Suppressive effects of mycoviral proteins encoded by Magnaporthe oryzae chrysovirus 1
strain A on conidial germination of the rice blast fungus

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26 Summary

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28 *Magnaporthe oryzae* chrysovirus 1 strain A (MoCV1-A) is the causal agent of growth
29 repression and attenuated virulence (hypovirulence) of the rice blast fungus, *Magnaporthe*
30 *oryzae*. We previously revealed that heterologous expression of the MoCV1-A ORF4 protein
31 resulted in cytological damage to the yeasts *Saccharomyces cerevisiae* and *Cryptococcus*
32 *neoformans*. Since the ORF4 protein is one of the components of viral particles, we evaluated
33 the inhibitory effects of the purified virus particle against the conidial germination of *M. oryzae*,
34 and confirmed its suppressive effects. Recombinant MoCV1-A ORF4 protein produced in
35 *Pichia pastoris* was also effective for suppression of conidial germination of *M. oryzae*.
36 MoCV1-A ORF4 protein sequence showed significant similarity to 6 related mycoviral
37 proteins; Botrytis dothidea chrysovirus 1, two *Fusarium graminearum* virus, *Fusarium*
38 *oxysporum* f. sp. dianthi mycovirus 1, *Penicillium janczewski* chrysovirus and *Agaricus*
39 *bisporus* virus 1 in the *Chrysoviridae* family. Multiple alignment of the ORF4-related protein
40 sequences showed that their central regions (210-591 aa in MoCV1-A ORF4) are relatively
41 conserved. Indeed, yeast transformants expressing the conserved central region of MoCV1-A
42 ORF4 protein (325-575 aa) showed similar impaired growth phenotypes as those observed in
43 yeasts expressing the full-length MoCV1-A ORF4 protein. These data suggest that the
44 mycovirus itself and its encoded viral protein can be useful as anti-fungal proteins to control
45 rice blast disease caused by *M. oryzae* and other pathogenic fungi.

46 (218 words)

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48 Keywords: Mycovirus; double-stranded RNA; MoCV1-A; anti-fungal protein; *Chrysoviridae*;
49 *Magnaporthe oryzae*

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51 Abbreviations:

52 MoCV1-A: Magnaporthe oryzae chrysovirus 1 strain A; MoCV1-B: Magnaporthe oryzae
53 chrysovirus 1 strain B

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56 **1. Introduction**

57 Hundreds of antifungal proteins and peptides produced by plants, bacteria, insects,
58 mollusks, fungi, and mammals have been reported, with more being discovered (Bull et al.,
59 1992; Chae et al., 1999; Charlet et al., 1996; Newman et al., 2000). Several antifungal proteins
60 and peptides are being tested for use as pharmaceutical agents for the treatment of human and
61 animal fungal diseases (Selitrennikoff, 2001). A well-known example of the antifungal proteins
62 is killer protein, which are the causal agent of killer phenomena in yeasts (Bajaj et al., 2003;
63 Magliani et al., 1997; Schmitt and Breinig, 2002; Wang et al., 2007). K₁ proteins encoded by
64 satellite M1-dsRNA are one of the best-known killer proteins which disrupt the electrochemical
65 ion gradient across the fungal plasma membrane (Vališ et al., 2006).

66 Fungal viruses, known as mycoviruses, have been found in almost all genera of fungi
67 (Bao and Roossinck, 2013; Dawe and Nuss, 2013; Ghabrial et al., 2013; Ghabrial et al., 2015;
68 Hillman and Cai, 2013). In some cases, mycovirus infection results in phenotypic changes of
69 host fungi including plant pathogenic fungi such as *Cryphonectria parasitica* (Nuss, 2005),
70 *Rosellinia necatrix* (Chiba et al., 2009; Kanematsu et al., 2004; Kondo et al., 2013), and
71 *Alternaria alternata* (Aoki et al., 2009). Magnaporthe oryzae chrysovirus 1 strain A
72 (MoCV1-A) is a mycovirus that causes impaired growth and abnormal pigmentation of the host
73 rice blast fungus, *M. oryzae*, which is the most serious and damaging of all the plant diseases in
74 rice production (Urayama et al., 2010; Komatsu et al., 2015). MoCV1-A classified as a member
75 of the *Chrysoviridae* has 5 dsRNA segments, and each dsRNA encodes a single ORF (ORF1 to

76 ORF5). In our previous study, we revealed that the addition of purified MoCV1-A particles to
77 *M. oryzae* in liquid culture led to aggregation of fungal hyphae (Urayama et al., 2010). Also,
78 expression of the MoCV1-A ORF4 protein (ORF4p), which is one of the structural proteins, in
79 *S. cerevisiae* by using a series of shuttle vectors which allow constitutive expression of
80 heterologous proteins resulted in growth defects, suggesting that ORF4p is one of the causal
81 agents of damage to the cell (Urayama et al., 2012). Furthermore, expression of the MoCV1-A
82 ORF4p in human-pathogenic *Cryptococcus neoformans* also caused fungal growth defects
83 (Urayama et al., 2014a).

84 This study was designed to obtain a further insight into MoCV1 virus particle and ORF4p
85 as antifungal proteins. We investigated the effects of purified MoCV1-A virus particles on
86 conidia germination of *M. oryzae* and confirmed that the addition of MoCV1-A particles
87 significantly suppressed conidia germination. We demonstrate that the suppressive effect on
88 conidia germination is attributed to ORF4p encoded in MoCV1-A by a heterologous expression
89 system in *Pichia pastoris*. Evaluation methods to characterize novel mycoviral proteins with
90 yeast model systems are introduced, and the potential application of mycoviral proteins as
91 antifungal proteins is discussed.

92

93 2. Materials and methods

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95 2.1 *Magnaporthe oryzae* strains and culture conditions

96 MoCV1-A-infected strain S-0412-II 1a (the original isolate), MoCV1-free strain
97 S-0412-II 1a (MoCV1-cured isolate) (Urayama et al., 2010) and isolate P2 of *M. oryzae*
98 (Kamakura et al., 1999) were grown on oatmeal agar (OMA) (Yang et al., 2009) for 2-3 weeks
99 at 25°C. For liquid cultures, mycelial plugs were inoculated in 0.5% yeast extract and 2%
100 glucose liquid broth (YG broth) with reciprocal shaking [60 strokes per min. (spm)] at 25°C in

a 3 L flask. Fermentation reactors (2.5 L; Able Biott) were also used for fungal cultivation in YG broth (1.5 L) at 25°C, with agitation at 100 rpm and air introduced at 1.5 L/min. For the measurement of conidial germination rate, MoCV1-A-cured isolate of *M. oryzae* was grown on OMA medium at 26°C for 14 days. After removing aerial hyphae, conidiation was induced by placing the plates under BLB lamps (FL-20S-BLB, Toshiba, Tokyo, Japan) for 3 days. The conidia were brushed off into distilled water. The spore suspension was then filtered through tissue paper (Kimwipe S-200) and adjusted to 4×10^4 spores/ml.

2.2 Detection of dsRNA

Purification of dsRNAs from mycelia or purified MoCV1-A particles was performed following published procedures (Okada et al., 2015; Urayama et al., 2010; Urayama et al., 2015).

2.3 Strains and plasmid constructs for expressing MoCV1-A ORF4 protein in *Pichia pastoris*

P. pastoris strains used for protein expression were PichiaPink strain 1 (*ade2*), strain 2 (*ade2, pep4*), strain 3 (*ade2, prb1*) and strain 4 (*ade2, pep4, prb1*), purchased from Invitrogen (CA, USA). *P. pastoris* strains were maintained on yeast extract-peptone-adenine-dextrose (YPAD) medium at 30°C according to the manufacturer's recommendations.

pPink α -HC-ORF4 was prepared by cloning ORF4 from a full length cDNA clone of the MoCV1-A genome into the *StuI* sites of pPink α -HC which was purchased from Invitrogen (using primers MO4 5w/oATG and MO4H3, see Supplementary Table 1 and 2). pPink α -HC encodes the *S. cerevisiae* α -mating factor signal sequence to permit the secretion of fusion gene products from *P. pastoris* cells into the culture medium. pPink α -HC-ORF4 and pPink α -HC (empty vector) were transformed into PichiaPink Strains 1-4 by the improved electroporation method (Wu and Letchworth, 2004), and plated on adenine dropout synthetic complete medium

(2% glucose, 2% agar, SC-ade) containing 1M sorbitol. White colonies, which were predicted to be multi-copy insert transformants, were picked up after a few days incubation at 30°C.

2.4 Culture of *P. pastoris* to express MoCV1-A ORF4 protein

Single colonies of transformants were individually inoculated into 10 ml of BMGY medium (Buffered Glycerol-complex Medium, 1% yeast extract; 2% peptone; 100 mM potassium phosphate, pH 6.0; 1.34% YNB-Yeast Nitrogen Base; 0.0004% biotin; 1% glycerol) in a 300 ml baffles-flask. Cells were grown for 2 days at 28°C with vigorous shaking at 200 rpm. Then, cultures were centrifuged at 1,500 × g for 5 min at room temperature, followed by resuspension of the cell pellet in 1 ml of BMMY medium (Buffered Methanol complex Medium, 1% yeast extract; 2% peptone; 100 mM potassium phosphate, pH 6.0; 1.34% YNB; 0.0004% biotin; 0.5% methanol) and cultured at 28°C with shaking at 200 rpm to induce the expression. After continuous cultivation for 24 h with daily addition of 40 % methanol, cells were harvested by centrifuging for 10 min at 1,500 × g. Supernatant was transferred to a separate tube and both the supernatant and cell pellet were stored at -80°C.

Collected transformants (0.3 g) were suspended in 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing a protease inhibitor cocktail (complete mini EDTA-free; Roche Diagnostics, Mannheim, Germany) and lysed by sonication. After the centrifugation at 15,000 × g for 10 min at 4°C, 10 µl of the supernatant fraction was separated by SDS-PAGE (8% gels) in 25 mM Tris-glycine and 0.1% SDS at 15 mA for 2 h.

2.5 Production of MoCV1-A ORF4 proteins by jar fermenter in *Pichia pastris*

Recombinant proteins were generated according to the manufacturer's instructions (PichiaPink Expression System, Invitrogen). Briefly, freshly transformed cells were individually inoculated into 50 ml of basal salts medium (BSM), which consisted of 26.7 ml

85% H₃PO₄, 0.93g CaSO₄, 18.2g K₂SO₄, 14.9g MgSO₄· 7H₂O, 4.13g KOH, and 40.0g glycerol per L in 200 ml flask. Prior to inoculation, pH was adjusted to 5.5 with concentrated ammonium hydroxide, followed by the addition of 8.7 ml of PTM1 trace salts (containing, per L, 6.0g CuSO₄· 5H₂O, 0.08g NaI, 3.0g MnSO₄· H₂O, 0.2g Na₂MoO₄· 2H₂O, 0.02g H₃BO₃, 0.5g CoCl₂, 20.0g ZnCl₂, 65.0g FeSO₄· 7H₂O, 0.2g biotin, and 5.0 ml H₂SO₄) and 0.25 ml of 1% Antifoam 204 per 50 ml as recommended by the product information (Sigma–Aldrich, USA). The cells were grown for 22.5 h at 28°C, with vigorous shaking at 140 rpm.

The cultures were diluted to an optical density reading at 600 nm (OD₆₀₀) of 0.2 in 1 L of the same medium used for pre-culture, including 5 ml of 1% Antifoam 204 per 1 L. Batch phase conditions were temperature, 28°C; pH 5.5 (controlled with concentrated ammonium hydroxide); agitation at 500 rpm; and 0.03 MPa pressure. These variables were monitored and maintained continuously by the fermentation unit. The end of batch phase was indicated by a spike in the dissolved oxygen (D.O.) caused by the exhaustion of glycerol. The fed-batch phase was performed under glycerol-limited conditions to increase cell mass and prepare the cells for induction. Fed-batch period was maintained by feeding the cells with 63 % glycerol (containing 12 ml/L PTM trace salts) at a rate of 24 ml/h for 13.7 h agitation at 750 rpm. After the end of the fed-batch phase, the transition phase was initiated by the addition of 100 % methanol at a feeding rate of 3 ml/h and temperature shift at 24°C. Cells were harvested by centrifuging for 10 min at 5,680 × g at 4°C. Supernatant was transferred to a separate tube and both the supernatant and cell pellet were stored at -80°C until ready for assay.

2.6 Measurement of conidial germination rate

Conidia from isolate P2 of *M. oryzae* were used for the tests (see above). Ten µL of conidia suspensions (4×10^4 conidia/ml) in distilled water and an equal volume of prepared sample solutions (the recombinant ORF4p or purified virus particles) were mixed on the

surface of polycarbonate plates (Sanplatec Corp. Osaka, Japan). Then, the plates were kept in a closed plastic chamber at 25°C with high humidity for 24 h. Alternatively, the mixture of conidia suspension and sample solutions was incubated in test tubes at 25°C, and then, conidia germination was evaluated using a light microscope after 24 h.

2.7 Production and purification of recombinant viral protein in *Escherichia coli*

In this study, a central region of ORF4p conserved across related viral proteins, which corresponds to 325-575 aa in MoCV1-A ORF4, was termed SUa fragment.

For recombinant expression of SUa peptide, it was cloned into pColdI (TaKaRa Bio) as described below. SUa fragments with BamHI/HpaI sites were amplified using oligonucleotides MO4SUBh5 and MO4SUH3, then, the amplified fragments were ligated into *Sma*I sites of pUC19 (TaKaRa Bio). pUC19-SUa was digested with *Bam*HI and *Eco*RI, and the resultant SUa fragment was cloned into the same restriction sites of pColdI.

E. coli BL21 (DE3) harboring pColdI-SUa was cultured in 2×YT rich medium containing 50 µg/ml ampicillin until the middle of the log phase, then induced by cold shock (15°C) and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 24 h. After the cells were collected by centrifugation, they were sonicated in 0.1 M sodium phosphate buffer (pH 7.4). Inclusion bodies were collected by centrifugation for 5 min at 31,200 × g, and solubilized in binding buffer (20 mM Tris, 500 mM NaCl, 5mM Imidazole) with 6 M urea. After centrifugation for 5 min at 31,200 × g, the solutions were applied to a Ni-NTA agarose column (Qiagen, Hilden, Germany). After washing the column with wash buffer (20 mM Tris, 500 mM NaCl, 60mM Imidazole) with 6 M urea, the proteins were eluted by Elute Buffer (20 mM Tris, 500 mM NaCl) containing 1 M imidazole and 6 M urea. Two ml of the eluted protein solution (0.93 mg/ml) were used to immunize rabbit, then, antiserum against SUa protein was obtained.

2.8 Protein analysis

Proteins were separated by 8% SDS-PAGE using 25 mM Tris-glycine and 0.1% SDS at 15 mA for 2 h. For protein staining, the gels were stained with Coomassie Brilliant Blue (Bio-Safe CBB; Bio-Rad). For western analysis, proteins were transferred to PVDF membranes after electrophoresis, and subjected to western blotting using an ECL Plus detection system (GE Healthcare). Rabbit antiserum raised against recombinant ORF4p or SUa was used to detect MoCV1-A ORF4p and its derivatives (Urayama et al., 2012; this study).

2.9 Preparation of MoCV1-A particles

Fermentation reactors (2.5 L, ABLE Biott) were used for fungal cultivation in YG broth (1.5 L) at 25 °C, agitated at 100 rpm and, with air introduced at 1.5 L min⁻¹, 20 g fresh mycelia was homogenized in a mixer with 10 vol of 0.1 M sodium phosphate buffer (pH 7.4) containing 0.2 M KCl (buffer A) at 4°C. The resultant suspension was subsequently subjected to French press (5501-M, Ohtake Works, Tokyo, Japan) at 160 MPa twice to efficiently disrupt the cell walls. The disrupted cells were dispensed into the centrifuge tubes and MoCV1-A virus particles were purified following the protocols described previously (Urayama et al., 2012).

2.10 Plasmids and growth conditions for *Saccharomyces cerevisiae*

Plasmids expressing ORF4-EGFP fusion protein in *S. cerevisiae* strain W303-1A (*MATa ura3 his3 leu2 trp1 ade2 can1* [L-A-o], Urayama et al., 2012) were constructed based on pRST426, which had a strong TDH3 promoter and terminator cassette. Plasmid expressing EGFP fused to the amino terminus of ORF4 protein was constructed as follows. EGFP cassettes eliminating stop codon with EcoRI linker sites were amplified using oligonucleotides EGFP E5 and EGFP E3delSTOP, then, the amplified cassettes were ligated into EcoRI site of pRST426-ORF4 (Urayama et al., 2012). Plasmid expressing EGFP fused to the carboxyl

terminus of ORF4 protein was constructed as follows. Overlap PCR using primers MO4E5, MO4EGFP, EGFP E5 and EGFP E3 was performed to create ORF4-EGFP cassette (Supplementary Fig. 1), then, the amplified cassettes were ligated into EcoRI site of pRST426. cDNA of the SUa fragment was inserted into the EcoRI/SmaI site of pRST426 (using primers MO4SUE5 and MO4SUSm3).

Standard yeast media were used (Sherman, 2002) and transformation was performed by the modified lithium acetate method (Gietz et al., 1995). Transformants were examined in a plate or a jar fermenter. When they were cultured in jar fermenter, freshly transformed cells were inoculated into 5 ml of SC-Ura in test tubes. Cells were grown for 16 h at 30°C with reciprocal shaking at 160 spm. Cultures were diluted to an optical density reading at 600 nm (OD₆₀₀) of 0.2 in 50 ml of the same medium used for pre-culture. Cells were grown for 12 h at 30°C with reciprocal shaking at 140 spm.

Cultures were diluted to an OD₆₀₀ of 0.03 in 1 L of the same medium for pre-culture. Conditions were temperature, 35°C; agitation 100 rpm. Number of viable cells per µL of culture was inferred by agar plate dilution method every 2 h from 7 h after culture. Then, cells were examined at × 1,000 magnification with a light microscope (Olympus IX71; Tokyo, Japan) and differential interference contrast (DIC) optics.

2.11 Analysis of conserved amino acids (aa) for MoCV1-A ORF4.

Viral proteins having significant similarity (BLAST hit, E-value $\leq 1 \times 10^{-5}$) with MoCV1-A ORF4 were selected and listed in Table 1. Multiple alignments of MoCV1 A - ORF4 and related aa sequences were made with the program MEGA5 (Tamura et al., 2011) using the aa residue comparison matrix MUSCLE (Edgar, 2004), and the resulting alignment was manually adjusted.

3. Results

3.1 Optimization of productivity of the MoCV1-A virus particles

For effective production of the MoCV1-A virus particle as an antifungal agent, we investigated the culture conditions of rice blast fungus infected with MoCV1-A. The inoculum of the MoCV1-A-infected strain S-0412-II 1a was cultured in 1 L of YG broth medium by a flask or a jar fermenter. The mycelium was harvested and its fresh weight was measured; the average amount of mycelium was 0.36 ± 0.08 g/day L (mean $\pm$ standard deviation) for 30 days cultivation in a flask, while the amount of hyphae was increased up to 2.95 ± 1.04 g/day L for 7 days cultivation in a jar fermenter.

To collect as many virus particles from the mycelium as possible, we applied the powerful cell-destruction step using French press, following mixer homogenization. After being subjected to French press, the amounts of MoCV1-A dsRNAs were increased in yield as much as $17.1 \mu\text{g/g}$ (dsRNA/fungal cells) (Fig. 1A, lane 2), while it was $2.8 \mu\text{g/g}$ by only mixer homogenization (Fig. 1A, lane 1). Filamentous structures were more effectively disrupted by additional French press process (Fig. 1C) rather than only by mixer (Fig. 1B). Collectively, the combination of fermenter and French press resulted in about 50 times yields of MoV1-A particle.

3.2 MoCV1-A particles suppress conidial germination of *M. oryzae*

Given that our previous experiments showed abnormal aggregation of mycelia by adding the MoCV1-A-containing culture supernatant to MoCV1-A-free strain of *M. oryzae* (Urayama et al., 2010), we examined suppressive effects on conidial germination by adding MoCV1-A particles. Purified MoCV1-A viral particles by sucrose gradient (Urayama et al., 2010) were suspended in 0.05 M phosphate buffer, pH 7.4, to prepare virus solution (approx. $10 \text{ ng}/\mu\text{l}$) (Fig.

2A, lane 1). We confirmed that the observed protein bands, from 116 kDa to 58 kDa, were components of MoCV1-A particles by western blot analyses using anti-MoCV1-A serum, as shown in our previous report (data not shown) (Urayama et al., 2012). Equal volumes of the purified MoCV1-A solution and conidia suspension (4×10^4 conidia/ml) prepared from isolate P2, which is a standard virulent strain of *M. oryzae*, were mixed on the surface of a polycarbonate plate, and then conidia germination was evaluated under the microscope after 24 h incubation at 25°C. Growth of germination tube was significantly suppressed (Fig. 2), and conidial germination was slightly suppressed (Supplementary Fig. 2) when the virus solution was mixed. The suppressive effects were also observed even when 10-fold diluted virus solution was applied (data not shown), while no growth suppression was observed when the control solution prepared from MoCV1-A-cured isolate was added to conidia suspension (Fig. 2A, lane 2 and 2C).

3.3 Expression of recombinant MoCV1-A ORF4 protein by *P. pastoris*

P. pastoris strains transformed with pPink α -HC-ORF4 were cultured and induced to express ORF4p by adding methanol in a 300 ml baffled flask. Two protein bands of estimated molecular weight 85 and 100 kDa, were detected in cell extracts by western analysis using anti-ORF4p antibodies after 24 h post induction. PichiaPink strain 1 (*ade2*) (Fig. 3A, lane 1) and strain 2 (*ade2 pep4*) (Fig. 3A, lane 2) produced larger amounts of ORF4p than those of strain 3 (*ade2 prb1*) (Fig. 3A, lane 3) and strain 4 (*ade2 pep4 prb1*) (Fig. 3A, lane 4). Since the ORF4p has 812 aa residues, a calculated molecular mass of ORF4p would be 85 kDa corresponding to the lower-sized band (Fig. 3A). The ORF4p (85 kDa) signals were hardly detected 48 h post induction, while the 100 kDa protein signals were slightly retained, especially in the cell extracts of PichiaPink strain 1 (*ade2*) (Fig. 3A, lane 5) and strain 2 (*ade2 pep4*) (Fig. 3A, lane 6). Both the 85 kDa and 100 kDa bands were hardly detected in culture

301 supernatant for all 4 strains (data not shown).

302 Then, secretory production levels of the ORF4p of the four ORF4-expressing strains
303 cultured in a fermenter were investigated. Unfortunately, we could not detect secreted ORF4
304 products in liquid medium for the transformants of strain 1, 2, and 4 (data not shown). In
305 contrast, in culture supernatant of strain 3 (*ade2 prb1*), the 85 kDa protein bands corresponding
306 to the full-sized ORF4p were gradually visible after 36 h post induction and increased until 84 h
307 (Fig. 3B lanes 2-6, arrow head). Strong intensities of the 100 kDa protein bands were also
308 detected by anti-ORF4p antibody after 36 h post induction (Fig. 3B lanes 2-6, arrow). These
309 data suggested that most of the recombinant ORF4p produced in *P. pastoris* cells were
310 subjected to some post-translational modifications. ORF4p signals were also detected near the
311 origin of electrophoresis by the western blots, especially in lanes 5 and 6 (Fig. 3B), suggesting
312 that the recombinant ORF4p might be an easily insoluble form. Yield of the secreted
313 recombinant ORF4p in liquid medium was estimated as 50 ng/5 μ l (10 μ g / ml) (Fig. 3C. see
314 arrow) by CBB staining. No ORF4p-specific band was detected in culture medium of the empty
315 vector samples (data not shown).

316 317 3.4 Suppressive effect of recombinant ORF4p on conidial germination

318 To examine whether the recombinant ORF4p had suppressive effects on conidial
319 germination, its biological activity as a potential antifungal agent was investigated. In the
320 course of freeze-thawing of the stock solution dissolved in recombinant ORF4p, most of the
321 ORF4p were fractionated as precipitates by centrifugation for 5 min at $15,000 \times g$ at 4 °C (Fig.
322 3D, lane 1), while a few of the dissolved ORF4p were retained in the solution (Fig. 3D, lane 2).
323 We applied this soluble ORF4p to the conidial germination assay using the isolate P2, which is
324 a standard virulence strain of *M. oryzae*. Even though the soluble ORF4p was reduced by less
325 than one-tenth after freeze-thawing and centrifugation, conidia germination was suppressed by

the retained ORF4p in supernatant (approx. 0.5 ng / μ l in test tube) (Fig. 4A). Indeed, 196 out of 662 conidia were counted as germinated in the ORF4p solution although almost of all conidium germinated in the control solution (Supplementary Fig. 3). Although several conidia were slightly germinated in the solution, their growths being apparently suppressed compared with the control solution, culture supernatant of PichiaPink strain 3 cells transformed with an empty vector of pPink α -HC (Fig. 4B). These results suggest that recombinant ORF4p exhibited suppressive effects against conidial germination at low concentrations.

In order to investigate the localization of ORF4p, we transformed yeast cells of *S. cerevisiae* W303-1A strain with the plasmid carrying ORF4-EGFP fusion cassette and observed the transformants expressing ORF4-EGFP. Aggregated green dots were often observed in transformants expressing EGFP fused to the carboxyl terminus of ORF4 protein (Fig. 5, lower panels), while green emission was observed in transformants in the entirety of the cell only when they were expressing EGFP proteins (Fig. 5, upper panels). In transformants expressing EGFP fused to the amino terminus of ORF4 protein, less aggregated green dots were seen (data not shown).

3.5 Conserved domains of ORF4-related protein sequences among tentative chrysovirus

Recently, several MoCV1-A related viruses, which are tentatively classified into the family *Chrysoviridae*, were isolated and completely sequenced: *Magnaporthe oryzae* chrysovirus 1 strain B (MoCV1-B) (Urayama et al., 2014b), *Botrytisphaeria dothidea* chrysovirus 1 (BdCV1) (Wang et al., 2014), *Fusarium graminearum* viruses (FgV-2 and FgV-ch9) (Darissa et al., 2011; Yu et al., 2011), *Fusarium oxysporum* f. sp. *dianthi* mycovirus 1 (FodV1) (Lemus-Minor et al., 2015), *Penicillium janczewskii* chrysovirus (PjCV1 and PjCV2) (Nerva et al., 2015), and *Agaricus bisporus* virus 1 (AbV1) (Van der Lende et al., 1996). We compared aa sequences of these virus proteins with MoCV1-A for each ORF by

BLAST algorithm (Table 1). Among the 5 ORFs, MoCV1-A ORF1 (RdRp) and ORF4 showed significant similarities to those of other tentative chrysovirus (E-value $> 1 \times 10^{-5}$). MoCV1-A ORF2 showed significant similarities to those of MoCV1-B, BdCV1, PjCV1 and PjCV2 but not to the other 4 viruses. The ORF3 was also comparatively conserved among the listed tentative chrysovirus except for AbV1. MoCV1-A ORF5 showed high similarity to MoCV1-B but no significant similarity to the 7 tentative chrysovirus. These results suggested that, in addition to the ORF1, the ORF4 would be essential for these tentative chrysovirus. Blast analysis (Altschul and Lipman, 1990) of the 811 aa gene product encoded by MoCV1-A ORF4 revealed significant similarity to 8 known viral proteins, which are shown in Table 2. In order to identify conserved region(s), a statistically significant alignment was generated with MoCV1-A ORF4 and other sequences by using MUSCLE algorithm (Edgar, 2004) (Fig. 6). Multiple alignment of amino acid sequences of these 8 other viral proteins showed that the central regions (210–591 aa in MoCV1-A ORF4) were relatively conserved, while the N- and C-terminal regions were not as conserved (Supplementary Fig. 4). Among the 35 conserved aa residues through the entire protein, 30 aa were hydrophobic (Supplementary Fig. 4) with a high frequency of conserved Gly and Leu (7/35 and 7/35, respectively). These data implied that the central region of ORF4, especially the conserved hydrophobic residues, might be important for suppressive effects of ORF4p on fungal conidial germination.

3.6 Heterologous expression of conserved regions of ORF4-induced cytological damage to the yeast *Saccharomyces cerevisiae*.

We previously reported that expression of the MoCV1-A ORF4p in *S. cerevisiae* resulted in cytological changes, such as an enlargement of the central vacuole. In this study, to investigate the effects of the conserved region of MoCV1-A (325-575 aa in MoCV1-A ORF4, designated as SUa region) to the cells of *S. cerevisiae*, we introduced expression vectors based

on a high-copy-number plasmid, pRST426, carrying the full-sized ORF4 or the SUa fragment into the *S. cerevisiae* strain W303-1A (L-A-o), and then examined and compared cell morphology and growth rates of the resultant 2 transformants.

Overexpression of these 2 proteins caused severe growth inhibition at 37°C (Fig. 7A upper panel). Morphological changes were also found under a light microscope. Extremely enlarged vacuoles were observed in both cells overexpressing ORF4 and SUa proteins, while unusually-shaped vesicles were rarely seen in cells carrying the pRST426 empty vector after 13 h of liquid culture at 35°C (Fig. 7A lower panel). Viable cell counts of the yeast carrying pRST426-ORF4 and pRST426-SUa decreased to approximately one third as compared with the control cells carrying the empty vector (Fig. 7B, Supplementary Table 3). We did not find significant differences of suppressive effects against *S. cerevisiae* between full-sized ORF4 and SUa proteins. When we assessed total cell lysates of the transformed cells by western blotting using anti-ORF4 and anti-SUa antiserum to detect ORF4 and SUa protein, respectively, we distinctly detected ORF4 and SUa proteins in their cell extracts (Fig. 7C lanes 2 and 6). The antiserum against recombinant full-sized ORF4p showed weak reactivity with SUa proteins (Fig. 7C lane 3) and vice versa (Fig. 7C lane 5). These results suggested that SUa proteins had similar effects for growth inhibition to yeast cells as the full-sized ORF4 protein.

4. Discussion

It is critical to establish a mass production system for the host fungus infected with MoCV1-A when we attempt to use MoCV1-A itself as an effective bio controller. Productivity of rice blast *biomass*, *Magnaporthe oryzae*, was apparently increased by culture in a fermenter with agitation and aeration. In general, free oxygen was often a limiting component in fermentations because of its low solubility and the low volumetric mass transfer in bioreactors (Wang et al., 2005). Cui et al. reported that the dissolved oxygen tension may affect

productivity, cell autolysis, and fungal morphology (Cui et al., 1998). These facts suggest that the increase in oxygen transfer rate in the fermenter might increase productivity levels of the rice blast fungus rather than flask culture, even when the fungus was infected by MoCV1-A.

In the case of heterologous expression of MoCV1-A ORF4p in *E. coli* and *S. cerevisiae*, most of the ORF4p was produced as an insoluble form (Urayama et al., 2012). In *P. pastoris*, recombinant full-sized ORF4p (84 kDa) and its modified form (100 kDa) were successfully secreted into the culture media (Fig. 4. B and C). However, in the course of freeze-thawing, most of the secreted ORF4p became insoluble, suggesting that ORF4p easily becomes aggregated (Fig. 4D). These features of ORF4p may explain the difficulty in purifying this recombinant protein in a soluble form, especially in *E. coli*. Indeed, several cysteine residues involved in disulfide bond formation were also found in ORF4p sequences (Supplementary Fig. 4).

To identify a minimum effective domain responsible for growth inhibition in the entire ORF4p, we made ORF4 deletion constructs and succeeded in obtaining a candidate short-ORF4 fragment by using *S. cerevisiae* heterologous expression system. Actually, the central region of the ORF4 encoded by MoCV1-A, which is conserved in proteins encoded by related chrysovirus (325-575 aa in MoCV1-A ORF4, Fig. 6), showed strong growth inhibition to a similar extent as the entire ORF4p (Fig. 7).

In this study, anti-ORF4p antibody recognized 84 and 100 kDa proteins when the ORF4p was expressed in *P. pastoris*. The 100 kDa protein derived from the ORF4p was not found in MoCV1-A infected *M. oryzae* (Urayama et al., 2012), nor in *S. cerevisiae* and *Cryptococcus neoformans* expressing ORF4p (Urayama et al., 2012; Urayama et al., 2014a). It is possible that the ORF4p would be glycosylated in *P. pastoris* during heterologous expression (Cereghino et al., 2002), since a secretion signal sequence of MAT α -factor is attached to the ORF4p. *P. pastoris* is able to add both O-linked and N-linked carbohydrate moieties to secreted proteins,

although those proteins are not normally glycosylated in the native host (Cereghino and Cregg, 2000). Indeed, the ORF4p has a potential N-glycosylation site (NXS/T) at 352-354 aa (NDS), indicating that the secreted ORF4p could be glycosylated, which could increase its molecular mass to 100 kDa.

Conidial germination of *M. oryzae* was efficiently suppressed by co-incubation with MoCV1-A particles, of which ORF4p was one of the structural components (Urayama et al., 2012), or with culture supernatant of *P. pastoris* containing recombinant ORF4p. Darissa et al. reported that one of the FgV-ch9 proteins was a viral surface component, which has significant similarity with MoCV1-A ORF4p (Darissa et al., 2011). Generally, it is a rare case that structural proteins of viruses act as toxins against host cells. Human immunodeficiency virus type-1 (HIV-1) coat protein gp120 is a representative example of a toxic structural protein (Dreyer et al., 1990).

It remains a mysterious and controversial issue to understand mechanisms of the toxic effects and mode of action of ORF4p against the host fungus, *M. oryzae*. High-molecular weight proteins could pass cell walls by cellular mechanisms such as micropinocytosis (Liu et al., 2008). Some effector proteins encoded by plant pathogens have shown to be secreted from the pathogens and to be translocated into plant cells independently of fungal factors (Rafiqi et al., 2010; Zhang and Xu, 2014). We did not observe the conserved aa sequences in ORF4p responsible for cell membrane passage. However, the fluorescence images revealed that ORF4-EGFP fusion proteins in live cells appear to become aggregated; this could be the cause of the observed cytological damages (Fig. 5). Conserved hydrophobic residues in ORF4p, especially the SUa domain, might be involved in the toxic effects of ORF4p as well as viral propagation

It is noteworthy that MoCV1-A viral particles, or ORF4p itself, have advantageous potential as a biological control material against *M. oryzae*. Yu et al. reported that purified

particles of a DNA mycovirus, *Sclerotinia sclerotiorum* hypovirulence-associated DNA virus 1 (SsHADV-1), are infectious when applied extracellularly to its host *S. sclerotiorum* and that the virus can suppress the development of lesions when applied to leaves (Yu et al., 2013), although the active agent of these phenomenon has not yet been identified.

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7. Figure titles and legends

Fig. 1. Comparison of hyphae-disruption methods to extract MoCV1-A dsRNAs in the fungi, *Magnaporthe oryzae*, strain S-0412-II 1a. (A) Agarose gel electrophoresis of dsRNAs purified from suspensions of (B) (lane 1) and (C) (lane 2), after staining with ethidium bromide. dsRNAs were extracted from 35 mg fresh weight of fungal cells cultured for 10 days in a fermenter. (B) Hyphae homogenized in a mixer with 0.1 M sodium phosphate buffer (pH 7.4) containing 0.2 M KCl at 4°C for 1 min. (C) Hyphae treated by French press at 160 MPa after the mixer disruption were observed under microscope (IX71, Olympus). Bars: 100 µm.

Fig. 2. Purified MoCV1-A particles suppressed the conidial germination of *M. oryzae*. Proteins of the collected virus fractions were separated by 8% SDS-PAGE (15 mA; 2 h), stained with CBB (A). Lane 1, MoCV1-A particles purified from 2-day cultures of MoCV1-A-infected mycelia by sucrose gradient; lane 2, control preparation purified from 2-day cultures of MoCV1-A-free mycelia. Conidia were suspended in distilled water to a final density of 4×10^4 conidia/ml, and the conidia suspension was mixed with the same quantity of MoCV1-A-containing (B), or MoCV1-A-free (C) fraction. After 24 h incubation on a polycarbonate plate at 26°C, conidial germinations were observed under the microscope (IX71, Olympus). Lane HM, high molecular mass protein makers [HMW-SDS, 116 kDa (0.16µg), 76 kDa (0.17µg), 53 kDa (0.18µg)], lane LM, low molecular mass protein markers [LMW-SDS, 97 kDa (0.34 µg), 66 kDa (0.42 µg), 45 kDa (0.735 µg), GE Healthcare Life Science].

Fig. 3. Expression of ORF4p by PichiaPink strains. (A) 24 and 48 h post methanol induction in a flask, cell extracts were separated by 8% SDS-PAGE (15 mA; 2 h) and immunoblotted with antiserum against *E. coli* expressed recombinant ORF4p. Genotypes of PichiaPink strains: lane 1; strain 1 (*ade2*), lane 2; strain 2 (*ade2 pep4*), lane 3; strain 3 (*ade2 prb1*), lane 4; (*ade2 pep4*

601 *prb1*). (B) (C) 0, 24, 36, 48, 60 and 84 h. post induction in fermenter culturing PichiaPink strain
602 3, 10 µl of culture medium were separated by 8% SDS-PAGE (15 mA; 2 h) and immunoblotted
603 with anti-ORF4p (B) or stained with CBB (C). (D) Insolubilized ORF4p after freeze-thawing.
604 Culture supernatant containing ORF4p was centrifuged at 10,000 rpm for 5 min at 4°C.
605 Resultant pellet (lane 1) and supernatant (lane 2) were separated by 8% SDS-PAGE (15 mA; 2
606 h) and immunoblotted with anti-ORF4p (D). Arrows indicate viral ORF4 structural proteins.

607
608 Fig. 4. Recombinant ORF4p suppressed the conidial germination of *M. oryzae*. Conidia were
609 suspended in distilled water to a final density of 4×10^4 conidia/mL, and the conidia
610 suspensions were mixed with same quantity of culture medium containing secreted ORF4p
611 after 84 h induction (A) or control culture medium. Control culture medium was prepared from
612 cells transformed with empty vector pPink α -HC. (B). After 24 h incubation in a test tube at
613 25°C, conidial germinations were observed. Similar results were obtained in 3 different
614 experiments prepared under the same conditions.

615
616 Fig. 5. Localization of ORF4-EGFP fusion proteins within transformants of the yeast
617 *Saccharomyces cerevisiae*, W303-1A strain. After yeast cells carrying pRST426-EGFP (upper
618 panel) and pRST426-ORF4-EGFP (lower panel) were pre-cultured in liquid SC-Ura medium at
619 30°C for 16 h, the cells were inoculated in fresh liquid SC-Ura medium at 30°C for 3 h.
620 Transformed cells were observed under light microscopy (IX71, Olympus) with a fluorescence
621 excitation filter (470 to 495 nm) and emission filter (510 to 550 nm) to visualize the localization
622 of EGFP or ORF4-EGFP fusion proteins.

623
624 Fig. 6. Multiple alignment of amino acid sequences of the conserved regions of ORF4 encoded
625 by MoCV1-A dsRNA4 with those of the corresponding regions of ORF4-related proteins of

MoCV1-B, BdCV1, FgV2, FgV-ch9, FodV1, PjCV1, PjCV2, and AbV1. Shading indicates: black, 100% identity; gray, 50%–80% identity. Numbers at the beginning and end of the sequences represent the aa position from the start of the predicted gene product. Underlines show SUa regions (325-575 aa in MoCV1-A ORF4).

Fig. 7. Heterologous expression of full-sized ORF4 and SUa protein in *S. cerevisiae*. (A) Growth tests on plates and individual cells of yeast strain W303-1A (L-A-o) harboring empty pRST426, pRST426-ORF4 or pRST426-SUa shuttle vectors. Transformants were streaked onto minimal medium (SC) without uracil and incubated at 37°C for 3 days (upper panel). The some transformants were also cultured in liquid medium at 35°C for 13 h and observed using a 1,000 × differential interference contrast objective (lower panel). (B) Comparison of viable cell counts among the 3 transformants. Transformants carrying empty pRST426 (chain line), pRST426-ORF4 (dashed line), and pRST426-SUa (solid line) were cultured in SC-Ura liquid medium at 35°C and viable cell numbers were counted every 2 h using plate culture at 30°C. Error bars denote the standard error of the mean from 3 independent experiments. (C) Western blot analysis. After growth in SC-Ura liquid medium for 16 hour-culture, proteins were extracted by Y-PER Yeast Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA), separated by SDS-PAGE (12% acrylamide, 20 mA 1.5 h), transferred to PVDF membranes, and probed with antiserum against recombinant ORF4 (lanes 1-3) and recombinant SUa (lanes 4-6). Lanes 1 and 4: cell extracts from transformants carrying empty pRST426, lanes 2 and 5: cell extracts from transformants carrying pRST426-full sized ORF4, lanes 3 and 6: cell extracts from transformants carrying pRST426-SUa.

Table 1

Pairwise comparisons of protein sequences for each MoCV1-A ORF with those of 8 related tentative chrysovirus.

		MoCV 1-B	BdCV1	FgV-2	FgV-ch9	FodV1	PjCV1	PjCV2	AbV1
MoCV 1-A	ORF1	0.0	0.0	2×10^{-165}	3×10^{-161}	2×10^{-163}	0.0	0.0	8×10^{-108}
	ORF2	0.0	1×10^{-22}	-	-	-	1×10^{-18}	8×10^{-21}	-
	ORF3	0.0	1×10^{-79}	2×10^{-50}	2×10^{-50}	5×10^{-54}	3×10^{-74}	2×10^{-44}	-
	ORF4	0.0	1×10^{-48}	4×10^{-15}	4×10^{-17}	3×10^{-20}	2×10^{-39}	2×10^{-42}	1×10^{-29}
	ORF5	0.0	-	-	-	-	-	-	-

-: not significant similarity (E-value $> 1 \times 10^{-5}$)

Table 2

GenBank/protein accession numbers for the phylogenetic analysis of the ORF4 sequences of MoCV1-A and 8 related tentative chrysoviruses.

Virus name	Abbreviation	Accession number	Size (aa)	Hydrophobic aa (%)
Magnaporthe oryzae chrysovirus 1-A	MoCV1-A	YP_003858288	811	56.1
Magnaporthe oryzae chrysovirus 1-B	MoCV1-B	YP_008914862	811	55.7
Botryosphaeria dothidea chrysovirus 1	BdCV1	AGZ84313	748	52.9
Fusarium graminearum dsRNA mycovirus-2	FgV-2	ADW08804	856	51.1
Fusarium graminearum mycovirus-China 9	FgV-ch9	ADU54125	856	50.8
Fusarium oxysporum f. sp. dianthi mycovirus 1	FodV1	YP_009158915	852	51.2
Penicillium janczewskii chrysovirus 1	PjCV1	YP_009182337	770	50.7
Penicillium janczewskii chrysovirus 2	PjCV2	ALO50150	783	54.9
Agaricus bisporus virus 1	AbV1	1906228C	788	49.6

Fig. 1

Fig. 2

Fig. 3

(A)

(B)

Fig. 4

Fig. 5

MoCV1-A 210 VF-VRMWLVHLSVLA-RQ--PVTVQV-VDPVLSAR-FA---NIAA-PA-ESD---AARAMRGVKINA-RG--L----- 265
MoCV1-B 210 VF-VRMWLVHLSVLS-RR--PVTVQV-VDPVLSAR-FA---NIAA-PA-ESD---AARAMRGVRIINA-RG--L----- 265
BdCV1 165 LLVV-MWLIHLDV-AAGAE-PV--VALDQ-MG-VHAGL--SN-VTDPADECEE--A-KSIDGVRLH-V----- 218
FgV2 213 VFTV-MWLLQLDV-AGGA--RV-ELGID-AND-MRVLV-PSS-VS-PLDEAD--AMKAVPGIRLHS-DA--L----- 269
FgV-ch9 213 VFTV-MWLLQLDV-AGGA--RV-ELGI-NAND-MRVLV-PSS-VS-PLDEAD--AMKAVPGIRLHS-DA--L----- 269
FodV1 209 IFSI-MWMIQLDI-AGG-N-PV-DINID-AGD-VRRVLV-PV-GLN-PLEEAE--AMKSQAGLRIH--R----- 262
PjCV1 164 VL-LRMWLLHLDVLA-GAD--V-SVRVDNT--QVRQQL-PTN-LTLE-ECEE--YA-KRLTGVRVH-VNS--L----- 220
PjCV2 170 LLTV-MWLLHLDV-AAGSEPRV-RVD--NQ--QVRRLVRSRGITQ--ECEEAKA-LP--GIRMH-V-----HSG 226
AbV1 198 -FT-RL-LWLLHIDI-ASGSLLTVRMDPSD-LEAQMTESTLKKLG--PS-E--LL-AM-----HSLEAVVLAGSEL 256

MoCV1-A 266 --TNT-AL-ALLVLGCSDT-S-QAAG-LHY-RARRYKFA-RSA-LSM-YGMQG--LGR---VAVALD-R--AEVTGLA 324
MoCV1-B 266 --SDT-AL-ALLVLGCSDT-S-QAAG-LHY-RARRYKFT-RSA-LSM-YGMQG-RI-R---LAVALD-R--AEVTGLA 324
BdCV1 219 NSCDN-TTNALL-AAACDY-NAIA-N-L-GRRAERYKWTP-S-RL-TLYG--G--L-MSASVNVPLTN-PDATAR--A 279
FgV2 270 --DPLA-YRLL-YTATHR-S--ALG-AASPWNRYRW-P-TTPL-TVY--SG-ALNP-GPYMQAFNGRP-AAAQ-LA 329
FgV-ch9 270 --DPLA-YRLL-YTATHR-S--ALG-AASPWNRYRW-P-TTPL-TVY--SG-ALNP-GPYMQAFNGRP-AAAQ-LA 329
FodV1 263 AALEPLALVML--YTATHR--AALT-QASPWLDRTYTW-P-DT-DMTVY--NG-PL-PAGTVGRQLHGRSDA-AQ-VA 325
PjCV1 221 --DDT-AR-ALCY-ACGL-S-QGDG-LSDRAA-RYIWEPID-LTF-YG--G-VL-PVAREVDLRD--P-A-GT SRC 278
PjCV2 227 NSTLR----ALLSAACDRTTVLGGLS-L--RAERYDW-PHVSLLT-LY--GILPASTEETLNSPD--A--TARA 285
AbV1 257 SAQGR----ALVAACTWRMS-GM-GT--RVKRYKFKQ-HQLFYLTGY-----KR--ERVELSD-AK-A--TLHV 310

MoCV1-A 325 --IVSLA--E--RYG--AEHA--CGAGLQ-TALMMYG-VND-SG-RYVQLKCEPEL----HDDVA---TTAGIRAL 380
MoCV1-B 325 --IVSLA--E--RYG--AEHA--CGAGLQ-TALMMYG-VND-SG-RYVRLKCEPEL----HDDVA---TTAGIRAL 380
BdCV1 280 ---INSFGHRFASPSE--MSAA--LE-TALLLYG-LED-GGTRVY-LDVPAPQL--HEDSHRLPMTTSGLAR- 338
FgV2 330 GQIVNIA--N--TY---DLRDTCSRAYSI-AFSLYG--DRCVNRNFRLHCGRQTLF--HEISNGQ-S---KAQV 387
FgV-ch9 330 GQIVNIA--N--TY---DLRDTCSRAYSI-AFSLYG--DRCVNRNFRLHCGRQTLF--HEISNGQ-S---KAQV 387
FodV1 326 AVIVGIA--N--TY---DLRDAACAGYRV-AQWLYG--DRCVNSSVRLDCGPQTLF--HEIENS-V---RNTM 383
PjCV1 279 --IVAF--E--KYGVVLECG--E-ALRM--ALHMYGSV-NPANKLSLSL--GVTRLHQQTH--QKQMVNR--AN-- 334
PjCV2 286 --ILSFA--E--SFGAIPFA--SAALS--TALVLYGVE--HPGTR-AVL-DVGLPALHEQTH--RVTGG---AAGL 342
AbV1 311 --ILHLAQRL-----DATAAC-SRGLQTAMWLFGGNMPVKS IK--LMQSKSKVYE-RHGGGVSLQG---VGML 369

MoCV1-A 381 SVKSYSDLSDNR-L-LSLSLFVGRAWRQSA-GHLL-RSSTMQTTT--ADIDAVVN---TLLPSQ---GALIKAVGSA 445
MoCV1-B 381 SVRSYSDLSDNR-L-LSLSLFVGRAWRQAA-GHLL-RSSTMQTTT--ADIDAVVN---TLLPSQ---GALIKAVGCA 445
BdCV1 339 ---TRELAGE--AVALSLFLGRAYMQVA-GAVL-RS-AVLTG--AKDLHGAEHFL-RR---NHELL--QVAA 396
FgV2 388 PQMCW-ALG-SYQLPMAAK-FNGYANAEMYR-D-LCRS-TI-TAGLRIRDRDAV---RRAVDQIDVHAL-K-TVQQ 451
FgV-ch9 388 PQMCW-ALG-SYQLPMAAK-FNGYANAEMYR-D-LCRS-TI-TAGLRIRDRDAV---RRAVDQIDVHAL-K-TVQQ 451
FodV1 384 PQMCW-ALS-SSQLALASQ-FIGYANAEMYR-D-LPRS-TVVH-ALRVRDAHAM---RRALSNDVHAL--ETIVD 447
PjCV1 335 -YRCH-ELGGRELVALAMLIGEGID--SAL-GHTL-HSAVRKM-G--LYDTPNMPYPLEPR-----ARLL-KDAA-D 395
PjCV2 343 VRRELAP-S-LHALAM--FIGRAYSQAAMV-L-RSAIRAT-G--ANDIPGAEVYL--RQCQ---KLMM-DCGAA 405
AbV1 370 LGAQY-AD-KAIAN-LSCYIA--RAMEQHIAELMHEY-VAQGVEA--DDDEGTRYLMEK---HMGAVVEIKD---- 431

MoCV1-A 446 HAR---AMG-W--VA---PLV-DTV---SYVE-QNY-RLLWEERGIVH--CLALGLR--VP-N-SVLEEATAV-- 497
MoCV1-B 446 HAR---AMG-W--VA---PLV-DTV---SYVE-QNY-RLLWEERGIVH--CLALGLR--VP-N-SVLEEATAV-- 497
BdCV1 397 GAHL--AQ-TWGQLH---P--NYIHHGTMLHV--NFVR-AWQQLGVHL--ALMAG--CVTEG-SIFE--T-VTN 451
FgV2 452 --RF-EE-GQQ--LT-GALP-I-DYR---HYVKH-GI-RRAWVVRGILH--NLSLG-RA-VPAAIS-GS---AVKQ 505
FgV-ch9 452 --RF-EE-GQQ--LT-DALP-I-DYR---HYVKH-GI-RRAWVVRGILH--NLSLG-RA-VPAAIS-GS---AVKQ 505
FodV1 448 --RY-IA-GQT-L-L-SD-FP-V-DFA---HHVR-LGL-RRSWVEKGVH--NLALG-RA-VGASIS-GSC---VER 501
PjCV1 396 SLRV--NE--W--L-SNFF--VAD-A---YVRR-NV-RYLYSKEGVH--AMCLG--IVVAG--SLLEDATQP-- 447
PjCV2 406 FSRE-----WNAL-R--PTLLNHA--DYIYV--NA-RRLWLQSGIVH-AVL-GG--IMAQG--SIAEEACQP-- 458
AbV1 432 --RWVDVVKTY--L-C--PAV-NWL---SYII-DNMAS-IWIVEG--RELALALG--MLVAGGVT--EMM-AVGI 486

MoCV1-A 498 -IEVPYPP-A-L-SPS-D-DPRSAGP-RA--GSLATLG--LV-ESLLSSSG-EGLCGS-ARARGRRQ-AG-LVAVP-- 557
MoCV1-B 498 -IDVPYPP-A-L-SPS-D-DPLSAKP-RA--NSLATLG--LV-ESLLSSSG-ESLCGS-ARARGRRQ-AG-LVAVP-- 557
BdCV1 452 ALELGT--A-LDTPVTD-SPSLGQS-RA--DEMVTWYIL-SE-LLRDGG-EHLNGS-RAA-IKRS-LG-ASTRLH- 512
FgV2 506 -LRMPSE--AEL--ADTDVLAYGKE--RAESEAN--AW--LINE-LIRNGGTS-LCQSVDEA-IPRS--GATAVL-HT 566
FgV-ch9 506 -LRMPSE--AEL--ADTDVLAYGKE--RAESEAN--AW--LINE-LIRNGGTS-LCQSVDEA-IPRS--GATAVL-HT 566
FodV1 502 -LEMP-DQ-AML--AETDVLAGSAHARA--T-AW--LVNE-LIRNGG-EHLCASLDEA-VPKS--GATTQM-HV 562
PjCV1 448 -FEVPSRG-AGQCT-D-DVLTASQAS--DDASR--W-LLLSAMLDDGLGCESG-TRAAMAKVADP--DEA-L-DD 508
PjCV2 459 -LKLGMLSA-A--NP-SD-IPAAGK-DRV--DDMIS-WQ-LLSH-LISDAG-ENLVGS-RDAMK-RGLGGRIPL--H 519
AbV1 487 N--LP-ER-ASEHAN--D-DEYV-E-T--GKQNV-R-AWW-LANQ-LAKDAGKKITHYTLKVEV--RGVE--QIPLIDH 545

MoCV1-A 558 AQVVA--LAG-H-RVQFTLLSVA---SGVAVR--VEELPTRP-L 591
MoCV1-B 558 AQVVA--LAG-H-RVQFTLLSVA---SGVAVK--VEELPTRP-L 591
BdCV1 513 AE--TRQYAG-T-KARFIISGSG--APLLLR--MVDNSTLA-L 546
FgV2 567 -D--CRYMGMD--LQYKIVGFG--NDM--RIKY---NRP-L 595
FgV-ch9 567 -D--CRYMGMD--LQYKIVGFG--NDM--RIKY---NRP-L 595
FodV1 563 -D--CSYMGMT--LQFMVVGFG--NDMV--LKY---HRP-V 591
PjCV1 509 -D--CPMYAGARVKM--GIVCFG--GTTVLRR-LA---KT-- 537
PjCV2 520 RD--VRLFEQS--TAQ--LVFAG--IGSPVFVNPAAE--TKSEL 553
AbV1 546 ID--C--LVG--QKLKYVFIGFGKSTLYSGEAFKV---TMP-A 578

Fig. 6

Fig. 7