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

Koki Fujisaki (k-fujisaki@ibrc.or.jp)
Iwate Biotechnology Research Center https://orcid.org/0000-0002-8247-6381
Chika Tateda
Iwate Biotechnology Research Center
Yoshiko Abe
Iwate Biotechnology Research Center
John Jewish A. Dominguez
Iwate Biotechnology Research Center
Mari Iwai
Iwate Biotechnology Research Center
Kazue Obara
Iwate Biotechnology Research Center
Taiki Nakamura
Iwate Agricultural Research Center
Yasuya Iwadate
Iwate Agricultural Research Center
Masanori Kaido
Setsunan Daigaku - Hirakata Campus
Kazuyuki Mise
Kyoto University Graduate School of Agriculture Faculty of Agriculture: Kyoto Daigaku Nogaku Kenkyuka Nogakubu

Research Article

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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 GVCPr1 (5'-

TTAAGGGAATGTGACCGAGTTTAT-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'-GGGCTGCATTTCTGCAATGT-3') and ReverTra Ace (TOYOBO). The full-length cDNA of SWBV genomic RNA was amplified by PCR using the primer pair GtSWBV-FW (5'-AAGCTTGCATGCCTGCAGGAAATTCTCCAGGATTTCTC-3') and GtSWBV-RV (5'-ACCCGGGGATCCTCTAGAACGCGTGGGCTGCATTTCTGCAATGT-3'). pUC19 was digested with *Pst*I and *Xba*I, 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'-AAGCTTGCATGCCTGCAGTAATACGACTCACTATAGGAAATTCTCCAGGATTTCTC-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

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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.

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Tables

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Figures

(Fig.1)

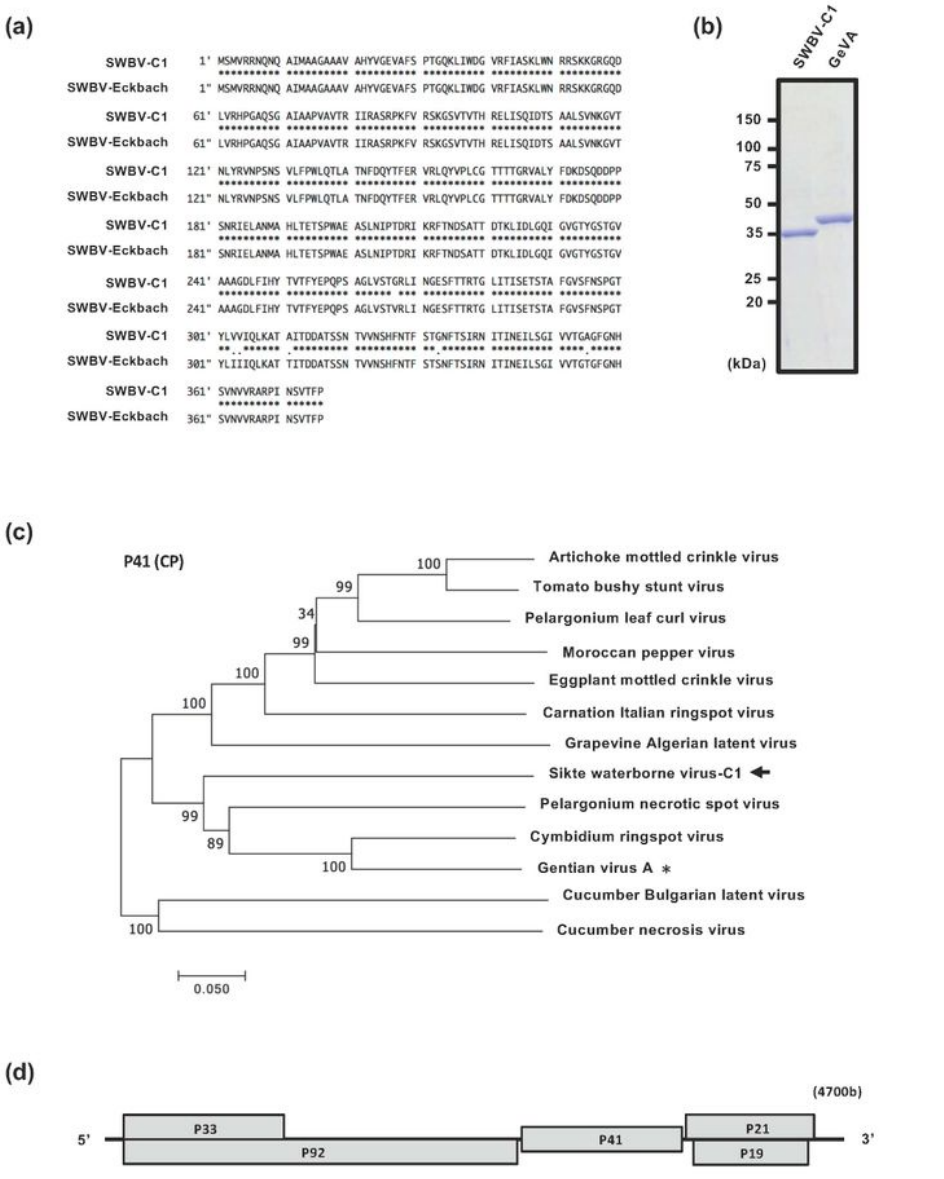

Figure 1

Genomic structure and analysis of the coat protein (CP) of Sikte waterborne virus strain C1 (SWBV-C1) isolated from Japanese gentian. (a) Alignment of the deduced amino acid sequences of SWBV-C1 and SWBV-Eckbach CPs (Accession no. AY500889). Alignment data were obtained using GENETYX-Mac ver.17 (GENETYX Corporation). (b) CPs of purified SWBV-C1 and gentian virus A (GeVA). Purified virions of SWBV-C1 and GeVA were subjected to SDS-PAGE, and proteins were stained by Coomassie Brilliant

Blue. The positions of molecular weight markers are indicated at the left. (c) Phylogenetic analysis of SWBV and 12 other tombusvirus CPs. The evolutionary history was inferred using the Neighbor-Joining method [21], and the analysis was conducted in MEGA7 [22]. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches [23]. The arrow and asterisk indicate the positions of SWBV-C1 and GeVA, respectively. Sequence information for Artichoke mottled crinkle virus (Accession no. X62493), Carnation Italian ringspot virus (Accession no. KP888563), Cucumber Bulgarian latent virus (Accession no. NC_004725), Cucumber necrosis virus (Accession no. M25270), Cymbidium ringspot virus (Accession no. NC_003532), Eggplant mottled crinkle virus (Accession no. NC_023339), GeVA (Accession no. LC373507), Grapevine Algerian latent virus (Accession no. NC_011535), Moroccan pepper virus (Accession no. NC_020073), Pelargonium leaf curl virus (Accession no. NC_030452), Pelargonium necrotic spot virus (Accession no. NC_005285) and Tomato bushy stunt virus (Accession no. M21958) were obtained from the NCBI database. (d) Schematic diagram of the SWBV-C1 genome. Five protein-coding regions conserved in tombusviruses are indicated by grey boxes, and non-coding regions are indicated by horizontal bars

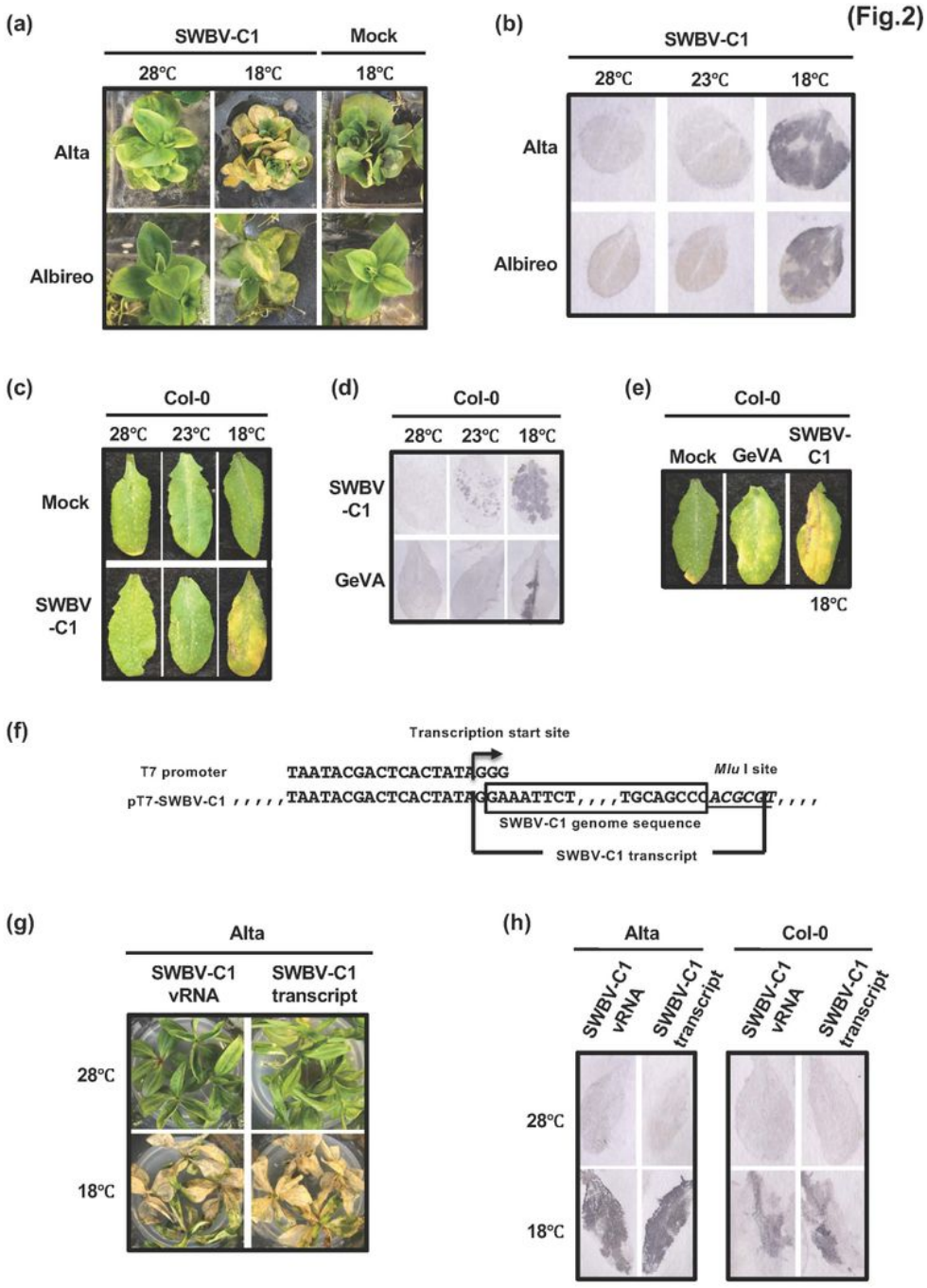

Figure 2

Virulence and infectivity of Sikte waterborne virus strain C1 (SWBV-C1) in Japanese gentians and Arabidopsis thaliana ecotype Columbia-0 (Col-0) at different temperatures. (a) Symptoms induced by SWBV-C1 on Japanese gentian cultivars ‘Alta’ and ‘Albireo’ grown in vitro. SWBV-C1 virion RNAs (0.5 mg/ml) were mechanically inoculated into gentians, and plants were photographed at 4 weeks post-inoculation (wpi). (b) SWBV-C1 infections in gentian leaves. SWBV-C1-inoculated leaves of ‘Alta’ and

‘Albireo’ were harvested at 10 d post-inoculation (dpi). SWBV-C1 were detected using press-blot assays with a SWBV-specific antiserum. The temperatures at which data were collected are indicated. (c) Symptoms induced by SWBV-C1 on inoculated leaves of Col-0. Leaves were photographed at 2 wpi. (d) SWBV-C1 infections in Col-0. Inoculated leaves were harvested at 10 dpi, and SWBV-C1 multiplication was detected as described in (b). (e) Comparison of symptoms between SWBV-C1- and gentian virus A (GeVA)-inoculated Col-0 leaves. Leaves were photographed at 2 wpi. (f) Partial nucleotide sequences of full-length cDNA clones (pT7-SWBV-C1) of SWBV-C1 genomic RNA. The SWBV-C1 genomic RNA sequence’s region is indicated by an open box, and the MluI site for the linearization of cloned plasmids is represented by italicized, underlined residues. The T7 promoter sequence and its transcription start site are also indicated. (g) Comparison of the virulence of SWBV-C1 virion RNA (vRNA) and its transcript in ‘Alta’. ‘Alta’ plants grown in vitro were independently inoculated with SWBV-C1 vRNA (0.5 mg/ml) and transcripts (0.5 mg/ml), and plants were photographed at 4 wpi. (h) SWBV-C1 infections in transcript-inoculated ‘Alta’ and Col-0 leaves. Leaves were harvested at 10 dpi, and SWBV-C1 infections were detected as described in (b). SWBV-C1 vRNA was used as a control. For all the data, the sample temperatures are indicated.

Supplementary Files

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