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

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The nucleotide sequence data of GRF1V-M and GRF1V-S have been deposited in the DDBJ Data Library under accession nos. AB558119 and AB558120, respectively.

## 1 INTRODUCTION

2

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

13           Fungal viruses (mycoviruses) have been found in a variety of fungi, including  
14 the Ascomycetes, Basidiomycetes, and Deuteromycetes (Sasaki *et al.*, 2006). Their  
15 genomes are composed of double-stranded RNA (dsRNA) in most cases (Fauquet *et al.*,  
16 2005) and unexceptionally possess an RNA-dependent RNA polymerase (RdRp) gene  
17 for replication (Ghabrial and Suzuki, 2009). They do not have an extracellular infection  
18 route (Buck, 1998) but are transmissible vertically via asexual spores (not, or less  
19 efficiently, via sexual spores) and horizontally via anastomosis (Hillman *et al.*, 2004).  
20 Because no universal artificial infection/elimination system for mycoviruses has been

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

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

41 but more than 70 pots are required to collect about 1 g of mycelia in this system. On the  
42 other hand, purity of fungal material is best in the *in vitro* monoxenic culture (Bécard  
43 and Fortin, 1988), which has been widely employed for, e.g., physiological (Bago *et al.*,  
44 2002; Nielsen *et al.*, 2002) and molecular biological (Maldonado-Mendoza *et al.*, 2001)  
45 studies, although fungal growth is generally poorer than in open culture. To overcome  
46 these problems we designed the ‘two-step strategy’ in combination with the  
47 next-generation sequencing technology in the present study: dsRNA viral genomes  
48 were first extracted from fungal material produced in the open pot culture and  
49 sequenced ‘deeply’ without purifying a single viral genome, and subsequently, the  
50 presence of the viruses was verified using material produced in the *in vitro* monoxenic  
51 culture by RT-PCR.

52

## 53 **RESULTS**

54

### 55 **Detection of dsRNA**

56

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

61 several dsRNA segments that differed in size, which were termed as *Glomus* sp. RF1  
62 virus-like-small dsRNA (GRF1V-S, 2.5 kbp), -medium dsRNA (GRF1V-M, 4.5 kbp),  
63 -large dsRNA 1 (GRF1V-L1, > 10 kbp), and -large dsRNA 2 (GRF1V-L2, > 10 kbp)  
64 (Fig. 1). Among them, GRF1V-M was chosen for further analysis because the segment  
65 was relatively abundant, which was expected to be readily characterized. For this  
66 purpose we employed FLX pyrosequencing, one of the next-generation sequencing  
67 technologies, instead of the traditional shotgun library-based sequencing, because at  
68 least the three dsRNA segments were coexist with GRF1V-M in the fraction. dsRNAs  
69 were reverse-transcribed with the 6N-anchored primer (Supplementary Table S2),  
70 amplified with the anchor primer, and then subjected to pyrosequencing. More than  
71 45,000 reads with an average length of 285 bp were obtained and assembled, and eight  
72 large (> 1 kbp) contigs showed amino acid sequence similarities to RdRp and other  
73 proteins of four different viruses: 2.5-kbp contig, *Botrytis cinerea* debilitation-related  
74 virus in the genus *Mitovirus*; 4.5-kbp contig, *Giardia canis* virus in the genus  
75 *Giardiavirus*; 2.6-, 3.7-, and 5.9-kbp contigs, *Phytophthora endornavirus* 1 in the genus  
76 *Endornavius*; 1.0-, 1.3-, and 4.2-kbp contigs, *Phlebiopsis gigantea* mycovirus dsRNA 1  
77 that was an unclassified totivirus (Supplementary Table S1). To examine the identity  
78 between these contigs and the dsRNA segments detected in the gel, GRF1V-S,  
79 GRF1V-M, and GRF1V-L1/L2 were re-extracted from the gel separately. GRF1V-L1  
80 and -L2 were collected together because the two could not be clearly separated on the

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

94

#### 95 **Molecular characterization of GRF1V-M**

96 To obtain full-length sequence of GRF1V-M about 100 bp of each of the  
97 5'/3'-terminal sequences were determined/confirmed by RACE. The segment is 4,557  
98 nucleotides (nt) in length and consists of a 5'-untranslated region (UTR) of 40 nt, two  
99 ORFs (ORF 1 and 2) encoding polypeptides of 927 and 537 aa, respectively, and a  
100 3'-UTR of 102 nt that includes a 19-nt poly(A) tail in the plus-sense strand (Fig. 2). No

101 notable ORF was found in the minus-sense strand. A preliminary BLAST search  
102 revealed that the protein encoded by ORF 1 had similarities to RdRps of the members  
103 of the family Totiviridae, but surprisingly, the highest score was observed with RdRps  
104 of protozoan viruses, followed by those of the viruses found in salmon, fruit fly,  
105 mosquito, and shrimp, which were included in subsequent phylogenetic analysis. The  
106 ORF 1 protein conserved a domain of the viral RdRp superfamily (Pfam accession no.  
107 PF02123) and its motifs I–VIII, including the SG---TS/T and GDD motifs (Ghabrial,  
108 1998; Chiba *et al.*, 2009) (Fig. 3A). The protein encoded by ORF 2 showed similarity to  
109 the S7 viral core protein of the rice dwarf virus (*Phytoreovirus*) that is suggested to  
110 have binding activity to the genomic RNA, RdRp, and other viral structural proteins  
111 (Ueda *et al.*, 1997; Hagiwara *et al.*, 2003). The domain of the *Phytoreovirus* S7 protein  
112 superfamily (Pfam accession no. PF07236) was also conserved in the ORF 2 protein  
113 (Fig. 3B). We considered, therefore, that ORF 2 might encode a structural protein that  
114 binds to and protects the genomic RNA.

115         Because the genome organization of GRF1V-M, in which an RdRp gene was  
116 encoded by the 5'-proximal ORF, was unique and had not been described within the  
117 members of Totiviridae, the region between 3'-end of ORF 1 and 5'-end of ORF 2 was  
118 amplified by nested PCR with the specific primers (Fig. 2 and Supplementary Table S2),  
119 cloned, and sequenced, which resulted in confirmation of the sequence obtained by  
120 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

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

147 Spore productivity, which could be assessed only during symbiosis with plants,  
148 was employed as an index of fungal reproductive/competitive potential. Sorghum, one  
149 of the greatest spore producers (reviewed in Smith and Read, 2008), was inoculated  
150 with either the GRF1V-M-free or -positive line and grown on sand culture in a  
151 greenhouse, and spores were harvested 20 weeks after sowing at which maximum spore  
152 production was expected. The GRF1V-M-free line produced at a density of more than  
153 130 spores g<sup>-1</sup> sand, but the GRF1V-M-positive lines produced only 37 – 63% (50 – 85  
154 spores g<sup>-1</sup> sand) of that of the free line (Fig. 5).

155 The impact of GRF1V-M on plant symbiotic growth was assessed both under  
156 acidic (pH 3.4) and non-acidic (pH 5.7) conditions, because *Glomus* sp. RF1 was  
157 originally isolated from acidic soil and highly acid-tolerant (unpublished observation).  
158 As a host plant, *Miscanthus sinensis* that is a C<sub>4</sub> perennial grass species was chosen.  
159 Although this species grows more slowly and produces less spores than sorghum, it is  
160 highly acid-tolerant (Kayama, 2001; Maki *et al.*, 2008) and thus more suitable for the

161 assessment than sorghum of which the growth is largely limited by soil acidity (Caniato  
162 et al., 2007). *M. sinensis* was inoculated either with the GRF1V-M-free or -positive line  
163 and grown in the greenhouse for 8 weeks, by which time plant growth was not limited  
164 by the pot size (though sporulation was minimum). Both the free and positive lines  
165 promoted plant growth to the same extent at pH 5.7, compared with the  
166 non-mycorrhizal control (Fig. 6). At pH 3.4 shoot growth was greater in those  
167 inoculated with the free line than with the positive line, although no significant  
168 difference in root growth was observed between the two lines. The level of mycorrhizal  
169 colonization was not different between the free and positive lines both at pH 5.7 and  
170 3.4.

171

## 172 **DISCUSSION**

173

174 The present study demonstrates that the obligate plant symbionts AM fungi  
175 harbored diverse dsRNAs, which are highly likely to be mycoviruses, for the first time.  
176 The ‘two-step strategy’, in which molecular characterization was conducted using  
177 material from the open culture and the presence was verified by RT-PCR using a small  
178 amount of material produced *in vitro*, was indispensable for the successful detection  
179 and characterization of dsRNA in AM fungi, in addition to next-generation sequencing.  
180 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

201 observations are in favor of dsRNA. For example, the RdRp gene was most closely  
202 related to dsRNA viruses, and both the RdRp and S7 core protein motifs of dsRNA  
203 viruses were conserved. In a preliminary experiment, cDNA of the viral genome could  
204 be synthesized and amplified from total RNA with the 3'-end oligo(dT) primer in  
205 combination with the 5'-end primers using the boiled (denatured) RNA preparation, but  
206 not without boiling, suggesting that no (or minimum) ssRNA form of GRF1V-M was  
207 present in the preparation. Purification of the viral particles, however, may be necessary  
208 to exclude the possibility that GRF1V-M is an ssRNA virus, if the particles really exist.  
209 We assume that the genome may be encapsulated by the S7 core protein (encoded by  
210 ORF 2) or, more likely, only associated with the protein to protect the virus from  
211 nucleases, because the S7 core protein may not be a real capsid protein (Ueda *et al.*,  
212 1997; Hagiwara *et al.*, 2003). To understand the structure, the first step we are planning  
213 is immunoprecipitation of GRF1V-M using antibodies raised against the S7 core protein.

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

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

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

240           Although the molecular mechanism underlying the phenotypic alteration by

241 GRF1V-M is obscure, ecological relevance of the reduced spore productivity and  
242 plant-growth promoting effect of *Glomus* sp. RF1 is of interest. It has been suggested  
243 that considerable photosynthetic carbon is allocated to the fungal symbiont for  
244 sporulation (Smith and Read, 2008). The reduction in spore productivity in *Glomus* sp.  
245 RF1 therefore likely leads to the idea that GRF1V-M improves overall carbon economy  
246 of the association via saving excess consumption of carbon. On the other hand, we  
247 consider that the impact on plant growth may be limited, because the  
248 GRF1V-M-positive line could still improve plant growth compared with the  
249 non-mycorrhizal plants under the acidic conditions and the percentage of mycorrhizal  
250 colonization, which may reflect infectivity of the fungus, was not significantly different  
251 between the GRF1V-M-positive and -free lines. Given that soil acidity is the major  
252 selection pressure on AM fungi (An et al., 2008), it is likely that the acidic stress (pH  
253 3.4) in the experimental conditions was so severe for the fungus that a negative impact  
254 of GRF1V-M, e.g., a decline in fungal fitness via exploitation of cellular resource due  
255 to GRF1V-M proliferation, might be enhanced. Accordingly, further investigation is  
256 necessary to elucidate direct relevance of the reduced spore productivity to plant  
257 competitiveness with respect to soil acidity.

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

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

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

279

## 280 **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 (26/20°C, 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 25°C 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<sup>-1</sup>.  
300 The mesh bag system consisted of the MC and hyphal compartment (HC) that were

301 separated by a cone-shaped 37  $\mu\text{m}$  nylon mesh bag, and both compartments were filled  
302 with autoclaved river sand. The pore size of the nylon mesh was small enough to  
303 prevent *L. japonicus* roots from passing but large enough to allow AM fungal hyphae to  
304 pass through. The plants were grown in a growth chamber at a photosynthetic photon  
305 flux of 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (16-h day length, 25°C) for a week only with deionized water,  
306 thinned to two plants  $\text{pot}^{-1}$ , and further grown with low-P nutrient solution (4 mM  
307  $\text{NH}_4\text{NO}_3$ , 1 mM  $\text{K}_2\text{SO}_4$ , 75  $\mu\text{M}$   $\text{MgSO}_4$ , 2 mM  $\text{CaCl}_2$ , 50  $\mu\text{M}$  Fe-EDTA, and 50  $\mu\text{M}$   
308  $\text{KH}_2\text{PO}_4$ ) for 7 weeks. After removing the MC, extraradical mycelium in the HC was  
309 harvested from 72 pots by wet sieving, blotted on filter paper, weighed, frozen in liquid  
310 nitrogen, and stored at  $-80^\circ\text{C}$  for dsRNA extraction. In this process, 0.4 – 0.9 g FW  
311 mycelia could normally be collected.

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

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

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

324

### 325 **dsRNA extraction, sequencing, and virus detection**

326 dsRNA was extracted from the frozen mycelia (0.4 – 0.9 g) by the SDS-phenol  
327 method (Suzuki *et al.*, 2003), purified by incubating with 0.4 units  $\mu\text{L}^{-1}$  DNase I and  
328 3.4 units  $\mu\text{L}^{-1}$  S1 nuclease (Takara Bio, Tokyo, Japan) at 37°C overnight according to  
329 Osaki *et al.* (2002), and electrophoresed on 0.8% agarose gel. For sequencing, dsRNA  
330 segments were eluted from the gel pieces in the GeBAflex dialysis tube (25 kDa cut-off,  
331 Gene Bio-Application, Kfar Hanagide, Israel), purified again by overnight incubation  
332 with DNase I and S1 nuclease, reverse-transcribed using the 6N-anchored primer  
333 (Supplementary Table S1), and amplified using the anchor primer with *Taq* polymerase.  
334 The program of the thermal cycler was as follows: initial denaturation at 94°C for 2 min,  
335 followed by 40 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 2 min, with a final  
336 extension at 72°C for 10 min. The PCR product was sequenced using the Genome  
337 Sequencer FLX system with a 1/8-scale gasket (Roche Diagnostics, Tokyo, Japan) and  
338 assembled using the SeqNova Data Analysis System at Hokkaido System Science Co.,  
339 Ltd. (Sapporo, Japan). The sequences of the extreme ends of the dsRNA segment were  
340 amplified using the specific primers (Supplementary Table S1), cloned, and sequenced

341 by the dideoxy-cycle sequencing method.

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

351

### 352 **Sequence analysis and phylogeny**

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

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

370

### 371 **Impact of GRF1V-M infection on fungal phenotype**

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

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

387

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389

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396

## 397 **LITERATURE CITED**

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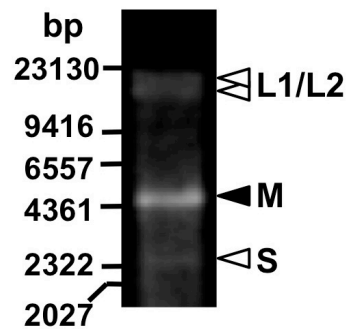
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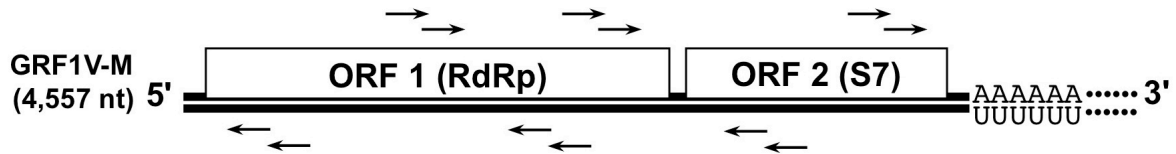
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**Fig. 1.** dsRNA segments found in *Glomus* sp. RF1. Total nucleic acid was extracted from mycelia by the SDS-phenol method, treated with DNase I and S1 nuclease, and subjected to electrophoresis on 0.8% agarose gel. S, *Glomus* sp. RF1 virus-like-small 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).

### A) Viral RdRp motifs

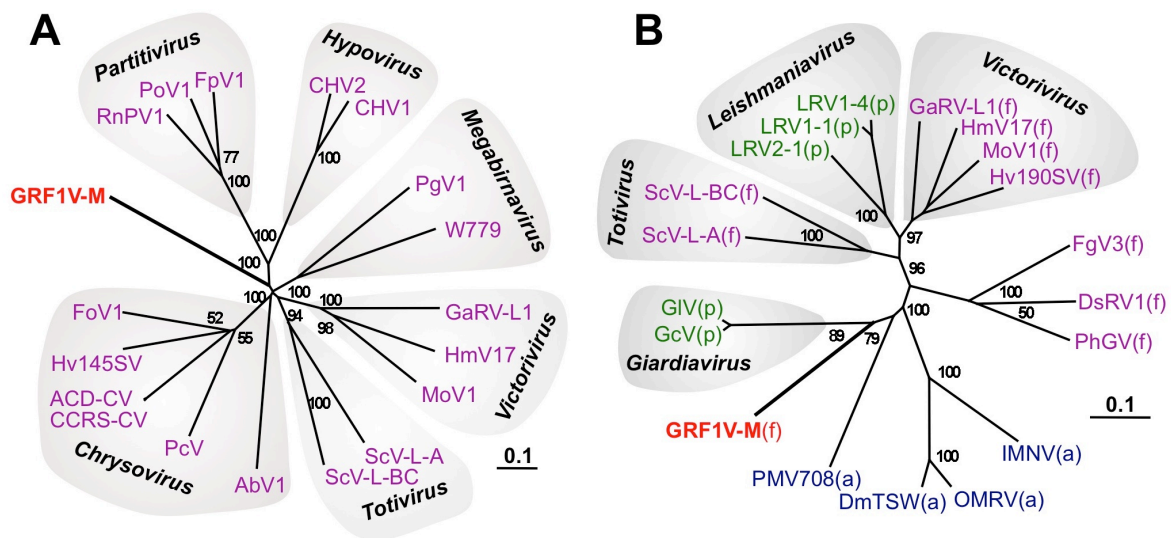
	motif I	motif II	motif III	motif IV	motif V	motif VI	motif VII	motif VIII
<b>GRF1V-M</b>	VVKVFN 65	KVKWDROG 49	DVKHKYLLQDVF 92	IPLDQSGFD 258	GIL <b>SG</b> HWIAEMDTLINIGEV 19	AQ <b>GDD</b> DDLLI 18	KINPAKFFIKNIIDEYLR 8	LGYPARMLITFFESNP
<b>GaRV-L1</b>	VLLGRA 62	RWAWAVNG 49	KLEAGK-TRAF 52	MMLDYDDN 50	TLM <b>SG</b> HRDTTFINSVLNKAYL 15	HV <b>GDD</b> VYFGV 18	RNNRMKQSVGHVSTEFRL 8	YGYFARAVASTVSGNW
<b>Hv190SV</b>	TLQGRY 58	RWLWCVNG 46	KLENGK-DRAIF 52	LMLDYDNN 49	TLM <b>SG</b> HRATTFINSVLNAAAI 15	HA <b>GDD</b> VYLR 18	RNNPTKQSIGYTGAEFLR 8	IGYLCRAIASLVSGSW
<b>ScV-L-A</b>	VIMNRG 54	RWEWVEGG 50	KYEWGK-QRAIY 49	FCEDYDDN 56	TLL <b>SG</b> WRLTTEMNTVLNWAYM 15	HN <b>GDD</b> VMSL 18	RAQPAKCNL-FSISEFLR 12	AQYLSRSCATLVHSRI
<b>PcV</b>	LLVGRG 70	RASMLIKG 63	KYEVGKDKRLL 49	VLYDWADN 53	GLY <b>SG</b> WRGTITWINTVLNFCIV 20	HC <b>GDD</b> IDLGL 18	KANKKQMGFTRSEFERN 7	YASPTRALASEVAGDW
	* *	+ * *	* +	* *	**+ *+ +**	*** +	+ *	+ *

### B) *Phytoreovirus* S7 protein motifs

	motif I	motif II	motif III	motif IV	motif V
<b>GRF1V-M</b>	NILRRKMLWINDLSRKVLLCHSPDQL	PSGVRVLCIINSNSLNWVK	3 SLQNRHSL	WENYRD 2	NYFEVQSNKVMMLDTII
<b>RdV</b>	SETISKGVSDGSYGNRVLISHMMSRL 1	NGGVKLIGRFKLSDENTVK 12	SRSGEIDS 4	WEALSG	NGELVDSNISMLHDKI
<b>WTV</b>	NQLVRKYLRCQSYGNKVLGHHPDNL 1	ENGISLIGVYALDSESNLE 7	SLKNRDL 4	WKEIRD	KITVWAPTIQELHHLI
	* +	+++ *	*	+++	* *

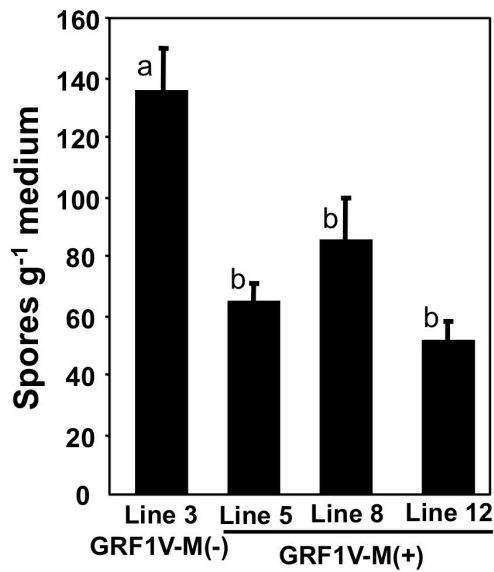
**Fig. 3.** Protein motifs found in the predicted ORFs in GRF1V-M. **A**, Alignment of viral RdRp motifs I–VIII (Pfam accession no. PF02123) encoded by GRF1V-M and the members of *Victorivirus* (GaRV-L1 and Hv190SV), *Totivirus* (ScV-L-A), and *Chrysovirus* (PcV). The SG---TS/T and GDD motifs are highlighted 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).



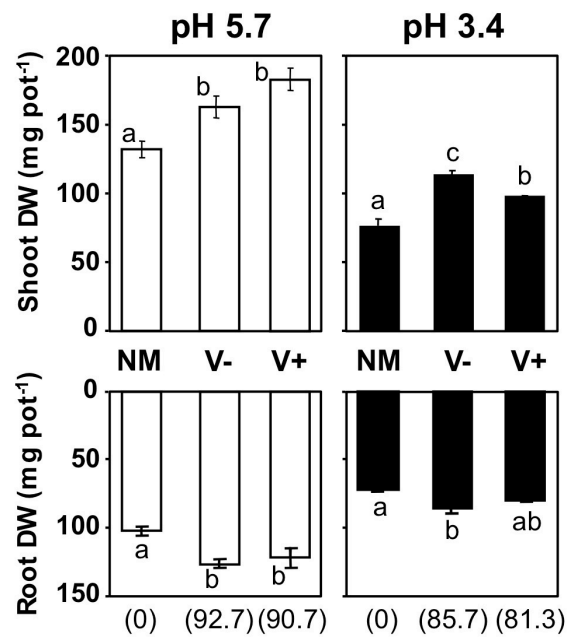


**Fig. 4.** Phylogenetic analysis of GRF1V-M based on the amino acid sequences of RNA-dependent RNA polymerase. **A**, Analysis with all genera of dsRNA mycoviruses. Note that PgV1 has not been confirmed to be a member of *Megabirnavirus*. **B**, Analysis with the animal (a), protozoan (p), and fungal (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<sup>a</sup>.

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

<sup>a</sup>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 <i>et al.</i> , 2007)
Anchor-1	CCT GAA TTC GGA TCC TCC	-	cDNA amplification (Márquez <i>et al.</i> , 2007)
4.5-R1 <sup>†</sup>	ATA GAC CGC AAT GGG GTA AAG C	1875-1896	1st sense primer for the detection of GRF1V-M
4.5-F1 <sup>†</sup>	CAC AGG TGC GGA GTG CTA CG	2368-2387	1st antisense primer for the detection of GRF1V-M
4.5-R2 <sup>†</sup>	ATA AGG GCG GTA CTT GGA ATG G	1922-1943	2nd sense primer for the detection of GRF1V-M
4.5-F2 <sup>†</sup>	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 <sup>*</sup>	CCG ATG GCA TTA GCA AGA CTA CC	2264-2286	1st sense primer for the detection of GRF1V-L1
5.9-R1 <sup>*</sup>	AGA CGC GAA AGA TGT TGT CAG G	2791-2812	1st antisense primer for the detection of GRF1V-L1
5.9-F2 <sup>*</sup>	TTC GAA ACA CCC TCA TAC AGC AC	2339-2361	2nd sense primer for the detection of GRF1V- L1
5.9-R2 <sup>*</sup>	TTC CTT GTC AAT GGC TCA CTA CG	2601-2623	2nd antisense primer for the detection of GRF1V- L1
L2-F4 <sup>§</sup>	ACA CCT TGC CTC CAA TGA AAC C	789-810	1st sense primer for the detection of GRF1V-L2
L2-R18 <sup>§</sup>	ACT CGA CAG AGG CCC GTC TT	1090-1109	1st antisense primer for the detection of GRF1V-L2
L2-F6 <sup>§</sup>	GCA GCG CCT CAG GAT ACA GAC	844-864	2nd sense primer for the detection of GRF1V- L2
L2-R19 <sup>§</sup>	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) <sub>16</sub> 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 <sup>†</sup>	GAT TGG TTG TGT GGG AGA TTG	4240-4260	1st amplification of cDNA for 3' RACE
4.5Ra5'-2nd <sup>†</sup>	CAT TAT CTG GAA ATT TGC TGG AGC	131-154	1st amplification of cDNA for 5' RACE
4.5Ra3'-3rd <sup>†</sup>	GTT GAC TAT GGG ATG CTT AAG TC	4357-4379	2nd amplification of cDNA for 3' RACE
4.5Ra5'-3rd <sup>†</sup>	GGG TTT AAA AGG TGG TAA CCG C	43-64	2nd amplification of cDNA for 5' RACE

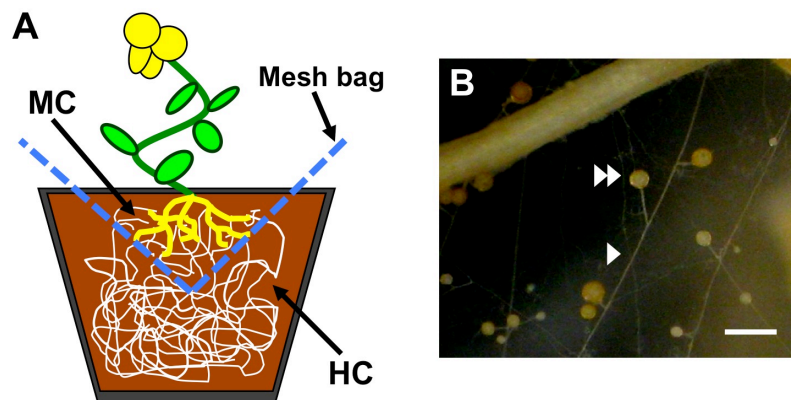
Supplementary Table S2 (*continued*).

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

<sup>†</sup>Relative positions of the primers are indicated in Fig. 2.

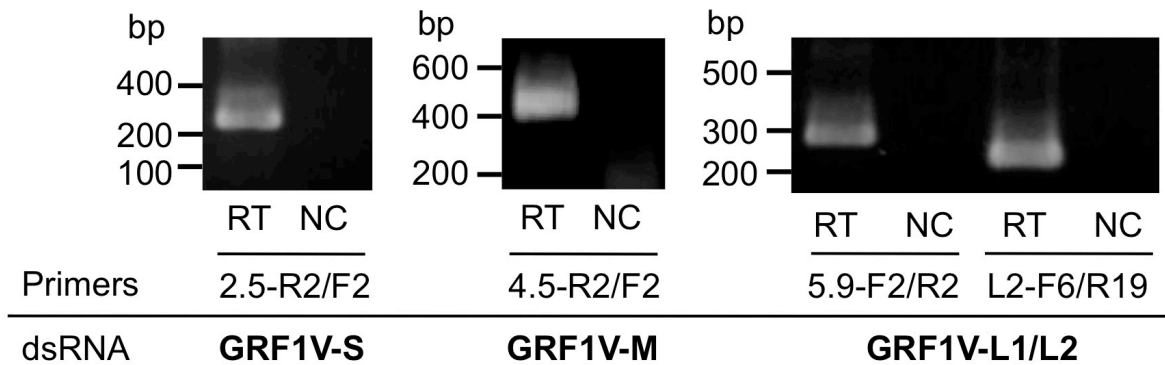
\*Primers designed based on the sequence of 5.9-kbp *Endornavirus*-like contig (Supplementary Table S1).

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

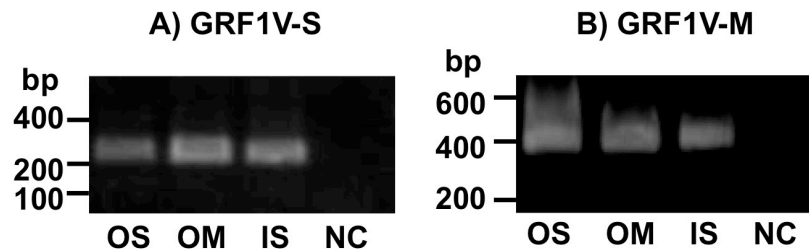


**Supplementary Fig. S1.** Open and *in vitro* culture systems of AM fungi. **A**, Mesh bag-separated open pot culture system with *Lotus japonicus* L. cv. Miyakojima MG-20. The dotted line represents the 37  $\mu\text{m}$  mesh bag that separates the mycorrhizal (upper part, MC) and hyphal (lower part, 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).