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A novel toti-like virus from a plant pathogenic oomycete *Globisporangium splendens*

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ABSTRACT

We investigated virus infection in the plant pathogenic oomycete *Globisporangium splendens*, formerly classified as *Pythium splendens*, in Japan. From 12 strains investigated, three strains contained virus-like double-stranded (dsRNA). Next-generation sequencing revealed that the *G. splendens* strain MAFF 425508 and MAFF 305867 contained a virus related to toti-like viruses, that we named Pythium splendens RNA virus 1 (PsRV1). PsRV1 has a *ca.* 5700 nt-length genome encoding two overlapping open reading frames (ORFs). The ORF2 encodes an RNA-dependent RNA polymerase (RdRp). Phylogenetic analysis with deduced RdRp amino acid sequences indicated that PsRV1 was closely related to Pythium polare RNA virus 1 (PpRV1) from *G. polare* infecting mosses in the Arctic. PsRV1 was vertically transmitted through the hyphal swellings, vegetative organs of *G. splendens*, in a temperature-dependent manner. Also, we showed that PsRV1 infected in a symptomless manner.

1. Introduction

Viruses that infect fungi are known as mycoviruses. Since a mycovirus was first discovered in mushrooms (Hollings, 1962), many mycovirus have been identified (Ghabrial et al., 2015; Roossinck, 2019). Most mycoviruses infect their host latently, but some inhibit the growth and pathogenicity of their host fungi (Ghabrial and Suzuki, 2009; Ghabrial et al., 2015). For example, Cryphonectria hypovirus 1 infection decreases the virulence of plant pathogenic fungus *Cryphonectria paracitica* and protects the chestnut tree from blight disease caused by *C. paracitica* (Dawe and Nuss, 2013). Thus, mycoviruses may be used as biological control agents against plant diseases caused by fungi.

Oomycetes are a fungal-like organism that belongs to a phylogenetic lineage distinct from the true fungi. They belong to the phylum Heterokontophyta (the "stramenopiles") in the kingdom Chromalveolata, to which brown and golden algae and diatoms also belong. *Globisporangium*, which is classified as *Pythium*, is a diverse genus of the oomycetes with over 30 species which can be saprophytes and plant parasites (Uzuhashi et al., 2010). Virus-like particles and/or double-strand RNAs (dsRNAs) have been reported in *Pythium butleri* (Buck, 1986) and *Globisporangium irregulare* (Klassen et al., 1991; Gillings et al., 1993). Recently, we identified a novel gammapartitivirus (Pythium nunn virus 1) from mycoparasitic *G. nunn* (Shiba et al., 2018) and three virus-like sequences, Pythium polare RNA virus 1 (PpRV1), Pythium polare RNA virus 2 (PpRV2) and Pythium polare bunya-like RNA virus 1 (PpBRV1) from *G. polare* infecting mosses in the Arctic (Sasai et al., 2018). PpRV1 is closely related to an unclassified arthropod toti-like virus (Sasai et al., 2018). On the other hand, viruses infecting these important plant pathogens have seldom been investigated.

Globisporangium splendens is an important plant pathogen that causes root rot and stem rot disease in many crops and ornamental plants worldwide (Waterhouse, 1968; van der Plaats-Niterink, 1981). *G. splendens* forms hyphal swellings, spherical-like structures on internal hyphae, as vegetative organ, but fails to produce sporangia and zoospores (Waterhouse, 1968; van der Plaats-Niterink, 1981). *G. splendens* is predominantly heterothallic, which requires opposite mating types to reproduce sexually (van der Plaats-Niterink, 1981).

In this study, we identified an unclassified toti-like virus from the two strains of *G. splendens* and named it Pythium splendens RNA virus 1 (PsRV1). PsRV1 has a *ca.* 5700 nt-length dsRNA genome having a long 5′-untranslated region (UTR) and two overlapping open reading frames (ORFs). Phylogenetic analysis using the deduced amino acid sequence of RNA dependent RNA polymerase (RdRp) indicated that PsRV1 is closely related to PpRV1.

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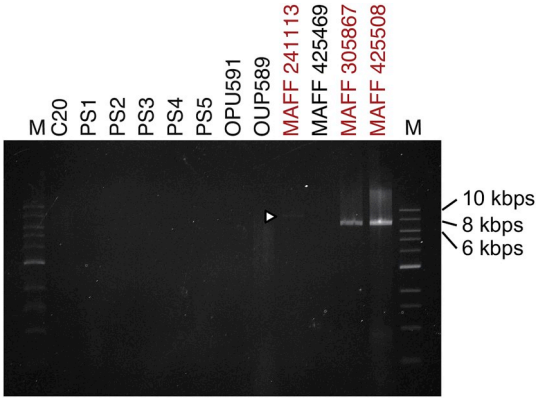

Fig. 1. A, Presence (red) or absence of virus-like dsRNA in *G. splendens* strains from Japan. dsRNAs purified by CF11 cellulose column chromatography were observed after 1% (v/v) agarose gel electrophoresis with ethidium bromide staining. M is a DNA marker (NEB, 1 kbps). Open arrowhead shows a faint dsRNA band between 8 and 10 kbp of the DNA marker from the strain MAFF 241113. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2. Results and discussion

2.1. Detection of virus-like dsRNA from G. splendens strains in Japan

We investigated the presence of viral-like dsRNA in the total nucleic acid extracted from 12 strains of *G. splendens* in Japan (Table S1) by CF11 cellulose column chromatography (Morris and Dodds, 1979; Sasai et al., 2018). A distinct dsRNA band between 6 and 8 kbp of the DNA marker was detected from strains MAFF 305867 and MAFF 425508, and a faint dsRNA band between 8 and 10 kbp of the DNA marker was observed in MAFF 241113 (Fig. 1). We confirmed that the dsRNAs from MAFF 305867 and MAFF 425508 were resistant to DNase I and S1 nuclease treatment (data not shown).

dsRNA extracted from *G. splendens* MAFF 425508 was used for full-length sequencing of the viral genome by the next-generation sequencing. The CF11-purified dsRNA with DNase I and S1 nuclease treatment was used for RNA-seq with a MiSeq sequencer. After RNA-seq and *de novo* assembly of trimmed raw reads, a large contig of 5168 nt showed similarity with viral sequences through a BLASTx search. This contig was assembled with 24,375 reads from a total 881,424 raw reads.

2.2. Sequence analysis of PsRV1

After determining the 5′ and 3′ ends of the contig of PsRV1 from MAFF 425508 by RNA ligase mediated rapid amplification of complementary DNA (cDNA) ends (RLM-RACE), full-length cDNA was generated from dsRNA by reverse transcription-polymerase chain reaction (RT-PCR) and was sequenced by the Sanger method using internal primers designed from the PsRV1 sequence (Table S2). The complete sequence of PsRV1 was determined to be 5705 nt with a CG content of 58%. The PsRV1 genome contained two overlapping large ORFs in different frames (Fig. 2A). Similar to PpRV1, PsRV1 had 1078 nt-long 5′-UTR (Fig. 2B) that contained ten AUG codons upstream from the first AUG codon predicted to initiate the large ORF1. We concluded that the ORFs from the AUG codons in the 5′-UTR are not translated because they are short. These AUG codons upstream of the predicted start codon could be used for translation initiation of ORF1 in a cap-independent manner (Cai et al., 2013). Although there is no AUG start codon at the beginning of ORF2, the shifty heptamer motif “ GCUUUUU” (3208–3214 nt) and RNA pseudoknot (3223–3254 nt) were predicted near the stop codon of ORF1. Thus, ORF2, which overlapped in the -1 frame with ORF1, is predicted to begin with leucine at 3214 nt in the shifty heptamer motif in near the end of ORF1 (Fig. 2A).

ORF2 (3214–5562 nt) encodes a protein with 783 amino acids (aa) with similarity to the RdRp of viruses from the Giardia lamblia virus (GLV)-like group (Liu et al., 2012), and showed the highest similarity with ORF2 of PpRV1 from *G. polare* (NC_040608; ident, 31%; Query cover, 92%; e-value 3e-82). RdRp_4 domain (274–501 aa, e-value, 1.2e-10) and the eight conserved motifs in the RdRps of dsRNA viruses (Bruenn, 1993, 2003; Chiba et al., 2009; Sasai et al., 2018) were found in ORF2 of PsRV1 (Fig. 2B), indicating that PsRV1 ORF2 encodes the RdRp of a dsRNA virus. ORF1 (1079–3217 nt) putatively encodes a protein with 711 aa, and no similar protein was found by a tBLASTn search. No conserved domains and motifs were found in ORF1 of PsRV1. We conclude that PsRV1 ORF1 encodes the coat protein (CP) because the 5′-ORF of toti and toti-like viruses generally encode the CP.

We determined the viral genome sequence of dsRNA from strain MAFF 305867 of *G. splendens*. Complete sequences of 5′-UTR, ORF1, and ORF2 were obtained. There were 9 nucleic acids differences and 1 gap in the 5′-UTR between PsRV1-305867 and -425508. Nucleic acids lengths of ORF1 and ORF2 were identical between PsRV1-305867 and -425508. Nucleic acids similarities were 99% (5 nt) in ORF1 and 99% (5 nt) in ORF2, respectively. Amino acids similarities were 99% (2 aa) in ORF1 and 99% (3 aa) in ORF2, respectively. These results indicate that PsRV1-305867 and -425508 are the same viral species.

G. splendens MAFF 305867 and MAFF 425508 were isolated from relatively nearby agricultural fields, in Miyazaki and Fukuoka prefectures on Kyushu, respectively (Table S1). Strains MAFF 305867 and MAFF 425508 were phylogenetically close; belonging to group B of *G. splendens* (Uzuhashi et al., 2019). This finding supports that the *G. splendens* strain infected with PsRV1 separately spread in the different regions of Kyushu in Japan.

2.3. Phylogenic analysis of PsRV1

We previously reported that the PpRV1 from *G. polare* had a phylogenetic relationship with the unclassified arthropod toti-like virus (Sasai et al., 2018). Thus, a maximum likelihood (ML) tree, rooted by *Totivirus* (ScV-L-A) and *Victorivirus* (RnVV1) in the family *Totiviridae* as reference, was constructed from the 580 sites of deduced RdRp amino acid sequences of PsRV1 and 25 confirmed and putative viruses from GLV-like and Infectious myonecrosis virus (IMNV)-like groups (Liu et al., 2012). Identical to the previous report (Sasai et al., 2018), PsRV1 and PpRV1 represented a monophyletic group in the GLV-like group with high approximate likelihood ratio tests (aLRT) value = 1. The same conclusion was also obtained by using an ML tree constructed from RdRps of 73 confirmed and putative viruses in the family *Totiviridae* (data not shown). According to the classification of *Pythium* by Lévesque and de Cock, 2004, *G. splndens* and *G. polare* belong to clade I and clade G in the 11 small clades of *Pythium* (Tojo et al., 2012), indicating that *G. spendens* and *G. polare* were not closely related. In fact, the amino acid similarity of RdRp between PsRV1 and PpRV1 is about 30%, and no evidence of homology was found between the CP sequences.

In order to further investigate the phylogenetic relationships of PsRV1, a tBLASTn search of RdRp of PsRV1 was conducted by using the transcriptome shotgun assembly (TSA) database for eukaryota (taxid: 2759) (Kondo et al., 2019). Some TSA sequences have significant similarities with putative RdRp amino acid sequence of PsRV1. Especially, TSA sequences from *Heterosiphonia pulchra* showed the highest similarity with PsRV1 RdRp (identity, 35.30%; query cover, 86%; e-value, 1e-109). We constructed an ML tree based on 519 sites of deduced RdRp amino acid sequences of viruses of the GLV-like group and viral-like TSA sequences containing the eight conserved RdRp motifs of dsRNA viruses, rooted by IMNV and Hubei diptera virus 22 (HDV22) of the IMNV-like group. Virus and TSA sequences were clearly grouped by host type (Fig. 3B). Interestingly, PsRV1, PpRV1, and TSA sequences from *Heterosiphonia pulchra* (red alga), *Nitzschia* sp. (diatom), and *Rhizopus oryzae* (fungi) were grouped as a monophyletic group and clearly

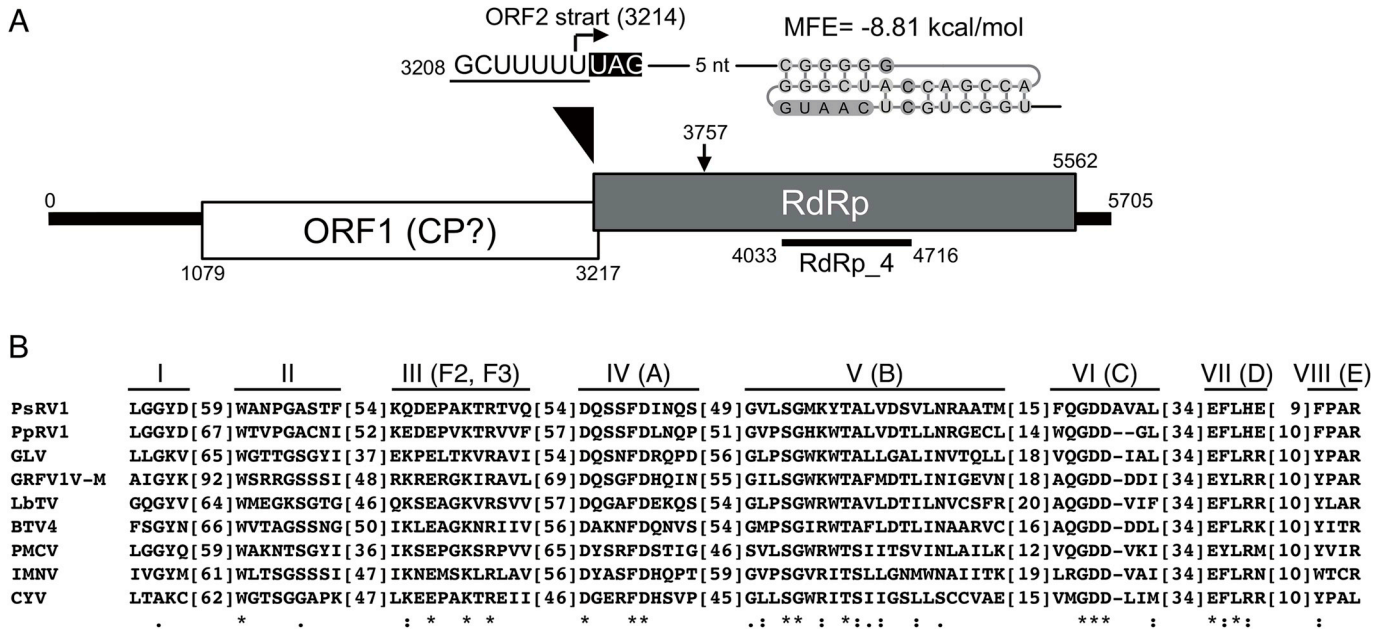

Fig. 2. A, The complete genome of PsRV1-425508. The underlined sequence shows putative slippage sites and putative H-type pseudoknot to the right. The boxed region shows two open reading frames (ORFs). **B,** Comparison of the eight conserved motifs in the RdRps of PsRV1, PpRV1, and other representative toti-like viruses (GLV, BTV4, LbTV, PMCV, GRF1V-M, and IMNV). Amino acid sequences were aligned using MAFFT 7.423. Consensus symbols were assigned according to the BLOSUM62 scoring matrix: *, identical; , nonidentical with score ≥1;., nonidentical with score = 0. PpRV1, Pythium polare RNA virus 1 (LC376044); GLV, *Giardia lamblia virus* (NP_620070); BTV4, Beihai toti-like virus 4 (YP_009336713); LbTV, Leptopilina boulardi toti-like virus (YP_009072448); PMCV, Piscine myocarditis virus (YP_004581250); GRF1V-M Rhizophagus sp. RF1 medium virus (BAJ23141); IMNV, Infectious myonecrosis virus (AAT67231).

divided from other GLV-like viruses from arthropod, fish, fungi, and excavate (Fig. 3B). Importantly, this ML tree indicated that PsRV1 was more closely related to TSA from a diatom and a red alga than PpRV1 (Fig. 3B), supporting that the PpRV1 is a common ancestor of PsRV1 and the putative virus sequence in the red alga and diatom. *Pythium* and diatoms are major groups of stramenopiles in the kingdom Chromalveolata, and red algae belong to the division of Rhodophyta in the Kingdom Plantae.

Transcriptome data from natural samples may be containing sequence reads from not only the target organism but also its associated microbes. Some *Pythium* species, such as *P. porphyrae*, infect red algae (Takahashi et al., 1977). Thus, our phylogenetic analysis using TSA sequences supports that the viruses infected the oomycetes that infected or was associated with red algae in nature. Indeed, some TSA sequences of the *H. pulchra* transcriptome showed similarity (> 80%) with the mitochondria genome of *G. ultimum* (NC_014280.1) or TSA sequences from *Phytophthora cactorum* (GBGX00000000.1) by a BLASTn search (data not shown). Alternatively, there is another possibility of cross-kingdom horizontal transmission of pythium viruses among stramenopile, fungus, and plant hosts. Recently cross-kingdom horizontal transmission of viruses between plants and fungi have been indicated (reviewed in Roossinck, 2019). For example, the cucumber mosaic virus infects cucumber, and is subsequently transmitted to *Rhizoctonia solani* that colonized on the cucumber roots (Andika et al., 2017).

2.4. Vertical transmission of PsRV1 through hyphal swellings

In fungi, mycoviruses are transmitted vertically through the asexual spores with varying efficiencies (Nuss, 2005). Vertical transmission through asexual spores is also known in oomycete. Cai et al. (2019) reported that Phytophthora infestans RNA virus 2 was transmitted through asexual-spore reproduction without exception in 29 single-spore cultures examined. Thus, vertical transmission of PsRV1 through the hyphal swellings, a vegetative organ of *G. splendens*, was examined. In *G. splendens*, progeny mycelium is generated from the hyphal swellings. Thus, we investigated the vertical transmission of PsRV1 through hyphal swellings of *G. splendens*.

Strain MAFF 425508 was cultured at 28 °C, nine hyphal swellings were isolated, and then progeny mycelia were recovered from the isolated hyphal swellings. Total nucleic acids were extracted from regenerated mycelia, and PsRV1 infection was assessed by dsRNA electrophoresis and RT-PCR with specific primers. PsRV1 was detected from all regeneration lines (Fig. 4A), indicating that PsRV1 is vertically transmitted through the hyphal swellings. Similar results were obtained by using strain MAFF 305867 (eight out of nine).

We previously reported that the viral dsRNA titer of PpRV1 was decreased by high-temperature cultivation (Sasai et al., 2018). Thus, we investigated the effect of high temperature on the vertical transmission efficiency of PsRV1. Strain MAFF 425508 was cultured at 31 °C, nine hyphal swellings were isolated, and then progeny mycelia were recovered. PsRV1 was not detected from five out of nine lines from the 31 °C culture by RT-PCR (Fig. 4B). Two independent experiments were conducted, and similar results were obtained, showing that the vertical transmission of PsRV1 through the hyphal swelling was temperature-sensitive.

Previously, we showed that the viral dsRNA titer of PpRV1 in the mycelia of *G. polare* was decreased at a higher temperature (Sasai et al., 2018). From this, it seems that the invasion rate of PsRV1 into the hyphal swellings was reduced by a lower viral titer in the mycelia at high temperature. PsRV1 was not detected from PS2 and PS3 strains in the Okinawa prefecture although these strains belonged to group B, which includes PsRV1-infecting strains MAFF 305867 and MAFF 425508 (Fig. 1 and Table S1). Transmission and/or the multiplication of PsRV1 may be low in strains PS2 and PS3 because Okinawa prefecture has a subtropical climate with high temperature.

2.5. Effect of PsRV1 infection on colony morphology and hyphal growth of G. splendens

To investigate the effect of PsRV1 infection on hyphal growth and colony morphology of strain MAFF 425508, we choose the isogenic 31-5 line as a virus-free isolate after high-temperature culture and hyphal

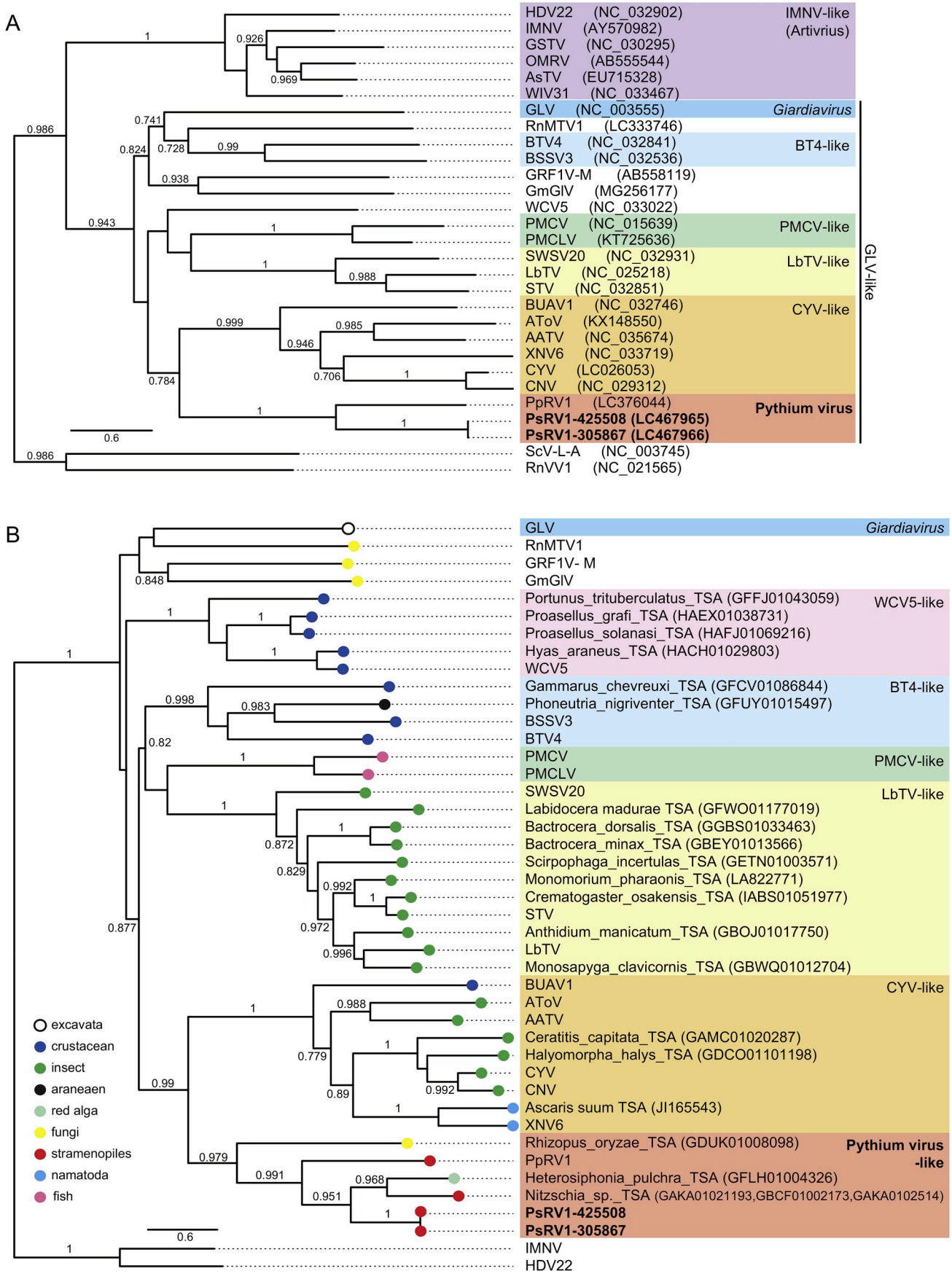

Fig. 3. A, The maximum likelihood tree (LG + I + Γ8 + F) based on amino acids of RdRp of viruses in the GLV-like and IMNV-like groups (Liu et al., 2012). RdRp of *Totivirus* (ScV-L-A) and *Victorivirus* (RnVV1) in the family *Totiviridae* was used for rooting. **B,** The maximum likelihood tree (LG + I + Γ8 + F) based on amino acids of RdRp of viruses of GLV-like group and TSAs. IMNV and HDV22 in the IMNV-like group were used for rooting. The numbers on branches indicate the results of SH-like approximate likelihood ratio tests (aLRT). Branch support values larger than 0.7 were shown. Scale bar shows substitutions per site.

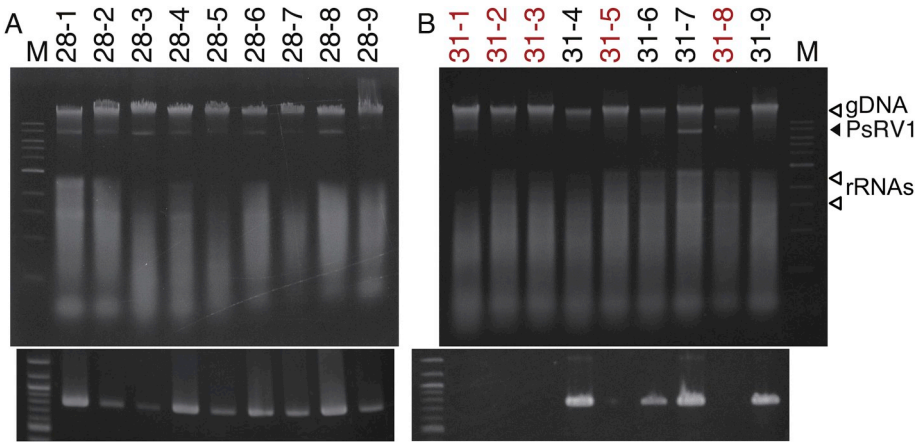

Fig. 4. dsRNA detection (upper panels) and RT-PCR detection (lower panels) of PsRV1 transmission through hyphal swellings. M, 1-kb DNA size marker; 1–9, single-hyphal swelling isolation, and subcultivation of *G. splendens* MAFF 425508. Precultivation before hyphal swellings isolation was conducted at 28 °C (**A**) or 31 °C (**B**).

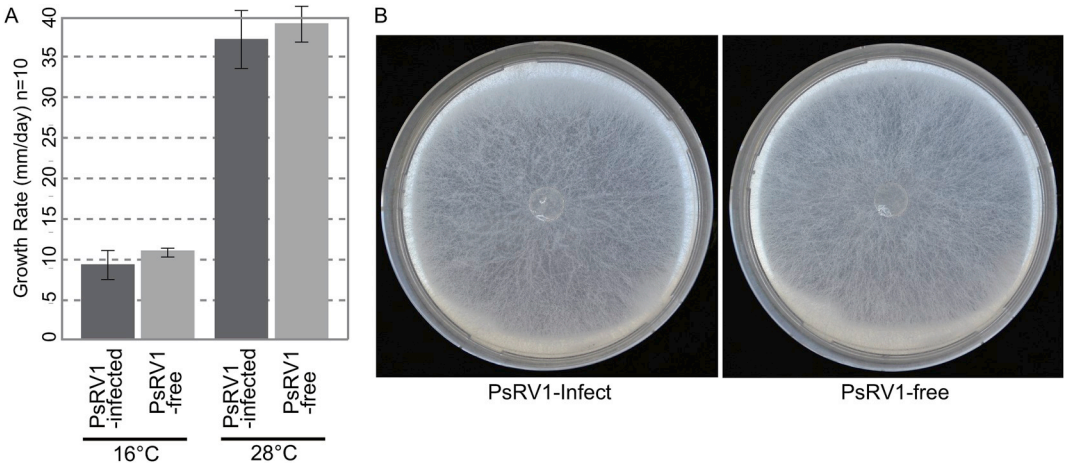

Fig. 5. The effects of PsRV1 infection on hyphal growth and colony morphology of *G. splendens* MAFF 425508. **A,** Hyphal growth rate of PsRV1-free and PsRV1-infected lines on potato carrot agar (PCA) plate medium at 16 °C and 28 °C (mean ± SD). **B,** Colony morphology of PsRV1-free and PsRV1-infected lines on PCA at 28 °C.

swelling isolations (Fig. 4B). Mycelia growth was measured at 16 °C and 28 °C, and no significant difference was found between PsRV1 infected- and -free lines (mean ± SD, n = 10, Student's t-test: 16 °C $P = 0.80 > 0.05$, 28 °C $P = 0.40 > 0.05$) (Fig. 5A). Apparent differences of colony morphology on potato carrot agar (PCA) medium at 28 °C were not observed between PsRV1 infected- and -free lines (Fig. 5B). These results indicate that PsRV1 infects in a symptomless manner in culture.

3. Materials and methods

3.1. Globisporangium splendens strains

Twelve strains of *G. splendens* (Uzuhashi et al., 2019) were used in this study (Table S1). They were maintained on 10% (v/v) V8 agar (V8A) medium at 20 °C in the dark.

3.2. dsRNA extraction

Mycelia were propagated on potato dextrose broth (PDB) in an autoclaved Ziploc container (156 × 117 × 53 mm, Asahi Kasei) at 20 °C for 7 days. After collecting mycelia and draining on paper towels, 400 mg mycelium was homogenized in liquid nitrogen with a mortar and pestle, and then the fine powder was kept at -80 °C. The fine powder was mixed with extraction buffer {0.1 M NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% (w/v) SDS }, and extracted with phenol-chloroform (1:1; w:w) twice. Then total nucleic acid was precipitated with 0.3 M sodium acetate and ethanol. dsRNAs were purified from total nucleic acids using CF-11 cellulose following previously described methods (Morris and Dodds, 1979; Sasai et al., 2018) and treated with S1 nuclease (Promega) and DNase I (Promega) at 37 °C for 30 min to remove DNA and single-stranded RNA residuals.

3.3. Determination of full-length sequence of dsRNA

The procedure of next-generation sequencing is described in Sasai et al. (2018). A cDNA library for next-generation sequencing was prepared from 91 ng of purified dsRNA from strain MAFF 425508 using NEBNext Ultra RNA Library Kit for Illumina and Index primer set 1 (New England BioLabs). Sequencing was performed on a MiSeq benchtop sequencer using a MiSeq Reagent Kit Nano V2 for 2 × 150 paired-end (Illumina). Trimmed raw reads were assembled by Velvet 1.2 (Zerbino and Birney, 2008) with a k-mer size = 31 and coverage cutoff = 100 options.

Sequences of the 5′ and 3′ ends were determined by RLM-RACE using a 5′-phosphorylated oligodeoxynucleotide primer and 5′ reverse- and 3′ forward-specific primers (Liu and Gorovsky, 1993). SuperscriptIII (Invitrogen) with 5′- and 3′-termini specific primers were used for the reverse transcription reaction. The full-length cDNA of dsRNA was amplified in two fragments of *ca.* 3.0 kbp by PrimeStar GXL DNA

polymerase (Takara). The amplicons were directly sequenced through the Sanger method using internal primers designed from the contig (Table S2). Sanger sequencing was carried out by macrogen Japan with ABI 3730 DNA sequencer (Applied Biosystems). Obtained sequences were assembled and analyzed by DNA Dynamo software (BlueTractor Software).

3.4. Sequence analyses of PsRV1

ORFs were predicted by DNA Dynamo software (BlueTractor Software). Predicted amino acid sequence were subjected to a tBLASTn search. HPknotter (Huang et al., 2005) was used to search the H- type pseudoknots with maximum free energy (MFE). RNAfold web-server (Gruber et al., 2008) was used to search the potential secondary structure with MFE in the 5′ and 3′-UTRs. The conserved domains and motifs in the ORF were searched by MOTIF Search (https://www.genome.jp/tools/motif/). The motifs in RdRp were detected in alignment by MAFFT 7.423 (Katoh and Standley, 2013) with an accurate option (L–INS–i).

3.5. Phylogenic analysis

Phylogenic analysis was done according to previous reports (Sasai et al., 2018). Accessions of viral RdRp and TSA sequences used for phylogenetic analysis are shown in Fig. 3. Multiple sequence alignments were implemented using MAFFT 7.423 (Katoh and Standley, 2013) with an accurate option (L–INS–i), and then the unreliable sites of alignment were removed by TrimAl 1.2 with the strict option (Capella-Gutiérrez et al., 2009). The best-fit amino acid substitution model was determined by ProtTest 3.4 (Abascal et al., 2005). The selected best-fit substitution model (LG + I + Γ8 + F) was used for the maximum likehood (ML) phylogenetic analysis by PhyML 3.1 (Guindon et al., 2010) with SPR moves algorithm and starting with BioNJ Tree. The branch supports were calculated by Simodaira-Hase-gawa-like procedure approximate likelihood ratio test (aLRT SH-like) (Guindon et al., 2010). The Newick tree was visualized by the Figtree software (http://tree.bio.ed.ac.uk/software/).

3.6. Vertical transmission analysis of PsRV1 through hyphal swellings

G. splendens was cultured in potato dextrose broth (PDB) at 28 °C and 31 °C for 1 week and the mycelia were homogenized in 150 mL Milli-Q water. One hundred fold-diluted homogenized mycelia solution was applied on a potato dextrose agar (PDA) plate and precultured at 28 °C and 31 °C for 1 week. Hyphal swellings were isolated from the PDA plate under a microscope and subcultured on the PDB at 28 °C for 1 week to generate the progeny mycelia. Total nucleic acids were extracted from the progeny mycelia and assessed for PsRV1 by dsRNA detection and RT-PCR with PrimeScript One-Step RT-PCR Kit Ver.2 (Takara) with specific primers for PsRV1 (Table S2).

3.7. Mycelia growth and colony morphology of G. splendens

The hyphal growth of virus-infected and virus-free lines of *G. splendens* was measured on a PCA plate at 16 °C and 28 °C. The hyphal growth was evaluated daily by visual measurement of the average increase of the colony along its longest perpendicular diameter. The measurements were repeated ten times. Colony morphology of virus-infected and virus-free lines was photographed at 28 °C on PCA plate.

3.8. Accessions

The genome sequences of PsRV1 from MAFF 425508 and MAFF 305867 were submitted to GenBank and given accession number LC467965 and LC467966, respectively.

Compliance with ethical standards

All the authors declare that they have no conflict of interests. This article does not contain any experiments with human participants or animals performed by any of the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.08.025.

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