). Additionally, the NGS contig sequence of 9,339 nt matched the complete ValMV-Nar genome. As a result of remapping onto the verified genome sequence as a reference using all NGS reads, a maximum coverage of 594 and a minimum coverage of 3 were observed at nt positions 48-9,386 (mean coverage, 60.94).

Phylogenetic analysis based on the CP coding region showed that ValMV-Nar belongs to the same clade as known ValMV isolates and is closely related to OrMV (Fig. 2A). Phylogenetic analysis based on polyprotein sequences confirmed the close relationship between ValMV-Nar and OrMV (Fig. 2B).

Pairwise sequence comparisons (Supplementary Table S2) showed that the CP coding region of ValMV-Nar from next-generation sequencing, and black lines represent amplification regions for each of the primers used to determine the complete ValMV-Nar genome sequence.
Complete genome sequence of vallota mosaic virus had > 98% nt and aa sequence identity to known ValMV isolates but shared low sequence similarity (61.5–71.9% nt and 57.5–76.8% aa sequence identity) with other potyviruses. The CP aa sequences were highly conserved between ValMV-Nar and known ValMV isolates, although the amino acid at position 59 was asparagine in ValMV-Nar and threonine in the other isolates. In contrast, the ValMV-Nar polyprotein shared 58.1–65.9% nt and 45.3–66.3% aa sequence identity with other potyviruses. Moreover, ValMV-Nar had 58.1–65.4% nucleotide sequence identity to other potyviruses in the whole genome, 49.5–53.4% in the P1 coding region, and 51.9–68.9% in the other eight mature protein regions. For all 10 mature protein regions and the polyprotein, ValMV-Nar had the highest sequence similarity to OrMV.

In this study, we characterized ValMV based on the complete genome sequence, whereas prior studies had used only partial sequences [8, 9]. The species demarcation criteria for the family Potyviridae are as follows: (i) < 76% nt and < 82% aa sequence identity in the viral polyprotein, (ii) < 76–77% nt and < 80% aa sequence identity in the CP coding region, or (iii) < 58% nt sequence identity in the P1 coding region and < 74–78% nt sequence identity in other protein coding regions [5]. Therefore, the nt and aa sequence identity values are above the species demarcation threshold when examining CP coding regions of ValMV-Nar and other isolates [8, 9] but below the threshold when examining complete genomes and individual protein regions of ValMV-Nar and other potyviruses (Supplementary Table S2). Moreover, phylogenetic analysis showed that ValMV-Nar was grouped with other potyviruses (Fig. 2). The available data indicate that ValMV-Nar belongs to the same species as existing ValMV isolates, which together represent a distinct species in the genus Potyvirus.

This is the first report of the complete genome sequence of ValMV, and the first to detect ValMV in Narcissus sp. However, because the analysed plant was coinfected with NLSYV, we cannot ascertain whether the symptoms were caused by ValMV infection alone. We therefore recommend further research investigating specific disease symptoms that are attributable to ValMV in narcissus plants.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00705-022-05406-w.
Author contributions All authors contributed to the study conception and design. Hironobu Yanagisawa supervised its execution. Material preparation was performed by Yuya Imamura, Moritsugu Oishi, and Yuji Fujiwara. Data collection and analysis were performed by Yuya Imamura. The first draft of the manuscript was written by Yuya Imamura, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding No funding was received for conducting this study.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval This article does not contain any experiments involving humans or animals, and no ethical approval was required.

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