Synergism between a mycoreovirus and a hypovirus mediated by the papain-like protease p29 of the prototypic hypovirus CHV1-EP713

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Infection of the chestnut blight fungus, Cryphonectria parasitica, by the prototypic hypovirus Cryphonectria hypovirus 1-EP713 (CHV1-EP713) or by the type member, Mycoreovirus 1-Cp9B21 (MyRV1-Cp9B21), of a novel genus (Mycoreovirus) of the family Reoviridae results in hypovirulence, but with a different spectrum of phenotypic changes. The former virus depresses pigmentation and conidiation dramatically, whilst the latter virus has little effect on these processes. This study showed that double infection by the two viruses resulted in a phenotype similar to that of CHV1-EP713 singly infected colonies, but with further decreased levels of host conidiation and vegetative growth and increased levels of MyRV1-Cp9B21 genomic dsRNA accumulation (twofold) and vertical transmission (sixfold). In contrast, CHV1-EP713 RNA accumulation was not altered by MyRV1-Cp9B21 infection. It was also found that the papain-like cysteine protease p29, encoded by CHV1-EP713 ORF A, contributes to the phenotypic alterations and transactivation of MyRV1-Cp9B21 replication and transmission. Chromosomally expressed p29 was able to increase MyRV1-Cp9B21 vertical transmission by more than twofold and genomic RNA accumulation by 80%. Transactivation was abolished by Cys→Gly mutations at p29 residues 70 and 72 located within the previously identified symptom-determinant domain required for suppression of host pigmentation and sporulation and p29-mediated in trans enhancement of homologous Δp29 mutant virus RNA replication. Transactivation was not altered by Ser substitutions at the p29 protease catalytic residue Cys162. These results indicated a link between p29-mediated enhancement of heterologous virus accumulation and transmission and p29-mediated host symptom expression. The role of p29 as a suppressor of RNA silencing is discussed.

INTRODUCTION

The observation that individual plant or animal hosts can be infected naturally by multiple viruses is well established (e.g. Pruss et al., 1997; Tsatsralt-Od et al., 2006). Although naturally occurring mixed virus infection has also been reported for a variety of fungal hosts (e.g. Ikeda et al., 2004; Peever et al., 1997; Tuomivirta & Hantula, 2005), the consequences of these multiple infections are less well studied, partly due to limitations in the experimental manipulation of mycoviruses and their fungal hosts. In this regard, the chestnut blight fungus, Cryphonectria parasitica, and associated mycoviruses provide one of the few tractable experimental systems for examining mycovirus–fungal host interactions.

C. parasitica has been shown to support the replication of members of five RNA virus families: Hypoviridae, Reoviridae, Narnaviridae, Partitiviridae and Chrysoviridae (Hillman & Suzuki, 2004). DNA-mediated transformation of C. parasitica is very efficient (Churchill et al., 1990), allowing targeted disruption of genes within the haploid genome (reviewed by Dawe & Nuss, 2001). An infectious cDNA-based reverse-genetics system has been developed for several members of the family Hypoviridae, providing the opportunity to manipulate the genomes of both a mycovirus and its fungal host (Chen & Nuss, 1999; Choi & Nuss, 1992b). Protocols have also been developed to initiate infection of C. parasitica spheroplasts with mycovirus particles purified from infected C. parasitica cultures (Hillman et al., 2004).

The family Hypoviridae comprises four species, Cryphonectria hypovirus 1 (CHV1), CHV2, CHV3 and CHV4,
which are distinguished by genome organization and symptom induction (Hillman & Suzuki, 2004; Nuss et al., 2005). The prototype hypovirus, CHV1-EP713, is the most intensively studied of the *C. parasitica*-infecting viruses (Dawe & Nuss, 2001). This hypovirus attenuates *C. parasitica* virulence significantly and severely reduces fungal conidiation, pigmentation and female fertility. These macroscopic changes are accompanied by modulation of approximately 13·4% of the *C. parasitica* transcriptome (Allen et al., 2003) and alteration of several cellular signal-transduction pathways (reviewed by Nuss, 2005).

The CHV1-EP713 genome is 12·7 kbp in size and contains two continuous open reading frames (ORFs), A and B. Each ORF encodes a papain-like protease, p29 and p48, respectively, responsible for self-cleavage from the respective polyproteins. The papain-like protease p29 is derived from the N-terminal portion of the ORF A polyprotein, p69, which is a multifunctional protein (Dawe & Nuss, 2001). Choi et al. (1991a, b) demonstrated p29-associated autoproteolytic activity and mapped the catalytic cysteine and histidine residues to the C-terminal region of the protein. The p29 protein was also shown to contribute both *in cis* and *in trans* to reduced conidiation and pigmentation and to enhance replication and vertical transmission of the homologous virus, CHV1-EP713 (Choi & Nuss, 1992a; Craven et al., 1993; Suzuki et al., 1999, 2003). Thus, transgenic expression of p29 results in suppression of orange pigment production and asexual sporulation, whilst deletion of 88% of the p29 coding domain in the context of an infectious cDNA of CHV1-EP713 partially alleviates suppression of pigmentation and conidiation. Replication and transmission through conidia of a Δp29 deletion mutant virus are enhanced by expression of p29, either from the fungal chromosomes (*in trans*) or from the CHV1 genome (*in cis*). Moreover, Segers et al. (2006) showed recently that p29 suppresses small interfering RNA (siRNA)-mediated, sequence-specific RNA degradation (RNA silencing) in the fungal host and also in a non-host plant. As RNA silencing is considered to be a host defence response against virus infection, p29 also appears to function as a counter-defensive element, as described for a number of RNA silencing suppressors of plant viruses (e.g. Anandalakshmi et al., 1998; Kasschau & Carrington, 1998).

The 9B21 strain of *Mycoreovirus 1* (MyRV1-Cp9B21) is the type member of the newly established genus *Mycoreovirus* within the family *Reoviridae* (Mertens et al., 2005; Suzuki et al., 2004). Purified MyRV1 particles are multi-shelled, characteristic of reoviruses, and are infectious when transfected into virus-free protoplasts (Hillman et al., 2004). The virus attenuates virulence of the host fungus to an even greater extent than hypovirus CHV1-EP713, but affects pigmentation and asexual sporulation only minimally (Hillman et al., 2004). Sequence analysis and partial biochemical analysis of the genomic segments have identified segments S1, S3 and S6 as encoding the RNA-dependent RNA polymerase, guanylyltransferase and NTP-binding protein, respectively (Hillman et al., 2004; Supyani et al., 2007; Suzuki et al., 2004).

Here, we report the synergistic effects of mixed infection of *C. parasitica* by the two distinct mycoviruses MyRV1-Cp9B21 and CHV1-EP713. We also present evidence that CHV1-EP713 p29, independent of its protease activity, augments the transmission and replication of an unrelated heterologous reovirus, but not of the related CHV2-NB58 hypovirus. The mechanism underlying this phenomenon is considered in view of the role of p29 as a suppressor of RNA silencing.

**METHODS**

**Fungal strains and culturing.** Three *C. parasitica* field isolates, EP155, NB58 and 9B21, belonging to different vegetative compatibility groups were used in this study. Strain EP155 transformed with the coding domain for wild-type p29 (Twtp29) or with mutation of Cys162 (T/Cys162) or Cys162 (TCys70Cys162) has been described previously (Suzuki et al., 2003). TCys162 is an EP155 transformant containing a p29 coding domain with a Cys→Ser substitution at the protease catalytic residue 162, whilst TCys70Cys162 is a transformant containing p29 with a Gly substitution at Cys70 and a Ser substitution at Cys162. Isolate NB58 infected with CHV2-NB58 and its virus-free isogenic strain NB58-19 were a generous gift from Dr Bradley Hillman of Rutgers University, NJ, USA (Hillman et al., 1994). *C. parasitica* strain 9B21, naturally infected with reovirus MyRV1-Cp9B21, was provided by Dr William MacDonald of West Virginia University, WV, USA, and Dr Bradley Hillman. An isogenic virus-free isolate 9B21-ss1 was derived from the parental strain by single conidial isolation (Hillman et al., 2004).

Fungal colonies were grown for 5–10 days on a bench top at 24–26 °C on potato dextrose agar (PDA; Difco) for morphological observation and in potato dextrose broth (PDB; Difco) when mycelia were used for RNA extraction. For maintenance, strains were cultured on regeneration plates (Churchill et al., 1990) and stored at 4 °C in a refrigerator until use.

**Virus transmission assay.** Frequencies of vertical transmission through asexual spores were determined as described by Suzuki et al. (2003). Fungal strains infected with MyRV1-Cp9B21 were cultured for 2 weeks on the bench top. Asexual spores were liberated in distilled water and spread on 10 cm PDA plates at appropriate dilutions. Single conidial germlings were transferred to new PDA plates at a density of 10 germlings per plate and cultured for 2 weeks. The number of infected colonies was scored based on visual observation of distinctive virus infection-associated colony morphologies.

The ability to distinguish CHV1-EP713 singly infected and CHV1-EP713/MyRV1-Cp9B21 doubly infected conidial isolates visually was complicated by an unexplained atypical phenotypic variation occasionally observed for CHV1-EP713-infected strains. CHV1-EP713-infected EP155 colonies grown on PDA typically produce aerial hyphae and expand slightly slower than the uninfected strain. Colonies exhibiting the atypical phenotype do not produce aerial hyphae and expand much slower than the uninfected strain, with hyphae at the colony margin tending to penetrate deep into the solid media. The basis for the atypical colony morphology is unknown and a switch back to the typical colony morphology can occur spontaneously.
Consequently, dsRNA was isolated from the single conidial isolates derived from doubly infected strains to ensure accurate measurement of MyRV1-Cp9B21 transmission.

**Construction of mutant virus cDNAs.** Site-directed substitution of Ser for Cys\(^{162}\) within the p69 coding domain was carried out by a PCR-based, overlap extension method (Sambrook & Russell, 2001). The Afl\(_II\)–Eco\(_R\) fragment (map positions 450–2506) of pLDST (Choi & Nuss, 1992b) containing the coding sequence for wild-type p69 was cloned into the baculovirus transfer vector pAcYM1 (Matsuura et al., 1987) to form pAcwtp69. The N-terminal and C-terminal regions of the p69 coding region were amplified separately from pAcwtp69 with the thermostable KOD DNA polymerase (Toyobo) and the mutagenic primer sets pAc1 (5′-ATGGTGGCTGATATCATGAGATA-3′) and pAc-2 (5′-CAACACAAGGCTACAGTTATATCCTGCGC-3′); the bold nucleotide causes a Cys→Ser codon change) and C162S-FW (5′-GGGCCAGAGAT-AACGTTGACCTGCCCCAATGCT-3′); the bold nucleotide causes a Ser codon change) and C162S-RV (5′-GTTATCTCTCGGCC-3′); the bold nucleotide causes a Ser codon change).

The resulting PCR fragment was gel purified and cloned into pGEM-T Easy (Promega). After digestion with SacI and SpH\(_I\), the coding sequence for p69 with a Cys→Ser mutation at p29 Cys\(^{50}\) (p69C162S) was cloned into the fungal expression vector pCPXHY1 (Craven et al., 1993). To obtain p69 double mutants at residues Cys\(^{162}\) and Cys\(^{70}\) or Cys\(^{72}\) (p92C70C162S and p92C72C162S, respectively), the same method was utilized as for p69C162S, except that template was the CHV1-EP713 cDNA containing the Gly substitution at Cys\(^{70}\) or Cys\(^{72}\) [virus Cys\(^{70}\) (or virus Cys\(^{72}\), respectively; Suzuki et al., 1999]. These mutant p69 coding sequences were cloned into pFastBac Dual (Invitrogen) for expression in insect cells. The mutant p29 coding domains were amplified from the p69 mutants using primers NS26 and NS27 (Suzuki et al., 2003) and cloned into pCPXHY1 for expression in fungal cells. All clones were examined for PCR misincorporation by sequencing.

**Baculovirus expression of ORF A-encoded proteins.** The wild-type and mutant coding sequences of p29, p40 and p69 were cloned into pAcYM1 (Matsuura et al., 1987) or pDualBac (Invitrogen). The transfer vector pAcYM1 with inserts was transfected with BD BaculoGold baculovirus DNA (BD Biosciences Pharmingen) into Spodoptera frugiperda (S\(\Phi\)9) cells, whilst inserts in pFastBac Dual were transfected to baculovirus DNA present as bacmics in DH10Bac Escherichia coli cells and then transfected into S\(\Phi\)9 cells according to the manufacturer’s protocol (Bac-to-Bac Baculovirus Expression System; Invitrogen). Cell culture and subsequent protein analysis were performed according to the methods of Matsuura et al. (1987) and Suzuki et al. (1994).

**Virulence assay.** Virulence of fungal colonies was measured with the apple assay, as described by Fulbright (1984) and Hillman et al. (2004). Commercially available apples were washed with ethanol, inoculated with plugs of freshly grown mycelia of different fungal strains after and incubated on a bench top (25–27°C). Parafilm was used to cover the inoculation sites and was removed after 1 week. Lesions were measured at days 10 and 14 post-inoculation.

**Transfection and transformation of C. parasitica spheroplasts.** Spheroplasts of the C. parasitica virus-free strains 9B21s1, NB58-19 and EP155 were prepared by the method of Churchill et al. (1990) and transformed with cDNA encoding wild-type or mutant p29 coding regions containing a Cys→Gly substitution at residue 70 or 72 (Suzuki et al., 2003) or a Cys→Ser substitution at position 162. The wild-type and resulting transformant strains were transfected with purified particles of MyRV1-Cp9B21 (Hillman et al., 2004) or with synthetic transcripts of CHV1-EP713 (Chen et al., 1994). CHV2-NB58 was transmitted into these virus-free transformants with the NB58-19 backgrounds by anastomosis with NB58 (Hillman et al., 1994).

**RNA preparation and viral dsRNA quantification.** Total RNA was prepared from C. parasitica mycelia cultured in 20 ml PDB as described by Suzuki & Nuss (2002). Harvested mycelia were homogenized with a pestle and mortar in the presence of liquid nitrogen. Nucleic acids were isolated by two rounds of phenol/chloroform extraction in 4 ml 100 mM Tris/HCl (pH 8.0), 200 mM NaCl, 4 mM EDTA, 4% SDS and precipitated by the addition of 2 vols ethanol. To eliminate fungal chromosomal DNA, extracted nucleic acids were treated twice with RNA DNase I (Promega), followed by phenol, phenol/chloroform and chloroform extractions and ethanol precipitation. The final RNA concentration was adjusted to an absorbance reading of 25 at 260 nm and used for agarose-gel electrophoresis.

Viral genomic dsRNA was quantified by densitometry (Suzuki et al., 2003). Total RNA extracted as above was electrophoresed in a 1–4% agarose gel in 1 x TAE [40 mM Tris/acetate (pH 7.8), 1 mM EDTA] buffer system and stained with ethidium bromide. RNA bands visualized using a UV lamp under a transilluminator were photographed digitally at different exposures. Images were read and analysed in an Atto densitometer using software provided by the manufacturer.

Relative amounts of MyRV1 genomic RNA were estimated by measuring the amount of S3 RNA segment normalized to the amount of host fungal 185 rRNA. The level of CHV2-NB58 genomic RNA accumulation was quantified similarly.

**Northern blot analysis.** Total ssRNA (8 μg) obtained by LiCl fractionation was electrophoresed in a 1% non-denatured agarose gel in 1 x TAE. This gel system can be used to distinguish viral mRNA from genomic dsRNA that might contaminate the ssRNA fraction. Fractionated RNA in an agarose gel was capillary-transferred on to Hybond-N+ nylon membrane (Amersham Biosciences) and denatured in 50 mM NaOH, 10 mM NaCl for 5 min. After washing twice in 2 x SSC and baking at 80°C for 2 h, the membrane was probed with digoxigenin (DIG)-11-dUTP-labelled DNA fragments amplified from cDNA of MyRV1-Cp9B21 S3 by PCR according to the method recommended by the manufacturer (Roche Diagnostics). Pre-hybridization and hybridization were carried out with the DIG Easy-Hyb Granules kit according to the instructions provided by the supplier (Roche). Hybridized bands were detected with a DIG detection kit and a CDP-Star kit (Roche). Chemiluminescent signals were visualized on film or in a Hamamatsu Photonics real-time image processor (model Argus-50; Hamamatsu Photonics KK).

**RESULTS**

**Hypovirus symptom expression is dominant over that of a mycoreovirus, MyRV1** To determine whether synergism was found in a mixed infection by a hypovirus, CHV1-EP713, and a distinct reovirus, MyRV1-Cp9B21, the two viruses were introduced into C. parasitica strain EP155. As reported previously (Hillman et al., 2004), MyRV1 infection resulted in enhanced production of brown-coloured pigments and no significant reduction in conidiation, reaching the level of 10\(^8\) conidia ml\(^{-1}\), similar to virus-free EP155. In contrast, CHV1 infection caused a severe reduction in asexual sporulation and pigmentation (Fig. 1a, c). The reduction in growth rate caused by CHV1 was similar to that caused by

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MyRV1. Doubly infected colonies were whitish in colour with no orange pigments and significantly reduced in growth compared with singly infected colonies. Asexual spore production was also severely reduced to the level of less than $10^{3}–10^{4}$ conidia ml$^{-1}$, which was one to two orders of magnitude less than that exhibited by CHV1-infected colonies (Fig. 1c). Under high light conditions, which generally alleviate the CHV1-induced reduction in sporulation and pigmentation (Hillman et al., 1990), conidiation levels were several logs lower for the doubly infected strain compared with CHV1-infected colonies (data not shown).

When assayed with apples, cankers induced by the virus-free EP155 strain were approximately 12 cm$^{2}$ in size, whilst cankers caused by MyRV1-infected EP155 were much smaller (Fig. 1b, c). Lesions caused by doubly infected colonies (~3 cm$^{2}$) were significantly larger than those caused by MyRV1-Cp9B21-infected colonies (~1 cm$^{2}$) and were comparable to those caused by CHV1-infected EP155. Thus, with the exception of conidiation and vegetative growth rate, which were reduced relative to either singly infected strain, most biological properties of doubly infected colonies were similar to those exhibited by colonies infected singly with CHV1, showing that CHV1 was dominant in symptom development.

**Mycoreovirus replication and transmission are elevated by co-infection with the hypovirus CHV1**

An increase in MyRV1-Cp9B21 dsRNA levels was observed for colonies infected with the two viruses (Fig. 2a, compare lanes 2 and 3 with lanes 4 and 5). Quantification of MyRV1 genomic RNA accumulated in fungal strains infected with both viruses revealed a 90% increase in MyRV1 dsRNA relative to the level in singly infected colonies (Fig. 2b). By contrast, no apparent elevation of CHV1-EP713 dsRNA levels was observed in the co-infected strain (compare lanes 4 and 5 with lanes 6 and 7). These results suggest that CHV1 co-infection results in enhancement in trans of MyRV1 replication.

In the case of CHV1 Δp29 and Δp69 mutant viruses, genomic RNA accumulation levels correlated well with their vertical transmission through conidia (Suzuki et al., 2003). We tested whether the increase in MyRV1 genomic RNA accumulation...
observed in doubly infected colonies also resulted in an altered MyRV1 transmission rate. Conidial isolates recovered from doubly infected colonies all contained virus, either CHV1 alone or CHV1 and MyRV1. The frequency of MyRV1 transmission was 65-9 % for doubly infected colonies, which was much greater than the transmission frequency of MyRV1 for singly infected colonies (9-4 %) (Table 1).

**Hypovirus p29 contributes to symptom expression of mixed infections**

Based on previous observations that the papain-like protease p29 encoded by CHV1-EP713 suppresses host pigmentation and conidiation, irrespective of whether it is expressed from the virus genome or from host chromosomes (Craven et al., 1993; Suzuki et al., 1999, 2003), we anticipated that p29 might also contribute to the symptoms displayed in the mixed infection. To test this possibility, we transfected p29 transformants with MyRV1-Cp9B21. Transformants with the wild-type p29 coding domain had reduced pigment production and sporulation in the absence of virus (Fig. 3). Transformants infected with MyRV1 displayed colony morphology intermediate between EP155 strains transformed with the p29 coding domain (Fig. 3a, virus-free Twtp29) and infected with the mycoreovirus (Fig. 3a, MyRV1-infected EP155). Thus, the p29 transformants infected with MyRV1 were reduced in pigmentation relative to EP155 infected with MyRV1 and had more aerial mycelia. Transgenic expression of p29 also resulted in enlargement of lesions on apples induced by MyRV1 (Fig. 3b, c; compare MyRV1-infected EP155 and Twtp29). However, MyRV1-infected Twtp29 produced conidia to a level of $10^7$–$10^8$ ml$^-1$, which was similar to the levels ($10^8$ ml$^-1$) exhibited by MyRV1-infected EP155 (Fig. 3c). Therefore, p29 contributes to the symptoms observed for doubly infected colonies, but does not appear to be responsible for the severe repression in conidiation observed for the mixed infection.

**Hypovirus p29 elevates mycoreovirus RNA accumulation and transmission**

Suzuki et al. (2003) showed previously that transgenic expression of p29 complemented defects in mutant virus Δp29 replication and transmission, increasing genomic dsRNA accumulation from approximately 40 to 90 % relative to that of wild-type CHV1-EP713 and enhancing the mean frequency for the mutant virus transmission through conidia from approximately 50 to 90 %. Thus, it was of interest to determine whether p29 elevated RNA accumulation and transmission of the unrelated virus MyRV1-Cp9B21. Total RNA was isolated from p29-transformed mycelia infected with MyRV1 and subjected to 1-4 % agarose-gel electrophoresis. Although MyRV1 has 11 dsRNA segments, S1–S11, ranging from 4127 to 732 bp in size, segments S5 and S6, S7 and S8, and S9 and S10 co-migrated under the gel conditions used (Hillman et al., 2004). Importantly for this study, none of the viral segments co-migrated with the rRNA that was used to normalize viral dsRNA accumulation measurements. As shown in Fig. 4(a), MyRV1 genomic dsRNA accumulated to higher levels (~80 % higher relative to that in non-transformants) in transformant strains expressing the wild-type p29 coding sequence (Fig. 4a, compare lane Twtp29 with EP155; Fig. 4b). Although some variation was observed in MyRV1 mRNA levels, as represented by the S3 mRNA (Fig. 4c), MyRV1 mRNA levels were consistently higher in...
Table 1. Efficiency of MyRV1 transmission through conidia

Strains are described in Supplementary Table S1 (available in JGV Online).

<table>
<thead>
<tr>
<th>Host strain</th>
<th>No. infected/no. tested spore germlings</th>
<th>Total</th>
<th>% (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP155</td>
<td>3/100, 1/50, 20/106</td>
<td>24/256</td>
<td>9.4 (±9.5)</td>
</tr>
<tr>
<td>EP155 doubly infected*</td>
<td>11/17, 12/18, 52/80, 12/17</td>
<td>87/132</td>
<td>65.9 (±2.7)</td>
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<tr>
<td>Twtp29†</td>
<td>9/60, 42/144, 48/150, 19/79 54/149</td>
<td>169/582</td>
<td>29.0 (±8.3)</td>
</tr>
<tr>
<td>TCys70‡</td>
<td>2/60, 17/89, 22/150, 6/100</td>
<td>47/399</td>
<td>11.8 (±7.4)</td>
</tr>
<tr>
<td>TCys72‡</td>
<td>4/70, 20/139, 22/130, 6/117</td>
<td>50/456</td>
<td>11.0 (±6.0)</td>
</tr>
<tr>
<td>TCys162‡</td>
<td>45/180, 44/180</td>
<td>89/360</td>
<td>24.7 (±0.4)</td>
</tr>
<tr>
<td>9B21ss1</td>
<td>6/90, 7/140, 3/66, 2/100</td>
<td>18/396</td>
<td>4.5 (±1.9)</td>
</tr>
<tr>
<td>9Bwtp29</td>
<td>40/100, 14/100, 50/150, 25/100</td>
<td>129/450</td>
<td>28.7 (±11.1)</td>
</tr>
</tbody>
</table>

†EP155 transformed with the CHV1-EP713 gene encoding wild-type p29 and infected with MyRV1-Cp9B21.
‡EP155 transformed with the CHV1-EP713 gene encoding the respective mutant proteins p29C70G (TCys70), p29C72G (TCys72) or p29C162S (TCys162) and infected with MyRV1-Cp9B21.

Fig. 3. Colony morphology and virulence of p29 transformants infected with MyRV1-Cp9B21. (a) The transformant lines Twtp29, TCys70 and TCys72 were obtained previously by introduction of the coding domains for wild-type p29 and mutant p29 harbouring Cys→Gly changes at positions 70 or 72, respectively (Suzuki et al., 2003). These strains plus the non-transformant EP155 were infected with MyRV1-Cp9B21, cultured for 1 week on the bench top and photographed (bottom row). Uninfected EP155 and p29 transformants were cultured in parallel (top row). Photographs were taken 1 week after inoculation of PDA plates. (b) Apples were inoculated with plugs of PDA cultures of virus-free or MyRV1-infected EP155 and Twtp29 strains. Cankers on apples were photographed at day 10 post-inoculation. (c) Means ± SD of canker sizes, taken from three measurements, are shown for strains EP155 and Twtp29, either uninfected or infected with MyRV1-Cp9B21. Filled bars, virulence; empty bars, conidiation.
p29 transformants (Twp29) (Fig. 4c, lanes 5 and 6) and doubly infected strains (Fig. 4c, lanes 3 and 4) than in wild-type EP155 (Fig. 4c, lanes 1 and 2).

MyRV1 was transmitted at a rate of 29% in conidia derived from infected colonies of the p29-expressing transformants (Twp29) compared with a rate of 9\%\(\pm\)4% for the mean transmission frequency in conidia derived from the corresponding infected untransformed strain (Table 1). The increase found in Twp29 was statistically significant (\(P<0.05\)). The combined results shown in Figs 3 and 4 and Table 1 indicated that, as observed for the homologous CHV1-Dp29 mutant virus, CHV1 p29 can function \textit{in trans} to enhance the transmission and genomic RNA accumulation of the mycoreovirus MyRV1-Cp9B21.

The effects of p29 expression on MyRV1 infection were also examined in the original MyRV1-infected strain subsequently freed of virus, strain 9B21ss1. A similar increase in MyRV1 transmission from 4.5 to 28.7% (Table 1) and in genome RNA accumulation (data not shown) was observed in the 9B21ss1 host background expressing wild-type p29 (9Bwtp29) as was observed in the EP155 background. Thus, the p29-mediated enhancement \textit{in trans} of MyRV1 replication and transmission is not restricted to the EP155 genetic background.

\textbf{Cys}^{70} and \textbf{Cys} \textsuperscript{72} are required for p29-mediated enhancement of mycoreovirus viral RNA accumulation and transmission

Suzuki \textit{et al.} (1999, 2003) showed by site-directed mutagenesis that \textbf{Cys}^{70} and \textbf{Cys} \textsuperscript{72} play pivotal roles in p29-mediated suppression of pigmentation and sporulation as a symptom determinant and enhancer activities in virus replication and transmission in the homologous CHV1-EP713 Dp29 mutant virus system. Thus, it was interesting to know whether these two residues were also required for enhancement in the heterologous system. As shown in Fig. 4(a, b), MyRV1-Cp9B21 dsRNA accumulated to similar levels in untransformed strain EP155 and transformants containing the p29C70G (TCys70) and p29C72G (TCys72) mutant p29 coding regions. Consistent with these results, vertical transmission of MyRV1 was similar for infected EP155, TCys70 and TCys70 colonies (Table 1). Moreover, transgenic expression of the site-directed mutants of p29 also failed to alter the phenotype of MyRV1-infected colonies, causing the same phenotype as observed for non-transformants infected with MyRV1 (Fig. 3). Thus, the colony morphology of TCys70 and TCys72 infected with MyRV1-Cp9B21 was identical to EP155 non-transformants infected with MyRV1-Cp9B21 (Fig. 3). Lesions
induced on apples by TCys70, TCys72 and EP155 were also similar (data not shown). Thus, we concluded that Cys70 and Cys72 are essential for p29-mediated phenotypic changes in heterologous virus-infected colonies and the augmentation of MyRV1 replication and transmission.

**Cysteine protease activity of p29 is dispensable for transactivation**

The C-terminal region of CHV1-EP713 p29 contains a cysteine protease catalytic domain that is responsible for cleavage of the p69 polyprotein to form p29 and p40 (Choi et al., 1993). As independent functional domains of a single polypeptide can influence the activity of each other (e.g. subtilisin proteases) (Ueda et al., 2003; Zhou et al., 1998), it was of interest to see whether the Cys70 and Cys72 mutations that altered p29-mediated enhancement of MyRV1-Cp9B21 replication and transmission might also alter p29 protease activity. The protease activities of p29 variants were monitored by self-cleavage of its precursor, p69, in insect cells. As shown in Fig. 5, wild-type p69 (Fig. 5, lane 8) was cleaved into p29 and p40, which migrated to the same positions as independently expressed p29 and p40 (Fig. 5, lanes 1 and 2). Baculovirus expression of p69C70G and p69C72G (Fig. 5, lanes 6 and 7) in insect cells resulted in a similar profile to that observed for the wild-type p69 expression (Fig. 5, lane 8). In contrast, mutation at the catalytic residue Cys162 abolished the catalytic activity, regardless of whether Cys70 or Cys72 was changed (Fig. 5, lanes 3–5). These results indicated that loss of p29-mediated transactivation due to mutations at Cys70 or Cys72 is not linked to loss of p29 proteolytic activity.

We next asked whether a p29 mutant in which the catalytic Cys at position 162 was substituted with a Ser residue (p29C162S) to eliminate proteolytic activity would retain the ability to enhance MyRV1 replication and transmission. As shown in Fig. 6(a), transformation of EP155 with the p29C162S coding sequence (TCys162) resulted in reduction of pigmentation and conidiation, consistent with the report by Craven et al. (1993) that the p29 catalytic activity is not required for these symptoms. The colony morphology of p29C162S transformants infected with MyRV1 (Fig. 6a) was indistinguishable from Twtp29 colonies infected with MyRV1, exhibiting more aerial mycelia than the MyRV1-infected untransformed strain. TCys162 colonies infected with MyRV1-Cp9B21 also induced larger lesions on apples than MyRV1-infected non-transformants (EP155) (Fig. 6b), as observed for Twtp29 infected with MyRV1-Cp9B21 (Fig. 3b). The p29C162S transformants also resembled the Twtp29 transformants in supporting enhanced levels of MyRV1 RNA accumulation (Fig. 6c) and significantly enhanced virus transmission (P<0.1) (Table 1). These combined results indicated that p29-mediated transactivation of MyRV1 is independent of p29 protease activity.

**CHV1-EP713 p29 does not enhance replication of the hypovirus CHV2-NB58**

The observation that p29 augmented the replication and transmission of a heterologous mycovirus, MyRV1-Cp9B21, prompted us to examine whether p29 acts on other members of the family Hypoviridae. As observed in C. parasitica strains EP155 (Fig. 3) and 9B21 (not shown), wild-type p29 reduced the orange pigmentation of C. parasitica strain NB58-19, before or after infection with hypovirus CHV2-NB58 (Fig. 7a). Transformants with the coding sequence of mutant p29C72G had an identical colony morphology to untransformed NB58-19. Due to the unavailability of transfection protocols for CHV2, anastomosis of transformant lines of NB58-19 with the p29 coding domain and the CHV2-containing strain NB58 was used to initiate infection. These infected strains were tested for virus genomic RNA accumulation levels. In contrast to the case of the EP155 transformant infected with MyRV1, NB58-19 transformants with the coding domain for wild-type p29 (NBwt29) and p29C72G mutant (NBwC72S) showed the same or slightly lower levels of genomic RNA, suggesting that p29 did not enhance the RNA replication of CHV2-NB58 (Fig. 7, b, c).
DISCUSSION

Antagonistic or synergistic interactions between viruses in single hosts often occur in plants and animals, resulting in milder or more severe disease development and alterations in the levels of accumulation of one or both viruses (Hull, 2002; Mbopi-Keou et al., 2002; Murphy & Bowen, 2006). Such interactions between viruses in mixed infections of fungal hosts (e.g. Ikeda et al., 2004; Peever et al., 1997; Tuomivirta & Hantula, 2005) have not been studied in detail. Taking advantage of transformation and transfection protocols available for C. parasitica hypoviruses and mycoreoviruses, we examined synergism between members of the two virus families and identified a hypovirus-encoded factor that contributes to the synergistic interaction.

Figs 2 and 4(c) and Table 1 showed clearly that CHV1-EP713 enhanced MyRV1-Cp9B21 replication and transmission in trans, whilst CHV1-EP713 replication and transmission appeared to be unaffected by MyRV1-Cp9B21 co-infection. This type of one-way synergistic effect has been described for several plant viruses. For example, potyviruses elevate replication of potex- and tombusviruses, but not vice versa (Goldberg & Brakke, 1987; Vance, 1991). CHV1-EP713 and MyRV1-Cp9B21 belong to two different virus families, Hypoviridae and Reoviridae. CHV1-EP713 is associated with the picornavirus superfamily in which members have positive-sense, ssRNA genomes and include poliovirus and plant-infecting potyviruses, whilst MyRV1-9B21 is a ‘true’ dsRNA virus. CHV1-EP713 is reported to replicate in host-derived membranous lipid vesicles without producing virus particles (Fahima et al., 1994; Jacob-Wilk et al., 2006). Intracellular transcription and replication of reoviruses are believed to occur in virus core particles contained within virus infection-induced viroplasms (Fields, 1996). This difference in replication strategy between the two viruses suggests that CHV1-EP713 enhances MyRV1-Cp9B21 replication indirectly rather than by directly participating in the MyRV1 replication cycle.

Dual infection induced more severe reductions in growth rate and sporulation relative to single infection by CHV1-EP713 or MyRV1-Cp9B21. Symptoms caused by dual infection were, however, more similar to those caused by MyRV1-9B21 alone than to those caused by MyRV1-Cp9B21 alone with respect to pigmentation, production of aerial mycelia and virulence. Thus, CHV1-EP713 seems to be dominant in symptom expression. It was slightly surprising that no additive effects on hypovirulence were seen by double infection. Lesions on apples induced by doubly infected strains were larger than those induced by colonies singly infected with MyRV1-Cp9B21 (Fig. 1). There may be a correlation between virulence and the ability to produce aerial mycelia that is enhanced by CHV1 co-infection (Fig. 1) or by transgenic expression of p29 (Fig. 3). Consistent with this view is the observation that infection by CHV2-NB58 and MyRV1-Cp9B21 resulted in extremely low levels of virulence when examined using the
The CHV1-EP713-encoded papain-like protease p29 clearly contributes to the synergistic effects observed for mixed infection, as shown in Figs 3 and 4 and in Table 1. Thus, transgenic expression of p29 in MyRV1-Cp9B21-infected colonies resulted in a colony phenotype similar to that caused by infection with the two viruses (Fig. 3). In addition to symptom development, p29 contributed to the elevated levels of MyRV1 genomic RNA accumulation and virus transmission through conidia found in the double infection (Fig. 4 and Table 1). Previous studies on p29 have revealed at least three functional domains involved in several aspects of the virus life cycle. The N-terminal 24 codons are necessary for virus replication, whilst the remaining 88% of the coding region is dispensable (Craven et al., 1993; Suzuki et al., 2000). The papain-like protease catalytic region is located in the C-terminal half containing the catalytic residues Cys162 and His215 (Choi et al., 1991a, b). As a symptom determinant, p29 is involved in suppression of pigmentation, sporulation and laccase activities, and the suppressive activity has been mapped to the N-terminal region downstream of the essential domain, at aa 25–73 (Suzuki et al., 1999). Recently, this region was also shown to play critical roles in augmentation of replication and transmission of the homologous virus, irrespective of whether p29 was expressed in cis (from the virus genome) or in trans (from host chromosomes) (Suzuki et al., 2003). The current study extends the functional analysis of p29 by showing that Cys70 and Cys72 residing in the symptom determinant are required for in trans enhancement of replication and virus transmission of the heterologous mycoreovirus MyRV1-Cp9B21.

It is well documented that a functional domain can influence the activity of another functional domain located in another region of the same polypeptide. Choi & Nuss (1992a) and Craven et al. (1993) previously showed that the proteolytic activities of p29 are not directly required for suppression of pigmentation and sporulation, i.e. p29 must be released from the p69 precursor in order to suppress host functions, but the suppressive activity of the released p29 is independent of its proteolytic activity. The current study showed that the p29 proteolytic activity is also dispensable for the elevation of MyRV1 replication and transmission, as the protease-defective p29 mutant, p29C162S, was still active in for enhancement in trans (Fig. 6 and Table 1). Furthermore, the in trans enhancement-defective mutants, p29C70G and p29C72G, were able to cleave p69 (Fig. 5).

Taken together with previous results (Craven et al., 1993; Suzuki et al., 1999, 2003), the current study suggests a correlation between p29-mediated suppression of pigmentation and conidiation and enhanced replication of heterologous and homologous viruses, with both functions mapped to the N terminal region of p29. In contrast, the synergistic effects caused by p29 are clearly independent of p29 autoproteolytic activity. These data will contribute to our understanding of structure/function relationships of this multi-functional protein when its three-dimensional structure is determined.
Segers et al. (2006) recently reported that p29 acts as an RNA silencing suppressor in plants and in C. parasitica. The current observation that p29 enhances the replication of a heterologous virus is consistent with a role for p29 as a suppressor of RNA silencing. The synergistic interactions between CHV1-EP713 and MyRV1-Cp9B21 can be considered to be analogous to those found in plant-infecting potyviruses and other viruses (Hull, 2002). The synergism conferred by potyviruses is thought to be the consequence of strong suppression of RNA silencing as an anti-virus defence response (Kasschau & Carrington, 1998) by a virally encoded, multi-functional protein, HC-Pro (Maia et al., 1998; Kasschau & Carrington, 1998), has been shown to transactivate replication of simultaneously infecting viruses, such as potex-, cucumo-, tombus- and luteoviruses. Although the HC-Pro activity domain for the synergism mapped to the central region (Shi et al., 1996) does not show amino acid sequence similarities to any region of hypovirus p29, the two proteins do share striking similarities, as reported previously (Choi et al., 1991a; Koonin et al., 1991). Similarities include amino acid sequence similarities found between the N- and C-terminal regions of HC-Pro and almost the entire p29 coding region, papain-like cysteine protease activities mapped to the C-terminal region of the two proteins, sequence similarities flanking the respective cleavage sites and the ability to suppress RNA silencing. Another parallel is that both proteins cause developmental alterations in their respective hosts when expressed transgenically in the absence of virus replication (Anandalakshmi et al., 2000; Craven et al., 1993; Suzuki et al., 2003). HC-Pro-mediated morphological changes in the host appear to be due to perturbation of the microRNA (miRNA) pathways (Kasschau et al., 2003). Although a miRNA pathway has not been reported in fungi, it is interesting to consider the possibility that p29 perturbs miRNA pathways to modify developmental processes leading to reduced sporulation and pigmentaion, as does HC-Pro in plant hosts (Kasschau et al., 2003).

In this study, p29 failed to enhance in trans the replication of another member of the family Hypoviridae, CHV2-NB58. CHV2 has a dicistronic genome possessing ORF A and ORF B like CHV1 (Hillman et al., 1994). However, unlike CHV1, CHV2 ORF A does not encode a papain-like protease, CHV2 ORF A and ORF B encode p50, a homologue of CHV1 p40, and p52, a homologue of CHV1 ORF B-encoded p48, considered to be a parologue of p29. It is noteworthy that the N-terminal region of p50, whose biological activities are unknown, seems to be homologous to the corresponding region of CHV1 p29 (Smart et al., 1999). No functional roles, other than papain-like protease activities responsible for co-translational cleavage from the ORF B precursor protein, have been ascribed to p48 or p52. The failure of p29 to transactivate the replication of CHV2-NB58 raises the possibility that CHV2 encodes an RNA silencing suppressor that may act in the same way as p29 and mask the in trans enhancement by ectopic expression of CHV1-EP713 p29.

Studies examining this possibility are in progress.

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