A Novel Virus-like Double-Stranded RNA in an Obligate Biotroph Arbuscular Mycorrhizal Fungus: a Hidden Player in Mycorrhizal Symbiosis

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INTRODUCTION

Arbuscular mycorrhizal (AM) fungi that belong to the phylum Glomeromycota are ubiquitous in terrestrial ecosystems and form mutualistic associations with most land plants (Smith and Read, 2008). The main benefit of the symbiosis for plants is facilitated uptake of immobile nutrients, especially phosphorus (P), through the hyphal networks constructed in the soil, and thus the fungi play a significant role in P cycling in terrestrial ecosystems. The origin of glomeromycotan fungi is quite ancient: they diverged from other fungi 600 million years ago (Redecker et al., 2000b), and AM associations coincided with the appearance of primitive land plants about 400 million years ago (Simon et al., 1993; Redecker et al., 2000a). Biological characteristics of the fungi, however, are largely unexplored due to their obligate biotrophic nature.

Fungal viruses (mycoviruses) have been found in a variety of fungi, including the Ascomycetes, Basidiomycetes, and Deuteromycetes (Sasaki et al., 2006). Their genomes are composed of double-stranded RNA (dsRNA) in most cases (Fauquet et al., 2005) and unexceptionally possess an RNA-dependent RNA polymerase (RdRp) gene for replication (Ghabrial and Suzuki, 2009). They do not have an extracellular infection route (Buck, 1998) but are transmissible vertically via asexual spores (not, or less efficiently, via sexual spores) and horizontally via anastomosis (Hillman et al., 2004). Because no universal artificial infection/elimination system for mycoviruses has been

established (Wickner, 2001), viral etiologies of altered fungal phenotypes must be understood on each individual species/strain of fungus. Infections of mycoviruses are asymptomatic in many cases, but often have a significant impact not only on phenotypic expression of the host fungus, (e.g., van Diepeningen et al., 2006), but also on higher order biological interactions, e.g., attenuation of virulence of a plant pathogenic fungus (Choi and Nuss, 1992) and conferring plant thermal tolerance via an endophytic fungus (Márquez et al., 2007). Recently, three mycoviruses, mitovirus (Stielow et al., 2011b), totivirus (Stielow and Menzel, 2010), and endornavirus (Stielow et al., 2011a) have been found in the ectomycorrhizal fungus *Tuber aestivum*, although their impact on phenotypic expression of the host fungus has yet to be elucidated. These observations led us to hypothesize that AM fungi harbor mycoviruses, which have a major impact on symbiotic interactions, but no information about viruses in AM fungi has so far been reported.

One technical limitation for virological study in AM fungi was the difficulty in obtaining a sufficient amount of pure fungal material for purification of viral particles. Recently, we have established the mesh bag-separated open pot culture system for mass production of AM fungal mycelia and applied to studies on cell fractionation (Tani et al., 2009) and polyphosphate dynamics (Hijikata et al., 2010). It seems unlikely, however, that a sufficient amount of material for virus purification could be obtained even by this system: 30 to 50 g of tissue are usually used for viral particle purification

but more than 70 pots are required to collect about 1 g of mycelia in this system. On the other hand, purity of fungal material is best in the *in vitro* monoxenic culture (Bécard and Fortin, 1988), which has been widely employed for, e.g., physiological (Bago et al., 2002; Nielsen et al., 2002) and molecular biological (Maldonado-Mendoza et al., 2001) studies, although fungal growth is generally poorer than in open culture. To overcome these problems we designed the 'two-step strategy' in combination with the next-generation sequencing technology in the present study: dsRNA viral genomes were first extracted from fungal material produced in the open pot culture and sequenced 'deeply' without purifying a single viral genome, and subsequently, the presence of the viruses was verified using material produced in the *in vitro* monoxenic culture by RT-PCR.

RESULTS

Detection of dsRNA

dsRNA was extracted from 0.4 – 0.9 g (FW) of *Glomus* sp. RF1 extraradical mycelia produced in the mesh bag-separated open pot culture (Supplementary Fig. S1), treated with DNase I and S1 nuclease that digest DNA and single-stranded RNA, respectively, and subjected to gel electrophoresis. The fungus was found to harbor

several dsRNA segments that differed in size, which were termed as *Glomus* sp. RF1 virus-like-small dsRNA (GRF1V-S, 2.5 kbp), -medium dsRNA (GRF1V-M, 4.5 kbp), -large dsRNA 1 (GRF1V-L1, > 10 kbp), and -large dsRNA 2 (GRF1V-L2, > 10 kbp) (Fig. 1). Among them, GRF1V-M was chosen for further analysis because the segment was relatively abundant, which was expected to be readily characterized. For this purpose we employed FLX pyrosequencing, one of the next-generation sequencing technologies, instead of the traditional shotgun library-based sequencing, because at least the three dsRNA segments were coexist with GRF1V-M in the fraction. dsRNAs were reverse-transcribed with the 6N-anchored primer (Supplementary Table S2), amplified with the anchor primer, and then subjected to pyrosequencing. More than 45,000 reads with an average length of 285 bp were obtained and assembled, and eight large (> 1 kbp) contigs showed amino acid sequence similarities to RdRp and other proteins of four different viruses: 2.5-kbp contig, *Botrytis cinerea* debilitation-related virus in the genus *Mitovirus*; 4.5-kbp contig, *Giardia canis* virus in the genus *Giardiavirus*; 2.6-, 3.7-, and 5.9-kbp contigs, *Phytophthora endornavirus* 1 in the genus *Endornavius*; 1.0-, 1,3-, and 4.2-kbp contigs, *Phlebiopsis gigantea* mycovirus dsRNA 1 that was an unclassified totivirus (Supplementary Table S1). To examine the identity between these contigs and the dsRNA segments detected in the gel, GRF1V-S, GRF1V-M, and GRF1V-L1/L2 were re-extracted from the gel separately. GRF1V-L1 and -L2 were collected together because the two could not be clearly separated on the

gel. Nested PCRs were then conducted by using these dsRNAs and the primers specific to each contig (Supplementary Table S2) after reverse-transcription with the 6N primer. As expected, the amplified fragments had the sequences identical to the 2.5-kbp *Mitovirus*-like contig, 4.5-kbp *Giardiavirus*-like contig, 5.9-kbp *Endornavius*-like contig, and 4.2-kbp totivirus-like contigs, which were obtained from the fractions of GRF1V-S, GRF1V-M, GRF1V-L1/L2, and GRF1V-L1/L2, respectively (Supplementary Fig. S2). Theses results thus confirmed the direct linkage between the contigs and the dsRNAs. Then the presence of the virus-like dsRNAs in the *in vitro* monoxenic culture (Supplementary Fig. S1) was further examined by RT-PCRs using the dsRNA fraction prepared from the in vitro-produced spores as template. Target PCR fragments were successfully obtained with the primer sets specific to GRF1V-S and -M (Supplementary Fig. S3), but not with those specific to GRF1V-L1 and -L2, indicating that at least GRF1V-S and -M were of AM fungal origin.

Molecular characterization of GRF1V-M

To obtain full-length sequence of GRF1V-M about 100 bp of each of the 5'/3'-terminal sequences were determined/confirmed by RACE. The segment is 4,557 nucleotides (nt) in length and consists of a 5'-untranslated region (UTR) of 40 nt, two ORFs (ORF 1 and 2) encoding polypeptides of 927 and 537 aa, respectively, and a 3'-UTR of 102 nt that includes a 19-nt poly(A) tail in the plus-sense strand (Fig. 2). No

notable ORF was found in the minus-sense strand. A preliminary BLAST search revealed that the protein encoded by ORF 1 had similarities to RdRps of the members of the family Totiviridae, but surprisingly, the highest score was observed with RdRps of protozoan viruses, followed by those of the viruses found in salmon, fruit fly, mosquito, and shrimp, which were included in subsequent phylogenetic analysis. The ORF 1 protein conserved a domain of the viral RdRp superfamily (Pfam accession no. PF02123) and its motifs I–VIII, including the SG---TS/T and GDD motifs (Ghabrial, 1998; Chiba et al., 2009) (Fig. 3A). The protein encoded by ORF 2 showed similarity to the S7 viral core protein of the rice dwarf virus (*Phytoreovirus*) that is suggested to have binding activity to the genomic RNA, RdRp, and other viral structural proteins (Ueda et al., 1997; Hagiwara et al., 2003). The domain of the *Phytoreovirus* S7 protein superfamily (Pfam accession no. PF07236) was also conserved in the ORF 2 protein (Fig. 3B). We considered, therefore, that ORF 2 might encode a structural protein that binds to and protects the genomic RNA.

Because the genome organization of GRF1V-M, in which an RdRp gene was encoded by the 5'-proximal ORF, was unique and had not been described within the members of Totiviridae, the region between 3'-end of ORF 1 and 5'-end of ORF 2 was amplified by nested PCR with the specific primers (Fig. 2 and Supplementary Table S2), cloned, and sequenced, which resulted in confirmation of the sequence obtained by pyrosequencing.

121

122 **Phylogeny of GRF1V-M**

123 To clarify the evolutionary position of GRF1V-M, phylogenetic analyses were
124 conducted based on the amino acid sequence of RdRp genes, the most highly conserved
125 gene among RNA viruses (Ghabrial, 1998). A phylogenetic tree was constructed first
126 with the representative members from all genera of dsRNA mycoviruses. As expected
127 from the preliminary BLAST search, GRF1V-M formed no robust cluster with any of
128 them (Fig. 4A). Therefore, subsequent analysis was conducted with the members of
129 Totiviridae, which includes not only mycoviruses but also protozoan and animal viruses.
130 Again GRF1V-M formed no robust cluster, but as predicted by the preliminary search
131 was more closely related to the protozoan (*Giardiavirus*) and animal viruses than the
132 mycoviruses (Fig. 4B).

133

134 **Impact of GRF1V-M infection on *Glomus* sp. RF1 phenotype**

135 To assess the impact of GRF1V-M on phenotypic expression of the host
136 fungus by comparing GRF1V-M-free and -positive fungal cultures, single spore culture
137 lines (open culture) were raised, which has often been employed in other fungi to obtain
138 virus-free culture (Hillman et al., 2004) and also in an AM fungus to cure
139 endosymbiotic bacteria (Lumini et al., 2007). Twelve culture lines were successfully
140 established (first generation), and the presence/absence of GRF1V-S, -M, -L1, and -L2

in each line was examined by RT-PCR on the dsRNA fractions prepared from the spores. GRF1V-S, -L1, and L2 were present in all lines, but fortunately, one of the lines (line 3) was found to be GRF1V-M-free. The second generation of the lines was raised from 100 – 200 spores produced in the first generation to obtain enough spores for subsequent inoculation experiments, and the presence/absence of the dsRNAs in the generation was further confirmed by RT-PCR.

Spore productivity, which could be assessed only during symbiosis with plants, was employed as an index of fungal reproductive/competitive potential. Sorghum, one of the greatest spore producers (reviewed in Smith and Read, 2008), was inoculated with either the GRF1V-M-free or -positive line and grown on sand culture in a greenhouse, and spores were harvested 20 weeks after sowing at which maximum spore production was expected. The GRF1V-M-free line produced at a density of more than 130 spores g^{-1} sand, but the GRF1V-M-positive lines produced only 37 – 63% (50 – 85 spores g^{-1} sand) of that of the free line (Fig. 5).

The impact of GRF1V-M on plant symbiotic growth was assessed both under acidic (pH 3.4) and non-acidic (pH 5.7) conditions, because *Glomus* sp. RF1 was originally isolated from acidic soil and highly acid-tolerant (unpublished observation). As a host plant, *Miscanthus sinensis* that is a C_4 perennial grass species was chosen. Although this species grows more slowly and produces less spores than sorghum, it is highly acid-tolerant (Kayama, 2001; Maki et al., 2008) and thus more suitable for the

assessment than sorghum of which the growth is largely limited by soil acidity (Caniato et al., 2007). *M. sinensis* was inoculated either with the GRF1V-M-free or -positive line and grown in the greenhouse for 8 weeks, by which time plant growth was not limited by the pot size (though sporulation was minimum). Both the free and positive lines promoted plant growth to the same extent at pH 5.7, compared with the non-mycorrhizal control (Fig. 6). At pH 3.4 shoot growth was greater in those inoculated with the free line than with the positive line, although no significant difference in root growth was observed between the two lines. The level of mycorrhizal colonization was not different between the free and positive lines both at pH 5.7 and 3.4.

DISCUSSION

The present study demonstrates that the obligate plant symbionts AM fungi harbored diverse dsRNAs, which are highly likely to be mycoviruses, for the first time. The 'two-step strategy', in which molecular characterization was conducted using material from the open culture and the presence was verified by RT-PCR using a small amount of material produced *in vitro*, was indispensable for the successful detection and characterization of dsRNA in AM fungi, in addition to next-generation sequencing. Traditionally, molecular characterization of a new virus starts from purification of viral

181 particles, followed by random amplification of cDNA fragments from the viral genome,
182 and sequencing of the shotgun library. Recently, FLX pyrosequencing has been applied
183 to the detection or genome sequencing of new viral pathogens in medical (e.g., Feng et
184 al., 2008; Spatz and Rue, 2008; Nakamura et al., 2009) and entomological (Cox-Foster
185 et al., 2007) sciences but for the first time in fungal virology, so far. The combination
186 of open and *in vitro* cultures with the next-generation sequencing could be a
187 breakthrough for discovery and molecular characterization of new viruses in
188 unculturable fungi from which a sufficient amount of material for purification of viral
189 particles is difficult to obtain.

190 We consider that GRF1V-M is highly likely to be an RNA mycovirus for the
191 following two reasons: i) ORF 1 was predicted to encode the replicase RdRp, which
192 may be responsible for genome replication, and ii) the GRF1V-M segment disappeared
193 in one of the single-spore culture lines, but remained in the other lines in which the
194 segment was inherited in the next generation. These observations strongly suggest that
195 GRF1V-M is “self-replicative” and “transmissible from generation to generation”, and
196 thus meet the prerequisite features of (myco)viruses.

197 Although the GRF1V-M segment was found in the dsRNA fraction, it has not
198 yet been confirmed whether GRF1V-M possesses a dsRNA genome or a
199 single-stranded RNA (ssRNA) genome, because a replicative form (RF) of ssRNA
200 viruses could also coexist in a dsRNA fraction. We consider, however, that our

observations are in favor of dsRNA. For example, the RdRp gene was most closely related to dsRNA viruses, and both the RdRp and S7 core protein motifs of dsRNA viruses were conserved. In a preliminary experiment, cDNA of the viral genome could be synthesized and amplified from total RNA with the 3'-end oligo(dT) primer in combination with the 5'-end primers using the boiled (denatured) RNA preparation, but not without boiling, suggesting that no (or minimum) ssRNA form of GRF1V-M was present in the preparation. Purification of the viral particles, however, may be necessary to exclude the possibility that GRF1V-M is an ssRNA virus, if the particles really exist. We assume that the genome may be encapsulated by the S7 core protein (encoded by ORF 2) or, more likely, only associated with the protein to protect the virus from nucleases, because the S7 core protein may not be a real capsid protein (Ueda et al., 1997; Hagiwara et al., 2003). To understand the structure, the first step we are planning is imunoprecipitation of GRF1V-M using antibodies raised against the S7 core protein.

GRF1V-M is evolutionarily distinct. Although the phylogenetic analysis indicated that the RdRp gene of GRF1V-M is related to Totiviridae, GRF1V-M cannot be assigned to Totiviridae due to the unique genome structure: an RdRp gene is encoded by the 5'-proximal ORF in GRF1V-M but by the 3'-proximal ORF in Totiviridae, and a 3'-poly(A) tail is present in GRF1V-M but absent in Totiviridae (Fauquet et al., 2005). On the other hand, the protein encoded by ORF 2 conserves the domain of a structural protein of the plant dsRNA virus *Phytoreovirus*. These results, in

addition to the fact that glomeromycotan fungi are ancient and have a long history of coevolution with land plants, support the idea that GRF1V-M might have been generated through extensive recombination events among ancestors of the protozoan, animal, and plant viruses, which occurred during the era of eukaryogenesis (Koonin et al., 2008).

Extensive phenotypic variations have been observed not only among AM fungal species but also within the individual species, and this has generally been considered to be due to the genetic diversity of AM fungi as a consequence of the multigenomic nature (Koch et al., 2004). The intracellular symbiotic bacterium '*Candidatus* Glomeribacter gigasporarum' in the AM fungus *Gigaspora margarita* has been shown to be a non-genetic, but a heritable (transmissible) component that altered phenotypic expression of the host fungus (Lumini et al., 2007; Salvioli et al., 2010). The alteration of phenotype by the bacterium, however, was observed only in the presymbiotic stages, not in the symbiotic stages. The present study demonstrates that the GRF1V-M altered the symbiotic phenotypes i.e. spore productivity and the plant-growth promoting effect of the host fungus *Glomus* sp. RF1, providing a new possible interpretation for the extensive phenotypic variability in AM fungi: mycoviruses would be a biologically active-transmissible component and definitely a potential player responsible for the phenotypic variability.

Although the molecular mechanism underlying the phenotypic alteration by

GRF1V-M is obscure, ecological relevance of the reduced spore productivity and plant-growth promoting effect of *Glomus* sp. RF1 is of interest. It has been suggested that considerable photosynthetic carbon is allocated to the fungal symbiont for sporulation (Smith and Read, 2008). The reduction in spore productivity in *Glomus* sp. RF1 therefore likely leads to the idea that GRF1V-M improves overall carbon economy of the association via saving excess consumption of carbon. On the other hand, we consider that the impact on plant growth may be limited, because the GRF1V-M-positive line could still improve plant growth compared with the non-mycorrhizal plants under the acidic conditions and the percentage of mycorrhizal colonization, which may reflect infectivity of the fungus, was not significantly different between the GRF1V-M-positive and -free lines. Given that soil acidity is the major selection pressure on AM fungi (An et al., 2008), it is likely that the acidic stress (pH 3.4) in the experimental conditions was so severe for the fungus that a negative impact of GRF1V-M, e.g., a decline in fungal fitness via exploitation of cellular resource due to GRF1V-M proliferation, might be enhanced. Accordingly, further investigation is necessary to elucidate direct relevance of the reduced spore productivity to plant competitiveness with respect to soil acidity.

We could detect the large dsRNAs GRF1V-L1 and -L2 in the open-culture spores, but the presence of the large segments in the *in vitro* culture has not yet been confirmed. Although relative abundance of the dsRNAs in the fungus has not been

estimated, it seems that GRF1V-L1 and -L2 are much less abundant than GRF1V-M (Fig. 1), which may be one reason why they could not be amplified from a small amount of material. An attempt to increase the yield and purity of dsRNA from a small amount of material is being undertaken for successful detection of GRF1V-L1 and -L2 in the *in vitro* culture.

In conclusion, the present study sheds new light on AM symbiosis from the viewpoint of virology. The demonstration of the technical feasibility of dsRNA manipulation, at least elimination, implies that improvement/innovation of the symbiotic performance of AM fungi would be possible without genetic manipulation of the fungi. It is expected that the presence/absence and/or molecular feature of mycoviruses would be applicable as a biological marker for identification of geographical isolates and anastomosis groups, because spore formation and anastomosis are the only likely routes of mycovirus transmission. The fact that a novel viral dsRNA was discovered in our very first and limited survey suggests that viruses in AM fungi, at least some of them, may have evolved under unique selection pressures and that further discovery of new viral dsRNA from AM fungi seems highly probable, which will contribute to understanding the coevolution between viruses and glomeromycotan fungi.

MATERIALS AND METHODS

281

282 **Fungal isolates**

283 *Glomus* sp. RF1 (MAFF520086) was isolated from the rhizosphere of *Petasites*
284 *japonicus* var. *giganteus* grown in acid sulfate soil (< pH 4) in Japan, and the small
285 subunit rRNA gene (SSU rDNA) sequences (GenBank accession no. AB220173)
286 showed similarity to those of *G. manihotis* and *G. clarum* in the *Glomus*-group A. The
287 stock culture of the isolate was established from multiple spores (approx. 50 spores)
288 and has been maintained in sand culture in a temperature (26/20ºC, day/night) /light
289 (14-h day length)-controlled greenhouse using sorghum and groundnut as host plants.

290

291 **Open and *in vitro* culture systems**

292 To obtain fungal material for dsRNA extraction and characterization, the mesh
293 bag-separated open pot culture system was employed (Tani et al., 2009)
294 (Supplementary Fig. S1a). *Lotus japonicus* L. cv. Miyakojima MG-20 (National
295 Bioresource Project Legume Base,
296 http://www.legumebase.agr.miyazaki-u.ac.jp/index.jsp) was pregerminated on
297 moistened filter paper at 25ºC in the dark for 24 h. Three seedlings were transplanted to
298 the mycorrhizal compartment (MC, 26 ml in vol) of the mesh bag culture system in a
299 120 ml plastic pot (6 cm in diam) and inoculated with the fungus at 1,000 spores pot^{-1}.
300 The mesh bag system consisted of the MC and hyphal compartment (HC) that were

separated by a cone-shaped 37 µm nylon mesh bag, and both compartments were filled with autoclaved river sand. The pore size of the nylon mesh was small enough to prevent *L. japonicus* roots from passing but large enough to allow AM fungal hyphae to pass through. The plants were grown in a growth chamber at a photosynthetic photon flux of 150 µmol m^{-2} s^{-1} (16-h day length, 25ºC) for a week only with deionized water, thinned to two plants pot^{-1}, and further grown with low-P nutrient solution (4 mM NH_4NO_3, 1 mM K_2SO_4, 75 µM $MgSO_4$, 2 mM $CaCl_2$, 50 µM Fe-EDTA, and 50 µM KH_2PO_4) for 7 weeks. After removing the MC, extraradical mycelium in the HC was harvested from 72 pots by wet sieving, blotted on filter paper, weighed, frozen in liquid nitrogen, and stored at –80ºC for dsRNA extraction. In this process, 0.4 – 0.9 g FW mycelia could normally be collected.

To obtain single spore culture lines of *Glomus* sp. RF1 *L. japonicus* seedlings were pregerminated, transplanted to the 6 cm plastic pot filled with autoclaved river sand without the mesh bag, inoculated with a single spore produced in the stock culture, and grown in the growth chamber for two months under the same conditions as those for the mesh bag culture system (first generation). For further proliferation, sorghum was inoculated with 100 - 200 spores produced in the first generation and grown in the sand culture in the greenhouse for four months (second generation).

The *in vitro* monoxenic culture of *Glomus* sp. RF1 was raised from five spores produced in the stock culture using the Ri T-DNA transformed carrot roots (Bécard and

Fortin, 1988) and maintained at 27˚C in the dark (Supplementary Fig. S1b). For dsRNA detection, spores were collected after melting the medium by shaking in 0.5 M EDTA solution.

dsRNA extraction, sequencing, and virus detection

dsRNA was extracted from the frozen mycelia (0.4 – 0.9 g) by the SDS-phenol method (Suzuki et al., 2003), purified by incubating with 0.4 units μL^{-1} DNase I and 3.4 units μL^{-1} S1 nuclease (Takara Bio, Tokyo, Japan) at 37ºC overnight according to Osaki et al. (2002), and electrophoresed on 0.8% agarose gel. For sequencing, dsRNA segments were eluted from the gel pieces in the GeBAflex dialysis tube (25 kDa cut-off, Gene Bio-Application, Kfar Hanagide, Israel), purified again by overnight incubation with DNase I and S1 nuclease, reverse-transcribed using the 6N-anchored primer (Supplementary Table S1), and amplified using the anchor primer with *Taq* polymerase. The program of the thermal cycler was as follows: initial denaturation at 94ºC for 2 min, followed by 40 cycles at 94ºC for 15 s, 55ºC for 30 s, and 72ºC for 2 min, with a final extension at 72ºC for 10 min. The PCR product was sequenced using the Genome Sequencer FLX system with a 1/8-scale gasket (Roche Diagnostics, Tokyo, Japan) and assembled using the SeqNova Data Analysis System at Hokkaido System Science Co., Ltd. (Sapporo, Japan). The sequences of the extreme ends of the dsRNA segment were amplified using the specific primers (Supplementary Table S1), cloned, and sequenced

by the dideoxy-cycle sequencing method.

For specific detection of each dsRNA segment in the open culture material, dsRNA prepared from the electrophoretic gel pieces or from 500 – 1,000 spores was used as RT-PCR template, whereas for the detection from the in vitro culture, the template dsRNA was prepared from 200 – 300 spores. dsRNA was reverse-transcribed with the 6N-random primer, amplified by nested PCR using the specific primers (Supplementary Table S2) with the following thermal cycle program: initial denaturation at 94ºC for 2 min, followed by 40 cycles at 94ºC for 15 s, 62ºC for 30 s, and 72ºC for 45 s, with a final extension at 72ºC for 10 min. The PCR products were subjected to gel electrophoresis, cloned, and sequenced for confirmation.

Sequence analysis and phylogeny

The contigs obtained by pyrosequencing were subjected to BLAST searches (http://www.ncbi.nlm.nih.gov/). ORF and motif searches were performed with the Vector NTI (Invitrogen, Tokyo, Japan) and the Position-Specific Iterated BLAST (PSI BLAST) against the Pfam database (http://pfam.sanger.ac.uk/), respectively. Multiple sequence alignments were implemented by Clustal X (Thompson et al., 1997), which was carefully edited/modified by eyes as described previously (Chiba et al., 2009). Neighbor-joining trees were constructed based on the alignments of RdRp sequences and displayed by TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). For

analysis with the representative mycovirus members, an alignment of the RdRp sequences from motifs III to VIII was employed due to the absence of motifs I and II in the members of the families Partitiviridae and Hypoviridae, and an alignment of the sequences from motifs I to VIII was employed for analysis with the members of Totiviridae. Tree topologies were confirmed by comparing with maximum-likelihood trees constructed using the best-fit model selected by the ProtTest 2.4 following the Akaike information criterion (Abascal et al., 2005). The GenBank accession numbers of viral sequences used in the motif and phylogenetic analyses were listed in Supplementary Table S3.

Impact of GRF1V-M infection on fungal phenotype

To examine spore productivity, sorghum was inoculated with 100 spores of either the GRF1V-M-free or -positive culture lines of *Glomus* sp. RF1 and grown in 9 cm plastic pots (350 mL in vol) filled with river sand in the greenhouse for 20 weeks, during which the low-P nutrient solution was applied every other day (2 plants pot^{-1}, n = 3). Spores produced in the pots were collected by wet sieving and counted under a stereomicroscope. For assessment of the plant-growth promoting effect of the GRF1V-M-free and -positive culture lines, acid sulfate soil (pH 3.4) collected from Rankoshi, Hokkaido, Japan (An et al., 2008), was autoclaved and divided into two parts: one part was mixed with calcium carbonate at 12 g kg^{-1} to adjust the pH to 5.7

and the other was not. *M. sinensis* was sown on either of the soils in 9 cm plastic pots, inoculated with 1,000 spores of the free or positive culture line and grown in the greenhouse for 8 weeks, during which the plants received only tap water (10 plants pot^{-1}, $n = 5$). After harvest, shoot and root dry weights were measured, and percentages of mycorrhizal colonization were assessed by the gridline intersection method (Giovannetti and Mosse, 1980).

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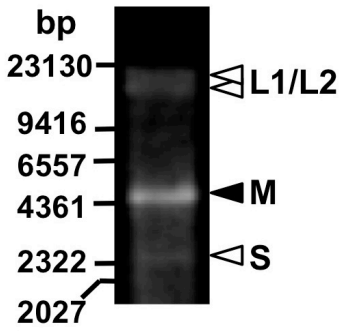


Fig. 1. dsRNA segments found in *Glomus* sp. RF1. Total nucleic acid was extracted from mycelia by the SDS-phenol method, treated with DNase I and S1 nuclease, and subjected to electrophoresis on 0.8% agarose gel. S, *Glomus* sp. RF1 virus-like-small dsRNA (GRF1V-S, 2.5 kbp); M, -medium dsRNA (GRF1V-M, 4.5 kbp); L1/L2, -large dsRNAs 1 and 2 (GRF1V-L1 and -L2, > 10 kbp). GRF1V-M was characterized in detail.

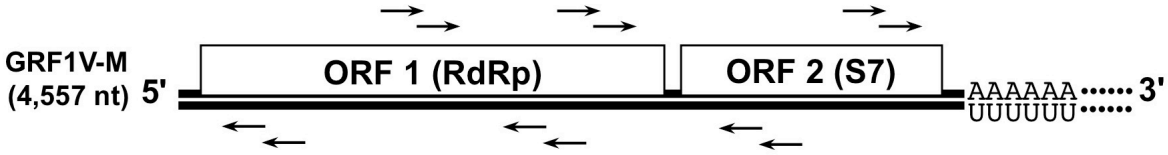


Fig. 2. Genome organization of GRF1V-M. The segment is 4,557 nt in length and possesses ORF 1 (927 codons) and ORF 2 (537 codons) that are predicted to encode RNA-dependent RNA polymerase (RdRp) and a S7 structural protein (S7), respectively. A 19-nt poly(A)-tail is present at the 3'-UTR. Relative positions of primers used for 5'/3' RACE, for detection from the *in vitro* culture (in the middle of ORF 1), and for confirmation of the genome organization (between 3'-end of ORF 1 and 5'-end of ORF 2) are indicated (absolute positions are indicated in Supplementary Table S2).

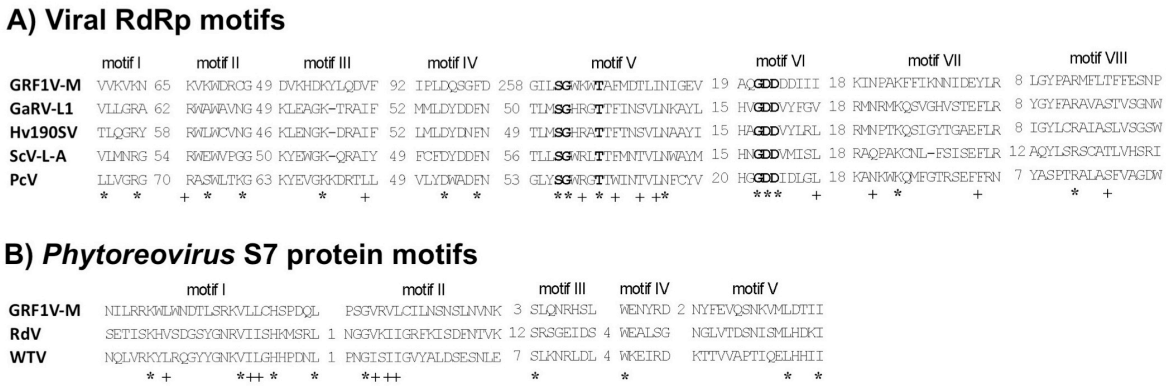


Fig. 3. Protein motifs found in the predicted ORFs in GRF1V-M. **A,** Alignment of viral RdRp motifs I–VIII (Pfam accession no. PF02123) encoded by GRF1V-M and the members of *Victorivirus* (GaRV-L1 and Hv190SV), *Totivirus* (ScV-L-A), and *Chrysovirus* (PcV). The SG---TS/T and GDD motifs are highlighted by bold letters. **B,** Alignment of *Phytoreovirus* S7 protein motifs I–V (Pfam accession no. PF07236) encoded by GRF1V-M and the members in the *Phytoreovirus* (RdV and WTV). Abbreviated viral names and accession numbers: GaRV-L1, *Gremmeniella abetina* RNA virus L1 (AAK11656); Hv190SV, *Helminthosporium victoriae* virus 190S (AAB94791); PcV, *Penicillium chrysogenum* virus (YP_392482); ScV-L-A, *Saccharomyces cerevisiae* virus L-A (NP_620495); RdV, Rice dwarf virus (NP_620543); WTV, Wound tumor virus (P12325).

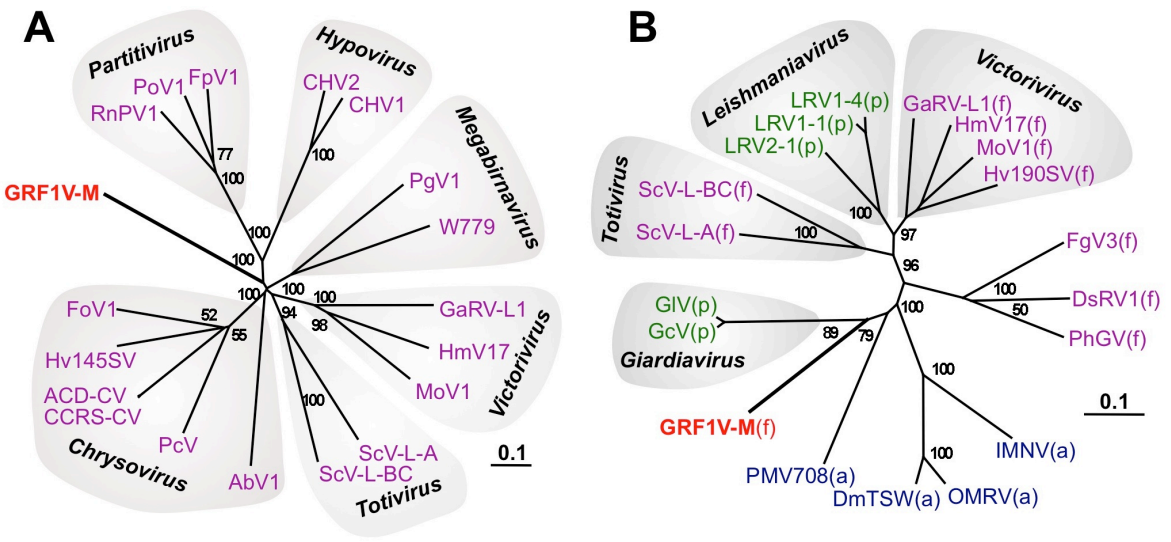


Fig. 4. Phylogenetic analysis of GRF1V-M based on the amino acid sequences of RNA-dependent RNA polymerase. **A,** Analysis with all genera of dsRNA mycoviruses. Note that PgV1 has not been confirmed to be a member of *Megabirnavirus*. **B,** Analysis with the animal (a), protozoan (p), and fungal (f) viruses in the family Totiviridae and with the unclassified dsRNA fungal viruses (FgV3, DsRV1, and PhGV) to which GRF1V-M RdRp showed similarities. Multiple sequence alignments were implemented by Clustal X and modified manually, and the neighbor-joining trees were drawn by NJplot. The tree topologies were confirmed by comparing with the maximum-likelihood trees constructed by the LG + I + G model following the Akaike information criterion. Numbers at the nodes represent bootstrap values (%) for 1,000 trials. The scale bars represent 0.1 amino acid substitutions per site corresponding to branch length. Abbreviated virus names and accession numbers: AbV1, *Agaricus bisporus* virus 1 (CAA64144); ACD-CV, Amasya cherry disease associated chrysovirus (YP_001531163); CCRS-CV, Cherry chlorotic rusty spot associated chrysovirus

(CAH03664); CHV1, *Cryphonectria* hypovirus 1-EP713 (Q04350); CHV2, *Cryphonectria* hypovirus 2-NB58 (AAA20137); DmTSW, *Drosophila melanogaster* totivirus SW-2009a (YP_003289293); DsRV1, *Diplodia scrobiculata* RNA virus 1 (YP_003359178); FgV3, *Fusarium graminearum* dsRNA mycovirus-3 (YP_003288789); FoV1, *Fusarium oxysporum* chrysovirus 1 (ABQ53134); FpV1, *Fusarium poae* virus 1 (AF047013); GaRV-L1, *Gremmeniella abetina* RNA virus L1 (AAK11656); GcV, *Giardia canis* virus (ABB36743); GlV, *Giardia lamblia* virus (AAB01579); HmV17, *Helicobasidium mompa* No.17 dsRNA virus (NP_898833); Hv145SV, *Helminthosporium victoriae* 145S virus (YP_052858); Hv190SV, *Helminthosporium victoriae* virus 190S (AAB94791); IMNV, Penaeid shrimp infectious myonecrosis virus (YP_529549); LRV1-1, *Leishmania* RNA virus 1-1 (NP_041191); LRV1-4, *Leishmania* RNA virus 1-4 (NP_619653); LRV2-1, *Leishmania* RNA virus 2-1 (NP_043465); MoV1, *Magnaporthe oryzae* virus 1 (NP_624349); OMRV, Omono River virus (BAJ21511); PcV, *Penicillium chrysogenum* virus (YP_392482); PhGV, *Phlebiopsis gigantea* mycovirus dsRNA 2 (CAJ34335); PgV1, *Phlebiopsis gigantea* mycovirus dsRNA1 (CAJ34333); PMV708, *Piscine myocarditis* virus AL V-708 (ADP37187); PoV1, *Pleurotus ostreatus* virus 1 (ACX43951); RnPV1, *Rosellinia necatrix* partitivirus 1-W8 (NC_007537); ScV-L-A, *Saccharomyces cerevisiae* virus L-A (NP_620495); ScV-L-BC, *Saccharomyces cerevisiae* virus L-BC (NP_042581); W779, *Rosellinia necatrix* megabirnavirus 1/W779 (YP_003288763).

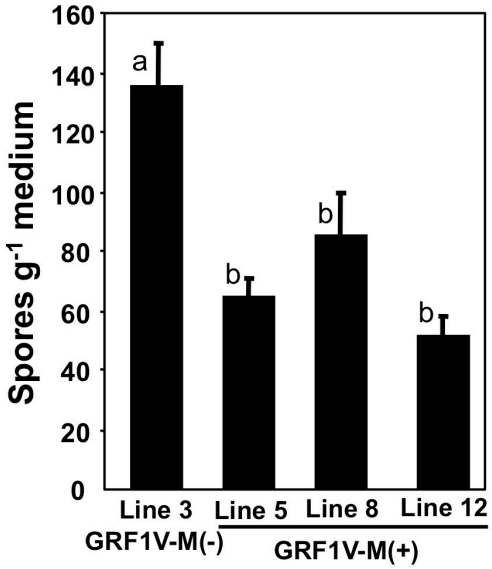


Fig. 5. Spore productivity of the GRF1V-M-free and -positive culture lines of *Glomus* sp. RF1. The free (Line 3) and positive (Lines 5, 8, and 12) culture lines were grown in the sand culture with sorghum in a greenhouse for 20 weeks, and spores formed in the medium were extracted by the wet sieving, and counted under a stereomicroscope. Different letters indicate significant difference ($P < 0.05$, Tukey–Kramer test). Vertical bars indicate SE ($n = 3$).

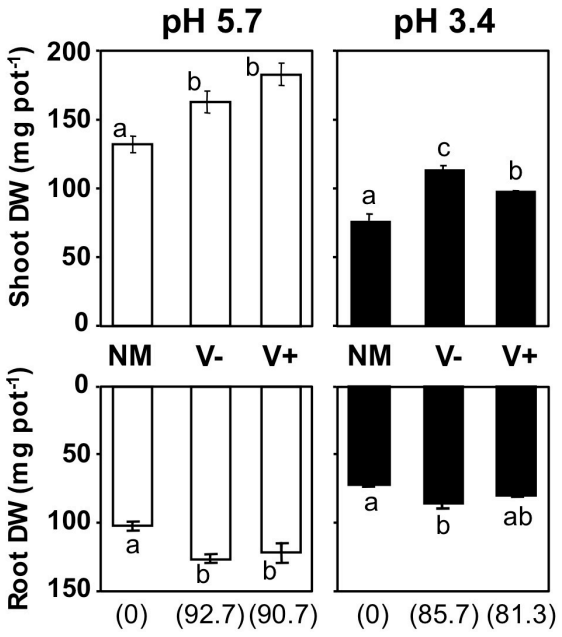


Fig. 6. Plant growth-promoting effect of the GRF1V-M-free and -positive culture lines of *Glomus* sp. RF1 with respect to soil pH. The pioneer grass species *Miscanthus sinensis* was inoculated either with the free (V–, line 3) or positive (V+, line 8) culture line and grown on acidic (pH 3.4) or non-acidic (pH 5.7) soil for 8 weeks. Non-mycorrhizal plants (NM) were grown as controls. Different letters indicate significant difference ($P < 0.05$, Tukey–Kramer test). Vertical bars indicate the SE ($n = 5$). Numbers in parentheses are the levels of mycorrhizal colonization (%) at 8 weeks. No significant difference in the levels of colonization was observed between the free and positive lines at $P < 0.05$.

SUPPLEMENTAL INFORMATION

Supplementary Table S1. List of top hit proteins encoded by ORFs found in the contigs obtained by FLX pyrosequencing of the *Glomus* sp. RF1 dsRNA fraction[a].

Contig ID	Length (bp)	No. of read	Top hit protein	Similarity	Accession no.
293	2517	630	RNA-dependent RNA polymerase (*Botrytis cinerea* debilitation-related virus)	42% (119/281)	ABQ65153
372	4573	329	RNA-dependent RNA polymerase (*Giardia canis* virus)	29% (76/270)	ABB36743
362	3705	284	p31 protein (*Phytophthora endornavirus* 1)	39% (25/63)	CAQ03373
556	2662	161	hypothetical protein PEV1p1 (*Phytophthora endornavirus* 1)	35% (73/131)	YP_241110
364	5947	361	hypothetical protein PEV1p1 (*Phytophthora endornavirus* 1)	24% (78/318)	YP_241110
6	1030	58	RNA-dependent RNA polymerase (*Phlebiopsis gigantea* mycovirus dsRNA 1)	30% (82/268)	YP_003541123
2696	1268	33	RNA-dependent RNA polymerase (*Phlebiopsis gigantea* mycovirus dsRNA 1)	22% (74/184)	YP_003541123
2855	4186	565	RNA-dependent RNA polymerase (*Phlebiopsis gigantea* mycovirus dsRNA 1)	40% (56/139)	YP_003541123

[a]More than 45,000 reads with an average length of 285 bp were obtained, and contigs longer than 1 kbp were subjected to BLAST searches.

Supplementary Table S2. PCR and reverse-transcription primers used in this study.

Primer names	Sequence (5' to 3')	Position	Notes
6N-Anchor	CCT GAA TTC GGA TCC TCC NNN NNN	-	1st strand cDNA synthesis (Márquez *et al*., 2007)
Anchor-1	CCT GAA TTC GGA TCC TCC	-	cDNA amplification (Márquez *et al*., 2007)
4.5-R1†	ATA GAC CGC AAT GGG GTA AAG C	1875-1896	1st sense primer for the detection of GRF1V-M
4.5-F1†	CAC AGG TGC GGA GTG CTA CG	2368-2387	1st antisense primer for the detection of GRF1V-M
4.5-R2†	ATA AGG GCG GTA CTT GGA ATG G	1922-1943	2nd sense primer for the detection of GRF1V-M
4.5-F2†	GCT GTC CAT TTC CAA CCG CTC	2311-2331	2nd antisense primer for the detection of GRF1V-M
2.5-R1	CAA CGG GTG GGC CTC TAT TC	1241-1260	1st sense primer for the detection of GRF1V-S
2.5-F1	GGG TTG AAG CGG CTA ATC TCC	1589-1609	1st antisense primer for the detection of GRF1V-S
2.5-R2	TCC TTA CTC CAA ATG GCG ACC	1276-1296	2nd sense primer for the detection of GRF1V-S
2.5-F2	ACA CCA AGA CAC CGC ATT ATC G	1480-1501	2nd antisense primer for the detection of GRF1V-S
5.9-F1*	CCG ATG GCA TTA GCA AGA CTA CC	2264-2286	1st sense primer for the detection of GRF1V-L1
5.9-R1*	AGA CGC GAA AGA TGT TGT CAG G	2791-2812	1st antisense primer for the detection of GRF1V-L1
5.9-F2*	TTC GAA ACA CCC TCA TAC AGC AC	2339-2361	2nd sense primer for the detection of GRF1V- L1
5.9-R2*	TTC CTT GTC AAT GGC TCA CTA CG	2601-2623	2nd antisense primer for the detection of GRF1V- L1
L2-F4§	ACA CCT TGC CTC CAA TGA AAC C	789-810	1st sense primer for the detection of GRF1V-L2
L2-R18§	ACT CGA CAG AGG CCC GTC TT	1090-1109	1st antisense primer for the detection of GRF1V-L2
L2-F6§	GCA GCG CCT CAG GAT ACA GAC	844-864	2nd sense primer for the detection of GRF1V- L2
L2-R19§	AGT GGT TCC AGC GGA ACC AGA A	1049-1070	2nd antisense primer for the detection of GRF1V- L2
Oligo d(T)-anchor	GAC CAC GCG TAT CGA TGT CGA $(T)_{16}$ V	-	1st amplification of cDNA synthesis for 3'/5' RACE
Anchor-2	GAC CAC GCG TAT CGA TGT CGA C	-	Anchor primer for cDNA amplification
4.5Ra3'-1st	TGA TGA GAG CTA AGG ATG AGG	4130-4150	1st strand cDNA synthesis for 3' RACE
4.5Ra5'-1st	ACC TGT TCC AAC GAC AGC TG	286-305	1st strand cDNA synthesis for 5' RACE
4.5Ra3'-2nd†	GAT TGG TTG TGT GGG AGA TTG	4240-4260	1st amplification of cDNA for 3' RACE
4.5Ra5'-2nd†	CAT TAT CTG GAA ATT TGC TGG AGC	131-154	1st amplification of cDNA for 5' RACE
4.5Ra3'-3rd†	GTT GAC TAT GGG ATG CTT AAG TC	4357-4379	2nd amplification of cDNA for 3' RACE
4.5Ra5'-3rd†	GGG TTT AAA AGG TGG TAA CCG C	43-64	2nd amplification of cDNA for 5' RACE

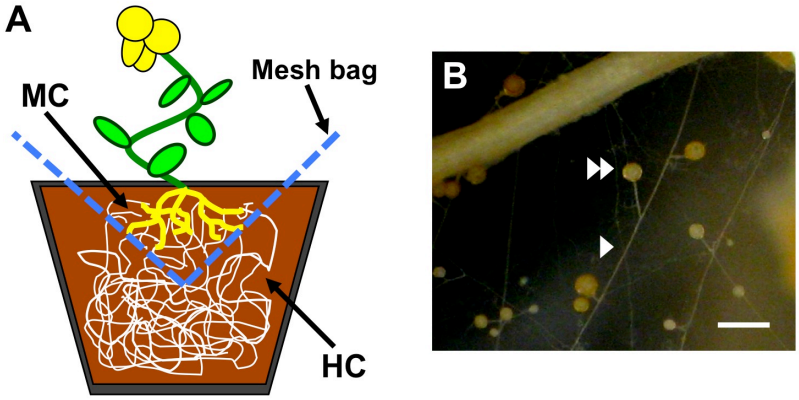
Supplementary Table S2 (*continued*).

Primer names	Sequence (5' to 3')	Position	Notes
4.5C-R1[†]	AAC GTA GCA CTC CGC ACC TG	2366-2385	1st sense primer for confirming the genomic organization of GRF1V-M
4.5C-F1[†]	AAC TGA TCT GGA CTG TGG CAT AAC	3093-3116	1st antisense primer for confirming the genomic organization of GRF1V-M
4.5C-R2[†]	AGA TGC TCA AGG TGA TGA TGA CG	2419-2441	2nd sense primer for confirming the genomic organization of GRF1V-M
4.5C-F2[†]	ATC GTT CCA TAA CCA CTT CCT ACG	3049-3072	2nd antisense primer for confirming the genomic organization of GRF1V-M

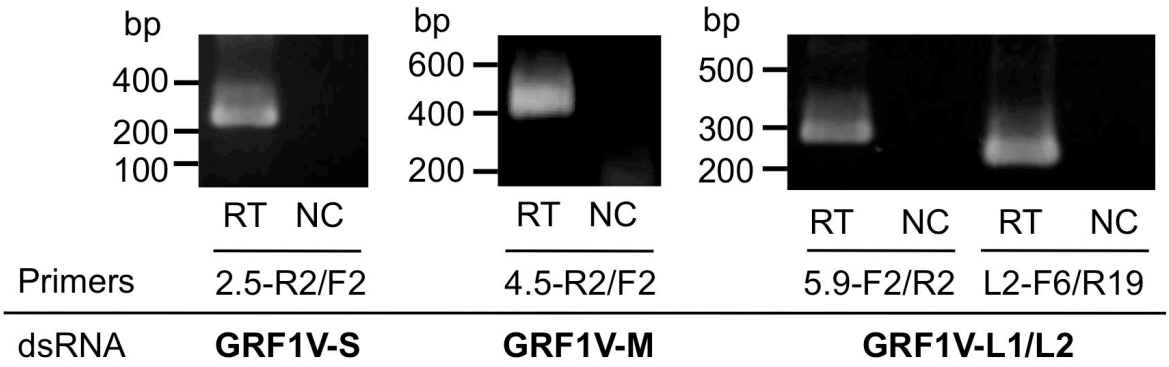
[†]Relative positions of the primers are indicated in Fig. 2.

[*]Primers designed based on the sequence of 5.9-kbp *Endornavirus*-like contig (Supplementary Table S1).

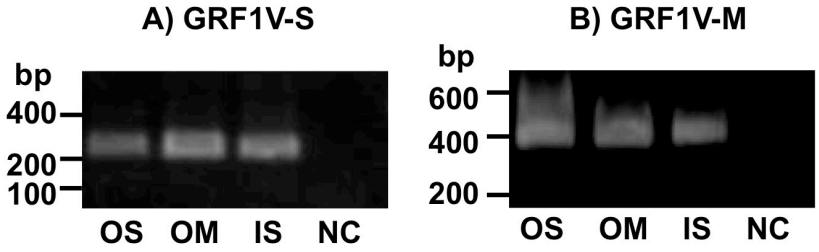
[§]Primers designed based on the sequence of 4.2-kbp totivirus-like contig (Supplementary Table S1).



Supplementary Fig. S1. Open and *in vitro* culture systems of AM fungi. **A,** Mesh bag-separated open pot culture system with *Lotus japonicus* L. cv. Miyakojima MG-20. The dotted line represents the 37 µm mesh bag that separates the mycorrhizal (upper part, MC) and hyphal (lower part, HC) compartments. **B,** *In vitro* monoxenic culture of *Glomus* sp. RF1 with Ri T-DNA transformed carrot roots. The arrowhead and double arrowhead indicate an extraradical hypha and a spore, respectively. Bar, 1.0 mm.



Supplementary Fig. S2. Identity between the contigs obtained by pyrosequencing and the dsRNA segments detected in electrophoresis. GRF1V-S, GRF1V-M, and GRF1V-L1/L2 were re-extracted separately from the electrophoretic gel (GRF1V-L1/L2 were collected together). Nested PCRs were then conducted by using these dsRNAs and the primers specific to each contig after reverse-transcription with 6N primer (RT). The primer pairs 2.5-R2/F2, 4.5-R2/F2, 5.9-F2/R2, and L2-F6/R19 were designed based on the sequences of 2.5-kbp *Mitovirus*-like contig, 4.5-kbp *Giardiavirus*-like contig, 5.9-kbp *Endornavius*-like contig, and 4.2-kbp totivirus-like contigs, respectively (Supplementary Table S2). The amplicons were cloned and sequenced for confirmation. dsRNA without reverse-transcription was used as negative control (NC).



Supplementary Fig. S3. Confirmation of the presence of GRF1V-S (**A**) and -M (**B**) in the *in vitro* culture of *Glomus* sp. RF1. dsRNAs were prepared from the open-culture spores (OS), open-culture mycelia (OM), and *in vitro* spores (IS), reverse-transcribed with 6N-random primer, and subjected to nested PCR with the specific primers (Supplementary Table S2). The amplicons were cloned and sequenced for confirmation. dsRNA without reverse-transcription was used as negative control (NC).