Characterization of *Magnaporthe oryzae* Chrysoivirus 1 Structural Proteins and Their Expression in *Saccharomyces cerevisiae*

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*Magnaporthe oryzae chrysoivirus 1* (MoCV1), which is associated with an impaired growth phenotype of its host fungus, harbors four major proteins: P130 (130 kDa), P70 (70 kDa), P65 (65 kDa), and P58 (58 kDa). N-terminal sequence analysis of each protein revealed that P130 was encoded by double-stranded RNA1 (dsRNA1) (open reading frame 1 [ORF1] 1,127 amino acids [aa]), P70 by dsRNA4 (ORF4; 812 aa), and P58 by dsRNA3 (ORF3; 799 aa), although the molecular masses of P58 and P70 were significantly smaller than those deduced for ORF3 and ORF4, respectively. P65 was a degraded form of P70. Full-size proteins of ORF3 (84 kDa) and ORF4 (85 kDa) were produced in *Escherichia coli*. Antisera against these recombinant proteins detected full-size proteins encoded by ORF3 and ORF4 in mycelia cultured for 9, 15, and 28 days, and the antisera also detected smaller degraded proteins, namely, P58, P70, and P65, in mycelia cultured for 28 days. These full-size proteins and P58 and P70 were also components of viral particles, indicating that MoCV1 particles might have at least two forms during vegetative growth of the host fungus. Expression of the ORF4 protein in *Saccharomyces cerevisiae* resulted in cytological changes, with a large central vacuole associated with these growth defects. MoCV1 has five dsRNA segments, as do two *Fusarium graminearum* viruses (FgV-ch9 and FgV2), and forms a separate clade with FgV-ch9, FgV2, *Aspergillus* mycovirus 1816 (AsV1816), and *Agaricus bisporus* virus 1 (AbV1) in the *Chysovirusidae* family on the basis of their RdRp protein sequences.
proteins. Processing of MoCV1 structural proteins occurs after the expression of full-size viral proteins. The fifth dsRNA segment of MoCV1, which we were not able to separate by agarose gel electrophoresis in earlier experiments but could separate by PAGE, was sequenced in this study. We used a yeast (Saccharomyces cerevisiae) expression system to identify gene products of MoCV1 involved in cytological damage to host cells; the open reading frame 4 (ORF4) protein of MoCV1 caused cytological changes and growth inhibition in yeast. The data presented here suggest that MoCV1 products may have roles in regulating fungal growth and development.

MATERIALS AND METHODS

Fungal strains and culture methods. The MoCV1-infected strain S-0412-II 1a (the original isolate) of Magnaporthe grisea and an MoCV1-free strain S-0412-II 1a (MoCV1-cured isolate) were grown on potato dextrose agar (PDA) for 2 weeks at 25°C. For liquid cultures, mycelial plugs were inoculated in 0.5% yeast extract and 2% glucose liquid broth (YG broth) with reciprocal shaking (60 strokes per min [rpm]) at 25°C in a flasks. Fermentation reactors (2.5 liters; Able Biott) were also used for fungal cultivation in YG broth (1.5 liters) at 25°C, with agitation at 100 rpm and air introduction at 1.5 liters min⁻¹.

Yeast strains and media. Saccharomyces cerevisiae strain W303-1A (MATa ura3 his3 leu2 trp1 ade2 can1 [L-A]) was used in this study and was a gift of A. Toh-e. We isolated an L-A virus-free strain of W303-1A by centrifugation at 112,700 g for 10 min, and the supernatant was centrifuged at 37,400 × g for 1 h. The supernatant was ultracentrifuged at 148,400 × g for 1 h, and the resultant precipitate was suspended in buffer A at 4°C. The suspension was applied to sucrose density gradients (100 to 400 mg ml⁻¹) in buffer A and centrifuged at 112,700 × g for 2.5 h. The virus-containing fractions were combined and diluted with buffer A. The solution was ultracentrifuged (148,400 × g for 1 h), and the pellets were resuspended in the same buffer (29). Proteins of the purified viral preparation were analyzed by 8% SDS-PAGE with 25 mM Tris-glycine and 0.1% SDS at 15 mA for 2 h. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (BioSafe CB; Bio-Rad). In order to obtain anti-MoCV1 antiserum, rabbits were immunized with 0.75 mg of the gradient-purified MoCV1 proteins.

Purification of the virus particles associated with full-size ORF3 and ORF4 was performed in the same manner, except that centrifugation at 5,100 × g for 10 min and 37,400 × g for 1 h replaced centrifugation at 22,000 × g for 15 min.

Plasmids. For expression vectors in Escherichia coli, pET-22b(+) ORF3 was prepared by cloning ORF3 without a stop codon from a full-length dsRNA clone of the MoCV1 genome into the NdeI/Xhol sites of pET-22b(+) (Novagen, Madison, WI). pET-22b(+) ORF4 was constructed in the same manner by cloning ORF4 into the NdeI/NolI sites. The NdeI/Xhol sites were added to the ORF3 PCF product by amplifying it using primers MO3N5 and MO3Xb3, and ORF4 with added KpnI/EcoRI sites by amplifying it using oligonucleotides MO4N5 and MO4E3 (see Tables S1 and S2 in the supplemental material). For expression vectors in yeast, plasmids were constructed by insertion of the TDH3 cassette into the NdeI/Xhol sites of pRS316 (3). pRS315, pRS316, and pRS425 were constructed by insertion of a NotI/ApaI fragment of a TDH3 cassette into pUC19. The cloned PCR products were digested by BlnI and ligated. Plasmids pRST315, pRST316, and pRST425 were constructed in the same manner by cloning ORF3 or ORF4 into the NdeI/Xhol sites.

Phylogenetic analysis. Molecular phylogenetic analysis using the deduced amino acid sequence of the putative RdRp gene of MoCV1 dsRNA1 was carried out using ClustalX, GeneDoc, and MEGA4 software (27, 28). A bootstrap test was conducted with 100 resamplings for a neighboring NJ tree.

Protein sequence analysis. For sequencing of internal peptides of MoCV1 P130, gradient-purified MoCV1 proteins were separated by 8% SDS-PAGE and stained with Coomassie Brilliant Blue (Bio-Safe CB; Bio-Rad). The part of the gel containing the dsRNA5 band was excised. Purified dsRNA5 was used as the template for cdNA synthesis, and a series of overlapping cdNA clones was obtained. These cdNA clones were confirmed to be derived from the fifth dsRNA segment of isolate S-0412-II 1a by Northern hybridization (data not shown). To obtain PCR clones that corresponded to the terminal regions of dsRNA5, 5′ rapid amplification of cdNA ends (RACE) was used; the 5′-end regions of the dsRNA were amplified by inverse PCR using 5′ phosphorylated primers following the manufacturer’s protocol (5′-Full RACE core set; Takara Bio, Kyoto, Japan).

Protein sequence analysis. For sequencing of internal peptides of MoCV1 P130, gradient-purified MoCV1 proteins were separated by 8% SDS-PAGE and stained with Coomassie Brilliant Blue (Bio-Safe CB; Bio-Rad). The part of the gel containing the P130 band was excised and separated by reverse-phase high-performance liquid chromatography (HPLC) on an analytical C18 column. One of the resolved peptides was selected for amino acid sequencing by automated Edman degradation.

Purification of recombinant ORF3 and ORF4 in E. coli. E. coli host BL21(DE3) harboring the ORF3 expression vector pET-22b(+) ORF3 or the ORF4 expression vector pET-22b(+) ORF4 was cultivated in Luria-
augmented liquid media.
Bertani medium containing 50 μg ml⁻¹ ampicillin until mid-log phase and then induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. After the cells were collected by centrifugation, they were disrupted by BugBuster Protein Extraction Reagent (Novagen). Inclusion bodies were collected by centrifugation for 20 min at 20,000 × g and solubilized in binding buffer (10 mM Tris, 1 mM EDTA, pH 8.0) with 6 M urea. After centrifugation for 20 min at 20,000 × g, the solutions were applied to a Ni-nitrilotriacetic acid (NTA) agarose column (Qiagen, Hilden, Germany). After the column was washed with 50 mM sodium phosphate buffer (pH 8.0) and 300 mM NaCl, the proteins were eluted by imidazole (250 mM).

The expression of recombinant ORF3 or ORF4 was induced in the pColdI-ORF3 or pColdI-ORF4 transformants, respectively, according to the pCold vector manufacturer’s instructions (TaKaRa Bio). After the cells were collected by centrifugation, purification of the recombinant proteins was performed in the same manner.

Detection of MoCV1 viral proteins. A fungal mat of rice blast fungus cultured for 9, 15, or 28 days was collected and pulverized in a mortar with pestle in liquid nitrogen. The resultant powder was added to 0.1 M sodium phosphate buffer (pH 7.4) containing protease inhibitor cocktail (complete mini EDTA-free; Roche Diagnostics, Mannheim, Germany) and centrifuged for 10 min at 20,000 × g at 4°C; the supernatant fraction was retained. Each extract contained about 2 μg ml⁻¹ protein, and 10 μl of each one was subjected to SDS-PAGE.

Samples were prepared by 8% SDS-PAGE using 25 mM Tris-glycine and 0.1% SDS at 15 mA for 2 h, transferred to PVDF membranes, and subjected to Western blotting using an ECL Plus detection system (GE Healthcare). To enhance signals in immunoassays, we used Can Get Signal (Toyobo, Osaka, Japan) during immunoreactions. Rabbit antisera raised against a synthetic peptide corresponding to the C-terminal 14 amino acids (aa) of the protein encoded by ORF3 and guinea pig antiserum raised against recombinant ORF3 or ORF4 were used to detect MoCV1 viral proteins.

Light microscopy and DAPI nuclear staining of yeast cells. S. cerevisiae strain W303-1A (MATα leu2 his3 ura3 trp1 ade2 can1 [L-A-o]) was used for the following experiments. Transformants of W303-1A (L-A-o) harboring the appropriate expression vector were streaked to form single colonies and grown at 30°C on plates containing synthetic complete medium lacking either uracil or leucine (SC-Ura or SC-Leu, respectively) for 4 to 5 days. Some of the single colonies were grown in the selective liquid medium at 28°C for 14 h. The transformants were suspended in 0.1 M sodium phosphate buffer (pH 7.4) containing protease inhibitor cocktail and lysed by sonication; then extracts were centrifuged in the same manner as the fungal powder; these extracts also contained about 2 μg ml⁻¹ protein, and 10 μl of each one was subjected to SDS-PAGE.

RESULTS

Nucleotide sequence of dsRNAs associated with MoCV1 particles. In our previous experiments, four separated dsRNA bands were visible following agarose gel electrophoresis (29); however, a fifth dsRNA (dsRNAs) band was found by PAGE in this study (Fig. 1A). dsRNAs purified from MoCV1 particles were electrophoresed in 5% (wt/vol) polyacrylamide gels, and the dsRNAs molecule was collected from the gels and used as a template for cDNA synthesis. The complete nucleotide sequence of the dsRNAs was determined (AB700631); it has a single ORF (Fig. 1B). The untranslated sequences conserved among the other four
dsRNAs (dsRNA1, -2, -3, and -4) of MoCV1 were also found in dsRNA5 (see Fig. S1 in the supplemental material). While the sequence of dsRNA3 (3,074 nucleotides [nt]) was longer than that of dsRNA4 (3,043 nt), dsRNA3 showed higher mobility than dsRNA4 on a 5% native PAGE gel (Fig. 1A and B). On the basis of RdRp protein sequences, MoCV1 forms a separate clade with FgV-ch9, FgV2, AsV1816, and AbV1 in the Chrysoviridae family (Fig. 1C). The FgV-ch9 and FgV2 genomes are composed of five dsRNA segments.

MoCV1 particles consist of four major structural proteins encoded by dsRNA1, dsRNA3, and dsRNA4. Coomassie blue staining of SDS-PAGE gels showed that a purified fraction of MoCV1 particles extracted from mycelia cultured for 4 weeks consisted of four major polypeptides, P130, P70, P65, and P58 (Fig. 2A). P130 was subjected to Edman degradation; however, no phenylthiohydantoin (PTH) amino acid derivatives were observed, indicating that the N terminus of P130 was blocked (data not shown). Purified P130 was digested with trypsin, and the internal peptides were analyzed by HPLC. A tryptic digested peptide in chromatographic peaks was subjected to Edman degradation; the sequence of the peptide (TMIDYYRQVG) matched the amino acid sequence of ORF1 at amino acids [aa] 1,025 to 1,034 (nt 3,191 to 3,220). The ORF has 1,127 aa residues, leading to a calculated molecular mass of 125 kDa, consistent with the size of P130 estimated by SDS-PAGE (Fig. 2A). P70 was also subjected to Edman degradation. The sequence of the peptide (RIDQG) matched the amino acid sequence of ORF4 positioned at aa 15 to 19 (nt 204 to 218). The first methionine of ORF4 was positioned at nt 162 to 164, indicating that 14 amino acids of the N terminus of P70 would be degraded by posttranslational cleavage. P65 was confirmed to be a derivative of P70 by Western blotting using anti-ORF4 antiserum (see below).

P58 was also subjected to Edman degradation. The sequence of the peptide (GLTLD) matched the amino acid sequence of ORF3 at aa 2 to 6 (nt 148 to 162). An N-terminal methionine was not detected in the P58 fragments, suggesting that the first methionine might have been removed posttranslationally. In addition to the main sequence, we also detected a minor peptide sequence (RIDQG) that matched the amino acid sequence of ORF4 at aa 15 to 19 (nt 204 to 218), indicating that the degraded P70 migrates at around 58 kDa. These data indicated that P130 is encoded by dsRNA1, P70 and P65 are encoded by dsRNA4, and P58 is encoded by dsRNA3.

P58 lacks the C-terminal region of the deduced full-size ORF3. Although the N-terminal sequence of P58 was consistent

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FIG 2 Characterization of P58 of MoCV1 particles. (A) SDS-PAGE analysis of the purified MoCV1 particles stained with CBB. MoCV1 particles were purified from 28-day mycelia cultured in a flask. Arrows indicate viral structural proteins. (B) The deduced amino acid sequence of ORF3 is written in one-letter code. Partial amino acid sequences of P58 digested by trypsin were determined by peptide mass fingerprinting analysis and are boxed. The N-terminal amino acid sequence of P58 is indicated by underlining. The deduced amino acid sequence of ORF3, indicated by dashed underlining (residues 786 to 799) is the sequence of the synthetic peptide antigen. (C) MALDI-TOF mass spectrum for purified MoCV1 particles. (D and E) MoCV1 particles, purified from 28-day flask-cultured mycelia of strain S-0412-II 1a, were separated by SDS-PAGE and immunoblotted. Antiserum raised against purified MoCV1 particles (D) or against synthesized ORF3 C-terminal peptide (shown in dashed line of panel A) (E) was used for immunoblotting. Lanes 1, MoCV1 particles; lanes 2, control preparation purified from 28-day cultures of MoCV1-free S-0412-II 1a.
FIG 3 Detection of ORF3 and ORF4 products from crude mycelial extracts. Aqueous crude extracts of mycelia of MoCV1-infected or MoCV1-free S-0412-II 1a were subjected to SDS-PAGE and immunoblot analysis. A 20-μg aliquot of protein from each extract was used. Antiserum against recombinant full-size ORF3 (A), ORF3 C-terminal peptide (B), or recombinant full-size ORF4 (C) was used for immunoblotting. Lanes 1, extracts from MoCV1-free isolate cultured for 9 days; lanes 2, extracts from MoCV1-infected isolate cultured for 9 days; lanes 3, extracts from MoCV1-free isolate cultured for 15 days; lanes 4, extracts from MoCV1-infected isolate cultured for 15 days; lanes 5, extracts from MoCV1-free isolate cultured for 28 days; lanes 6, extracts from MoCV1-infected isolate cultured for 28 days.

with the sequence of ORF3 encoded by dsRNA3, its apparent molecular mass (58 kDa) was smaller than the deduced molecular mass of ORF3 (799 aa; 84 kDa). Then, we performed MALDI-TOF peptide mass fingerprinting analysis of P58. Mass spectrometry of tryptic peptides of P58 identified peptides specific to the deduced amino acid sequence of ORF3, but no peptide sequence mapped to the C terminus of the deduced amino acid sequence of ORF3 (M565 to L799) (Fig. 2B). MALDI-TOF MS was performed to determine the molecular mass of P58. A resultant ion signal at m/z 62,559 was observed (Fig. 2C). The molecular mass of the deduced amino acid sequence from residues G1 to V590 was 62,530 and from residues G1 to Q591 was 62,658. These results suggested that the deduced C-terminal end of P58 might be located around residues M565 to Q591 of ORF3. An ion signal at m/z 31,307 corresponding to a divalent ion was also observed.

We carried out Western analysis using anti-MoCV1 antiserum to detect full-size ORF3 protein. Signals of full-size ORF3 (84 kDa) were not detected in purified MoCV1 preparations (Fig. 2D, lane 1) from mycelia cultured for 28 days in the absence of a protease inhibitor. No specific signal was detected from preparations (Fig. 2D, lane 2) of the MoCV1-free isogenic strain. Rabbit antiserum, which was raised against a synthesized peptide corresponding to residues S785 to L798 (C55DAGSSG5RGEEL) of the full-size ORF3, did not detect P58 protein or the full-size ORF3 of MoCV1 particles prepared from an MoCV1-infected strain (Fig. 2E, lane 1). These data were consistent with the data of MALDI TOF-based peptide mass fingerprinting (Fig. 2B), indicating that P58 of MoCV1 particles is the C-terminal truncated form of the full-size ORF3.

Expression of ORF3 and ORF4 proteins in Escherichia coli. MoCV1 ORFs were heterologously expressed in E. coli. Expression of ORF3 in E. coli using pET-22b(+)–ORF3 or pColdI–ORF3 yielded protein products of 84 kDa, which is consistent with the predicted molecular mass of ORF3 and was confirmed by Western analysis with anti-MoCV1 antiserum (see Fig. S2 in the supplemental material). Expression of the ORF4 protein in E. coli using pET-22b(+)–ORF4 or pColdI–ORF4 yielded protein products of 85 kDa, which is consistent with the predicted molecular mass of ORF4 and was confirmed by Western analysis with anti-MoCV1 antiserum (see Fig. S2 in the supplemental material). We used the ORF3 and ORF4 recombinant proteins to immunize guinea pigs, and the resultant antisera were tested for specific reactivity with MoCV1 structural proteins by immunoblotting. ORF1 and ORF2 were also cloned into pET-22b(+) and pColdI vectors, but no protein product was detected. We also confirmed that recombinant ORF3 and ORF4 were detected by anti-His6 antibody. Anti-ORF3 C-terminal peptide antiserum detected the recombinant ORF3. No signal was detected in cell lysates harboring empty vectors using anti-MoCV1 antiserum (data not shown).

Detection of full-size ORF3 and ORF4 proteins in mycelial crude extracts. We performed Western analysis to detect MoCV1 viral proteins in mycelial extracts. P58 was detected by anti-MoCV1 antiserum in extracts of mycelia cultured for 28 days (see Fig. S3, lane 6, in the supplemental material) but was hardly detectable in mycelia cultured for 9 or 15 days (see Fig. S3, lanes 2 and 4).

Using anti-ORF3 antiserum, a protein band corresponding to the full-size ORF3 (84 kDa) was detected in the 9-, 15-, and 28-day extracts (Fig. 3A, lanes 2, 4, and 6). P58 was also detected in 28-day extracts to the same extent as the full-size 84-kDa ORF3 (Fig. 3A, lane 6), but a relatively small amount of P58 was detected in 9- and 15-day extracts (Fig. 3A, lanes 2 and 4). Full-size ORF3 was also detected in mycelial extracts using anti-ORF3 C-terminal antiserum, but no signal for P58 was detected (Fig. 3B, lanes 2, 4, and 6), indicating that P58 was generated through processing involving C-terminal cleavage of the full-size ORF3. As the culture duration increased, the amount of the P58 was gradually accumulated in the mycelial extracts, while the full-size ORF3 (84 kDa) was reduced.

The ORF4 protein is also associated with MoCV1 virus particles. Using anti-ORF4 antiserum, full-size ORF4 was detected in mycelial extracts (Fig. 3C, lanes 2, 4, and 6). P70 and P65 were also detected in 28-day extracts (Fig. 3C, lane 6), but only very small amounts of them were detected in 9- and 15-day extracts (Fig. 3C, lanes 2 and 4). No signal was detected in mycelial extracts prepared from the S-0412-II 1a MoCV1-free strain by any of the three antisera (Fig. 3A, B, and C, lanes 1, 3, and 5).

Isolation of MoCV1 particles associated with full-size ORF3 and ORF4. In mycelia cultured for 28 days, full-size ORF3 and ORF4 proteins were detected in crude extracts (Fig. 3A, B, and C) but not in purified MoCV1 particles (Fig. 2D and E). To
investigate whether the full-size proteins are components of MoCV1 particles, we attempted to purify the particles from fresh mycelia grown in a fermentation reactor. We also improved our purification methods for MoCV1 particles (see Materials and Methods).

Isometric virus particles with buoyant densities of 1.38 to 1.40 g cm$^{-3}$ in CsCl and a diameter of about 35 nm were observed by electron microscopy in fractions purified from mycelia grown in a fermentor for 2 days (Fig. 4A and C). SDS-PAGE analysis of the pooled fractions revealed six protein bands of 130, 85 (84), 75, 65, and 63 kDa (Fig. 4E, lane 1). Anti-ORF3 antiserum detected an 84-kDa protein corresponding to full-size ORF3 in addition to 75-, 70-, 67-, and 63-kDa proteins (Fig. 4F, lane 1). Anti-ORF4 antiserum detected an 85-kDa protein corresponding to full-size ORF4 as well as a 70-kDa protein (Fig. 4G, lane 1). These results indicated that full-size ORF3 and ORF4 might be components of MoCV1 particles. The other protein bands smaller than 84 or 85 kDa must be degraded forms of the full-size proteins.

We also isolated MoCV1 particles from mycelia grown in the fermentor for 14 days. The buoyant densities of the isometric virus particles ranged from 1.38 to 1.40 g cm$^{-3}$, which was almost the same as the particles obtained from mycelia grown in a fermentor for 2 days or in flasks for 28 days (Fig. 4B and D) (29). SDS-PAGE and Western analysis of the pooled fractions showed that the protein band patterns observed on staining with CBB (Fig. 4E, lane 2) and the bands recognized by the anti-ORF3 (Fig. 4F, lane 2) and anti-ORF4 (Fig. 4G, lane 2) antisera were almost the same as those observed for 28-day cultures, except for a 55-kDa protein detected by anti-ORF4.

No degradation of ORF1 protein (P130) was observed. We performed RdRp assays using [γ-$^{32}$P]UTP on the purified MoCV1 particles from mycelia cultured for 2 or 14 days (12). Autoradiography of the native PAGE gel revealed radioactive signals from both types of MoCV1 particles (see Fig. S4, arrow, lanes 3 and 6, in the supplemental material). No radioactivity was detected in preparations from the MoCV1-free strain (data not shown).
Heterologous expression of ORF4 induced cytological damage to the yeast *Saccharomyces cerevisiae*. We previously reported that MoCV1 affects the fungal host phenotype of isolate S-0412-II 1a, causing irregular mycelial growth, unusual pigmentation, and abnormal vesiculation (29). We attempted to identify which gene products of MoCV1 are involved in these types of cytological damage by utilizing an *S. cerevisiae* expression system. First, each of the MoCV1 ORFs was expressed using five types of shuttle vector: pRSA313 (HIS3), pRSA314 (TRP1), pRSA315 (LEU2), pRSA316 (URA3), and pRSA317 (LYS2). These shuttle vectors have a single-copy CEN6 replication origin, and the expression of the ORFs was controlled by an ADH1 promoter, which leads to moderate expression levels. Yeast transformed with each of the shuttle vectors bearing ORF1, -2, -3, -4, and -5 was grown on the appropriate selective agar medium. The transformants were grown at 25°C, 30°C, and 37°C and were investigated for growth and morphological changes.

No change was observed in the yeast transformants, except for those expressing ORF4. Colonies of the transformants expressing ORF4 were smaller than those of the transformants expressing ORF1, ORF2, ORF3, or ORF5 on selection medium when grown at 37°C (data not shown).

To test the effects of overproduction of ORF4 protein on yeast growth, we introduced single-copy (CEN6 and TDH3-ORF4) or high-copy-number (2μm and TDH3-ORF4) plasmids into strain W303-1A (L-A-o), in which the expression of ORF4 was con-
the ORF4 expression level. Overexpression of ORF3 by pRSA315- 
nuclear fragmentation, and the phenotypic severity depended on 
observations suggested that heterologous overproduction of the 
were easily stained without detergent permeabilization. These ob-
randomly distributed nuclear fragments (Fig. 6A). Enlarged cells 
showed strong chromatin condensation with fragments or several 
(Fig. 5D, right panel), while few vesicles were observed in cells 
enlarged vacuoles that occupied the majority of the cell volume 
scope. The cells overexpressing ORF4 protein showed extremely 
ting using antiserum against ORF4 protein (Fig. 5B).

ORF4 protein was overproduced was obtained by Western blot-
growth inhibition at both 30°C and 37°C. Confirmation that 
overexpression of ORF4 protein by pRST426-ORF4 caused severe 
impediment of DIC and DAPI fluorescence data. Scale bar, 10 

DIC panels show differential interference light microscopy data. DAPI panels show DNA staining. Merged panels show super-

FIG 6 DIC and DAPI-stained fluorescence micrographs of S. cerevisiae cells harboring shuttle vectors. Cells were grown on SC-Ura (A) or SC-Leu (B) plates at 30°C for 3 days. DIC panels show differential interference light microscopy data. DAPI panels show DNA staining. Merged panels show super-imposition of DIC and DAPI fluorescence data. Scale bar, 10 µm. (A) Yeast cells were transformed with empty vectors (pRSA316, pRST316, or pRST426) or ORF4-expressing vectors (pRSA316-ORF4, pRST316-ORF4, or pRST426-

proteases in senescent cells. Although full-size ORF3 and ORF4 being 
assembled as viral structural proteins might be degraded by cellular 
tracts, we could not purify MoCV1 particles consisting of only 
full-length ORF3 and ORF4 proteins, suggesting that proteolytic 
degradation of intact MoCV1 particles might occur rapidly during 
purification or prolonged liquid culture, leading to truncated 
ORF3 (P58) and ORF4 (P70 and P65) proteins.

processing or prolonged liquid culture period of 28 days (29). The MoCV1 par-
ticles were also found in culture medium during a prolonged liquid culture period of 28 days (29). The MoCV1 par-
ticles released into liquid medium also showed a 58-kDa band as a 

Morphological changes were also observed with a light microscope. The cells overexpressing ORF4 protein showed extremely 
enlarged vacuoles that occupied the majority of the cell volume 
(Fig. 5D, right panel), while few vesicles were observed in cells 
carrying pRST426 empty vector (Fig. 5D, left panel).

DAPI staining of W303-1A (L-A-o) harboring pRST426-ORF4 showed strong chromatin condensation with fragments or several 
randomly distributed nuclear fragments (Fig. 6A). Enlarged cells 
were easily stained without detergent permeabilization. These ob-
servations suggested that heterologous overproduction of the 
ORF4 protein in S. cerevisiae resulted in cytological changes and 
nuclear fragmentation, and the phenotypic severity depended on 
the ORF4 expression level. Overexpression of ORF3 by pRSA315-

controlled by the strong promoter of TDH3 (22). As shown in Fig. 5A, 
overexpression of ORF4 protein by pRST426-ORF4 caused severe 
growth inhibition at both 30°C and 37°C. Confirmation that 
ORF4 protein was overproduced was obtained by Western blot-
ting using antiserum against ORF4 protein (Fig. 5B).

As described above, we obtained two types of particles, partially 
processed MoCV1 and fully processed MoCV1. This means that 
at least two types of viral particles might exist in infected 
fungal cells. We could not observe any differences in viral struc-
tural proteins. A similar observation was reported 
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closely related to MoCV1 (5).

Processing of MoCV1 proteins was investigated in crude 
extracts of mycelia grown in flasks; the full-size ORF3 (84 kDa) and 
ORF4 (85 kDa) proteins were detected as major components of 
MoCV1 particles until 15 days of culturing, while the processed 
ORF3 (58 kDa) and ORF4 (70 and 65 kDa) proteins gradually 
accumulated after 15 days (Fig. 3A and C). The processed ORF3 
and ORF4 proteins were hardly detected in 9-day cultures, sug-

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RdRp protein sequences, MoCV1 forms a separate clade with FgV-ch9, FgV2, AsV1816, and AbV1 in the Chrysosporiaceae family (Fig. 1C). The FgV-ch9 and FgV2 genomes are composed of five dsRNA segments (5, 37).

We detected several dsRNA segments ranging from 3.0 to 3.5 kb in eight isolates collected in Vietnam (29). Now, we have confirmed that some of these mycoviruses, including MoCV1, have five dsRNA segments. We also realized that one of these mycoviruses, which was detected in isolate S-0412-II 2a, has four or five dsRNA segments. During cultivation of the host fungus, we succeeded in separating a mycovirus bearing five dsRNA segments and a mycovirus bearing four dsRNA segments, lacking the fifth dsRNA segment. Therefore, the fifth dsRNA segment might be satellite RNA and dispensable for maintenance of the viruses.

In this study, a yeast expression system was used to evaluate the effects of the MoCV1 gene products on host cells. Overexpression of the ORF4 protein in S. cerevisiae caused remarkable growth inhibition (Fig. 5) and led to abnormally enlarged cells and vacuoles with signs of membrane damage. Nuclei in the ORF4-overexpressing cells showed condensation and fragmentation (Fig. 6), suggesting that heterologous expression of ORF4 induces apoptosis-like cell death, with the severity depending on expression level. To observe the effect of ORF4 in its natural host, ORF4 expression in M. grisea is being studied.

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