A Host Factor Involved in Hypovirus Symptom Expression in the Chestnut Blight Fungus, Cryphonectria parasitica

M. Iqbal Faruk, Ana Eusebio-Cope, and Nobuhiro Suzuki*

Agrivirology Laboratory, Research Institute for Bioresources, Okayama University, Kurashiki, Okayama 710-0046, Japan

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The prototype hypovirus CHV1-EP713 causes virulence attenuation and severe suppression of asexual sporulation and pigmentation in its host, the chestnut blight fungus, Cryphonectria parasitica. We identified a factor associated with symptom induction in C. parasitica using a transformation of C. parasitica strain EP155 with a full-length cDNA clone from a mild mutant virus strain, Cys72. This was accomplished by using mutagenesis of the transformant fungal strain TCys72-1 by random integration of plasmid PhygR, conferring hygromycin resistance. The mutant, nam-4 (after nami-gata, meaning wave shaped), showed an irregular fungal morphology with reduced conidiation and pigmentation while retaining similar levels of virulence and virus accumulation relative to TCys72-1 or Cys72-infected strain EP155. However, the colony morphology of virus-cured nam-4 (VC-nam4) was indistinguishable from those of EP155 and virus-cured TCys72-1 [VC-TCys72-1]. The phenotypic difference between VC-nam4 and VC-TCys72-1 was found only when these strains infected with the wild type or certain mutant CHV1-EP713 strains but not when infected with Mycoreovirus 1. Sequence analysis of inverse-PCR-amplified genomic DNA fragments and cDNA identified the insertion site of the mutagenic plasmid in exon 8 of the nam-1 gene. Nam-1, comprising 1,257 amino acids, shows sequence similarities to counterparts from other filamentous fungi and possesses the CorA domain that is conserved in a class of Mg²⁺ transporters from prokaryotes and eukaryotes. Complementation assays using the wild-type and mutant alleles and targeted disruption of nam-1 showed that nam-1 with an extension of the PhygR-derived sequence contributed to the altered phenotype in the nam-4 mutant. The molecular mechanism underlying virus-specific fungal symptom modulation in VC-nam4 is discussed.

Induction of symptoms by a virus in its host is a consequence of alterations in complex physiological processes that involve interactions between host and viral factors at the molecular level. Thus, it is difficult to investigate the molecular mechanisms underlying macroscopic phenotypic alterations. This is particularly true for mycoviruses/filamentous fungus systems, because genetic manipulation is often unavailable for host fungi and/or viruses, and even virus introduction into host cells is not always easy to perform. In this regard, the mycoviruses and Cryphonectria parasitica are placed in a unique position.

C. parasitica is an ascomycetous fungus that is the causal pathogen of chestnut blight disease. This fungus is the host of a number of viruses, and some confer hypovirulence to the host fungus, thus being of potential or practical use as biological control agents (2, 19). Efficient DNA-mediated transformation is available for C. parasitica (9), which allows multiple transgenic expressions of endogenous and exogenous genes and targeted gene disruption by homologous recombination-mediated gene replacement (11, 32). Infectious cDNA clones are available for a few members of the family Hypoviridae (5, 8, 23), while a transfection protocol with purified virus particles has been established for members of the genus Mycoreovirus (18). These established techniques provide a foundation to study fungal host-virus interactions.

The prototype hypovirus CHV1-EP713 moderately attenuates virulence (hypovirulence) of the chestnut blight fungus. Hypovirulence-associated phenotypic traits include severe reduction in pigmentation, asexual sporulation, and loss of female fertility. In this virus-host combination, viral symptom factors have been studied using transformation and transfection analyses. The viral double-stranded RNA genome of CHV1-EP713 is 12.7 kb in size with two continuous open reading frames (ORFs), ORF A and ORF B (11). The ORF A-encoded polyprotein p69 is cleaved into a papain-like protease, p29, and a basic protein, p40. The p29 protein, associated with membranous vesicles derived from the trans-Golgi network (20), plays multifunctional roles; that is, p29 contains an essential domain for virus viability (38), contributes to a reduction in pigmentation and sporulation (7, 10, 34, 35, 37), suppresses RNA silencing (31), enhances the replication of the homologous and heterologous viruses (34, 37), and is responsible for self-cleavage (6). The symptom determinant and replication-enhancing activity domain resides in the N-terminal region containing critical cysteine residues at positions 70 and 72. The substitution of a glycine for Cys70 and Cys72 that induce profoundly altered phenotypes and symptoms similar to those of a p29 null mutant Δp29 virus (35). Transgenic expression of wild-type p29 in the absence of virus replication results in reductions in pigmentation and sporulation, while mutation at Cys70 or Cys72 abolishes its suppressive activities (37). The other ORF A-encoded protein, p40, is also involved in virus RNA accumulation and symptom...
induction only when provided in cis (from the CHV1-EP713 genome (36)).

Perturbation of signaling pathways, e.g., the heterotrimeric G protein pathway, is implicated in symptom expression by CHV1-EP713. Support for this comes from the pleiotropic effects of CHV1 infection on the fundamental physiology in the host fungus and similarities in transcription profiles between fungal strains infected with the virus and defective in a signaling pathway. Using a catalogued expressed-sequence-tag (EST) library (13), Allen et al. (1) previously performed microarray analysis of CHV1-EP713-infected and virus-free fungal strains to identify host genes that are responsive to infection by the virus. Those studies showed that the macroscopic changes induced by infection with CHV1-EP713 are accompanied by either the up- or down-regulation of at least 295 host genes (13.4% of 2,200 genes tested). Many of the responsive genes are affected by the disruption of the G protein subunits in the same direction (12). Furthermore, the disruption of a C. parasitica homologue of the Ste12 transcription factor that is down-regulated by CHV1-EP713 infection results in virulence attenuation and female sterility (14). Ste12 is a part of the mitogen-activated protein kinase signaling cascade regulating mating and pseudohyphal growth in Saccharomyces cerevisiae.

Here, we describe the development of a genetic screening protocol entailing the transformation of a wild-type fungus with a cDNA clone of the CHV1-EP713 variant Cys(72), mutation by random plasmid integration, isolation of mutant strains, and identification of mutated host genes by inverse PCR (1-PCR). We report the characterization of a mutant C. parasitica strain, termed nam4 (nami-gata, meaning wave shaped), showing unusually altered symptoms upon infection with the hypovirus, but not with a distinct mycoreovirus, compared to its parental fungal strain. Furthermore, we characterized a mutated host gene encoding a CorA Mg2+ transporter domain, which contributed to the mutant phenotype.

**TABLE 1. Viral and fungal strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHV1-EP713</td>
<td>Prototype of the family Hypoviridae</td>
<td></td>
</tr>
<tr>
<td>Cys(72)</td>
<td>CHV1-EP713 with a Cys-Gly substitution at p29 residue 72</td>
<td>35</td>
</tr>
<tr>
<td>Δp29</td>
<td>CHV1-EP713 lacking 87.5% of the p29 coding domain</td>
<td>35</td>
</tr>
<tr>
<td>Δp40b</td>
<td>CHV1-EP713 lacking 99.5% of the p40 coding domain</td>
<td>36</td>
</tr>
<tr>
<td>Δp69b</td>
<td>CHV1-EP713 lacking 96.1% of the p69 coding domain</td>
<td>36</td>
</tr>
<tr>
<td>MyRV1-Cp9B21</td>
<td>Prototype of the genus Mycoreovirus</td>
<td>18</td>
</tr>
</tbody>
</table>

**Fungal**

EP155                           | Virus-free field isolate                                                    | ATCC 38755          |
TCys(72)-1                      | Transformant of EP155 with full-length cDNA of Cys(72)                      | This study          |
VC-TCys(72)-1                   | Cys(72) virus-cured strain of TCys(72)-1                                   | This study          |
nam4                           | Mutant of TCys(72)-1 obtained by random plasmid integration                | This study          |
VC-nam4                        | Cys(72) virus-cured strain of nam4                                          | This study          |
VC-nam4/p29                    | Transformant of VC-nam4 with the p29 coding sequence                        | This study          |
VC-nam4/p40                    | Transformant of VC-nam4 with the p40 coding sequence                        | This study          |
nam4/namC1                     | Transformant of nam4 with complementing plasmid pBSDnamC1                  | This study          |
nam4/namC2                     | Transformant of nam4 with complementing plasmid pBSDnamC2                  | This study          |
nam4/namC3                     | Transformant of nam4 with complementing plasmid pBSDnamC3                  | This study          |
TCys(72)-1/Δnam1/1−TCys(72)-1/Δnam1-2 | nam-1 disruptant of TCys(72)-1                                          | This study          |
VC-TCys(72)-1/Δnam1/1−VC-TCys(72)-1/Δnam1-2 | Cys(72)-cured strains of TCys(72)-1/Δnam1-1 and TCys(72)-1/Δnam1-2 | This study          |
TCys(72)-1/Δnam1/nam1-HygR1     | Transformant of TCys(72)-1/Δnam1-1 with a nam-1 mutant allele of nam4       | This study          |

**MATERIALS AND METHODS**

**Viruses.** Table 1 shows the virus strains used: the prototype hypovirus CHV1-EP713 (28); its deletion mutants Δp29, Δp40b, and Δp69b (36); and the site-directed mutant Cys(72) (35). The type member of the genus Mycoreovirus, MyRV1-Cp9B21, was described previously (19, 39).

**Fungal strains and culturing.** The field fungal isolate EP155 (ATCC 38755) and transfectants with the EP155 background were infected or uninfected with viruses (Table 1). Fungal colonies were cultured at 25°C to 27°C for 3 to 7 days in potato dextrose broth (PDB) (Difco) for RNA/DNA extraction and spheroplast preparations or on potato dextrose agar (PDA) (Difco) for phenotypic observation. Fungal strains were cultured on regeneration plates (9) for maintenance and stored at 4°C in a refrigerator until use.

**Transfection and transformation of C. parasitica spheroplasts.** Spheroplasts were prepared from fungal strains with the EP155 backgrounds that were cultured in PDB as described previously by Churchill et al. (9) and transformed with full-length cDNA clones of Cys(72) or genomic DNA fragments as described previously by Choi and Nuss (8). Transformation of EP155 with in vitro-synthesized RNA from cDNA of CHV1-EP713 and its mutant strains or purified virus particles of MyRV1 was carried out according to methods described previously by Suzuki et al. (35) and Hillman et al. (18), respectively.

**Phenotypic measurements of fungal colonies.** Virulence was measured in apples (cv. Jonathan Gold) as areas of lesions induced by fungal strains as described previously by Fulbright (15) and Hillman et al. (17). Sporulation and pigmentation were evaluated using methods described previously by Hillman et al. (17, 18). Fungal strains were cultured for 14 days on PDA plates that were 60 mm in diameter: the first 4 days on the bench top and the last 10 days under moderate light of approximately 3,000 lx using a 16-h photoperiod. Produced spores were liberated in 6 ml of 0.15% Tween 20 and filtered through a double layer of Miracloth (Calbiochem). Conidia were counted with the aid of a hemocytometer. The mycelia (300 mg) were homogenized using a mortar and pestle under liquid nitrogen. After the addition of 3 ml ethanol, the absorbance at 454 nm was determined using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech) (18). Alternatively, pigment production on PDA cultures was visually estimated.

**Mutagenesis by random plasmid integration of a strain transformed with the virus cDNA clone.** A full-length cDNA clone of CHV1-EP713 mutant strain Cys(72) that has a Cys-to-Gly substitution at p29 at Cys72 and that caused phenotypic alterations similar to those induced by Δp29 in EP155 when transfected was described previously by Suzuki et al. (35). After being liberated from the pRF4-based plasmid by NotI, Cys(72) cDNA was transferred into a transformation vector, pCPXbn1 (14), with the benomyl-resistant gene as the select-
able marker to form pBn1-Cys(72). Recombinant DNA procedures were performed according to methods described previously by Sambrook and Russell (30).

One of the transformant strains with pBn1-Cys(72), TCys(72)-1, was mutated by random insertion with a plasmid (pHygR) that contained the hygromycin B phosphotransferase gene (hph) controlled by the Aspergillus nidulans trpC promoter (P<sub>trp</sub>) and terminator (T<sub>trp</sub>) (10) at the EcoRV site of pBluescript SK<sup>+</sup> (Stratagene) (sequence is available upon request). Spheroplasts of TCys(72)-1 were prepared as described above, retransformed with pHygR, and subjected to single conidal isolation to obtain homokaryons with insertion mutations. The resulting transformants were screened for mutants with phenotypes that were different from that of the parental strain TCys(72)-1. This screening procedure was based on the visual estimation of fungal phenotypes and comparison with TCys(72)-1 cultured on PDA plates in parallel.

The genetic stabilities of the phenotypes of TCys(72)-1 and mutated fungal strains were examined by comparing the colony morphologies and drug resistances of approximately 50 single conidal isolates and isolates passaged four times to those of the original isolates.

### DNA isolation and Southern blot analysis

Total nucleic acids were prepared from mycelia of fungal strains cultured in 20 ml PDB as described previously by Suzuki et al. (37). Chromosomal DNA was enriched by digestion with RNase A overnight at 37°C, followed by phenol-chloroform extraction and ethanol precipitation. Ten micrograms of DNA was digested with the appropriate restriction enzymes overnight at 37°C. The digested DNA was treated with phenol-chloroform, ethyl alcohol precipitated, and suspended in water. The DNA was applied to a well in 0.7% agarose gel, electrophoresed, and blotted onto a Hybond-N+ nylon membrane (Amersham Biosciences, Buckingham, England). The membrane was washed twice in 5× SSC (1× SSC is 0.15 M sodium chloride, 0.15 M sodium citrate [pH 7.0]), baked at 80°C for 10 min, and then probed with digoxigenin (DIG)-11-UTP-labeled DNA fragments amplified from genomic DNA or cDNA according to methods recommended by the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany). Prehybridizations and hybridizations were carried out at 42°C using the DIG Easy-Hyb Granules kit according to the manufacturer's instructions (Roche). Hybridized bands were detected with a DIG Luminescence kit and a CDP Star kit (Roche). Chemiluminescence signals were visualized on film.

### RNA preparation and Northern blot analysis

Total nucleic acids were prepared from mycelia of fungal strains cultured in PDB as described above. Total RNA was enriched by eliminating fungal chromosomal DNA with two-round digestion of the extracted nucleic acids with RQI Dnase I (Promega, Madison, Wis.) in the presence of an RNase inhibitor (40 units) (Toyobo, Tokyo, Japan) for 1 h at 37°C. After phenol, phenol-chloroform, and chloroform extractions and ethanol precipitation, total RNA was suspended in sterile distilled water at a final optical density at 260 nm of 25.

For Northern blot analysis, single-stranded nucleic acids were precipitated from total nucleic acids with LiCl (2 M) and treated with twice with RQI Dnase I. Total single-stranded RNA (ssRNA) was extracted twice by phenol-chloroform and chloroform extractions, and the resulting RNA was subjected to capillary transfered onto a Hybond-N+ nylon membrane (Amersham Biosciences, Buckingham, England) as described previously by Suzuki et al. (37). Procedures for prehybridization, hybridization, and detection of hybridization signals were described previously (37).

### Plasmid rescue and I-PCR

Genomic DNA (10 μg) obtained from fungal strains was cut with an appropriate restriction enzyme, which digested the inserted plasmid at one site. After extraction by phenol-chloroform, the digested products were self-linked by T4 DNA ligase (3 units) at 4°C overnight. For I-PCR, the ligated DNA (200 ng) was mixed with the primer set HG15 and HG18 (10 pmol) (see Table S1 in the supplemental material) and the long and accurate (LA) Taq polymerase (2.5 units) (Takara) in a 50-μl reaction solution (10 mM Tris-HCl [pH 8.0], 50 mM KCl, 2.5 mM deoxynucleoside triphosphate). Reactions consisted of 4 min of initial denaturation at 94°C followed by 35 thermal cycles: a denaturation step at 94°C for 30 s, an annealing step at 52°C for 30 s, an extension step at 72°C for 10 min, and a final extension step at 72°C for 10 min. When needed, nested PCR was performed on I-PCR products in which the primer set consisting of HG21 and HG12 was used under the same reaction conditions as those used for I-PCR.

### Nucleotide sequencing of genomic DNA and cDNA of nam-1

The sequence of genomic DNA containing a 8.5-kb region of the nam-1 gene was amplified by using a primer-walking method. First, I-PCR products were sequenced with hph-specific primers to obtain the sequences of the junction regions of pHygR integrated into chromosomes and the restriction enzyme sites used in I-PCR. The 6.5-kb DNA fragment that encoded the nam-1 gene was amplified by genomic PCR with the primer set Nan5′ and Nam25, whose sequences were obtained by sequencing of the I-PCR products, and purified by a Wizard SV gel and PCR clean-up system kit (Promega) according to the manufacturer's protocol. The PCR fragments or fragments cloned into the pGEM-T Easy vector (Promega) were sent to Macrogen, Inc. (Seoul, South Korea) to be sequenced with a series of Nam primers listed in Table S1 in the supplemental material.

An almost-full-length cDNA of nam-1 was synthesized by using SuperScript II reverse transcriptase (Invitrogen) and an oligo(dT) primer and amplified by two rounds of PCR with the primer set Nam1C and Nam1C15 and subsequently with the primer set Nam2C and Nam1C16. This fragment was sequenced using a series of Nam and NamC primers (see Table S1 in the supplemental material). The terminal sequences of nam-1 mRNA were determined by using an RLM-RACE kit (Ambion). For 3′ rapid amplification of cDNA ends (RACE), cDNA was synthesized from mRNA isolated as described above using the 3′ RACE adapter containing a 41-base primer: amplified by PCR with a primer set, the 3′ RACE outer primer and a gene-specific primer, Nam59, and subsequently amplified with the 3′ inner primer and Nam60. The protocol for 5′ RACE was described previously (39). The deoxyoligonucleotide supplied in the kit was ligated into mRNA treated with calf intestinal phosphatase and tobacco acid pyrophosphatase (decapping enzyme). The resulting RNA was subjected to cDNA synthesis using random decamers and SuperScript reverse transcriptase and PCR amplification with primer sets consisting of the 5′ RACE outer primer and Nam1 as well as the 5′ inner primer and Nam2.

Both genomic and complementary cDNAs were sequenced at least twice from two directions. The determined sequences were examined for PCR misincorporation.

### Complementation assay

A few plasmid clones were prepared for functional complementation of the nam-4 mutant. After determining the sequences of both flanking regions of the pHyr insertion sites, genomic DNA fragments of the wild-type allele were amplified on EP155 chromosomal DNA using LA PCR with oligonucleotide primer sets consisting of Nam5′ and Nam25, Nam5 and Nam30-C, and Nam5 and Nam42 (see Table S1 in the supplemental material for sequences). The resulting fragments of approximately 6.5 kb, 7.5 kb, and 8.5 kb, respectively, were cloned into pGEM-T Easy vector (Promega). After digestion with NotI, the DNA fragments were moved to vector pBSD1 to obtain the complementation constructs pBSDnamC1, pBSDnamC2, and pBSDnam3. pBSD1 contained a coding domain for blasticidin S deaminase that confers blasticidin S resistance at the EcoRV site of pBluescript pSll (Stratagene). Spheroplasts of the nam4 mutant were transformed with the complementing constructs as well as the empty vector pBSD1. The resulting transformants were selected on PDA containing 150 μg/ml blastidicin S (Nacalai Tesque, Kyoto, Japan) or a 1/10,000 (v/v) dilution of the blastidicin S-based fungicide Bla-S (Nihon Tokushu Nouyaku, Nihonmatsu, Japan).

### Construction of a replacement vector and targeted gene disruption

Targeted gene disruption was achieved by homologous recombination-based gene replacement in the yeast strain previously established for this fungus (16, 21). A disruption plasmid clone was constructed using pHygR with the pBluescript S(+) background. A genomic DNA fragment of approximately 1.7 kb (map positions 698 to 977) was amplified by PCR using chromosomal DNA of EP155 as the template and the primer set Nam5 and Nam10 (see Table S1 in the supplemental material) and cloned between the two NotI sites of the pGEM-T Easy vector (Promega). After liberation with NotI, the fragment was subcloned into the NotI site of pHygR. The 3′ 1.3-kb region of nam-1 (map positions 3851 to 5116) was amplified by PCR using primers Nam2-C and Nam25. This fragment was inserted into the HindIII and KpnI sites of the intermediate plasmid. The resulting replacement vector was then used to transform TCys(72)-1, the parental strain for mutagenesis.

### Nucleotide sequence accession number

The sequence reported in this study was deposited in the GenBank/EMBL/DDBJ database under accession number AB300388.

## RESULTS

Transformation of EP155 with a full-length cDNA clone of Cys(72). Several mutants of prototype hypovirus strain CHV1-EP713 were characterized previously (35, 36). Surmising that different host factors could be isolated using different viral mutants, we chose a site-directed mutant, Cys(72), that induced mild symptoms, allowing the host to sporulate relatively

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This moderate level of conidiation was important for the screening procedure that required single conidial isolation to obtain homokaryons. Full-length cDNA of Cys(72) was moved into pCPXBn1 to obtain pBn1Cys(72) and was used to transform EP155. More than 10 transformants with pBn1Cys(72) were selected on benomyl-containing PDA and examined for copy numbers of the viral cDNA integrated into the host chromosomes, phenotypic characteristics, and virus replication. Most benomyl-resistant strains showed symptoms typical of strains transfected with the in vitro-synthesized RNA of Cys(72) (35). However, they have different copy numbers of pBn1Cys(72), ranging from 1 to 3, and relative genetic stability when examined for their colony morphologies and abilities to grow on benomyl-PDA. Considering copy number and genetic stability, one of the transformants, TCys(72)-1, was selected for mutagenesis by plasmid insertion. In Southern blot analyses (Fig. 1), a probe hybridizing the p29 coding sequence detected only a single major band when the chromosomal DNA of TCys(72)-1 was digested by XbaI, KpnI, MfeI, and AfeI (Fig. 1). These enzymes digest the cDNA sequence of Cys(72) at one site (KpnI or XbaI) or more than one site (the rest of enzymes) downstream of the p29 coding sequence, while the foundation vector pCPXBn1 has no (AflI or MfeI), one (XbaI), or more than one (KpnI) cut site. Southern analysis indicated that TCys(72)-1 contained one copy of pBn1Cys(72) per genome. Integrated cDNA was maintained through the next generations in which virus infection could be launched by the transmitted Cys(72) or initially from transcripts of the chromosomally integrated Cys(72) cDNA and, subsequently, by autonomous replication by the viral RNA polymerase complex (27).

Screening of the mutant collection for phenotypes different from that of TCys(72)-1. Spheroplasts of TCys(72)-1 were transformed with pHygR for mutagenesis by random plasmid insertion and selected on hygromycin-containing PDA. A mutant population of homokaryonic single conidial isolates was created and observed for phenotypes differing from those of parental strain TCys(72)-1 showing symptoms caused by Cys(72). Of the 1,052 transformants containing pHygR, 109 showed altered phenotypes, while many of the remaining transformants manifested a phenotype that was indistinguishable from that of TCys(72)-1. One of the altered strains, designated nam4, was subjected to further characterization in this study. EP155 infected by CHV1-EP713 was severely reduced in...
sporulation and pigmentation relative to virus-free EP155, while the parental strain TCys(72)-1 was restored in pigmentation like EP155 infected by a p29 null mutant virus, p29, as previously reported (35). However, mutant nam4 had irregular margins with a number of “protrusions” and was reduced in pigment production and asexual sporulation. The nam4 phenotype resembled that of the EP67 strain containing a virus of Michigan origin reported previously by Anagnostakis (see Fig. 2).
Averages and standard deviations were calculated from six measurements for conidiation and virulence for each strain. In the virulence assay, the areas of the lesions induced on commercially purchased apples were measured at day 14 postinoculation.

**Phenotypic changes in VC-nam** is virus species and virus strain specific. As shown in Fig. 2, the namA mutant reverted to the wild-type (parental) phenotype upon virus elimination, and infection of VC-namA with certain strains of CHV1-EP713 was required to regain the phenotype. We next determined whether the phenotype alteration manifested by VC-namA was virus species specific. A relatively well-characterized mycoreovirus of *C. parasitica*, MyRV1, was chosen because a transfection protocol was available for this virus (18). Interestingly, unlike the VC-namA strain infected with Cys(72), no significant difference was observed in colony morphologies of VC-namA and EP155 or VC-TCys(72)-1 that were infected with MyRV1 (Fig. 2C). Thus, the mutated gene in VC-namA was considered to be involved in symptom induction by wild-type CHV1 but not by MyRV1.

Furthermore, several CHV1-EP713 mutants (Δp29, Δp40b, and Δp69b) were introduced into VC-namA and VC-TCys(72)-1 by fusing them with the donor strain EP155 infected with the respective virus strains. Upon infection with the wild type, CHV1-EP713, Δp29, or Δp40b, VC-namA showed a reduced growth rate and repressed pigmentation with irregular mycelial extensions (Fig. 2D). As observed with namA, levels of growth suppression and numbers and lengths of hyphal extensions were different between cultures of single strains infected with each virus. These phenotypic characteristics were very similar to those exhibited by namA, being distinguishable from VC-TCys(72)-1 or EP155 infected with the respective viruses that showed regular symptoms as previously reported (36).

Irregular colony morphology of virus-infected VC-namA was concurrent with the firm colony edge (Fig. 2D). Interestingly, no significant differences in colony morphology were, however, detected between VC-namA and EP155 or VC-TCys(72)-1 strains when infected with Δp69b (Fig. 2D).

Comparative measures of other biologic properties of VC-namA and EP155 infected with different viral strains are summarized in Table 2. Conidiation levels are usually decreased...
upon infection by CHV1-EP713 derivatives, although different viral strains have different magnitudes of impact (36). With abnormality of colony morphology, a further decrease in conidiation was found in virus-infected VC-nam. When infected with wild-type CHV1-EP713, Δp29, Δp40b, or Cys(72), VC-nam4 manifested a 1- to 2-log reduction in conidiation levels relative to those of EP155 or VC-TCs(72)-1 infected with the respective viruses. For example, VC-nam4 infected with wild-type CHV1-EP713 produced conidia at 2.2 × 10^4 conidia/ml, a reduction of over 2 magnitudes relative to EP155 infected by CHV1-EP713 (2.8 × 10^6 conidia/ml). However, no significant difference in conidiation was found in strains VC-nam4 and EP155 infected with either Δp69b or MyRV1. Uninfected VC-nam4 and EP155 also sporulated at similar levels (1.9 × 10^4 versus 1.6 × 10^6 conidia).

Pigment production was also reduced in VC-nam4 infected with Δp29 or Δp40b compared to EP155 infected with the respective viruses, while VC-nam4 showed a level of pigment production similar to those of EP155 and VC-TCs(72)-1 (Fig. 2A and D). Therefore, repression in pigment production and asexual sporulation were observed only in strains that showed altered colony morphologies upon virus infection (Fig. 2A and D and Table 2).

In contrast, no significant difference in virulence was observed between the VC-nam4 and EP155 backgrounds, regardless of virus infection, when assayed with apples (Table 2). Lesions of similar sizes (ranging from 7.46 to 9.47 cm^2) were formed on apples inoculated by the fungal strains EP155 or VC-nam4 that carried wild-type, Δp29, and Δp40b virus strains. Uninfected EP155 or VC-nam4 induced much larger lesions (17.15 and 17.43 cm^2), while Δp69b and MyRV1 caused a severe reduction in the size of the lesions formed by those fungal strains (2.49 to 2.85 cm^2).

Transgenic supply of either p29 or p40 confers the nam4 phenotype to VC-nam4 infected by Δp69. The observation that VC-nam4 shows an irregular phenotype similar to that of nam4 upon infection by Δp29 or Δp40, but not by Δp69, raises the possibility that the manifestation of the nam4 phenotype in VC-nam4 requires either p29 or p40. To test this, effects of transgenic expression of the proteins on the colony morphology of Δp69-infected VC-nam4 were investigated. Interestingly, transformants of VC-nam4 with the p29 coding domain (VC-nam4/p29) were similar in phenotype to nam4 when infected with Δp69 (Fig. 3). Likewise, a transgenic supply of p40 (VC-nam4/p40) (Fig. 3) or p69 (VC-nam4/p69) (data not shown) altered the phenotype of Δp69-infected VC-nam4 into the nam4 type. Δp69-infected VC-nam4/p29 and VC-nam4/p40 were similar to each other while slightly different in the shapes of mycelial extensions. In contrast, Δp69-infected VC-nam4 that had been transformed with the empty vector pCPXBSD1 showed symptoms that were identical to those of Δp69-infected EP155. These results indicate that either p29 or p40 is required for VC-nam4 to show the nam4 phenotype when infected with Δp69.

Determining the insertion site of the mutagenic plasmid HygR and the sequence of its flanking region. The mutant nam4 was expected to have two different plasmid clones, pBn1Cys(72) and the mutagenic plasmid pHyrR. Based on the assumption that a copy of the hph sequence detected by Southern blotting (Fig. 2B) contributed to the phenotype of nam4, we used I-PCR to amplify DNA fragments adjacent to the insertion site. As shown in Fig. 4A, I-PCR on HindIII-, Xhol-, and SacI-digested chromosomal DNA of nam4 and VC-nam4 with the primer set HGI5 and HGI8 allowed the amplification of a large DNA fragment. A series of hygromycin resistance gene-specific (HG) primers are specific to the hph sequence (see Table S1 in the supplemental material). The sizes of I-PCR-amplified fragments were approximately 9 kb and 8 kb for HindIII and SacI digests, respectively, of chromosomal DNA from nam4 or VC-nam4 (Fig. 4A). I-PCR on Xhol-digested DNA of nam4 and VC-nam4 amplified two major fragments of approximately 6 kb and 3.5 kb and one minor 4.5-kb fragment. DNA of the expected size of 6 kb was produced from mutagenic plasmid DNA by I-PCR (pHygR) (Fig. 4A), while no amplification was observed from genomic DNA from EP155. Nested PCR was performed on I-PCR products as shown in Fig. 4B. The nested PCR led to the amplification of large amounts of specific single DNA fragments (9 kb for HindIII, 4.5 kb for Xhol, and 8 kb for SacI) (Fig. 4B). The sizes of the nested-PCR-amplified fragments were in accordance with the sizes of the DNA fragment observed in the Southern blot analysis (Fig. 2B and 4B). The lack of differences in I-PCR and nested PCR profiles between nam4 and VC-nam4 provided additional evidence that pHyrR was retained stably in VC-nam4.

These inverse and nested PCR fragments were sequenced directly or after being cloned into the pGEM-T Easy plasmid. A chromosomal DNA fragment of approximately 8.5 kb spanning both sides of the integrated plasmid was sequenced. I-PCR and nested PCR on SacI-cleaved/self-ligated DNA provided the flanking region on the opposite side of that obtained by HindIII and Xhol DNA. The sequence of the 8.5-kb region, excluding the pHyrR sequence, and the plasmid insertion site are shown in Fig. S1 in the supplemental material. The corresponding region in the wild-type allele was also sequenced. The sequence of the inserted mutagenic plasmid is available upon request. Linearization of plasmid pHyrR at the AmpR coding domain and deletion of a small region of the plasmid occurred during the insertion event.

FIG. 3. Effects of transgenic expression of the ORF A-encoded proteins on the colony morphology of VC-nam4. Spheroplasts of VC-nam4 were transformed with pCPXBSD1 containing the coding domains for p29 (VC-nam4/p29) or p40 (VC-nam4/p40). To introduce Δp69b, independent transformants were fused with EP155 infected with the mutant viral strain. Virus-free transformed fungal colonies and corresponding Δp69b-infected fungal colonies were cultured on PDA for 10 days. A fungal strain transformed with the empty vector was grown in parallel (pCPXBSD).
To ensure insertion site and sequence integrity, genomic LA PCR was performed (Fig. 4C). When PCRs were conducted with the primer set Nam2 and Nam5 (see Fig. 5A for primer map positions), each specific to the endogenous sequence, all three fungal strains tested, EP155, nam-4, and VC-nam-4, gave products of the expected size of 5 kb (Fig. 4C). The primer set consisting of Nam5 and hph-specific primer HG21 amplified a 6-kb fragment in nam-4 and VC-nam-4 but not in EP155 that did not harbor pHygR.

**Complementation assay.** To determine whether the insertion mutation identified above was associated with the virus infection-specific phenotypic changes in nam-4 (Fig. 2A), three DNA fragments of approximately 6.5 kb, 7.5 kb, and 8.5 kb, containing the corresponding unaltered region in the insertion site, were LA PCR amplified from chromosomal DNA from wild-type EP155 (wild-type allele). These fragments were cloned into plasmid pBSD1 containing the blasticidin resistance gene as the selectable marker (pBSDnamC1, pBSDnamC2, and pBSDnamC3) (Fig. 5A) and transformed into mutant strain nam-4 to determine their abilities to complement the phenotypic abnormalities. For each transforming DNA clone, several transformants were obtained. Colony morphologies of representative isolates are shown in Fig. 5B. Consequently, all the plasmid clones complemented the altered phenotype of the mutant and conferred a TCys(72)-1 phenotype to nam-4; that is, nam-4 transformed with the DNA fragments manifested regular mycelial growth without hyphal extensions and restoration of pigment production to the level shown by TCys(72)-1 (nam-4/namC1, nam-4/namC2, and nam-4/namC3) (Fig. 5B). Transformants with pBSDnamC2 showed a slight increase in pigmentation compared to those with the other two complementing DNA clones. Transformation with the empty vector pBSD1 failed to restore the phenotype of TCys(72)-1 (nam-4/pBSD1) (Fig. 5B). Two independent transformants with pBSD1 were similar in pigmentation and growth rates but different in the irregular margins from each other (Fig. 5). These results indicated that the 6.5-kb DNA fragment was sufficient for the complementation of defects in a gene(s) involved in the nam-4 phenotype.

**Characterization of the nam-1 gene.** Based on data from the complementation assay (Fig. 5), the 6.5-kb fragment may contain sufficient sequence for a functional gene mutated in nam-4. cDNA from mRNA derived from the wild-type allele was synthesized, and its nucleotide sequence was determined to reveal the organization of the gene, termed nam-1. The exon-intron structure of nam-1, which is composed of eight exons and seven introns, is shown in Fig. 5A. An ORF starts at the AUG triplet at map positions 19 to 21 and ends with the TAG codon at map positions 4381 to 4383 (the transcription start site on the genomic DNA is numbered 1). Most splicing sites conform to the GT/AG rule, except for the first intron, where it is 5’-GT-AG-3’ rather than 5’-GT-GG-AG-3’. A putative CAAT box and a TATA box (see Fig. S1 in the supplemental material) are found at positions -95 to -98 and -155 to -158, respectively, and are more distantly located from the transcription start site than usual. Additional TATA and CAAT sequences are found in further upstream regions (see Fig. S1 in the supplemental material). The nucleotide sequences of the genomic DNA and cDNA of nam-1 mRNA and the deduced amino acid sequence of NAM-1 comprising
1,257 amino acids are shown in Fig. S1 in the supplemental material. To confirm the expression of the *nam-1* gene in wild-type strain EP155 and the parental strain TCys(72)-1, Northern analysis was pursued. It was, however, difficult to detect *nam-1* transcripts when total RNA fractions were used. Thus, poly(A)-enriched RNA fractions were prepared for Northern analysis. As a result, *nam-1* was shown to be expressed in the two *C. parasitica* strains (Fig. 6A). No significant difference in *nam-1* transcription levels between fungal colonies infected and uninfected with the wild-type CHV1 or Cys(72) was found, suggesting that *nam-1* was not responsive to hypovirus infection (Fig. 6A).

Dawe et al. (13) constructed a publicly available EST library containing 2,200 genes from *C. parasitica*. *nam-1* was not found in the EST collection. However, a BLAST search of the GenBank library with the deduced amino acid sequence of NAM-1 revealed similar hypothetical proteins from other ascomycetous, filamentous fungi including *Neurospora crassa*, *Gibberella zeae*, and *Chaetomium globosum*. The sequence similarities were found over the entire sequences (see Fig. S2 in the supplemental material). The levels of identities found in NAM-1 and its counterpart were 38%, with an E value of 2e-172, for *N. crassa*; 34%, with an E value of 3e-121, for *G. zeae*; and 34%, with an E value of 3e-121, for *C. globosum*. Highly conserved sequence stretches were detected in the middle and C-terminal regions. The equivalent, hypothetical proteins of *N. crassa* and *C. globosum* have a C-terminal extension of approximately 250 amino acids (aa) and a deletion of approximately 200 aa in the central region, respectively, thus being different in size from NAM-1 (see Fig. S2 in the supplemental material).
A search of motif databases with the NAM-1 sequence identified the CorA domain at its C-terminal region (aa 792 to 1170), as defined by PFAM family PF01544 (Fig. 6B), in addition to a number of consensus sequences including glycosylation sites, phosphorylation sites, myristoylation sites, and leucine zipper motifs that are frequently found in protein sequences. Interestingly, the CorA motif was conserved in the counterparts of filamentous fungi. The N-terminal portion of NAM-1 contains the PFAM DUF1777 domain at aa 133 to 253 shared by the counterpart of G. zeae but not by that of N. crassa or C. globosum. The CorA consensus sequence is found in prokaryotic and eukaryotic members of the CorA Mg$^{2+}$/H$^{+}$-transporter domain and a less characterized domain, DUF1777, are shown in the NAM-1 sequence. For the overall alignment, see Fig. S2 in the supplemental material. All proteins contain the CorA domain, while only the counterpart from G. zeae possesses the DUF1777 domain. Accession numbers for the GenBank database are SM_955371 for N. crassa, SM_381249 for G. zeae, SM_001226467 for C. globosum, Q4BIY7 for B. vietnamiensis, and Q4IVJ8 for A. vinelandii. The two transmembrane domains, TM1 and TM2, within the CorA consensus sequence are boxed. At the C terminus of TM1, the signature sequence GMN is strictly conserved. Asterisks refer to amino acids that are strictly conserved, while colons and dots indicate changes to chemically similar amino acids.
Disruption of nam-1 in TCys(72)-1 results in virus infection-specific alteration of the colony phenotype. To further confirm the involvement of mutations of the nam-1 gene in the phenotypic change found in nam4, a nam-1 disruption construct that contained an hph gene cassette inserted between the 5′ and 3′ sequences of nam-1 was prepared (Fig. 7A). The construct was designed to delete six exons in the central region of the nam-1 gene (Fig. 7A) based upon homologous recombination-based gene replacement. A population of approximately 120 transformants of TCys(72)-1 with the construct was screened for disruptants using selection on hygromycin-containing PDA and subsequently by genomic PCR. Several independent disruptant candidates, such as TCys(72)-1/Δnam-1-1 and TCys(72)-1/Δnam-1-2, were obtained. Targeted disruption was further confirmed by Southern blot analysis (Fig. 7B); that is, probe 1, spanning map positions 191 to 1381, hybridized to 5.3 kb genomic DNA was digested by XbaI. No signal was found in the disruptant candidates with probe 2 harboring the nam-1 sequence that was expected to be deleted, while a 5.3-kb band was detected in EP155 and TCys(72)-1. An hph probe (probe 3) detected the expected signals (2.2 kb) only in disruptant candidates [TCys(72)-1/Δnam-1-1 and TCys(72)-1/Δnam-1-2] but not in EP155 or TCys(72)-1. These data clearly indicated that TCys(72)-1/Δnam-1-1 and TCys(72)-1/Δnam-1-2 were nam-1 disruptants.

TCys(72)-1/Δnam-1-1 and TCys(72)-1/Δnam-1-2 showed similar colony morphologies (Fig. 7C). The disruptants had uneven, dense aerial hyphae and lacked the protrusions possessed by nam4 (Fig. 7C). The disruptants were also reduced in growth rate and pigmentation compared with TCys(72)-1 (Fig. 7C). The suppression in pigmentation found in the disruptants was pronounced when cultured in PDB (Fig. 8). Levels of pigmentation suppression were exhibited by the disruptants and were similar to those found in nam4, which were lower than those in TCys(72)-1 and much lower than those in EP155 or VC-nam4 (Fig. 8). These showed the involvement of nam-1 in host pigmentation. To determine the phenotype of virus-free nam-1 disruptants, TCys(72)-1/Δnam-1-1 and TCys(72)-1/Δnam-1-2 were cured of the Cys(72) virus as in the case of nam4 and TCys(72)-1 [VC-TCys(72)-1/Δnam-1-1 and VC-TCys(72)-1/Δnam-1-2]. In the absence of virus infection, the disruptants VC-TCys(72)-1/Δnam-1-1 and VC-TCys(72)-1/Δnam-1-2 were identical to VC-nam4 and EP155 (data not shown).
These results showed that the disruption of nam-1 in the TCys(72)-1 backgrounds had a virus infection-specific effect on colony morphology. The data also suggested that nam-1 was involved in the altered phenotypic changes in nam \( A \), but the disruption of the gene was not sufficient to reproduce its phenotype. Altered, longer versions of nam-1 transcripts contribute to the nam \( A \) phenotype. The observation that the morphologies of TCys(72)-1/\( \Delta \)nam1-1 and nam4 were not identical (Fig. 7C) suggested that the phenotype of nam4 may be due to expression of the modified nam-1 transcripts with altered function rather than because of a simple disruption of its function. To examine this possibility, EP155, nam4, and TCys(72)-1/\( \Delta \)nam1-1 were subjected to Northern analysis. Consequently, while no signal was observed in TCys(72)-1/\( \Delta \)nam1-1 at the expected size (approximately 4 kb), transcripts of nam-1 were detected in EP155 and nam4 (Fig. 9A). Nevertheless, transcripts of a slightly large size than that in TCys(72)-1 or EP155 were detected in nam4 (Fig. 6A and 9A, compare lane 1 to

FIG. 8. Comparison of pigment production of nam-1 disruption mutants with those of other strains. (A) Pigment production of a nam-1 disruptant [TCys(72)-1/\( \Delta \)nam1-1] in PDB medium. The disruption mutant was cultured in 20 ml PDB (Difco) in parallel with its parental strain, TCys(72)-1, Cys(72)-containing and VC-nam4 strains, and EP155 for 12 days on a laboratory bench at 25 to 27°C and photographed. (B) Relative levels of pigmentation of different fungal strains. Three hundred milligrams of harvested mycelia from each strain shown in Fig. 8A was pulverized under liquid nitrogen and homogenized in 3 ml absolute ethanol (18). An aliquot of the mixture was transferred into a 1.5-ml microcentrifuge tube, incubated at room temperature for 3 h followed by centrifugation at 12.5 krpm for 1 min, and photographed. (C) Graphical representation of relative levels of pigmentation of different fungal strains. Pigmentation was measured as the absorbance at 454 nm by a densitometer. Ten replicates for each isolate of nam4, TCys(72)-1/\( \Delta \)nam1-1, TCys(72)-1, EP155, and VC-nam4 were used for the measurements to calculate averages and standard deviations.

FIG. 9. Contribution of an altered longer version of nam-1 transcripts to the nam4 phenotype. (A) Northern hybridization analysis to detect nam-1 transcripts. Ten micrograms of poly(A)-enriched RNA isolated from the mycelia of wild-type strain EP155, the nam4 mutant, and the nam-1 disruptant with the TCys(72)-1 backgrounds, TCys(72)-1/\( \Delta \)nam1-1, was electrophoresed in a 1.4% gel under denaturing conditions and transferred onto a nylon membrane for hybridization with a nam-1-specific, DIG-11-dUTP-labeled probe (top). \( /H9252\)-tubulin mRNA was used as a loading marker. To detect \( /H9252\)-tubulin mRNA as a loading marker, 3 \( /H9262\)g of RNA from the same poly(A)-enriched fraction as that in the top panel was probed by a DIG-11-dUTP-labeled probe amplified on \( /H9252\)-tubulin cDNA (bottom). \( \beta \)-Tubulin mRNA was used as a loading marker. To detect \( \beta \)-tubulin mRNA as a loading marker, 3 \( /H9249\) of RNA from the same poly(A)-enriched fraction as that in the top panel was probed by a DIG-11-dUTP-labeled probe amplified on \( \beta \)-tubulin cDNA (bottom). (B) Effects of expression of modified nam-1 on the colony morphology of TCys(72)-1/\( \Delta \)nam1-1. A 6-kb genomic DNA fragment carrying the HygR sequence on exon 8 of nam-1 was amplified on the genomic DNA of nam4 by LA PCR using primer sets Nam5 and HG17 and cloned into the NotI site of pBSD1 (pBSD-nam1-HygR). Exons/introns of nam-1, the pHygR integration site, and primers' map positions are shown in Fig. 5A. Spheroplasts of the TCys(72)-1/\( \Delta \)nam1-1 strain were transformed with pBSD-nam1-HygR to generate TCys(72)-1/\( \Delta \)nam1-1/nam1-HygR1. The resulting transformant strain was cultured on PDA in parallel with the TCys(72)-1, TCys(72)-1/\( \Delta \)nam1-1, and the nam4 mutant strain for 8 days on the laboratory bench at 25 to 27°C.
lane 2). This may indicate that the plasmid insertion into the last exon of the nam-1 gene in nam4 (Fig. 5) leads to the production of extended transcripts containing the partial pHygR sequence. Data from 3' RACE supported this possibility and showed that the altered nam-1 transcripts in nam4 contained a deletion of the 3'-terminal region of approximately 150 nucleotides (nt) and an extension of approximately 450 nt derived from the pHygR plasmid sequence (see Fig. S3 in the supplemental material). The integration of pHygR induced an amino acid sequence change at the C terminus from ——RS TEGKPPGGRIKNGNIL [in EP155 or TCys(72)-1] to ——T NTVLLV (in nam4). Thus, it seemed likely that the generation of modified transcripts was involved in the nam4 phenotype.

To test this possibility, the nam-1–pHygR sequence was amplified from genomic DNA of nam4 by LA PCR with the primer pair Nam5 and HG17 (see Fig. 5A for primers' map positions) and cloned into the NotI site of pBSD1. The resulting plasmid, pBSD1-nam1-HygR, which covered the extended region of the altered nam-1 transcripts, was used to transform TCys(72)-1/Δnam1-1. Transformation of TCys(72)-1/Δnam1-1 with pBSD1-nam1-HygR resulted in colony morphology that resembled that of nam4 (Fig. 9B) and was characterized by an irregular margin with mycelial protrusions and reduced pigmentation. However, unlike in nam4, no firm colony edge was observed in TCys(72)-1/Δnam1-1 transformed with pBSD1-nam1-HygR. Fungal colonies of TCys(72)-1/Δnam1-1 cultured in parallel were reduced in pigment but increased in arial hyphae relative to TCys(72)-1 (Fig. 9B). These results showed that the extension of transcripts of the nam-1 gene contributed to the phenotype of nam4.

DISCUSSION

To understand virus-host interactions, we need to identify host factors involved in virus replication and macroscopic symptom induction. Allen et al. (1) and Deng et al. (14) previously used microarray analyses to identify hypovirus infection-responsive genes in C. parasitica and subsequently validated the approach. Here, we describe the development of a genetic screening protocol where a collection of fungal strains carrying artificially induced mutations is mined for host factors involved in hypovirus symptom expression. The method involved (i) the integration of the full-length cDNA to the genomic RNA of a hypovirus single-amino-acid substitution mutant, Cys(72), into the host genome, allowing the launch of the virus infection in a theoretically every fungal cell (7); (ii) mutagenesis by plasmid insertion; (iii) isolation of mutants of interest; and (iv) identification of causative mutated genes by PCR. This method enables the elimination of an inoculation step that usually requires tremendous labor and time in screening. Plasmid insertion for mutagenesis allows the direct identification of mutated genes by PCR or plasmid rescue. Furthermore, this mutagenesis approach is important because little is known about natural fungal host variations in symptom expression that are governed genetically. This is in contrast to plant viral hosts for which a number of natural mutants with different symptom responses to certain viruses and virus susceptibility are reported at the variety or ecotype level (26).

The nam4 mutant isolated by this method manifested a phenotype that was distinguishable from that of its parent, TCys(72)-1, while the virus-cured corresponding strains were identical in colony morphology [VC-nam4 compared to VC-TCys(72)-1 (Fig. 2A); that is, the irregular phenotype in nam4 was produced only upon infection by Cys(72)]. More intriguingly, the nature of the mutant phenotype is virus specific and virus strain specific. When infected with CHV1-EP713 or its mutant virus strain Δp29 or Δp40, VC-nam4 shows symptoms similar to those of nam4 but different from those of EP155 or VC-TCys(72)-1 infected with the respective virus strains. Colonies of VC-nam4 infected with the hypoviral strains were characterized by irregular colony morphology with mycelial extensions and reduced pigmentation and sporulation (Table 2 and Fig. 2A and D and 8) while retaining the ability to confer similar levels of hypovirulence (Table 2) and support similar levels of viral accumulation (see Fig. S4 in the supplemental material). However, infection with Δp69b results in the induction of symptoms that are indistinguishable between VC-nam4 and VC-TCys(72)-1 (Table 2 and Fig. 2D), which suggests a requirement of p29 or p40 for phenotypic differences between the two host backgrounds. This idea was proven to be correct by transformation analysis in which a transgenic supply of either p29 or p40 conferred the nam4 phenotype to VC-nam4 infected by Δp69b (Fig. 3). To our knowledge, this is the first description of such a virus-specific phenotype in filamentous fungal hosts. Δp69b and MyRV1 induce a similar set of phenotypic alterations: fungal colonies infected by the two viruses manifest severe reductions in the growth of aerial hyphae and attenuation of virulence with apple assay while retaining levels of pigmentation and conidiation comparable to those exhibited by virus-free colonies (18, 36). Previously, p29 and/or p40 was shown to contribute to the restoration of the growth of aerial hyphae of fungal colonies infected by Δp69b (36) and MyRV1 (34), while their modes of action were considered to be different from each other. These activities of p29 and p40 may be related to the infection-specific phenotypic alterations in VC-nam4 that require the growth of aerial mycelia.

We demonstrated that plasmid insertion into nam-1 contributed to the phenotype of nam4 by complementation and disruption assays. The wild-type allele of nam-1 of EP155 complemented the colony morphology of nam4, and the disruption of the nam-1 gene in the TCys(72)-1 background resulted in infection-specific phenotypic alterations similar to but distinguishable from the phenotype exhibited by nam4 (Fig. 7C). The observation that disruptants of nam-1 in the TCys(72)-1 background [TCys(72)-1/Δnam1-1] did not show a phenotype identical to that of nam4 may be accounted for in several ways that are not mutually exclusive. As expected from the insertion site of plasmid pHygR (Fig. 5A), Northern blot analysis revealed that sizes of transcripts different from those in EP155 were produced in nam4 (Fig. 9A). Sequence analysis of the 3′ RACE clones showed that the nam-1 transcripts in nam4 contained a deletion of 150 nt and an extension of approximately 450 nt derived from pHygR at the 3′ terminus that could direct the synthesis of a modified version of NAM-1 (see Fig. S3 in the supplemental material). This altered NAM-1 is considered to contribute to the irregular growth of mycelia and repressed pigmentation and conidiation in nam4 by playing a different role from that of wild-type NAM-1. This notion is supported by the data shown in Fig. 9B in which transformants
of TCys(72)-1/Δnam1-1 with the DNA fragment including the nam-1 and pHygR sequences of nam4 [TCys(72)-1/Δnam1/nam1-HygR] show a greater resemblance to nam4 in colony phenotype. TCys(72)-1/Δnam1/nam1-HygR exhibited still a slightly different phenotype from that of nam4.

Given that the mutation of this gene results in a severe reduction in pigmentation and sporulation in addition to abnormal growth characteristics (Fig. 2 and Table 2), it is likely that the expression of NAM-1 is involved in the alleviation of symptoms caused by hypoviruses. The molecular mechanism underlying the alleviation of hypovirus-mediated symptom expression has yet to be unraveled. However, some clues to its functional role were supplied by computer-assisted analysis. A BLAST search identified sequence similarities between NAM-1 and counterparts in other filamentous fungi that comprised 952 to 1,506 aa (see Fig. S2 in the supplemental material). A motif search showed that NAM-1 has a well-defined functional domain, CorA, as well as frequently observed consensuses. CorA is found at the C-terminal region of all the NAM-1 counterparts in filamentous fungi (Fig. 6) and in eukaryotic and prokaryotic CorA family members. CorA members are one of the classes of Mg2+-transporters to serve as membrane-associated Mg2+ transport systems that serve as Mg2+-dependent Mg2+ channels in the plasma membrane (25, 33).

Typical CorA homologues are 300 to 500 aa in length with two domains: a large N-terminal cytoplasmic domain and a shorter C-terminal transmembrane domain. Unlike other well-characterized CorA members, NAM-1 contains an extremely long N-terminal portion. S. cerevisiae also possesses two orthologs of CorA, ALR1 (encoding 859 aa) and ALR2 (encoding 858 aa), that are responsible for aluminum resistance and that also have larger N-terminal extensions. The Alr proteins possibly mediate Mg2+ uptake in Al3+-induced Mg2+ deficiency, and their N-terminal regions are presumably involved in Al3+-binding (25). However, no discernible sequence similarities between the N-terminal portions of NAM-1 and Alr1p, Alr2p, or other known sequences in the GenBank database were found, excluding the filamentous counterparts shown in Fig. 6 and Fig. S2 in the supplemental material. A motif search identified an additional domain, DUF1777 (PFAM), in the N-terminal region of NAM-1 whose functional role has yet to be assigned clearly (Fig. 6). The long N-terminal portion of NAM-1 is likely to be involved in unrecognized functions as in the yeast Alr proteins.

Mg2+ is involved in numerous enzymatic reactions as a cofactor and phosphotransfer influencing structures of nucleic acids, proteins, and membranes and controlling the activities of Ca2+ and K+ channels in the plasma membrane (25, 33). Therefore, defects in CorA member proteins would result in detrimental effects on fundamental cellular functions, e.g., lethality in pathogenic bacteria (29) and growth arrest in yeast (22). Although whether NAM-1 is a functional Mg2+ transporter like other CorA members remains to be elucidated biochemically, the conservation of the CorA domain containing the two transmembrane sequences and the GMN motif in NAM-1 (Fig. 6) strongly suggests that nam-1 encodes an Mg2+ uptake system most probably in the plasma membrane based on the PSORT prediction. The pHygR integration into nam-1 of nam4 causing a replacement of 18 aa with seven unrelated amino acids (see Fig. S3 in the supplemental material) may lead to the abnormal functionality of NAM-1. Therefore, the reduced pigmentation and sporulation found in nam4 and nam-1 disruptants (Fig. 8) may be a consequence of a perturbation in Mg2+ homeostasis. Coordinated hyphal growth of fungi is also regulated in a Ca2+-dependent manner (3). Addressing how phenotypic alterations specific to infection by CHV1 viral strains occur in nam4 and nam-1 disruptants and whether those alterations happen upon infection by other hypovirus species like CHV2, CHV3, and CHV4 (28) is an interesting challenge that warrants further investigation.

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REFERENCES

fungus *Cryphonectria parasitica* that is infectious as particles and related to the *Coltivirus* genus of animal pathogens. J. Virol. 78:892–898.