A novel virus transmitted through pollination causes ring-spot disease on gentian (Gentiana triflora) ovaries

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In this study, we identified a novel virus from gentian (Gentiana triflora) that causes ring-spots on ovaries. Furthermore, the virus causes unusual symptoms, ring-spots that appear specifically on the outer surface of the ovarian wall after pollination. Pollen grains carrying the virus were used to infect host plants by hand-pollination. RNA extracted from purified virions indicated that the virus had two segments, RNA1 and RNA2. The full-length cDNA sequence indicated that RNA1 had two ORFs: ORF1 had methyltransferase and helicase motifs, and ORF2 had an RNA-dependent RNA polymerase motif. RNA2 had five ORFs encoding a coat protein, triple gene block proteins 1–3 and a cysteine-rich protein. The length of RNA1 was 5519 bases and that of RNA2 was 3810 bases not including a polyU/polyA region between the first and second ORFs. Viral RNA does not have a polyA tail at the 3’ end. Sequence similarity and phylogenetic analysis suggested that the virus is closely related to members of the genera Pecluvirus and Hordeivirus but distinct from them. These combined results suggest that the causal agent inducing ring-spot symptoms on gentian ovaries is a new virus belonging to the family Virgaviridae but not to any presently known genus. We tentatively name the virus gentian ovary ring-spot virus.

INTRODUCTION

Plant viruses cannot directly infect intact host cells because 1) plant cells do not have receptors for viruses to enter the plasma membrane, and 2) viruses cannot penetrate physical barriers such as cell walls (Hull, 2013). In nature, plant viruses are transmitted by mechanical means including human activities or biological vectors such as fungi, nematodes, mites or insects. Transmission also occurs through pollen grains carrying the virus that can spread rapidly by wind, honeybees or humans.

Pollen-mediated virus transmission occurs vertically and/or horizontally (Card et al., 2007). At present, viruses of at least 17 genera appear to be transmitted by pollen horizontally and/or vertically (Hull, 2013). In horizontal transmission, viruses infect plants after pollination from pollen grains carrying virus to maternal tissue. In vertical transmission, the virus infects mother host plants and invades the reproductive organ. When virus infects a seed, a seedling that will grow from the seed may be infected with virus. If viruses are effectively transmitted by pollen horizontally, there can be significant problems, especially for perennial plants, because the viral infection persists in the following year; however, little is known about horizontal transmission. Effective transmission of viruses can cause significant economic loss. Therefore, identifying the transmission route and characterizing the mechanism for transmission are important factors towards developing approaches to control viral diseases.

Japanese gentians (Gentiana triflora, Gentiana scabra and their hybrids) are blue-flowered plants and one of the most important ornamental flowers in Japan. Japanese gentians are perennials and are usually cultivated in open fields. Therefore, gentians are continuously exposed to pathogen infections including viruses. Cucumber mosaic virus (genus Cucumovirus), Broad bean wilt virus and Gentian mosaic virus (Fahavirus), Clover yellow vein virus and Bean yellow mosaic virus (Potyvirus), Tobacco rattle virus (Tobravirus), Impatiens necrotic spot virus (Tospovirus) and Gentian Kobu-sho-associated virus (unassigned) have been reported as pathogens threatening gentian production in Japan (Atsumi et al., 2013; Kobayashi et al., 2005, 2013). If a virus infects gentians, it is likely that the viral infection is sustained in the following years. Therefore, it is quite important to control viral infections for stable gentian production. In 2009, a new disease with virus-like symptoms was observed in gentians cultivated for seed production in open fields in Iwate, Japan. Ring-spot symptoms developed on the ovaries after hand-pollination. The yield of seeds from...
diseased ovaries tended to be reduced, thus presenting a significant problem for stable gentian seed production.

In this study, we show that the virus is detected from tissue developing ring-spot symptoms. We also show that the virus effectively infects gentian maternal tissues when hand-pollinated using pollen grains carrying the virus. Furthermore, we show that the ring-spot symptoms develop on the outer surface of the ovarian wall after pollination. Finally, the characterized viral properties indicate that the identified virus is a novel virus that presumably belongs to a new genus in the family Virgaviridae.

RESULTS

Ring-spot symptoms on ovaries and virus identification

Ring-spot symptoms developed on the ovaries of hand-pollinated gentians grown in open fields for seed production in Iwate, Japan (Fig. 1a). Because ring-spot symptoms are typically caused by viruses, this phenotype was presumed to be virally induced. Previously, we developed a novel high-throughput procedure for disease diagnosis, the DECS method, that detects RNA viruses without having any prior sequence information (Kobayashi et al., 2009, 2013). Isolation of dsRNA from symptomatic gentian ovaries revealed that there were two different sizes of dsRNA (Fig. 1b). BLAST searches of the dsRNA-derived sequences showed limited similarity with sequences of Lychnis ringspot virus and Barley stripe mosaic virus, both of which belong to the genus Hordeivirus in the family Virgaviridae (data not shown).

Virus characterization

Crude sap prepared from ovaries showing ring-spot symptoms was mechanically inoculated onto Nicotiana benthamiana leaves. Inoculated leaves developed chlorotic and necrotic rings (Fig. 2a), and the upper non-inoculated leaves developed line patterns and yellowing (Fig. 2b). A single-lesion isolation was repeated several times from the inoculated leaves of N. benthamiana. We tentatively named the virus gentian ovary ring-spot virus (GORV) isolate S.

Virions were purified from N. benthamiana leaves infected with GORV. A single major band was observed near the 28 kDa protein marker after SDS-PAGE of the purified particles (Fig. 2c). N. benthamiana leaves inoculated with virions developed ring-spot symptoms within 5 days, indicating that the purified virions were infectious (data not shown).

LC-MS/MS analysis of purified virions revealed that all of the peptides were derived from ORF3 of RNA2 and shared homology with the coat proteins (CP) of members of the genera Pecluvirus and Hordeivirus by a BLAST search (Fig. 2d, Table S1, available in the online Supplementary Material). A single band near the 28 kDa size marker (similar in size to the purified virions) was detected specifically in immunoblots of infected-plant proteins using the antibody against a synthetic peptide (YGYVLSDLVTTR) from the deduced amino acid sequence of the CP gene (Fig. 2e). Viral RNA was isolated from purified virions. Denaturing agarose gel electrophoresis indicated that naked RNA was susceptible to RNaseA digestion (Fig. 2f). By contrast, the analysis of RNA from virions treated with RNaseA showed that the virions contained approximately 6000 nt and 4000 nt RNAs, which were similar in size to the dsRNAs isolated from diseased tissue (Figs 2f, 1b).

Transmission of the ring-spot disease phenotype by hand pollinating with pollen grains carrying GORV

To test our hypothesis that pollen carrying GORV was the source of transmission, uninfected gentian plants were hand-pollinated with pollen grains carrying GORV. At 11 days after the first pollination, ring-spot symptoms appeared on the outer surface of the ovarian walls. Three weeks after the first pollination, ring-spot symptoms were observed on 107 of 614 ovaries (from 14 out of 16 plants) pollinated with pollen grains carrying GORV, whereas no ring-spot symptoms were observed in 572 ovaries (16 plants) pollinated with healthy pollen grains. We randomly collected two ovaries (ovarian wall) per plant and checked for GORV infection by reverse transcription (RT)-PCR. GORV was detected in all samples showing ring-spot symptoms. Furthermore, all the ovaries pollinated with pollen grains carrying GORV but not showing ring-spot symptoms also tested positive for the virus by RT-PCR. By contrast, GORV was not detected in any ovaries pollinated with GORV-free pollen grains.
To investigate whether GORV induces ring-spot symptoms in other hosts, we inoculated GORV onto the leaves of Eustoma grandiflorum, petunia (Petunia hybrida), tobacco (Nicotiana tabacum), Capsicum annuum, Vigna unguiculata, Phaseolus vulgaris and Chenopodium quinoa. We found that GORV could induce ring-spot symptoms on petunia and tobacco leaves (Fig. S1a–d). Concentric rings of auto-fluorescence were clearly observed on the inoculated leaves (Fig. S1e–h), indicating cell death. GORV CP was detected in samples from all the inoculated leaves of petunia (five inoculated plants) and tobacco (five inoculated plants) by Western blot analysis using an antibody against GORV CP (Fig. S1i, j). Virus infections were not confirmed for plants not exhibiting ring-spot symptoms.

Full-length sequences of GORV RNAs

We determined the full-length sequences of GORV RNA1 and RNA2 using RNAs isolated from purified virions (see Methods). The full-length sequence of RNA1 was 5519 bases. For RNA2, we found that polyU/polyA (UnAn) sequences existed between the first and second ORFs (Fig. 3a, grey bar). The RT-PCR products of the UnAn region were cloned into a plasmid vector. Sequence analysis of 32 clones indicated that the number of uracils ranged from 10 to 24 (Fig. 3b). The mean number of uracils was 13.59 (SD was 2.72) and the median was 13. This finding suggested that the number of uracils in the UnAn region of RNA2 was heterogeneous in vivo. By contrast, we could not determine the number of adenine bases by sequencing PCR products or even plasmid clones because of technical problems associated with sequencing long polyA sequences. We concluded that the length of RNA2 was 3810 bases not including the UnAn region.

Genome organization

To investigate the genomic organization of GORV, ORFs were predicted from the sequences of RNA1 and RNA2 by NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/) with standard codon usages. We found two ORFs (ORF1 and 2) in RNA1 and five ORFs (ORF3–7) in RNA2 (Fig. 3a). Nucleotide sequences of the 5′- or 3′-untranslated regions (UTRs) of RNA1 and RNA2 were aligned by the CARNA program (http://rna.informatik.uni-freiburg.de/CARNA/Input.jsp) (Dal Palù et al., 2010; Sorescu et al., 2012). The analysis revealed that the 3′-UTRs have high nucleotide sequence similarity (Fig. 3c). An Rfam search (http://rfam.xfam.org/) (Burge et al., 2013) showed that the 3′-UTRs of RNA1 and RNA2 have a Tyomivirus/Pomovirus tRNA-like 3′-UTR element (RF00233) between nucleotides 201 and 277 (E value = 1.1e-7), and 232 and 307 (E value = 6.1e-06), respectively (Fig. 3c, grey bar). Secondary structures of the sequences homologous to tRNA-like elements were predicted by CentroidHomfold (http://www.ncbi.nlm.nih.gov/CarNA/Homfold) (Hamada et al., 2009). This result suggested that both regions form tRNA-like...
structures (Fig. 3d). To investigate whether GORV RNA has a polyA sequence at the 3' end, RNA isolated from virions was self-ligated by RNA ligase, and the circular RNA was converted to cDNA by reverse transcriptase. Sequencing the region between the 5' and 3' ends of RNA2 showed that a polyA sequence did not exist (24 clones were sequenced).

**Homology search of deduced amino acid sequences encoded by each ORF**

Sequences homologous with the deduced amino acid sequences encoded by each ORF were searched for by PSI-BLAST (Altschul et al., 1997). The results are summarized in Table S1. Proteins encoded in ORF1 of RNA1 had homology with proteins having methyltransferase and helicase motifs (MetHel): *Pecluvirus* [Indian peanut clump virus (IPCV); E value = 0, top hit], *Hordeivirus* [barley stripe mosaic virus (BSMV); 0], *Pomovirus* [beet soil-borne virus (BSBV); 1e-102], *Furovirus* [sorghum chlorotic spot virus (SrCSV); 3e-94], *Tobravirus* [pepper ringspot virus (PeprSV); 5e-65] and *Tobamovirus* [maracuja mosaic virus (MarMV); 2e-55] of the family *Virgaviridae*. ORF2 had homology with RNA-dependent RNA polymerase (RdRp): *Pecluvirus* (IPCV; 5e-175), *Hordeivirus* (BSMV; 0), *Pomovirus* (BSBV; 1e-161), *Furovirus* (SrCSV; 0), *Tobravirus* (PeprSV; 5e-126) and *Tobamovirus* [odontoglossum ringspot virus (ORSV); 1e-64]. ORF3 in RNA2 had homology with coat protein of *Pecluvirus* [peanut clump virus (PCV); 3e-44] and *Hordeivirus* [lychnis ringspot virus (LRSV); 6e-37]. These results were consistent with the results from LC-MS/MS analysis of purified virions (Fig. 2d). ORF4 had homology with Triple Gene Block (TGB) 1 of *Hordeivirus* [poa semilatent virus (PSLV); 3e-56], *Pecluvirus* (PCV; 4e-77) and *Pomovirus* [potato mop-top virus (PMTV); 8e-73]. Furthermore, ORF4 also had homology with the TGB1 of *Benyvirus* [rice stripe necrosis virus (RSNV); 4e-22] (family unassigned), *Allexivirus* [garlic virus C (GarV-C); 2e-06] and *Potexvirus* [pepino mosaic virus (PepMV); 5e-07] of the family *Alphaflexiviridae*, and *Carlaviridae* (4e-06) of the family *Betaflexiviridae*. ORF5 had homology with TGB2 of *Hordeivirus* (LRSV; 1e-29), *Pecluvirus* (IPCV; 1e-26) and *Pomovirus* (2e-30). ORF5 also shared homology with TGB2 of *Benyvirus* [burdock mottle virus (BdmMoV); 3e-14], *Potevirus* [alternanthera mosaic virus (AltMV); 1e-06], *Carlaviridae* [sweet potato C6 virus (SPC6V); 8e-07] and *Foveavirus* [grapevine rupestris stem pitting-associated virus (GRSPA); 1e-06] of the family *Betaflexiviridae*. ORF6 had homology with TGB3 of members of the family *Virgaviridae* including *Hordeivirus* (LRSV; 7e-12), *Pecluvirus* (IPCV; 5e-08) and *Pomovirus* [beet virus Q (BVQ); 3e-12]. ORF7 had homology only with a cysteine-rich protein (CRP) of *Pecluvirus* (IPCV; 5e-05).

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**Fig. 3.** Genome organization of GORV. (a) Schematic representation of the genome structure of GORV. RdRp, RNA-dependent RNA polymerase; CP, coat protein; TGB, triple gene block; CRP, cysteine-rich protein. (b) Distribution of the number of T bases in the cDNA sequences of the UnAn region cloned into plasmid vectors (n = 32). (c) Alignment of the 3’-terminal sequences of RNA1 and RNA2 using the CARNA program. The grey bar below the alignment indicates the *Virgaviridae* (family unassigned) 3′-UTR element (RF00233) detected by Rfam search. (d) Predicted secondary structures for the sequences of putative tRNA-like structures of RNA1 and 2.
Phylogenetic analysis

Phylogenetic relationships were inferred between GORV and other members of the family Virgaviridae (Adams et al., 2009). Deduced amino acid sequences of MetHel, RdRp, CP, TGB1-3 and CRP of GORV were aligned with the homologous proteins of each member of the family Virgaviridae listed in the study of Adams et al. (2009). Analyses indicated that confidences for TGB1-3 and CRP were low, and many branches had low bootstrap values in the maximum-likelihood trees. Thus, the trees were analysed only for MetHel, RdRp and CP. The tree reconstructed from the MetHel sequences revealed that GORV has the closest relationship with Pecluvirus (PCV and IPCV) (Fig. 4a). By contrast, the tree reconstructed from the RdRp sequences indicated that GORV was not clustered with any viruses (Fig. 4b). The tree reconstructed from CP sequences indicated that GORV was clustered with Hordeivirus (LRSV, BSMV and PSLV) in addition to Pecluvirus (PCV and IPCV) (Fig. 4c).

Cysteine-rich protein has RNA silencing suppression activity

CRP is conserved among the family Virgaviridae except for the majority of the genus Tobamovirus. Previous reports indicated that several CRPs have RNA silencing suppressor (RSS) activity, suggesting that the RSS activity of CRP is conserved among members of the Virgaviridae (Dunoyer et al., 2002; Martin-Hernández & Baulcombe, 2008; Yelina et al., 2002). RSS activity of CRP of GORV was assessed by an Agrobacterium-mediated transient assay in N. benthamiana leaf tissue. CRP tagged with 3 × HA peptide (CRP-3HA), GUS-3HA (negative control) or tomato bushy stunt virus p19 (positive control) were expressed with GFP and the inverted repeat sequence of GFP (inducer for silencing of GFP mRNA, IR-GFP). GFP fluorescence was not observed in the GUS-3HA-expressed area but was strongly present in the p19-expressed area (Fig. 5a). We also found GFP fluorescence in the CRP-3HA-expressed area (Fig. 5a). Northern blot analysis indicated that the GFP transcript level correlated with the amount of GFP fluorescence (Fig. 5b). CRP-3HA accumulation was confirmed by Western blot analysis using an antibody raised against the HA peptide (Fig. 5c). These results indicated that CRP encoded in GORV could suppress IR-triggered RNA silencing of mRNA.

DISCUSSION

Our investigation revealed that a virus was effectively transmitted horizontally by hand pollinating gentians with pollen grains carrying GORV. Furthermore, ring-spot disease developed efficiently (87.5 %) and rapidly (within 2 weeks), indicating that GORV could aggressively invade maternal tissues after pollination.

In this study, pollen grains carrying GORV were attached to stigma using a small ink brush in hand-pollination. Therefore, there is a possibility that GORV infects via wound sites caused by touching with the brush. It remains to be revealed whether GORV transmission occurred mechanically or required pollination/fertilization processes.

We showed that ring-spot symptoms developed on all but two of the plants that were hand-pollinated with pollen carrying GORV at the Iwate Agricultural Research Center (IARC) field. By contrast, the symptoms were not observed in any plants grown under growth chamber conditions (21–23 °C with a 16 h photoperiod) (data not shown). Fourteen out of 20 plants were GORV-positive in ovaries pollinated with pollen carrying GORV. These results suggest that environmental factors affect both symptom expression and virus transmissibility.

GORV is most likely a member of a new genus belonging to the family Virgaviridae for the following three reasons: 1) phylogenetic relationships and sequence identity, 2) genomic organization and 3) other viral characteristics. Phylogenetic analysis of GORV and other viruses belonging to the family Virgaviridae suggested that GORV had a close relationship with Pecluvirus (MetHel and CP) and Hordeivirus (CP) (Fig. 4). By contrast, amino acid identities of MetHel and CP between GORV and Pecluvirus or Hordeivirus were low. The highest sequence identity and similarity were 37.2 % and 54.3 % (gaps=14.5 %; IPCV of Pecluvirus) in MetHel; 41.8 % and 55.8 % (gaps=6.7 %; PCV of Pecluvirus), and 41.4 % and 58.6 % (gaps=3 %; LRSV of Hordeivirus) in CP, respectively (Table S1). These low identities and similarities indicate that GORV is a distinct member of the Virgaviridae family from the genera Pecluvirus and Hordeivirus.

The genomic organization observed in GORV has not been reported for any other members of the Virgaviridae family (Fig. 3a) (Adams et al., 2009). Members of the genus Pecluvirus have the most similar genomic organization (Adams et al., 2009). Pecluvirus encodes a replicase and CRP in RNA1 and encodes CP, P39 and TGB1-3 in RNA2. There are similarities in that the replicase unit is encoded in RNA1, and CP and TGB1-3 are in RNA2. By contrast, GORV did not encode a sequence homologous to P39, which is thought to be involved in the transmission of PCV by its fungal vector. Several peanut clump virus isolates propagated on N. benthamiana have long deletions in the P39 ORF, but even viruses with deletions could form virions and systemically infect plants (Manohar et al., 1993). For BVQ (genus Pomovirus), it is hypothesized that the coat protein readthrough domain, which has a crucial function in viral transmission by a protist such as Polymyxa, was shortened during serial passage in experimental host plants, Chenopodium quinoa (Crutzen et al., 2009; Koenig et al., 1998). In this study, we determined the sequence of RNA packaged within viromes, which was isolated from N. benthamiana leaves after serial passage. Therefore, there is a possibility that the ORF corresponding to P39 or new unknown ORF is deleted from the GORV genome. GORV encodes a CRP in RNA2 that is different from the CRP of
Fig. 4. Phylogenetic analysis of genes encoding methyltransferase/helicase, RdRp and CP. Maximum-likelihood trees of each gene encoding methyltransferase/helicase motifs (a), RdRp motif (b) and CP (c) were reconstructed using the deduced amino acid sequences of GORV and members of the Virgaviridae family. GORV is highlighted with grey. The majority-rule bootstrap consensus trees were reconstructed with a 70% cut-off value. The significance of the nodes was estimated with 1000 bootstrap replicates. Abbreviations and accession numbers of each virus are shown in Table S2.
members of the genus Pecluvirus encoded in RNA1. CRPs are encoded in the genomes of the family Virgaviridae (except for the majority of Tobamovirus). CRPs of members of the genera Hordeivirus, Pecluvirus and Tobravirus have RNA silencing suppressor activity (Dunoyer et al., 2002; Martín-Hernández & Baulcombe, 2008; Yelina et al., 2002). We showed that GORV CRP also has an ability to suppress RNA silencing using a transient assay in N. benthamiana (Fig. 5). Our results suggest that the GORV CRP is closely related to CRPs encoded by other members of the family Virgaviridae.

All viruses in the family Virgaviridae have tRNA structure and no polyA tail at the 3’ end (Adams et al., 2009). Rapid amplification of cDNA ends (RACE) analysis using circularized RNA revealed that RNA2 does not have a polyA sequence at the 3’ end. There is homology between RNA1 and RNA2 in the 3’ terminal sequences where a Tymovirus/Pomovirus tRNA-like 3’-UTR element was detected by an Rfam search (Fig. 3c, d). Another feature of the Virgaviridae is that virus particles are rod-shaped and 20–25 nm in diameter (Adams et al., 2009). Electron microscopic analysis of virions indicated that the GORV virions were rod-shaped and approximately 20 nm in width (data not shown). However, we could not estimate the length of virions because many different lengths of virions were observed under the present experimental conditions. We are attempting to isolate the intact virions and estimate their size. Taken together, our results indicate that GORV is most likely a member of a new genus belonging to the family Virgaviridae.

METHODS

Plant growth conditions and viral infection protocol. Plants used in this study [gentian (G. triflora), N. benthamiana, E. grandiflorum, petunia, tobacco, Capsicum annuum, V. unguiculata, P. vulgaris and Chenopodium quinoa] were cultivated at 21–23 °C with a 16 h photoperiod in a growth chamber or room. Virions or crude sap prepared from N. benthamiana leaves infected with GORV were inoculated mechanically onto plant leaves dusted with carborundum powder. Virions were suspended in inoculation buffer (10 mM phosphate buffer, pH 7.0, 1%, v/v, 2-mercaptoethanol). Crude sap was prepared by grinding the leaves with 10 volumes of inoculation buffer relative to leaf fresh weight.

DECS (dsRNA isolation, exhaustive amplification, cloning and sequencing) method. Total RNA was extracted from the ovaries or leaves by the AGPC (acid guanidinium thiocyanate–phenol/chloroform extraction) method (Chomczynski & Sacchi, 1987). dsRNA was isolated from total RNA using recombinant dsRNA-binding protein, DRB4* fused with GST (GST-DRB4*) (Kobayashi et al., 2009). The dsRNA was converted to cDNA and then amplified exhaustively. The amplified fragments were cloned into the pCR4-TOPO vector (Life Technologies) according to the manufacturer’s instructions. The sequences of fragments cloned in pCR4 vector were determined on a 3130 genetic analyser (Applied Biosystems) using M13-M3 or M13-RV primers (Table S3). Homologous sequences were obtained by BLAST searches.

RNA extraction, reverse transcription and PCR. Total RNA was isolated by the AGPC method or an RNAqueous kit (Life Technologies) according to the manufacturer’s instructions (Chomczynski & Sacchi, 1987). Total RNA was reverse-transcribed using ReverTra Ace (TOYOBO) or PrimeScript RTase (TaKaRa) according to the manufacturer’s instructions. For virus detection, GORV fragments or β-actin mRNA (positive control) was amplified by PCR using Ex-Tag DNA polymerase (TaKaRa). For determination of the full-length sequence of GORV, GORV fragments were amplified using high-fidelity PrimeSTAR GXL DNA polymerase (TaKaRa) according to the manufacturer’s instructions. Primers for GORV detection were 913 and 914 and those for β-actin mRNA were GtACT1-U50 and GtACT1-L555. Primers used in this study are listed in Table S3.

Purification of virus particles. GORV-infected N. benthamiana leaves were homogenized with 0.1 M sodium citrate buffer (pH 6.5) containing 10 mM EDTA and 0.25% (v/v) 2-mercaptoethanol using a mortar and pestle. The homogenate was centrifuged at 6000 g for 10 min; the supernatant was added with 10:1 volume of 20% (v/v) Triton X-100. The suspension was layered on a 20% (w/v) sucrose cushion and centrifuged at 125,000 g for 2 h. The pellet was thoroughly resuspended in 0.05 M sodium citrate buffer (pH 6.5) containing 5 mM EDTA, and the suspension was centrifuged at 12,000 g for 5 min. The supernatant was layered on a 20% (w/v)
DNA fragments were sequenced using a primer-walking method. For RNA1, four overlapping fragments were amplified using primer manufacturer’s instructions. The resulting fragment was cloned into a GeneRacer kit (Life Technologies) according to the manufacturer’s instructions and 3’-RACE of the GORV genome (RNA1 and RNA2) was conducted using a GeneRacer kit (Life Technologies). cDNA was synthesized and PCR was conducted using primers oriented to the 5’- and 3’-terminal regions: 574 and 575. The fragments were cloned into the pCR4-TOPO vector and sequenced using primers, M13-M3 and M13-RV.

For polyA analysis, RNA extracted from GORV virions was dephosphorylated by calf-intestinal alkaline phosphatase, followed by dephosphorylation with tobacco acid pyrophosphatase. The RNAs were treated with T4 RNA ligase to produce self (head-to-tail)-ligated forms using the enzymes included in the GeneRacer kit (Life Technologies). cDNA was synthesized and PCR was conducted using primers oriented to the 5’- and 3’-terminal regions (R14 or R15) and those oriented to the 3’- end in the 3’-terminal region (F4 or F10) of RNA2. PCR products were cloned into the pCR4-TOPO vector. Twenty-four clones were sequenced in total: six clones for R14-F4 fragment and R15-F4, five clones for R14-F10 and seven clones for R15-F10.

Primers used in this study are listed in Table S3.

**Microscopy.** An epifluorescence microscope (SZX16; Olympus) equipped with a filter unit (SZX2-FCFPHQ; Olympus), was used to detect the auto-fluorescence occurring during cell death. Images were recorded using a charge-coupled device (CCD) camera (DP70; Olympus).

**Phylogenetic analysis.** Multiple sequence alignment (MSA) was conducted using a web-base GUIDANCE server (http://guidance.tau.ac.il/) (Penn et al., 2010). All MSAs were created by MAFFT (Katoh et al., 2005). The confidences of the MSAs were estimated by the GUIDANCE algorithm, and trees were reconstructed by using the MEGA5 package (Tamura et al., 2011). The substitution models and rates among sites were rtREV+G+I+F, which was determined by the MEGA5 program. For reconstructing the trees, we used MSAs with columns cut below the 0.93 score (approx. 63 % columns remain) for MetHcl and cut below the 0.93 score (approx. 84 % columns remain) for RdRp using the GUIDANCE algorithm. As Tobravirus sequences lowered the confidence of the MSA for the CP sequence, their sequences were removed and columns below the 0.93 score were cut (approx. 40 % columns remain). The majority-rule bootstrap consensus trees were reconstructed with a 70 % cut-off value. The significance of the nodes was estimated with 1000 bootstrap replicates.

**Plant pollination.** Uninfected gentians were pollinated using healthy pollen grains or pollen grains carrying GORV. Pollen grains were attached to a stigma using a small ink brush. The plants were first tested by RT-PCR or Western blot analysis using an antibody raised against GORV CP to verify that they were not infected with GORV. Gentians were cultivated and hand-pollinated in an experimental field at the IARC from 15 August to 5 September 2013. Sixteen plants were pollinated using healthy (GORV-free) or GORV-carrying pollen grains. Plants were pollinated several times on different days. Symptoms were observed for 3 weeks.

**Full-length genomic sequence of GORV.** Based on the short contigs obtained by DECS analysis, viral fragments were amplified from cDNA synthesized from RNA extracted from virions, and 5’- and 3’- RACE of the GORV genome (RNA1 and RNA2) was conducted using a GeneRacer kit (Life Technologies) according to the manufacturer’s instructions. The resulting fragment was cloned into the pCR4-TOPO vector, and the consensus sequence was determined from three plasmid clones. For RNA1, four overlapping fragments were amplified using primer sets 604-605, 580-588, 606-579 and 612-616. The resulting DNA fragments were sequenced using a primer-walking method using primers M13-M3, M13-RV, 571, 578, 579, 580, 581, 585, 586, 587, 588, 601, 603, 604, 605, 606, 607 and 609. For RNA2, a fragment amplified using primer set F12 and R16 was sequenced using primers M13-M3, M13-RV, F3, F11, R12, F7, F2, F1, R1, F4, F10 and R10. To determine the number of U and A bases in the UnAn region of RNA2, the region was amplified using primer sets designed in adjacent regions: 574 and 575. The fragments were cloned into the pCR4-TOPO vector and sequenced using primers, M13-M3 and M13-RV.

**Western blot analysis.** Western blots were conducted as described previously (Sekine et al., 2012). In brief, proteins were dissolved in 12 % (w/v) NuPAGE Bistris gel (Life Technologies) in MES-SDS buffer, followed by electrophoresis to a PVDF membrane. To detect the GORV CP, rabbit IgG raised against a GORV CP peptide (GGYVLSDLVTTR) was used as the primary antibody and goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad Laboratories) was used as the secondary antibody. To detect the HA-tagged proteins, rat monoclonal antibody against the HA peptide (Roche Diagnostics) was used for the primary antibody and goat anti-rat IgG conjugated with horseradish peroxidase (Sigma-Aldrich) was used as the secondary antibody. The chemiluminescence signals were detected with the ECL Prime (Roche Diagnostics) using a LAS-4000 camera system (GE Healthcare). As a loading control, membranes after transfer were stained with Amido Black (Sekine et al., 2012).

**Phylogenetic analysis.** Multiple sequence alignment (MSA) was conducted using a web-base GUIDANCE server (http://guidance.tau.ac.il/) (Penn et al., 2010). All MSAs were created by MAFFT (Katoh et al., 2005). The confidences of the MSAs were estimated by the GUIDANCE algorithm, and trees were reconstructed by using the MEGA5 package (Tamura et al., 2011). The substitution models and rates among sites were rtREV+G+I+F, which was determined by the MEGA5 program. For reconstructing the trees, we used MSAs with columns cut below the 0.93 score (approx. 63 % columns remain) for MetHcl and cut below the 0.93 score (approx. 84 % columns remain) for RdRp using the GUIDANCE algorithm. As Tobravirus sequences lowered the confidence of the MSA for the CP sequence, their sequences were removed and columns below the 0.93 score were cut (approx. 40 % columns remain). The majority-rule bootstrap consensus trees were reconstructed with a 70 % cut-off value. The significance of the nodes was estimated with 1000 bootstrap replicates.

**Northern blot analysis.** Leaves were homogenized in liquid nitrogen, and total RNA was isolated as described above. Total RNA (3 μg) was dissolved in RNA denaturing pre-mix (1 x MOPS buffer, 50 %, v/v, formamide, 17.5 %, v/v, formaldehyde), heated at 70 °C for 15 min, separated in a 1.2 % (w/v) denaturing gel, and blotted to a nylon membrane (Hybond-N; GE Healthcare). The membrane was hybridized with a DIG-labelled RNA probe for a GFP sequence in hybridization buffer (DIG Easy Hyb; Roche Diagnostics).
The chemiluminescence signals were detected with CDP-Star Reagent (New England Biolabs) using a LAS-4000 camera system (GE Healthcare).

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