Infectious in Vitro Transcripts From cDNA Clone of a Japanese Gentian Isolate of Sikte Waterborne Virus, Which Shows Host-specific Low-temperature-preferred Multiplication

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Research Article

Keywords: Tombusviruses, cDNA clone, Japanese gentian, Sikte waterborne virus, Infectious in vitro transcripts, low-temperature-preferred multiplication

DOI: https://doi.org/10.21203/rs.3.rs-216392/v1
Abstract
Tombusviruses have been identified in several crops, which include gentian virus A (GeVA), in Japanese gentians. In this study, we isolated another tombusvirus, Sikte waterborne virus strain C1 (SWBV-C1) from Japanese gentian. Although SWBV-C1 and GeVA are not closely related among tombusviruses, SWBV-C1, like GeVA, showed host-specific low-temperature-preferred multiplication in gentians and Arabidopsis. The use of in vitro transcripts from full-length cDNA clones of SWBV-C1 genomic RNA as inocula confirmed these properties, which indicates that the identified genomic RNA sequences encode viral factors underlying characteristic SWBV-C1 features.

Introduction
Plant viruses form a group of pathogens that cause serious damage to economically important crops. Although many plant viral diseases have been reported in natural fields, new unknown viral diseases have arisen, owing, in part, to current agricultural practices, such as the cultivation of diverse new crops and environmental amendments.

Japanese gentians (Gentiana triflora, Gentiana scabra and their hybrids) are important ornamental flowering plants in Japan [1], and multiple viruses, including cucumber mosaic virus, broad bean wilt virus 2, clover yellow vein virus, impatiens necrotic spot virus, and gentian mosaic virus [2–4], infect Japanese gentians in farms. By investigating cultivated gentians in northeastern Japan that had symptoms of an unknown disease, novel plant viruses, such as gentian ovary ring-spot virus, gentian kobu-sho-associated virus, and gentian virus A (GeVA), have been identified [5–7]. Tombusviruses, including GeVA, have monopartite positive-sense RNA genomes that encode five proteins, p33 and its read-through product p92, which are replication proteins, as well as p41, p21, and p19, which represent a coat protein (CP), movement protein, and silencing suppressor, respectively [8]. A phylogenetic analysis of the amino acid sequences of these proteins suggests that GeVA is a novel tombusvirus [7]. In addition, GeVA efficiently multiplies at a low temperature (18°C) and induces symptoms in gentians and Arabidopsis, but GeVA multiplication and virulence have not been detected at 23°C [9]. To our knowledge, GeVA was the first tombusvirus reported to show virulence against Japanese gentians.

In this study, to understand more about tombusvirus-related diseases of gentians, we determined the presence of viruses in gentian plants using the double-stranded RNA (dsRNA) isolation, exhaustive amplification, cloning, and sequencing (DECS) method [10, 11]. In the DECS analysis, dsRNA was purified from the total RNA of gentians using glutathione S-transferase-tagged dsRNA-binding protein 4, and the cDNAs were cloned and sequenced as described previously [7]. Of the 96 clones produced by the DECS method from a Japanese gentian plant showing necrotic symptoms, 34 cDNA fragments were homologous to the nucleotide sequence of the Sikte waterborne virus (SWBV) isolate Eckbach CP gene (92.8% coverage). On the basis of the nucleotide sequences, cDNA fragments containing full-length CP-coding sequences were amplified by reverse transcription polymerase chain reaction (RT-PCR) using the primer pair GVCPf1 (5'-ATGTCGATGGTAAGAAGAAATCAG-3') and GVCPf1 (5'-
TTAGGGAATGTGACCGAGTTTAT-3′), and the resulting PCR products were directly sequenced using the Sanger method. The deduced CP amino acid sequence shared a 98.8% identity, which is greater than the 87% taxonomic criterion for tombusvirus species, with SWBV-Eckbach CP (Figure 1a) [12, 13], suggesting that the identified virus belongs to the same species as SWBV. Single lesion isolations were repeated three times from inoculated leaves of Chenopodium quinoa, and viruses were finally propagated in Nicotiana benthamiana and purified as described previously [6]. We named this virus SWBV strain C1 (SWBV-C1).

SWBV is a pathogen of Limonium sinuatum [13], but SWBV infections in gentians have not been reported. During SDS-PAGE, the CP band of purified SWBV-C1 migrated more rapidly than that of GeVA (Figure 1b), and a phylogenetic analysis using the deduced amino acid sequences of tombusvirus CPs (Figure 1c) revealed a distance between SWBV-C1 and GeVA (50.6% shared identity between CP amino acid sequences), indicating that GeVA and SWBV-C1 belong to different tombusvirus species. To characterize SWBV-C1, its genomic structure was determined. On the basis of the partial tombusvirus sequences identified using the DECS method, we designed the rapid amplification of cDNA ends (RACE) primers G-SWBV-RACErev (5′-CCTGCCGCCAGTCGCAATTG-3′) and G-SWBV-RACEfw (5′-AGCGTCTCATTGAGATGGCA-3′). SWBV-C1 virion RNA was polyadenylated by poly A polymerase (NEB), and the 5′ and 3′ terminal cDNA ends were amplified by the RACE method using a GeneRacer kit with Superscript III (ThermoFisher Scientific). A Zero Blunt TOPO PCR cloning kit (ThermoFisher Scientific) was used for the cloning and sequencing of the RACE fragments. Then, cDNA was synthesized using a reverse-transcription reaction with primer SWBV-3′Rv (5′-GGGCTGCTTCTGCAATGT-3′) and ReverTra Ace (TOYOBO). The full-length cDNA of SWBV genomic RNA was amplified by PCR using the primer pair GtSWBV-FW (5′-AAGCTTGCATGCCTGCAGGAATTCTCCAGGATTTCTC-3′) and GtSWBV-RV (5′-ACCCGGGGATCCTCTAGAAGCGTGGGCTGATTCTGCAATGT-3′). pUC19 was digested with PstI and XbaI, and a 4–5-kb cDNA fragment was cloned using an In-Fusion HD cloning Kit (TaKaRa). Cloned plasmids were amplified in Escherichia coli strain JM109, and plasmid vectors carrying SWBV cDNA were sequenced using the Sanger method.

The SWBV-C1 RNA genome is 4,700 nt (Accession no. MT988146) and encodes five proteins, p33, p92, p41 (CP), p21, and p19, as in other tombusviruses (Figure 1d). The 5′- and 3′-untranslated regions of the SWBV-C1 genome are 149 and 332 nt, respectively, and “Y-shaped” structures, which act as 3′ cap-independent translation enhancers [14], were found in a section (nt 4,397–4,580) of the latter. An internal replication element [15], a cis-element required for genome replication, is present in the p92-coding region (nt 1,359–1,419). Moreover, sequences identical to the tomato bushy stunt virus upstream linker (UL: 5′-UGGAGAGUCUG-3′) and its complementary downstream linker (DL: 5′-CAGACUCUUCA-3′), which mediate long-range RNA–RNA interactions required for RNA replication [16], were also found (UL, nt 1,438–1,448; DL, nt 4,352–4,362). These data indicated that the fundamental tombusvirus genomic structures are conserved in the SWBV-C1 genome. Currently, complete genome sequences of SWBVs isolated from L. sinuatum (FN strain) and Eustoma grandiflorum (MAFF strain) are available (Accession nos. LC564888 and LC564887, respectively) [17]. The amino acid sequences of SWBV-C1’s p92, p41, p21, and p19 proteins were determined to be highly homologous to their corresponding sequences in SWBV-FN and -
MAFF. The sequence identity levels of p92, p41, p21, and p19 proteins between SWBV-C1 and -FN were 98.7%, 98.1%, 97.8%, and 96.5%, respectively, while between SWBV-C1 and -MAFF, they were 99.0%, 99.2%, 98.4%, and 96.5%, respectively. When the four protein sequences were used independently to establish tombusvirus phylogenetic trees, the relationships between SWBV-C1 and other tombusviruses differed, and gentian-infecting SWBV-C1 and GeVA were not closely related among the tombusviruses (Figure 1c; Online Resource 1).

The low-temperature (18°C) multiplication preference of GeVA in gentians and *A. thaliana* ecotype Columbia-0 (Col-0) has been reported [9]. Therefore, we examined the effects of temperature on SWBV-C1 multiplication and virulence in gentians and Arabidopsis. For the infection assay, gentian plants grown in vitro, which are available all year, were inoculated with SWBV-C1 virion RNA (0.5 mg/ml). At 4 weeks post-inoculation, necrotic symptoms were observed on inoculated leaves of gentian cultivars ‘Alta’ and ‘Albireo’ at 18°C but not at 28°C (Figure 2a). To detect SWBV infections, rabbit SWBV-C1 virion-specific antiserum was prepared by Scrum Inc. (Tokyo, Japan), and press-blot assays [18] using the anti-SWBV-C1 antiserum were performed. At 10 days after inoculation, SWBV-C1 infections in the inoculated leaves of ‘Alta’ and ‘Albireo’ were detected at 18°C but not at 28°C (Figure 2b). The low-temperature-preferred multiplication and virulence of SWBV-C1 were observed in not only gentians but also in Arabidopsis (Figure 2c, d).

However, in other experimental plants, including *N. benthamiana*, *Nicotiana tabacum*, and *C. quinoa*, SWBV-C1 efficiently multiplied at both 18°C and 28°C (Table 1), which was consistent with previous findings for GeVA [9]. In comparison with GeVA, SWBV-C1-induced symptoms in gentians were similar or more severe (Online Resource 2). In particular, SWBV-C1 induced more severe symptoms than GeVA in the inoculated leaves of Col-0 (Figure 2e). Additionally, SWBV-C1 infections in Col-0 leaves but not gentian leaves were detected by the presence of small dots at 23°C (Figure 2b and 2d), while GeVA infections were not detected at either 23°C or 28°C (Figure 2d), suggesting that SWBV-C1 is more virulent than GeVA in Arabidopsis. Overall, although some differences between SWBV-C1 and GeVA were observed, these data highlight their common host ranges and viral multiplication properties. Thus, we speculated that a common mechanism may underlie the host-specific preference for low temperature associated with SWBV-C1 and GeVA multiplication. We then focused on further analyzing SWBV-C1.

To elucidate tombusvirus–gentian/Arabidopsis interactions, we established an infectious SWBV-C1 genomic RNA synthesis system to analyze viral factors. The full-length cDNA of SWBV-C1 genomic RNA was amplified using the primer pair T7-GtSWBV5′-FW (5′-AAGCTTGATGCTGCTGATATACGACTCTAATAGGAAATTCTCCAGGATTTTCTC-3′) and GtSWBV-RV, and the resulting cDNA fragment was inserted into the *Pst*I- and *Xba*I double-digested pUC19 vector using an In-Fusion HD Cloning Kit (TaKaRa) to construct pT7-SWBV-C1 (Figure 2f). The 5′ primer (T7-GtSWBV5′-FW) contained the T7 promoter sequence. On the basis of previous reports of biologically active bromovirus cDNA clones [19, 20], an extra G residue was added to the 5′ terminus of the SWBV-C1 genome to enhance the efficiency of in vitro transcription. The 3′ primer (GtSWBV-RV) contained a *Mlu*I site for the linearization of the cloned plasmid. The SWBV-C1 genomic sequence in pT7-SWBV-C1 was identical to that determined in the above experiments. pT7-SWBV-C1 was digested with *Mlu*I and transcribed by T7 RNA polymerase. The resulting in vitro transcripts contained extra residues at the 5′ and
3′ termini of the SWBV-C1 genomic sequence (Figure 2f). Because the addition of these extra residues did not have any detrimental effects on the infectivity of SWBV in *N. benthamiana* (Online Resource 3), we used the transcripts for further infection assays. After the Japanese gentian cultivar ‘Alta’ was inoculated with SWBV-C1 transcripts, severe necrotic symptoms were induced at 18°C but not 28°C (Figure 2g), and the resulting symptoms were similar to those produced by inoculation with SWBV virion RNA. Additionally, the low-temperature-preferred multiplication was detected in gentian and Arabidopsis leaves inoculated with the SWBV-C1 transcript (Figure 2h). Overall, the infectivity of the SWBV-C1 transcript were identical to those of the SWBV-C1 virion RNA in all the tested plants under the test conditions (Table 1). Thus, these data confirmed that viral factors underlying SWBV-C1 features (*e.g.*, host range, symptoms, and host-specific low-temperature-preferred multiplication) are encoded in the same genomic RNA sequence. The SWBV factors, especially those involved in SWBV-C1-characteristic features, will be analyzed using this biologically active SWBV cDNA clone in the future.

**Declarations**

**Acknowledgements**

We thank Masahiro Nishihara for plant materials, Sayaka Fujisaki for general assistance and Lesley Benyon, PhD, from Edanz Group (https://en-author-services.edanzgroup.com/) Edanz Group (https://en-author-services.edanz.com/ac) for editing a draft of this manuscript.

**Funding**

This study was funded by Iwate Prefecture, Japan and Japan Society for the Promotion of Science (JSPS) KAKENHI (Grant No. 18K05661).

**Conflict of interest**

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

**Code availability**

Not applicable.

**Authors’ contributions**

KF, MK, and KM designed the research. KF, CT, YA, JD, MI, and KO performed the research and analyzed the data. TN and YI located and identified diseased plant materials. KF, CT, MK, and KM wrote the paper.

**References**

1. Nishihara M, Tasaki K, Sasaki N, Takahashi H (2018) Development of basic technologies for improvement of breeding and cultivation of Japanese gentian. Breed Sci 68:14–24.
2. Nekozuka S, Hamada K, Katsube K (2005) Necrotic mottle disease of gentian caused by Impatiens necrotic spot virus. Jpn J Phytopathol 71:183–184.
3. Yamamoto H (2012) Detection of Broad bean wilt virus 2, Clover yellow vein virus, Cucumber mosaic virus and Gentian mosaic virus in gentian by multiplex RT-PCR. Ann Rept Plant Prot North Japan 63:107–109.
4. Kobayashi YO, Kobayashi A, Hagiwara K, Uga H, Mikoshiba Y, Naito T, Honda Y, Omura T (2005) Gentian mosaic virus: a new species in the genus Fabaviridae. Phytopathology 95:192–197. https://doi.org/10.1094/PHYTO-95-0192
5. Kobayashi K, Atsumi G, Iwadate Y, Tomita R, Chiba K, Akasaka S, Nishihara M, Takahashi H, Yamaoka N, Nishiguchi M, Sekine KT (2013) Gentian Kobu-sho-associated virus: a tentative, novel double-stranded RNA virus that is relevant to gentian Kobu-sho syndrome. J Gen Plant Pathol 79:56–63. https://doi.org/10.1007/s10327-012-0423-5
6. Atsumi G, Tomita R, Yamashita T, Sekine KT (2015) A novel virus transmitted through pollination causes ring-spot disease on gentian (Gentiana triflora) ovaries. J Gen Virol 96:431–439. https://doi.org/10.1099/vir.0.071498-0
7. Fujisaki K, Tateda C, Shirakawa A, Iwai M, Abe Y (2018). Identification and characterization of a tombusvirus isolated from Japanese gentian. Arch Virol 163:2477–2483. https://doi.org/10.1007/s00705-018-3888-5
8. Lommel SA, Sit TL (2008) Tombusviruses. In: Mahy BWJ, van Regenmortel MHV (eds) Encyclopedia of Virology, 3rd Academic Press, Waltham, MA, pp 145–151.
9. Fujisaki K, Abe Y, Tateda C, Iwai M, Kaido M, Mise K (2020) Host specific preference for low temperature in the multiplication of a tombusvirus, gentian virus A. Virus Res 286:198048. https://doi.org/10.1016/j.virusres.2020.198048
10. Atsumi G, Sekine KT, Kobayashi K (2015) A New Method to Isolate Total dsRNA. In: Uyeda I, Masuta C (eds) Plant Virology Protocols. Methods in Molecular Biology (Methods and Protocols), vol 1236. Humana Press, New York, NY. https://doi.org/10.1007/978-1-4939-1743-3_3
11. Kobayashi K, Tomita R, Sakamoto M (2009) Recombinant plant dsRNA-binding protein as an effective tool for the isolation of viral replicative form dsRNA and universal detection of RNA viruses. J Gen Plant Pathol 75:87–91. https://doi.org/10.1007/s10327-009-0155-3
12. Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (2005) Eighth Report of the International Committee on Taxonomy of Viruses. Academic Press, Waltham, MA, pp 907–935.
13. Koenig R, Verhoeven JThJ, Fribourg CE, Pfeilstetter E, Lesemann DE (2004) Evaluation of various species demarcation criteria in attempts to classify ten new tombusvirus isolates. Arch Virol 149:1733–1744. https://doi.org/10.1007/s00705-004-0331-x
14. Nicholson BL, Zaslaver O, Mayberry LK, Browning KS, White KA (2013) Tombusvirus Y-shaped translational enhancer forms a complex with eIF4F and can be functionally replaced by heterologous translational enhancers. J Virol 87:1872–1883. https://doi.org/10.1128/JVI.02711-12
15. Nicholson BL, Lee PKK, White KA (2012) Internal RNA replication elements are prevalent in Tombusviridae. Front Microbiol 3:279. https://doi.org/10.3389/fmicb.2012.00279

16. Wu B, Pogany J, Na H, Nicholson BL, Nagy PD, White KA (2009) A discontinuous RNA platform mediates RNA virus replication: Building an integrated model for RNA-based regulation of viral processes. PLoS Pathog 5:e1000323. https://doi.org/10.1371/journal.ppat.1000323

17. Uehara-Ichiki T, Urayama S, Hirai M, Takaki Y, Nunoue T, Fujinaga M, Hanada K (2021) Complete genome sequence of Sikte (Sitke) waterborne virus, a member of the genus Tombusvirus. Arch Virol in press. https://doi.org/10.1007/s00705-020-04949-0

18. Fujisaki K, Iwahashi F, Kaido M, Okuno T, Mise K (2009) Genetic analysis of a host determination mechanism of bromoviruses in Arabidopsis thaliana. Virus Res 140:103–111. https://doi.org/10.1016/j.virusres.2008.11.007

19. Fujisaki K, Hagihara F, Kaido M, Mise K, Okuno T (2003) Complete nucleotide sequence of spring beauty latent virus, a bromovirus infectious to Arabidopsis thaliana. Arch Virol 148:165–175. https://doi.org/10.1007/s00705-002-0909-0

20. Iwahashi F, Fujisaki K, Kaido M, Okuno T, Mise K (2005) Synthesis of infectious in vitro transcripts from Cassia yellow blotch bromovirus cDNA clones and a reassortment analysis with other bromoviruses in protoplasts. Arch Virol 150:1301–1314. https://doi.org/10.1007/s00705-005-0500-6

21. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

22. Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870-1874. https://doi.org/10.1093/molbev/msw054

23. Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783-791.

### Tables

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