Mycoviruses related to chrysovirus affect vegetative growth in the rice blast fungus *Magnaporthe oryzae*

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Mycoviruses causing impaired growth and abnormal pigmentation of the host were found in the rice blast fungus, *Magnaporthe oryzae*. Four dsRNAs, dsRNA 1 (3554 bp), dsRNA 2 (3250 bp), dsRNA 3 (3074 bp) and dsRNA 4 (3043 bp), were detected in isolate S-0412-II 1a of *M. oryzae*.

By picking up single conidia of S-0412-II 1a, cured strains of the fungus were isolated that had completely lost the mycovirus. The cured strains had normal mycelial growth and pigmentation, suggesting that this mycovirus modulates host traits. The buoyant densities of isometric virus particles (~35 nm diameter) containing these dsRNAs in CsCl ranged from 1.37 to 1.40 g cm$^{-3}$.

The single ORF (3384 nt) of dsRNA 1 encoded a gene product highly homologous to the viral RNA-dependent RNA polymerase of members of the family *Chrysoviridae*. It is noteworthy that mycovirus S-0412-II 1a was detected not only in host cells but also in culture supernatant. Furthermore, abnormal aggregation of mycelia was observed after adding the mycovirus-containing culture supernatant to an uninfected strain of *M. oryzae* and mycoviral dsRNAs were detectable from the aggregated mycelia. This novel dsRNA mycovirus was named *Magnaporthe oryzae* chrysovirus 1.

INTRODUCTION

The dsRNA components found in filamentous fungi are known as mycoviruses (Ghabrial, 1998). Although most known mycoviruses are apparently cryptic in nature, several have been shown to affect host growth rate, sporulation, pigmentation (Anagnostakis & Day, 1979) and enzymic activities (Rigling & Van Alfen, 1993). They are transmitted intracellularly during cell division, sporogenesis and cell fusion, but apparently lack an extracellular route for infection. Mycoviruses are found in all major groups of plant-pathogenic fungi. A well-known example of virus-mediated modulation is the chestnut blight fungus, *Cryphonectria parasitica*, infected with the ssRNA mycovirus CHV1-EP713, a member of the family *Hypoviridae* (Nuss, 2005). Members of the genus *Mycoreovirus* of the family *Reoviridae* have also been detected in *C. parasitica* (Hillman et al., 2004). *Penicillium chrysogenum* virus (PcV) is one of the first mycoviruses to be extensively studied at the biochemical, biophysical and ultrastructural levels (Ghabrial, 2008).

*Magnaporthe oryzae* (formerly known as *Magnaporthe grisea*, anamorph: *Pyricularia oryzae*) is a filamentous heterothallic ascomycete that causes rice blast disease and diseases of other cultivated gramineous plants by giving rise to spindle-shaped necrotic lesions on leaves or by attacking the aerial parts of the plant at any stage of growth. *M. oryzae* was the first plant-pathogenic fungus from which a virus with particles was found (Yamashita et al., 1971). Fifteen distinct dsRNA electrophoretic patterns have been described among 29 isolates of *M. oryzae*, and virus particles of ~35 nm in diameter have been partially purified. However, evidence of a relationship between dsRNA content and pathogenicity of *M. oryzae* has not been addressed (Hunst et al., 1986). Unencapsidated 1.6 and 1.8 kb dsRNAs have been found in *M. oryzae* strain MG01. These dsRNAs were inherited at low frequency during sexual reproduction (Chun & Lee, 1997). Two distinct viruses in the family *Totiviridae* were found in *M. oryzae*, *M. oryzae* virus 1 and 2 (MoV1 and MoV2) and the complete sequences of both have been determined (Yokoi et al., 2007; Maejima et al., 2008). Recently, mycovirus-
derived small interfering RNAs were found in MoV2-infected M. oryzae (Himeno et al., 2010).

In this study, we report novel types of mycoviruses in Vietnamese isolates of M. oryzae (Le et al., 2010), which cause impaired growth of host cells. These mycoviruses had segmented dsRNA genomes ranging from 1.2 to 3.6 kb. Sequence analysis of one of these mycoviruses, which we named Magnaporthe oryzae chrysovirus 1 (MoCV1), revealed that the mycovirus is related to chrysoviruses such as PcV. Surprisingly, virus particles containing the dsRNA segments of MoCV1 were found to accumulate gradually in the supernatant when grown in suspension culture. Furthermore, treatment of healthy mycelia of M. oryzae with the mycovirus-containing culture supernatant induced hyphal shrinkage, and dsRNAs of MoCV1 were detected in the aggregated hyphae.

RESULTS

Occurrence of dsRNAs in M. oryzae

Among the 58 isolates examined, 11 contained dsRNA. Three distinct dsRNA electrophoretic patterns were detected from these 11 positive isolates (Fig. 1). Pattern 1 (Fig. 1, lanes 1–6 and 8, seven isolates) consisted of four segments of dsRNA ranging from 3.0 to 3.5 kb. Pattern 2 (Fig. 1, lanes 9–11, three isolates) consisted of four dsRNA segments ranging from 1.1 to 2.2 kb. The third pattern consisted of both types of dsRNA segments, i.e. eight dsRNA segments ranging from 1.1 to 3.5 kb (Fig. 1, lane 7, one isolate). Because pattern 1 isolate S-0412-II 1a (Fig. 1, lane 2) maintained these dsRNAs stably during vegetative growth and the amounts of the dsRNAs seemed to be much higher (~10 μg (g fresh weight mycelia)^-1) than in the other isolates, we focused on this strain for further experiments. We refer to the dsRNAs from this isolate as dsRNA 1 (~3.5 kb), dsRNA 2 (~3.3 kb), dsRNA 3 (~3.1 kb) and dsRNA 4 (~3.0 kb) (Fig. 1, lane 2; see also Figs 3c and 4a).

Virus particles containing dsRNAs

To determine whether the dsRNAs in S-0412-II 1a were associated with virus particles, we attempted to purify the virus using standard methods without chloroform/butanol or carbon tetrachloride. After centrifugation at 35 000 g, the supernatant was subjected to centrifugation at 148 000 g. The resulting precipitate was then purified by sucrose gradient and CsCl density equilibrium centrifugation (Fig. 2). Virus particles with buoyant densities of 1.37–1.40 g cm^-3 in CsCl were isolated. dsRNA 1 was most concentrated in the fraction with a buoyant density of 1.40 g cm^-3 (Fig. 2a, lane 5), dsRNA 2 was most concentrated in the fraction with a buoyant density of 1.39 g cm^-3 (Fig. 2a, lane 6) and dsRNA 3 was most concentrated in the fraction with a buoyant density of 1.38 g cm^-3 (Fig. 2a, lane 7). The dsRNA 4 segment was barely detectable in the fraction with a buoyant density of 1.37 g cm^-3 (Fig. 2a, lane 8). Therefore, the buoyant densities of the particles depended on the size of the packaged dsRNA segments within them (Fig. 2a). This result suggested that the four dsRNA segments are packaged separately in individual virus particles, resulting in particles with different buoyant densities. Conserved sequences found in the 5' and 3' terminal regions of each dsRNA (see Fig. 4b) may include common packaging signals.

After purification by CsCl density equilibrium centrifugation, fractions 5–8 (representing buoyant densities of 1.375 to 1.40 g cm^-3; Fig. 2a) were pooled, concentrated by centrifugation at 148 000 g and examined via electron microscopy. Isometric virus particles with a diameter of ~35 nm were observed (Fig. 2b). Analysis of the pooled fractions using SDS-PAGE revealed a major band of ~58 kDa, which might be a coat protein. A larger protein of about 130 kDa, which might be the viral RNA-dependent RNA polymerase (RdRp), was also seen, encoded by dsRNA 1. The minor bands of around 70 kDa might be virion-associated proteins (Fig. 2c).

The mycovirus affects the fungal host phenotypes in isolate S-0412-II 1a

Isolate S-0412-II 1a had an abnormal phenotype, including irregular mycelial growth and unusual pigmentation in comparison with standard M. oryzae isolates (Fig. 3a). To investigate whether the mycovirus was responsible for this impaired growth, we attempted to eliminate the mycovirus from the host by single-spore isolation or exposure to...
cycloheximide during hyphal tip isolation. Total nucleic acids were extracted from candidate MoCV1-cured isolates, which exhibited normal mycelial growth and pigmentation (Fig. 3b). After digestion of the total nucleic acids with DNase I and S1 nuclease, the presence or absence of the mycoviral dsRNAs was assessed by agarose gel electrophoresis and ethidium bromide staining (Fig. 3c). Loss of the dsRNAs was confirmed by Northern blot hybridization with cDNAs derived from MoCV1 dsRNAs as probes (Fig. 3d). Among 73 single-conidium isolates, 16 had completely lost the mycovirus. These MoCV1-cured strains had a normal phenotype. No cured isolate was obtained by exposure to cycloheximide during hyphal tip isolation. In addition, abnormally enlarged vesicles were observed in mycelial cells of the virus-infected S-0412-II 1a; Fig. 3(e) represents those of the majority of 57 virus-infected isolates. Fewer vesicles were seen in mycelial cells of the virus-cured strain (Fig. 3f). This abnormal vesiculation appeared to increase in the virus-infected mycelial cells during liquid culture (Fig. 3e).

Molecular structure of the four dsRNA segments in S-0412-II 1a

The complete nucleotide sequences of the four dsRNA segments were determined from a series of cDNA clones spanning the entire length of each dsRNA. The genetic organization of the four dsRNAs is shown in Fig. 4(a). Each dsRNA encoded a single ORF. Both the 5' and 3'-terminal sequences of all four dsRNAs were highly conserved and the nucleotide sequences of the 5' and 3' non-coding regions might be involved in the replication cycle, gene expression or virion packaging (Fig. 4b).

Adenine-rich regions were found in the 3' untranslated regions (UTRs) of dsRNA 3 and dsRNA 4. The 3'UTR of dsRNA 3 was rich in adenine and uracil, whilst the 3'UTR of dsRNA 4 was rich in adenine and cytosine. These adenine-rich sequences were similar to interrupted poly(A) tails, which have been found at the 3' ends of partitiviruses (Strauss et al., 2000).

Phylogenetic analysis of the putative RdRp encoded by dsRNA 1 in S-0412-II 1a

BLAST analysis (Altschul et al., 1997) of the 1127 aa gene product encoded by dsRNA 1 revealed significant similarity to viral RdRps (Pfam02123, RdRp_4). The most similar sequence was the RdRp encoded by Aspergillus mycovirus 1816 (AsV1816; Hammond et al., 2008). The identity and similarity between amino acid sequences of the dsRNA 1 gene product and the RdRp of AsV1816 were significant (39.7 and 75.5 %, respectively) in their overall coding regions.

A multiple alignment of the amino acid sequences of the putative RdRp encoded by dsRNA 1 with RdRps of AsV1816 and of three selected mycoviruses in the family Chrysoviridae is shown in Fig. 5(a). Eight conserved motifs characteristic of RdRps of dsRNA mycoviruses were found in the predicted gene product of dsRNA 1 (Fig. 4a). This indicated that dsRNA 1 encodes an RdRp belonging to one of the viral RdRp families (RdRp_4, Pfam02123) and is one of the genomic segments of a dsRNA virus. Therefore, we named this novel mycovirus Magnaporthe oryzae chrysovirus 1 (MoCV1).

In order to analyse the phylogenetic positions of MoCV1 and AsV1816, which is as yet unclassified (Hammond et al., 2008), amino acid sequences of the RdRps of MoCV1, AsV1816 and 31 selected dsRNA viruses belonging to the families Totiviridae, Chrysoviridae, Partitiviridae and
Reoviridae, as well as ssRNA viruses belonging to the families Hypoviridae, Luteoviridae and Barnaviridae, were compared by molecular phylogenetic analysis. Many dsRNA mycoviruses have been classified into these four virus families, and RdRps encoded by mycoviruses in the four dsRNA virus families and the three ssRNA virus families have been reported to be phylogenetically related (Ghabrial et al., 2005a, b) and classified into RdRP_4 (PFam02123). In the resulting phylogenetic tree, MoCV1 clustered with representatives of the family Chrysoviridae and we therefore provisionally classified MoCV1 and AsV1816 in that family (Fig. 5b). The predicted gene products of dsRNA 2 and dsRNA 3 were not found to be significantly similar to other known sequences. The predicted amino acid sequence encoded by dsRNA 4 showed homology with the Agaricus bisporus virus 1 (AbV1) L3 segment (16.4 % identity, 65.3 % similarity).

Release of mycoviruses from the mycelium of mycovirus-infected M. oryzae into culture supernatant

Three mycovirus-infected isolates, S-0412-II 1a, S-0412-II 1c and S-0412-II 2a (Fig. 1), were cultured in liquid medium for 4 weeks. Total nucleic acids were extracted from the filtered supernatant and subjected to agarose gel electrophoresis without enzymic treatments. The mycoviral dsRNA genomes of the three strains were subjected to agarose gel electrophoresis and estimated to be approximately 50–250 ng RNA per 250 μl cultured supernatant (Fig. 6a). The culture supernatant was centrifuged at 20 000 g for 10 min and filtered through a 0.22 μm nylon filter (Millipore) prior to extraction of the dsRNAs. Therefore, the detected dsRNA genomes were not derived from suspended cells or cell debris, but from cell-free suspension. Viral proteins of MoCV1 were also detected by SDS-PAGE analysis stained with Coomassie brilliant blue (CBB) (Fig. 6b, lane 1). The molecular mass of the major band of the viral proteins in culture supernatant was 58 kDa, the same as that found when purified from mycelia. No virus-specific band was detected in the culture supernatant of an MoCV1-cured isolate (Fig. 6b, lane 2). Isometric virus particles (~35 nm; Fig. 6c) were also observed in samples precipitated by ultracentrifugation of culture supernatant of MoCV1-infected S-0412-II 1a.

Treatment of a virus-cured strain with MoCV1

The presence of free MoCV1 in the culture supernatant suggested that the released MoCV1 might be infectious to naive fungal cells. To investigate the effects of the released virus on M. oryzae, MoCV1-containing culture fluid was added to a 3-day cultured MoCV1-cured isolate of S-0412-II 1a. Culture fluid from an MoCV1-cured isolate of S-0412-II 1a was also used as a control inoculum. Abnormally aggregated hyphae were observed 2–3 days after treatment with cell-free culture fluid from the infected strain (Fig. 7a). Mycoviral dsRNAs were detectable from the shrunk hyphae after a further 9 days in culture (Fig. 7c, lane 2, arrow), but not from the cultured supernatant as inoculum (Fig. 7c, lane 3). The hyphae were washed with distilled water twice before the extraction process. To confirm these results, RT-PCR amplification was carried...
out using two specific primers, 5'-ACATGGAGAAGGAGCTGGCTGA-3' and 5'-AAGTTGTCTGTAATCCAGCATA-3', located at nt 1440–1461 and 2084–2106 in MoCV1 dsRNA 1, respectively. A 646 bp DNA fragment was amplified from the total nucleic acids of MoCV1-containing filtered supernatant as inoculum (Fig. 7d, lane 1) and the 12-day co-cultured aggregated hyphae (Fig. 7d, lane 3). No morphological change was observed when an MoCV1-cured culture supernatant was used as the inoculum (Fig. 7b) and no band was detected by RT-PCR (Fig. 7d, lanes 2 and 4).

A virus-containing solution prepared by partial purification from mycelia of MoCV1-infected S-0412-II 1a was used to test whether MoCV1 was able to induce hyphal shrinkage in naive M. oryzae. Abnormal, aggregated hyphae were observed after inoculation with purified MoCV1 particles, but not after control inoculation using supernatant from a cured strain (Fig. 7e–h).

**DISCUSSION**

Rice blast disease, caused by M. oryzae, is the most important disease of rice worldwide. M. oryzae is also an important model fungus to understand fungal pathogen–plant interactions, and its genome has been sequenced (Dean et al., 2005). In this study, we found at least two types of mycovirus that infect M. oryzae and demonstrated that one of these, MoCV1, causes impaired host growth. The complete nucleotide sequences of each of the four dsRNA segments of MoCV1 were determined. Phylogenetic analysis of the putative RdRp of MoCV1 showed that MoCV1 forms a sister clade with the chrysoviruses PcV and Helminthosporium victoriae 145S virus (Hv145SV). The MoCV1 virions were isometric particles ~35 nm in diameter, with buoyant densities in CsCl ranging from 1.37 to 1.40 g cm$^{-3}$. The four dsRNA segments (3.0–3.5 kb) were packaged separately (Fig. 3). These results indicated that MoCV1 is evolutionarily related to members of the family Chrysoviridae.

PcV and Hv145SV are representatives of the family Chrysoviridae (Jiang & Ghabrial, 2004; Ghabrial, 2008). Although these typical chrysoviruses have (CAA)$_n$ repeats in the 5'UTRs of all four dsRNA segments, analogous repeats are not found in MoCV1. The most abundant of the purified viral proteins, p58, is a candidate coat protein. As the predicted molecular masses of the proteins encoded by dsRNA 2, dsRNA 3 and dsRNA 4 are 98, 84 and 85 kDa, respectively, p58 may be a processed polypeptide derived from the one of the predicted proteins. Such a processing event has been found in the capsid protein of...
Fig. 5. (a) Multiple alignment of the deduced amino acid sequence of the RdRp region encoded by MoCV1 dsRNA 1 with those of AsV1816 and three mycoviruses in the family Chrysoviridae. The eight conserved motifs in the RdRps of dsRNA viruses are shown as numbers 1–8 (Ghabrial, 1998). Shading: black, 100 % amino acid identity; grey, 60–80 % amino acid identity. Numbers at the beginning and end of the sequences represent the amino acid positions from the start of the predicted gene product. (b) Phylogenetic analysis of the RdRp sequences of MoCV1 and 31 other selected viruses of the families Chrysoviridae, Totiviridae and Partitiviridae. An unrooted phylogenetic tree based on the neighbour-joining method was created using MEGA 4. Numbers at nodes represent bootstrap values as percentages estimated by 100 replicates. The GenBank sequences used in the analysis are given in Supplementary Table S1 (available in JGV Online). MoCV1, Magnaporthe oryzae chrysovirus 1; AsV1816, Aspergillus mycovirus 1816; AbV1, Agaricus bisporus virus 1; PcV, Penicillium chrysogenum virus; Hv145SV, Helminthosporium victoriae 145S virus; ACD-CV, Amasya cherry disease associated chrysovirus; Hv190SV, Helminthosporium victoriae virus 190S; SsRV1, Sphaeropsis sapinea RNA virus 1; SsRV2, Sphaeropsis sapinea RNA virus 2; GaRV-L1, Gremmeniella abietina RNA virus L1; AhPV, Atkinsonella hypoxylon partitivirus; HmPV-V1-1, Helicobasidium mompa partitivirus V1-1; RsV717, Rhizoctonia solani virus 717; FUPO-1, Fusarium poae virus 1; EbRV1, Eimeria brunetti RNA virus 1; TVV1, Trichomonas vaginalis virus 1; UmVH1, Ustilago maydis virus H1; HRV-C, Human rotavirus (group C, strain Bristol); HRV-B, Human rotavirus B strain Bang373; AoV, Aspergillus ochraceus virus; PeV-S, Penicillium stoloniferum virus S; DdV2, Discula destructiva virus 2; DdV1, Discula destructiva virus 1; CHV-EP713, Cryphonectria hypovirus 1-EP713; CHV-NB58, Cryphonectria hypovirus 2-NB58.
Helminthosporium victoriae virus 190S (Hv190SV), which belongs to the family Totiviridae (Soldevila et al., 2000). The apparent molecular mass of p58 is notably smaller than those of the PcV (109 kDa) and Hv145SV (100 kDa) coat proteins. Determination of the N- and C-terminal protein sequences of p58 will help clarify its provenance.

MoCV1 dsRNA 4 encoded an 85 kDa protein with homology to the AbV1 L3 segment (16.4% identity, 65.3% similarity), but neither the PcV nor the Hm145SV segment encodes a gene product with such a homology. As described in Results, the MoCV1 RdRp forms a sister clade with those of the chrysovirus PcV and Hv145SV (Fig. 5), a clade that includes AbV1 and AsV1816, both candidate chrysoviruses. These results suggest that MoCV1 may also be classified as a chrysovirus.

Although mycoviruses apparently lack an extracellular phase in general, virus particles including all four dsRNA segments of MoCV1 were detected in 4-week culture fluid of infected M. oryzae (Fig. 6). The presence of fungal viruses in culture fluid was first detected serologically (Hollings & Stone, 1969). However, only a few examples of virus release have been reported, notably for Penicillium stoloniferum and Penicillium chrysogenum (Hollings & Stone, 1969; Lemke et al., 1973; Lemke & Nash, 1974). As the release of MoCV1 into culture supernatant was prevalent in older cultures (Fig. 6a), it is probably a consequence of autolysis of M. oryzae mycelia, which may also be the explanation for the previous reports in Penicillium viruses. Significantly, treatment of a virus-cured strain with MoCV1-containing culture supernatant induced hyphal shrinkage (Fig. 7a). MoCV1 partially purified from mycelium produced a similar result (Fig. 7e). These data indicate that both extracellular and intracellular MoCV1 viruses can be the causal agents of hyphal shrinkage. Furthermore, mycoviral dsRNAs were detected in the aggregated mycelium (Fig. 7c, d).

In our previous report for Alternaria alternata virus 1 (AaV1), a high concentration of AaV1 induced the emergence of abnormally enlarged vesicles in the fungal host, while few such vesicles were seen in the cured strain (Aoki et al., 2009). Such abnormally enlarged vesicles were observed in MoCV1-infected S-0412-II 1a (Fig. 3e), but few vesicles were seen in the MoCV1-cured strain (Fig. 3f). These results suggest that the mycovirus in S-0412-II 1a may be responsible for the impaired fungal host growth. Putative mycoviral factors that affect host-cell growth or induce autophagy-like phenomena may be found through further experiments. MoCV1-cured isolates showed higher virulence on infected rice leaves, including both higher lesion numbers and larger lesions, compared with the MoCV1-infected original isolate S-0412-II 1a (T. Teraoka, personal communication). These results suggest that MoCV1 has potential as a biological control agent against M. oryzae.

**METHODS**

**Isolates of M. oryzae and culture methods.** All isolates of M. oryzae used in this paper were obtained from symptomatic rice leaves sampled in Vietnam (Le et al., 2010) (isolate S-0412-II 1a has been deposited in ATCC under accession no. PTA-9137). These isolates were grown on potato dextrose agar (PDA) for 2 weeks at 25 °C, with
a light period of 12 h day⁻¹. For liquid cultures, mycelial plugs were incubated in 0.5 % yeast extract and 2 % glucose liquid broth (YG broth) with reciprocal shaking (60 r.p.m.) for up to 4 weeks at 25 °C.

Detection and purification of dsRNAs from fungal mats. Total nucleic acids extracted from 50 mg fresh weight of fungal tissue were treated with S1 nuclease (Takara Bio) and DNase I (Takara Bio) to digest ssRNA and DNA, respectively, as previously described (Aoki et al., 2009). The remaining nucleic acids were subjected to electrophoresis in 1 % (w/v) agarose gels containing TAE buffer [40 mM Tris/acetate (pH 8.1), 2 mM EDTA] and 500 ng ethidium bromide ml⁻¹ (Fig. 1). To prepare template dsRNAs for cDNA cloning, fungal isolates were cultured in 25 ml YG medium in the dark at 25 °C for 2 weeks with shaking at 50 r.p.m. One gram of the resulting fungal mat was pulverized in a mortar in liquid nitrogen and then suspended in 2 × STE [0.1 M Tris/HCl (pH 6.8), 0.2 M NaCl, 2 mM EDTA]. The fungal dsRNAs were extracted with SDS/phenol and purified by chromatography on CF-11 cellulose (Whatman) as described by Morris & Dodds (1979), and contaminating DNA and ssRNA fragments were eliminated by treatment with DNase I and S1 nuclease, as previously above.

cDNA cloning. Four dsRNA segments isolated from M. oryzae isolate S-0412-II 1a were used as templates for cDNA synthesis, and a series of overlapping cDNA clones was obtained. These cDNA clones were confirmed to be derived from the four dsRNA segments of isolate S-0412-II 1a by Northern blot hybridization and RT-PCR (data not shown). To obtain PCR clones that corresponded to the terminal regions of each dsRNA, 5'-RACE was used; the 5'-end regions of the dsRNAs were amplified by inverse PCR using 5'-phosphorylated primers following the manufacturer’s protocol (5'-Full RACE Core Set; Takara Bio).

Phylogenetic analysis. Sequences similar to those encoded by the dsRNA ORFs were identified from the NCBI database using the BLAST program. Molecular phylogenetic analysis using the deduced amino acid sequences of the putative RdRp gene of MoCV1 dsRNA 1 were carried out using CLUSTAL_X, GeneDoc and MEGA 4 programs (Thompson et al., 1997; Tamura et al., 2007). A bootstrap test was conducted with 100 resamplings for the neighbour-joining tree.

Virus purification from mycelia. Approximately 20 g fresh mycelia was homogenized in a mixer with 200 ml 0.1 M sodium phosphate buffer (pH 7.4) containing 0.2 M KCl at 4 °C (Castón et al., 2003). The homogenate was subjected to centrifugation at 5100 g for 10 min and the supernatant was subsequently subjected to centrifugation at 34 700 g for 1 h. The supernatant was ultracentrifuged at 148 400 g for 1 h and the resultant precipitate was suspended in 0.05 M Tris/HCl (pH 7.8) containing 0.15 M NaCl at 4 °C (Castón et al., 2003). The suspension was subjected to centrifugation in sucrose density gradients (100–400 mg ml⁻¹) in 0.05 M Tris/HCl with 0.15 M NaCl and centrifuged at 112 700 g for 2.5 h. The virus-containing fractions were diluted with 0.05 M Tris/HCl with 0.15 M NaCl. The solution was subjected to ultracentrifugation (148 400 g for 1 h) and the pellets were resuspended in 0.05 M Tris/HCl with 0.15 M NaCl and purified by CsCl density equilibrium centrifugation at 133 800 g for 24 h. The virus-containing fractions were identified by monitoring

![Fig. 7. Hyphal shrinkage induced by MoCV1 inoculation. (a, b) Hyphae of MoCV1-cured S-0412-II 1a after inoculation with MoCV1-containing culture supernatant (a) or with MoCV1-free culture supernatant (b). During the first 3 days after inoculation, hyphal shrinkage was observed following inoculation with MoCV1, whilst no shrinkage of hyphae was observed following control inoculation. Bars, 0.5 mm. (c) Detection of MoCV1 dsRNAs. Total nucleic acids were extracted from 250 µl released MoCV1-containing medium used as inoculum (lane 1). Total nucleic acids were extracted from 100 mg mycelia, which were co-incubated with MoCV1-containing medium for 12 days. The sample was digested with S1 nuclease and DNase I (lane 2). Total nucleic acids were extracted from 250 µl co-cultured supernatant for 12 days (lane 3). Lane M, DNA marker. (d) Detection of MoCV1 dsRNA 1 by RT-PCR. Lanes: 1, MoCV1-containing filtered supernatant as inoculum; 2, MoCV1-cured cultured filtered supernatant as inoculum; 3, 12-day co-cultured aggregated mycelium; 4, 12-day cultured mycelium with MoCV1-cured culture supernatant. (e, f) Mycelium of MoCV1-cured S-0412-II 1a after inoculation with MoCV1 partially purified from infected S-0412-II 1a mycelium (e), or control inoculation purified from uninfected mycelium (f). During the first 3 days after inoculation, mycelium shrinkage was observed (e). No shrinkage of mycelium was observed when the MoCV1-free sample was used as inoculum (f). Hyphal shrinkage was also observed after inoculation with MoCV1 (g), but not following control inoculation (h). Bars, 0.5 mm.]

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for the presence of viral dsRNA. The collected virus fractions were diluted with 0.05 M Tris/HCl with 0.15 M NaCl. Isolated viral particles were stained with 2% uranyl acetate and observed using a transmission electron microscope (H7100; Hitachi). Proteins from the purified viral preparation were analysed by 8% SDS-PAGE with 25 mM Tris/glycine and 0.1% SDS, at 60 °C for 1 h and then blotted onto a Zeta Probe nylon membrane (Bio-Rad Laboratories). cDNA clones derived from dsRNAs 1, 2, 3 and 4 were used to make probes using a BcaBEST labelling kit (Takara Bio) and [α-32P]dCTP (GE Healthcare).

**Northern hybridization.** Total dsRNAs were purified from mycelia of isolate S-0412-II 1a cultured for 2 weeks in liquid YG medium. The dsRNAs were separated by electrophoresis on 1% nage agarose gels containing TAE buffer and 500 ng ethidium bromide ml−1, denatured in 50% (v/v) formamide, 25% (v/v) formamidethylene and 25% (v/v) TAE buffer at 60 °C for 1 h and then blotted onto a Zeta Probe nylon membrane (Bio-Rad Laboratories). cDNA clones derived from dsRNAs 1, 2, 3 and 4 were used to make probes using a BcaBEST labelling kit (Takara Bio) and [α-32P]dCTP (GE Healthcare).

**Curing** *M. oryzae* S-0412-II 1a of MoCV1. Mycelial plugs of MoCV1-infected S-0412-II 1a (original isolate) were inoculated on YG agar containing a low concentration (0.1 μg ml−1) of cycloheximide and incubated at 25 °C for 5–7 days. Hyphal tips were transferred on to YG agar without cycloheximide and incubated for 1 week at 25 °C. Plugs of YG agar 4 mm in diameter were inoculated into YG liquid medium and cultured for 2 weeks. Nucleic acids extracted from the resultant fungal mats were then analysed.

As another method, single-spore isolation was performed by means of micromanipulation. Conidia were collected from S-0412-II 1a infected with MoCV1 grown on PDA or oatmeal medium. After 14 days, each spore isolate was inoculated into YG liquid medium and the presence or absence of MoCV1 was confirmed by Northern blot hybridization using cDNA probes derived from MoCV1 dsRNAs as described above.

**Detection of MoCV1 dsRNAs or virus particles in culture supernatant.** S-0412-II 1a infected with MoCV1 was cultured in a 100 ml flask containing 50 ml YG liquid medium. After 4 weeks of culture with shaking at 50 r.p.m., a portion of the culture supernatant (250 μl) was centrifuged at 17 000 g for 5 min to remove conidia and free cells. The resultant supernatants were recovered and total nucleic acids were extracted using SDS/phenol and subjected to agarose gel electrophoresis. Alternatively, the liquid supernatants were filtered through a 0.22 μm nylon filter and the filtrates were recovered to extract the total nucleic acids.

To detect virus particles and viral proteins of MoCV1, 4 ml culture supernatant was centrifuged at 17 000 g for 5 min to remove conidia and free cells. The resultant supernatant was subjected to ultracentrifugation (148 400 g for 1 h) and the precipitate from the second centrifugation was suspended in 0.05 M Tris/HCl (pH 7.8) containing 0.2 M KCl at 4 °C. After discarding debris from the 5100 g and 34 700 g centrifugation steps as described above, the supernatant was subjected to ultracentrifugation at 148 400 g for 1 h and the resultant precipitate was recovered as partially purified virus and suspended in 10 ml YG medium. After 0.22 μm nylon filter sterilization, the viral solution was added to virus-cured strains of S-0412-II 1a cultured for 3 days in YG broth. After a further 3 days in culture, morphological changes in the inoculated mycelium were observed under a microscope. Culture supernatant from MoCV1-cured (virus-free) isolates of S-0412-II 1a or recovered precipitate by the three-step centrifugation method were used as controls for experiments.

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