In-tree behavior of diverse viruses harbored in the chestnut blight fungus, *Cryphonectria parasitica*[+]

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Abstract (<250 words)

The ascomycete *Cryphonectria parasitica* causes destructive chestnut blight. Biological control of the fungus by virus infection (hypovirulence) has been shown to be an effective control strategy against chestnut blight in Europe. To provide biocontrol effects, viruses must be able to induce hypovirulence and spread efficiently in chestnut trees. Field studies using living trees to date have focused on a select family of viruses called hypoviruses, especially prototypic hypovirus CHV1, but there are now known to be many other viruses that infect *C. parasitica*. Here we tested seven different viruses for their hypovirulence induction, biocontrol potential, and transmission properties between two vegetatively compatible but molecularly distinguishable fungal strains in trees. The test included cytosolically and mitochondrially replicating viruses with positive-sense single-stranded RNA or double-stranded RNA genomes. The seven viruses showed different *in-planta* behaviors and were classified into four groups. Group I including CHV1 had great biocontrol potential and could protect trees by efficiently spreading and converting virulent to hypovirulent cankers in the trees. Group II could induce high-levels of hypovirulence, but showed much smaller biocontrol potential, likely because of their inefficient virus transmission. Group III showed poor performance in hypovirulence induction and biocontrol, while efficiently being transmitted in the infected trees. Group IV could induce hypovirulence and spread efficiently, but showed poor biocontrol potential. Nuclear and mitochondrial genotyping of fungal isolates obtained from the treated cankers confirmed virus transmission between the two fungal strains in most isolates. These results are discussed in view of dynamic interactions in the tripartite pathosystem.

Importance (<150 words)

The ascomycete *Cryphonectria parasitica* causes destructive chestnut blight, which is controllable by hypovirulence-conferring viruses infecting the fungus. The tripartite chestnut/*C. parasitica*/virus pathosystem involves the dynamic interactions of their genetic elements, i.e., virus transmission and lateral transfer of nuclear and mitochondrial genomes between fungal strains via anastomosis occurring in trees. Here we tested diverse RNA viruses for their hypovirulence induction, biocontrol potential, and transmission properties between two vegetatively compatible but molecularly distinguishable fungal strains in live chestnut trees. The tested viruses, which are different in genome type (single-stranded or double-stranded RNA) and organization, replication site (cytosol or mitochondria), virus form (encapsidated or capsidless) and/or symptomatology, have been unexplored in the aforementioned aspects in

63 controlled conditions. This study showed intriguing different in-tree behaviors of the seven
64 viruses and suggested that to exert great biocontrol effects viruses must be able to induce
65 hypovirulence and spread efficiently in the fungus infecting the chestnut trees.
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Chestnut blight, caused by the ascomycete *Cryphonectria parasitica*, is one of the most destructive tree diseases (1, 2). The fungus infects the stem and branches of susceptible chestnut trees causing rapidly expanding bark lesions, called cankers (Rigling and Prospero, 2018). This disease could be controlled by naturally disseminating viruses conferring hypovirulence in certain areas of Europe or by treating cankers with hypovirus-infected fungal strains (3, 4). Another type of attempt to control chestnut blight in the US is to spray chestnut forests with engineered conidia that carry infectious cDNA of the prototype hypovirus CHV1 (Cryphonectria hypovirus 1) in their chromosomes and are able to allow for CHV1 replication upon gemination (5). The latter treatment is often impaired by the self/non-self intraspecies recognition system, so-called vegetative incompatibility, genetically governed by 6 diallelic loci (*vic1* to *vic4*, *vic6 and vic7*) (6). These loci have been molecularly characterized by Nuss and colleagues (7, 8), and their genotyping can be readily conducted by PCR-based methods (9, 10). To breach the restriction imposed by vegetative incompatibility, Zhang and Nuss prepared a “super donor” formula, a mixture of two independent quadruple disruptions of four out of 6 *vic* genes, which allows for lateral transmission of the prototype hypovirus CHV1-EP713 to any known fungal strain (11).

Other viruses besides CHV1 have been found in *C. parasitica* in natural settings. In North America, the hypovirus CHV3 has been the subject of several studies and releases for attempted biocontrol (12, 13), and CHV4 has been studied to a lesser extent (14). Local screening for presence of CHV2 was performed where it was first identified (15), and broader screenings have been performed in the northeast U.S. for viruses including CHV1, CHV2, CHV3, and CHV4 (16), but these latter studies did not include virus releases. Thus, CHV1 and CHV3 have been the subjects of most of the virus releases for purpose of controlling *C. parasitica* in natural settings, with CHV1 representing the great preponderance.

C. parasitica is also important as a virus host organism for studying virus/virus and virus/host interactions (17, 18). This fungus supports the replication of diverse viruses, not only those discovered in *C. parasitica* itself (referred to here as homologous viruses) (19), but also viruses originally detected in different fungi, as exemplified by several viruses from *Rosellinia necatrix*, which belongs to an order different from *C. parasitica* (referred to here as heterologous viruses) (19-24). These viruses all fall within the realm *Riboviria* accommodating all RNA viruses but retroviruses (25) and span over 10 families including *Hypoviridae*, *Reoviridae*, *Mitoviridae*, *Totiviridae*, and *Partitiviridae* with different genome organizations (19) (N. Suzuki, unpublished results). It is of interest to note that beside CHV1, other homologous viruses originally isolated from *C. parasitica* and some heterologous viruses isolated from different fungi also confer hypovirulence to

the chestnut blight fungus under laboratory conditions. Also of note is that while most viruses of *C. parasitica* are cytosolically replicating, the fungus was the first shown to host a mitochondrially replicating virus (26), now known to be common in fungi.

The tripartite chestnut/*C. parasitica*/virus pathosystem involves the dynamic interactions of their genetic elements that include virus transmission and lateral transfer of nuclear and mitochondrial genomes between fungal strains via anastomosis that occur in trees. Most studies aiming at exploring such tripartite interactions are limited to those with members of the family *Hypoviridae* (hypoviruses) including CHV1, and field-level or chestnut tree-level investigations of other viruses are scarce. CHV1 is able to reduce considerably the pigmentation, sporulation and virulence of host fungus; CHV1-infected fungi typically lose the ability to invade aggressively and kill chestnut trees. In-tree behavior of hypovirulent fungal strains was earlier investigated in American chestnut sprouts (*Castanea dentata*) inoculated in proximity by two vegetatively compatible fungal strains with distinct nuclear genetic backgrounds (27). The study investigated how the virus and host fungus spread in cankers and showed the transmission of an uncharacterized hypovirulence-inducing agent (virus) from the fungus used for challenge inoculation to the virulent fungus used as the primary inoculation. Another related interesting study in a natural setting showed dynamic exchange of genetic materials at the population levels in a chestnut coppice between virus-free resident and experimentally introduced CHV1-infected fungi (28). Long-term monitoring showed efficient spread of CHV1 as well as relatively less efficient spread of the mitochondrial genome of the introduced strain in the resident fungal population.

In the current study, we tested seven different viruses for their hypovirulence induction, biocontrol potential, and spread in the fungus using European chestnut trees (*Castanea sativa*). Biocontrol potential in this study refers to curative effects of virus-infected fungal strains against specific virulent cankers, rather than their biocontrol performance at field-level, which includes spontaneous dissemination of the virus in the pathogen population. This study showed different in-tree behaviors of the viruses and suggested that to exert great biocontrol effects viruses must be able to induce hypovirulence and spread efficiently in the fungus infecting the chestnut trees.

RESULTS

Phenotypes of virus-infected *C. parasitica* strains EP155 and PC7. Fungal and viral strains used in the current study are shown in Table 1. A total of seven RNA viruses were tested. Two dsRNA viruses, Rosellinia necatrix partitivirus 6 strain W113 (RnPV6-W113, bi-segmented dsRNA genome, genus *Betapartitivirus*, family *Partitiviridae*), mycoreovirus 1 (MyRV1-9B21, 11-segmented, genus

Mycoreovirus, family *Reoviridae*), four hypoviruses with positive strand RNA genomes, the prototype hypovirus CHV1 wild type (WT) strain EP713 (CHV1-EP713, genus *Hypovirus*, family *Hypoviridae*), an RNA silencing suppressor (RSS) deletion mutant of CHV1-EP713 (Δp69), Cryphonectria hypovirus 2 strain NB58 (CHV2-NB58, genus *Hypovirus*), Cryphonectria hypovirus 3 strain GH2a (CHV3-GH2a, genus *Hypovirus*), a mitochondrially replicating virus, Cryphonectria mitovirus 1 strain NB631 (CpMV1-NB631, genus *Mitovirus*, family *Mitoviridae*) were tested. In order to trace in-tree spread of both virus and fungus, we introduced viruses into two fungal strains, EP155 and PC7, that were genetically distinguishable based on nuclear genotype as well as mitochondrial haplotype, but belonging to the same vegetative compatibility (VC) type. The standard *C. parasitica* strain EP155 was infected by the above seven viruses, and the viruses were then transferred from EP155 to PC7 via anastomosis. Symptom induction profiles for CHV2-NB58 were similar to those reported earlier for the original CHV2-NB58-bearing *C. parasitica* strains, but were less severe than those reported for CHV3 (13, 29) as discussed below (Fig. 1). Symptom induction in EP155 by the other viruses was as described before (Fig. 1) (22, 30-32). See below for symptom description.

We confirmed through anastomosis experiments that the virulent, virus-free strain PC7 could receive viruses from EP155. All of the seven tested viruses could move from EP155 to the recipient PC7 strain on PDA plates and were stable in the new host. All of these fungal strains were confirmed to be infected by the respective viruses by RT-PCR. Symptoms induced by Δp69, CHV2-NB58 and MyRV1-9B21 were similar to one another and characterized by reduced growth of aerial mycelia and enhanced brown pigmentation. CHV1-EP713 reduced orange pigmentation and mycelial growth rate, while RnPV6-W113 caused severe growth reduction in culture. CHV3-GH2a and CpMV1-NB631 exerted little effect on culture morphology. However, it should be noted that MyRV1-9B21 and RnPV6-W113 were less efficiently moved to PC7. This phenomenon was not due to the host genotype difference but the intrinsic virus attributes, because it was also observed in their transfer from EP155 to EP155. Symptoms induced by these viruses in PC7 grown on PDA were indistinguishable from those in EP155 (Fig. 1) suggesting no significant difference in effects of viruses on virulence attenuation between the two fungal strains.

Virulence of virus-infected *C. parasitica* strain EP155. Three-year-old European chestnut trees (*Castanea sativa*) were used in three different assays to investigate I) virulence; II) in-tree spread of different viruses; and III) biocontrol effects of fungal strains infected with different viruses (see Fig. 2 and Materials and Methods for detail). In assay I, we tested the virulence levels of EP155 infected by the respective viruses using living chestnut trees under controlled conditions. As shown is Table 2,

166 virulence of fungal strains as measured by canker sizes 2, 4, and 6 weeks and 2 months post
167 inoculation varied depending on infecting viruses. Differences among the fungal strains were
168 pronounced as incubation time became longer. Virulence levels measured 2 months post inoculation
169 are shown in Fig. 3. The virulence levels determined by the current study were generally congruent
170 with those reported using their original fungal isolates and other assay systems with apple fruits and/or
171 chestnut dormant stems. Namely, CHV1-EP713, CHV1-Δp69, CHV2-NB58, and MyRV1-9B21
172 induced great hypovirulence, whereas RnPV6-W113-infected EP155 caused much greater cankers,
173 approaching to the level exhibited by virus free EP155. Our CHV3-GH2a-infected EP155 isolate also
174 caused greater sized cankers, indicating a difference between this isolate and the original CHV3-GH2
175 strain (33, 34). CpMV1-NB631, which had never been tested for virulence attenuation using isogenic
176 fungal strains, was shown to confer a level of hypovirulence similar to CHV1-EP713. It was
177 anticipated that RnPV6-W113 induced hypovirulence given the observation that the virus caused great
178 reduction in culture growth on PDA and on apple fruits (22). However, RnPV6-W113 did not
179 significantly cause hypovirulence in living chestnut trees. This was not due to spontaneous loss of
180 RnPV6-W113 during fungal growth in tree, because all of 22 fungal isolates recovered from three
181 cankers induced by RnPV6-W113-infected EP155 harbored the virus (data not shown). A difference
182 from the literature was noted for CHV3-GH2a; CHV3-GH2a did not induce great hypovirulence
183 (Table 2), whereas the virus had earlier been reported to cause hypovirulence (29, 33, 34). It should be
184 mentioned that CHV3-GH2 transferred to EP155 does not carry defective RNAs that was carried in
185 the original field-collected GH2 strain (35).
186 At least one, and in most cases two or three fungal isolates were obtained from each canker
187 induced by the respective virus-harboring fungi 74 days post inoculation. Approximately 30% of
188 fungal isolates derived from cankers induced by the MyRV1-9B21- and RnPV1-W113-harboring
189 fungal strains were virus-free, suggesting emergence of virus-free sectors within the canker. In the
190 other cases, all recovered fungal isolates were confirmed to be infected by expected viruses (Table 2)
191 with only a few exceptions. As expected, nine isolates recovered from three virus-free EP155-induced
192 cankers remained virus-free, confirming no virus contamination in the BSL3 greenhouse
193 environments.
194
195 **Virus spread in chestnut trees.** In assay II, two weeks following the inoculation of chestnut
196 trees with the virulent, virus-free strain PC7, the trees were challenged by EP155 strains infected with
197 different viruses at the lower end of the growing cankers (Fig. 2). Three and six weeks after the
198 challenge inoculation (Fig. 1), six bark plugs were taken from each of three rows of the single canker,

as shown in Fig. 1 and fungal isolates recovered were tested for virus infection. This experiment was repeated once as experiment I and II. Virus infection was determined by phenotypic observation and RT-PCR as shown in Fig. 4. Phenotypic diagnosis was useful for highly symptomatic viruses such as CHV1-EP713, CHV1-Δp69, CHV2-NB58, MyRV1-9B21 and RnPV6-W113, and fully agreed with that based on RT-PCR (see Fig. 4, RnPV6-W113 and CHV2-NB58). Virus detection rates were summarized in Table 3. Interesting differences in virus detection rates were observed. CHV1-EP713 and CHV3-GH2a were found to move to the top edge of the canker within 3 weeks, while RnPV6-W113 did not move up to the top in one of the cankers in experiment I (Fig. 4). It is, however, worth noting that the detection patterns of RnPV6-W113 and MyRV1-9B21 varied depending on cankers or experiments and that viruses were not necessarily detected more frequently at 6 weeks post challenge inoculation (Table 3). As summarized in Table 3, CHV1-EP713, CHV1-Δp69, CHV2-NB58, CHV3-GH2a and CpMV1-NB631 showed greater (>93.6%) virus detection rate, whereas the other two viruses, RnPV6-W113 and MyRV1-9B21 was detected at lower frequency (<75.8%). This was consistent with the results of bench virus transmission assay via anastomosis. Virus-free fungal isolates tended to be obtained frequently from the middle row in the spread assay with RnPV6-W113 and MyRV1-9B21 (Fig. 5A and B). A similar trend was observed for other efficiently spreading viruses.

These results clearly indicate that different viruses have different spread efficiencies in trees. The relatively lower spread rates of the two viruses RnPV6-W113 and MyRV1-9B21 may be associated with the lower detection rates of the viruses in assay II (virulence assay) (Table 2). Note that all of the fungal isolates cultured from the infected trees were confirmed to have the PC7 genetic background when tested for microsatellite markers (see below), indicating that lateral virus transmission had occurred from fungal strain EP155 to PC7 in trees, rather than simple overgrowth and re-isolation of the original virus-infected EP155 inoculum.

Biocontrol potential of viruses. The seven viruses tested in this study include dsRNA viruses and (+)RNA viruses whose biocontrol potential had not been explored comparatively in controlled conditions. As in the case for assay II, chestnut trees were primarily inoculated with the virulent, virus-free strain PC7, but the challenge inoculation was made at the eight periphery sites of the growing cankers in assay III (Fig. 2). The potential ability of these viruses to serve as biological control agents was measured by inhibition of canker expansion and reduction of mortality caused by the primary inoculation with the virulent chestnut blight fungus (Fig. 2). They showed great variation in biocontrol effects. Representative canker morphology is shown in Fig. 6A to illustrate how

challenge inoculation of the trees with each virus-infected fungal EP155 strain contributed to the repression of virulent or active cankers. Fig. 6B and Table 4 summarize the biocontrol effects of the respective viruses. CHV1-EP713, CHV1-Δp69 and CHV2-NB58 exerted great biocontrol effects, whereas CHV3-GH2a and RnPV6-W113 did not and allowed for canker expansion as for the treatment with agar (negative control). CpMV1-NB631 and MyRV1-9B21 inhibited canker expansion at a statistically significant, albeit a modest level (Fig. 6). Approximately three months post challenge inoculation with RnPV6-W113-, CHV3-GH2a-, and CpMV1-NB631-infected colonies, chestnut leaves were wilting, a sign of destroyed function of vascular tissue, as in the case for the treatment with agar (Fig. 7). No wilting symptoms were observed when virulent cankers were challenged with CHV1-EP713-, CHV2-NB58- or CHV1-Δp69 (Fig. 7, data not shown).

It is important to confirm that the aforementioned biocontrol effects resulted from conversion of the PC7 strain from virulent to hypovirulent strain by the transmitted virus. To this end, we isolated fungi from four to eight sites of each canker (see Fig. 2) and tested them for virus infection. As observed in the *in vitro* horizontal transmission assay above, CHV1-EP713, CHV1-Δp69, CHV2-NB58, CHV3-GH2a and CpMV1-NB631 were transmitted within trees more efficiently than others (MyRV1-9B21 and RnPV6-W113 (Table 5). Virus detection rates ranged from 66.7-73.3% for MyRV1 and RnPV6, and to 93.3-96.7% for the other 5 tested viruses (Table 2).

Nuclear and mitochondrial genotyping of fungal isolates recovered from cankers. From the perspective of field-level biocontrol of chestnut blight, it is important to know how introduced hypovirulent fungal strains disseminate in treated chestnut forests, particularly when transgenic hypovirulent strains are used. All of the fungal isolates, which were analyzed for virus infection in the virus spread assay, were examined for their nuclear genotype. It should be prefaced that in virus spread assay, a maximum of 18 fungal isolates were recovered from a larger area of an active canker relative to that of a hypovirulent canker (Fig. 1). At the time points of fungal re-isolation, 3 and 6 weeks post challenge inoculation, virulent cankers were expanding aggressively, while hypovirulent cankers, which were challenged by CHV1-EP713, CHV2-NB58, or CHV1-Δp69, stopped expanding (data not shown). As summarized in Tables 3 and 4, 3 weeks post challenge inoculation, most of the recovered isolates had the PC7 nuclear genotype, while at this time point all of the viruses moved within cankers though to different degree (see above). Six weeks post challenge inoculation, both genotypes were observed in a few cultures isolated from cankers challenged by CHV3-GH2a-infected EP155 (Fig. 5C). Also, a single recovered isolate from a canker challenged by CpMV1-NB631-infected EP155 was found to contain both the PC7 and EP155 genotypes.

For biocontrol assay, two months post challenge inoculation, fungal isolates were recovered from four bark samples obtained at the periphery of each treated canker. For cankers that were still expanding after biocontrol treatment (i.e., CHV3-GH2a, CpMV1-NB631, MyRV1-9B21, RnPV6-W113 treated cankers and the agar control) additional four samples were taken inside of the cankers near the treatment holes (Fig. 1). Table 5 summarizes data from 5 trees for each virus-infected fungus. The vast majority of the isolates obtained from the treated cankers had the PC7 genotype and were infected with the respective virus used in the biocontrol assay indicating overall successful virus transmission from EP155 into the PC7 induced cankers. Isolates with the EP155 genotype or a mixed genotype were only observed in expanding cankers treated with CHV3-GH2a, CpMV1-NB631, and RnPV6-W113.

For mitochondrial haplotyping we first tried PCR-fragment length polymorphism-based differentiation as reported by Shahi et al. (31). However, EP155 and PC7 showed identical profiles regardless of PCR primer sets used. Thus, a polymorphic gene for NADH-ubiquinone oxireductase subunit 5 (36) was sequenced and compared between the two strains. The EP155 NADH-ubiquinone oxireductase subunit 5 gene sequence was confirmed to be identical to that previously reported by Gobbi et al. (36). PC7 was shown to have a single nucleotide polymorphism at map position 22 from the termination codon (A of the initiation codon numbered 1). This SNP (T for EP155 and C for PC7) was utilized for mitochondrial haplotyping of fungal isolates obtained from trees inoculated with EP155 infected by the mitochondrially replicating CpMV1-NB631. We tested fungal isolates obtained from the virus spread assay with the mitochondrially replicating CpMV1-NB631 and those from the biocontrol assay with CHV3-GH2a. Consequently, a total of 68 isolates from the virus spread assay, largely carrying CpMV1-NB631, had the recipient (PC7) mitohaplotype rather than the donor's (EP155) (Table 3). For the seven isolates from the biocontrol assay, three had the EP155 mitochondrial genotype while four had the PC7 mitohaplotype. Importantly the four isolates with the EP155 mitohaplotype had the EP155 nuclear genotype. Likewise, two isolates with the PC7 mitohaplotype had the PC7 nuclear genotype. The remaining two isolates with the PC7 mitohaplotype had a mixture of both nuclear genotypes. These results suggest that no efficient exchange between the nuclear and mitochondria genomes occurred in this assay.

DISCUSSION

Biocontrol of chestnut blight involves the dynamic interactions of three organisms: chestnut tree, chestnut blight fungus and viruses infecting the fungus. Various studies have explored fungal and viral factors governing success or failure in population-level biocontrol and suggested VC type diversity

298 and ecological fitness of virus infected fungi as major determinants (37). Such studies largely involved
299 hypoviruses, especially CHV1-EP713, because the viruses have served as biocontrol agents in some
300 areas of Europe. The characterization of many other viruses infecting *C. parasitica* in the last few
301 decades prompted the current study. Here we monitored in-tree behaviors of seven viruses with
302 dsRNA or (+)RNA genomes that were harbored in the chestnut blight fungus by assessing their
303 biocontrol potential, horizontal virus transmission, and virus spread in chestnut blight cankers. This
304 study highlighted different behaviors of the seven viruses and provided interesting insights into
305 biocontrol of chestnut blight. It should be noted that the current study focused on European chestnut,
306 *C. sativa*, as host plant, whereas some other studies in the literature have focused on American
307 chestnut, *C. dentata*, as host. While differences in blight resistance between these two tree species are
308 not dramatic, they are measurable, with *C. dentata* being slightly more blight-susceptible than *C.*
309 *sativa* (38).

310 All hypoviruses but CHV3-GH2a showed a similar in-tree behavioral profile, although they
311 induced different symptoms on PDA (Fig. 2). CHV1-EP713, its deletion mutant CHV1-Δp69, and
312 CHV2-NB58 were able to confer great hypovirulence, be horizontally transmitted efficiently and have
313 high potential as biocontrol agents (Figs. 3 & 6, Tables 2-4). CHV1-Δp69 lacks the ORF coding
314 region encoding the RNA silencing suppressor (32, 39, 40) and a basic protein p40. Therefore, this
315 study confirms the previous notion that p29 plus p40 are dispensable for hypovirulence induction.
316 Although both viruses can confer hypovirulence to the fungal host, they differ in symptom induction;
317 the wild-type CHV1-EP713 reduces pigmentation and conidiation more than Δp69. Another difference
318 is the inducibility of the antiviral RNA silencing genes; Δp69 activates RNA silencing via
319 transcriptional up-regulation while the wild-type CHV1-EP713 does not (41, 42). By contrast, the
320 CHV3-GH2a isolate in this study was predicted to be a poor performer as a biocontrol agent (Fig. 6),
321 even though it was transmitted efficiently as were the other hypoviruses (Table 3). The speeds at
322 which these hypoviruses spread in chestnut blight cankers are estimated to be > 100 ~ 200 μm/h, being
323 greater than that of the cell-to-cell movement of plant virus (~5 μm/h) (43) and smaller than that of the
324 long distance movement (~ cm/h) (44). Like CHV1-EP713 and unlike CHV1-Δp69 and CHV2-NB58,
325 CHV3-GH2a is unable to upregulate antiviral RNA silencing transcriptionally (45).

326 The other two viruses spread less efficiently than the hypoviruses: the homologous virus
327 MyRV1-9B21 originally isolated from strain 9B21 of *C. parasitica* (mean virus detection rate: 70.3%),
328 and the heterologous virus RnPV6-W113 originally isolated from strain W113 of *R. necatrix* (mean
329 virus detection rate: 75.8%), were substantially lower than the aforementioned hypoviruses (mean
330 virus detection rate: over 94%) (Table 3). Given the observation that most fungal isolates from trees

had the PC7 genotype (primary inoculum; see below), virus was considered to be transmitted in trees via inter- and intra-strain anastomosis rather than predominant colonization by virus-infected EP155 (secondary inoculum). Although CHV1-EP713 was recently shown to replicate in an annual plant (46), this is unlikely to have occurred in chestnut trees because systemic infection by CHV1-EP713 in plant requires supply of a movement protein from a plant virus. It is unknown why the two viruses moved less efficiently than hypoviruses. It should be noted that while RnPV6-W113 causes severe growth reduction on PDA (Fig. 2), this virus did not reduce fungal virulence much in the trees (Fig. 3, Table 2). CHV1-Δp69, CHV2-NB58 and MyRV1-9B21 induce similar symptoms such as reduced growth of aerial hyphae, enhanced production of brown pigments (Fig. 2). Despite similar symptom induction pattern (Fig. 2), only MyRV1-9B21 spread less efficiently in trees than the others. A property distinguishing the less efficiently moving from efficiently moving viruses is an encapsidated (RnPV6-W113 and MyRV1-9B21) or capsidless nature (hypoviruses).

CpMV1-NB631 is different from all other tested viruses in that it replicates in mitochondria and utilize mitochondrial translational codes (26, 47). In this sense, it is of great interest to know how mitochondria and its virus CpMV1-NB631 spread in cankers on living chestnut trees. This study clearly showed that CpMV1-NB631 spreads as efficiently as the hypoviruses (Table 3). A previous study showed that CpMV1-NB631 has a relatively narrow host range and is not targeted by RNA silencing (31). Also suggested by the same authors is compatibility between the nuclear and mitochondrial genotypes and two modes of transmission: an extra-, and intra-mitochondrial mode beside colonization of CpMV1-NB631-infected mitochondria in the recipient (31). While studying interspecies and intraspecies transmission of CpMV1-NB631 via protoplast fusion, it was shown that only CpMV1-NB631 moved into recipient strains that maintain their original donor mitochondrial haplotypes. Note that in that study the recipient nuclear genotypes were selected on drug-amended media. In the current study we could not detect the introduced EP155 mitohaplotype in fungal isolates recovered from treated cankers (Table 3), indicating that CpMV1-NB631 alone could readily transmit to the mitochondria of the primary inoculum PC7. Previous studies showed the presence of mitovirus-derived small RNAs likely generated in the non-mitochondrial cytoplasm (48). Currently, no mitochondrial heteroplasmy was observed. Collectively these observations favor the idea that CpMV1-NB631 could be transmitted extra-mitochondrially while not negating the possibility of fusion-based intra-mitochondrial transmission.

The dissemination and exchange of three genetic elements – viruses, mitochondria and nuclei – were previously examined at the canker and population levels. Shain and Miller (27) studied within-canker spread of an uncharacterized hypovirulence-inducing virus in an American chestnut (*C.*

dentata) tree. The authors showed that it took six weeks for the virus to move from one single challenge inoculation site, at the lower end of a virulent canker, to the top edge of the canker about 10 cm apart from the challenge site. The current study showed CHV1 and other hypoviruses to move slightly more quickly (100 ~ 200 μm/h) than the unidentified virus reported by Shain and Miller (40~110 μm/h) (27). In this study, CHV1-EP713 was detected in almost all fungal isolates recovered from the treated virulent cankers 3 weeks post challenge inoculation. An interesting similarity is that the nuclear genotype of the virulent strain, PC7 (primary inoculum) for this study and a methionine-requiring auxotroph (Met-) for the previous study, was dominant in the fungal isolates obtained from the treated cankers in both studies. These results demonstrate not only efficient horizontal transmission of the viruses into treated cankers but also efficient replication and spread within canker.

At the population level, the three genetic elements disseminated at different speeds during a two-year biocontrol experiment, in which a CHV1-infected fungal strain was used to treat active cankers induced by resident virulent fungi. Simultaneous transfer of CHV1 and the mitochondrial haplotype into the resident nuclear genotype were observed in an almost half of CHV1-infected fungal isolates recovered from treated cankers one year post challenge (28). Although the present study did not investigate the mitochondrial haplotype of isolates obtained from cankers challenged with CHV1-EP713-infected fungi, no transfer of the mitochondrial genome of the introduced strain would be expected from the results with isolates from cankers challenged by the CpMV1-NB631-infected strain (Table 3). In Hoegger et al. (28), almost one third of the isolates from treated cankers had the same mitochondrial and nuclear genomes as those of the introduced strain, a pattern not observed in the current study. The rest carried the same mitochondrial and nuclear genomes as those of the resident strain, meaning that only CHV1 was transmitted. The nuclear and mitochondrial genotypes of introduced EP155 could seldom be detected and resident ones remained predominant in fungal isolates from cankers treated with fungi infected by CHV1-EP713 or other viruses in the current study. This noticeable difference in detection pattern may be accounted for by different time scales of the experiments. The nuclear genotype of challenged EP155 could only be detected occasionally from fungal isolates recovered from bark of expanding canker areas of trees challenged by viruses without biocontrol potential (Table 5). It should be noted that CHV3-GH2a infection appeared to facilitate karyon transmission as observed in the two independent assays with three or five biologic replicates (Tables 3 and 5). Although its mechanism remains largely unknown, it is of interest to speculate a positive effect of CHV3-GH2a on anastomosis and spread of infected host fungi in tree. Such effects of CHV3-GH2a could also be observed *in vitro* cultures (N. Suzuki, unpublished data).

Conclusions.

This study suggests that CHV1-EP713, CHV2-NB58, and CHV1-Δp69 are likely more robust as biological control agent than the other viruses tested in this study (Table 6, Fig. 6). MyRV1-9B21 confers strong hypovirulence to its fungal host strain when tested on apple fruits previously and with live chestnut trees in the current study. Therefore, the failures of the virus in biocontrol against the virulent PC7 appear to be due to their inefficient transmission into PC7 (Table 3). These results allow us to conclude that to serve as robust biocontrol agents, viruses must be able to induce hypovirulence and efficiently spread into chestnut blight cankers (Table 6). Why CpMV1-NB631 has poor biocontrol potential despite its ability to confer hypovirulence and spread efficiently (Table 6, Fig. 6) remains unknown. Discussion of biocontrol agent potential at the field level requires cautious consideration of many factors other than VC type diversity as discussed earlier (37). For example, CHV1-EP713 considerably reduces not only the virulence but also asexual sporulation of its host fungi, which would affect ecological fitness of infected host fungi at the field level. This point was previously noted by several studies (see Milgroom and Cortesi (37) for review). While this study did not explore effects of viruses on sporulation, it underlines the importance of horizontal virus transmissibility and in particular virus spread within chestnut blight cankers.

MATERIALS AND METHODS

Viral and fungal strains. Fungal and viral strains used in the current study are shown in Table 1. A total of seven RNA viruses were tested: RnPV6-W113(22), MyRV1-9B21(30, 49), CHV1-EP713 (50), CHV1-Δp69) (32), CHV2-NB58 (51), CpMV1-NB631(26), and CHV3-GH2a, a derivative of CHV3-GH2 (29). Note that these viruses are diverse molecularly and biologically. Even the hypoviruses are different from each other in many properties, including their origins, effects on fungal phenotype and ability to confer hypovirulence (19) as well as their induction of antiviral silencing genes (45). Another noteworthy point is that CHV3-GH2a, derived during subculturing of the infected fungus by the original CHV3-GH2, lacked the defective RNA termed RNA2 but retained two satellite RNAs (RNA3 and RNA4), while the original contains all the three sub-viral RNAs (52). Two strains of *C. parasitica*, PC7 (Swiss Federal Institute for Forest, Snow and Landscape Research (WSL) fungal collection accession number: M1334) originally isolated in 1993 from Bergamo, Italy (53), and EP155 (ATCC 38755) originally isolated in 1977 from Bethany, Connecticut, U. S. A., both belonging to the EU5 VC group, were used as virulent strains (54, 55). These two strains are readily distinguishable by microsatellite markers as described below.

Virus inoculation and horizontal and vertical transmission. Many viruses from *Cryphonectria* spp. and other fungi could be experimentally introduced into the standard EP155 genetic background using several previously developed methods. Different methods were used depending primarily on whether viruses of interest form virus particles and whether infectious RNAs or DNAs are available for them. CHV1-Δp69 was introduced to strain EP155 by spheroplast transformation with an infectious cDNA clone pCPXHY-CHV1-Δp69 (56) or *in vitro* synthesized RNA derived from pRFL4 (57). EP155 infected by capsidless CHV2-NB58, CHV3-GH2a, and CpMV1-NB631 were previously obtained by protoplast fusion (31, 45). The remaining encapsidated viruses, RnPV6-W113 and MyRV1-9B21 were transfected into EP155 earlier (22, 30). Horizontal viral transmission was examined by co-culture of viral-donor and -recipient fungal strains on a PDA plate (9 cm in diameter) as described previously (56).

Inoculation of chestnut trees with fungal strains. Three-year-old, approximately 2.5 m high, and approximately 2.5 cm wide (diameter) chestnut trees (*C. sativa*, provenance Maggiatal, Switzerland) were purchased from a Swiss nursery and grown outside for a few months from April to July, 2019. They were moved to a biosafety level 3 (BSL3), temperature-controlled greenhouse (25 C) greenhouse at WSL and acclimated to the greenhouse environment for a week. These trees were used in three different assays (I, II, III) (Fig. 2). All inoculations were done using mycelial plugs from actively growing cultures as described by Dennert et al. (58). For assay I to investigate virulence, strain EP155 infected by different viruses was inoculated into the chestnut trees. Three biological replicates were used for each virus-infected strain and for a virus-free control. For assay II to examine in-tree spread of different viruses, two trees each were singly inoculated with the virulent strain PC7 inoculated in two consecutive experiments. For assay III to estimate biocontrol potential, five trees were singly inoculated with PC7. Two weeks following these primary inoculations with PC7, challenge inoculations were made with the virus-infected EP155 strains (Fig. 2). For assay II, one single inoculation at the lower end of each virulent canker was made. For assay III, eight inoculations regularly distributed along the periphery of a virulent canker were carried out. After inoculation, the holes were sealed with LacBalsam (Frunol Delicia, Delitzsch, Germany) to prevent desiccation. At the time of challenge inoculation in assay II and III, the typical canker size was approximately 43 mm x 21 mm. Length and width of the treated cankers were bi-weekly measured and the canker area calculated using the formula of an ellipse. The degree of canker expansion after the biocontrol treatments was used to assess the biocontrol effects of the different mycoviruses. Linear model with

Scheffe post hoc test (calculated using DataDesk 6.3, DataDescription Inc, Ithaca, NY) was applied to test for significant differences in mean canker expansion between viruses.

Fungal isolation from cankers on chestnut trees. All cankers were sampled in the end of the experiment to verify virus infection and fungal identity (Fig. 1). For assay I, three bark samples (top, middle, and bottom of the canker) were taken from each canker using a bone marrow biopsy needle (diameter 2 mm; Jamshidi 11 gauge, BD, Franklin Lakes, NJ). For assay II, four bark samples at the periphery of each cankers were taken two months after the challenge inoculations. For expanding cankers, four additional samples were taken inside of the cankers between the four biocontrol inoculation holes. For assay III, two cankers were sampled for each virus 3 and 6 weeks after the biocontrol inoculations. At each time point, six bark samples were taken along three rows from the lower to the upper end of the cankers, resulting in 18 samples per canker (Fig. 1). Bark plugs were surface-sterilized using 70% ethanol and placed on water agar plates containing streptomycin at a concentration of 40 mg/L. Outgrowing mycelia were transferred on potato dextrose agar (PDA; 39 g/L, BD Difco™) plates, which were then incubated at room temperature in the dark for 10 to 14 days. Under this condition, cultures infected with CHV1-EP713, CHV1 (Δp69), CHV2-NB58, MyRV1-9B21, and RnPV6-W113 developed a virus-specific culture morphology that was used as an indicator for virus infection (Fig. 2). Results based on culture morphology were confirmed for subsamples by virus-specific RT-PCR as described below. The presence of CHV3-GH2a and CpMV1-NB631 was exclusively verified by RT-PCR, as these two viruses did not induce a specific cultural phenotype in infected fungal strains (see Fig. 4 for some examples).

DNA extraction, RT-PCR, and nuclear and mitochondrial genotyping. The simplified and reliable one-step RT-PCR technique developed by Urayama et al. (59) for virus detection in *Magnaporthe oryzae* without nucleic acid extraction was employed to detect virus in fungal isolates obtained from cankers. The method entails stabbing the freshly growing region of mycelial colony on PDA by a toothpick, and dipping the toothpick into a 10-µl pre-mixed RT-PCR reaction mixture prepared according to the protocol for PrimeScript® One Step RT-PCR Ver.2 (Dye Plus) (Takara Bio, Inc, Shiga, Japan). Importantly this technique was found to be applicable to the *C. parasitica*/viruses (59, 60). The PCR was programmed as described in the manufacturer's protocol. The sequences of primers for detection of respective viruses are available upon request.

For nuclear genotyping, DNA was extracted from lyophilized fungal mycelium using the KingFisher 96 Flex (Thermo Fisher, Waltham, MA) according to the manufacturer's protocol. The

multiplex PCR reaction 3 described by Prospero and Rigling (61) was used to distinguish the two strains PC7 and EP155. In this PCR, strain PC7 yields a 130 bp long DNA fragment at microsatellite locus CPE1 and a 252 bp long fragment at locus CPE5. The corresponding DNA fragments in strain EP155 are 148 bp and 260 bp long.

Mitochondrial genotyping or mito-haplotyping was based on a single nucleotide polymorphism detected on the coding region of NADH-ubiquinone oxireductase subunit 5. This region was amplified using the primers MtF2 and MtR2 (sequences available upon request) and the PCR fragments were sequenced by the Sanger method.

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Author contributions: NS and DR designed research; NS, CC, AA, SS and DR performed research; NS, BIH and DR analyzed data; NS, DR and BIH wrote the manuscript.

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Figure legends

FIG. 1. Colony morphology of PC7 and EP155 infected by different viruses. EP155 (top row) and PC7 (bottom row) infected by the seven different virus strains were prepared as described in Materials and Methods. Introduced viruses, listed in Table 1, are shown on the top and bottom of the panel. Colonies were grown on PDA for one week on the bench top approximately at 23 C, and photographed. Virus-free EP155 and PC7 were grown in parallel.

FIG. 2. Schematic representation of the experimental procedures to assess biocontrol potential and in-tree spread of viruses.
For both assays, the European virulent strain PC7 was inoculated into European chestnut trees (shown by blue circles) with the aid of a cork borer (A). Two weeks post inoculation, the trees were challenged (shown by yellow circles) with EP155 colonies infected with different viruses (Table 1) at one (for virus spread assay II) or eight sites (for biocontrol assay III) of the periphery of virulent cankers (shown by brownish ovals) induced by PC7 (B) with the aid of a cork borer. For fungal isolation, bark plugs were obtained from a total of 18 sites (for virus spread assay) or 4 sites inside the original canker area plus 4 sites in expanded areas (for biocontrol assay) for challenge inoculation with CHV3-GH2a, RnPV1-W113, CpMV1-NB631, and MyRV1-9B21-infected EP155 (C). For challenge inoculation with the remaining virus-infected colonies, only four inside plugs were utilized, because the original virulent cankers became inactive and failed to expand. Bark sampling was performed three and six weeks (for virus spread assay) and two months (for biocontrol assay) post challenge inoculation with the aid of a bone marrow needle. Isolated fungi were examined for virus infection, and nuclear and mitochondrial genotypes.

FIG. 3. Virulence of EP155 infected by different viruses. Three trees per virus-infected strain were inoculated. Virulence levels were expressed by areas of cankers induced by the respective fungal strains that were measured two months post inoculation. Virus-free EP155 was also inoculated into three chestnut trees in parallel. (A) shows mean canker areas and standard deviations calculated from (B) in a graph, while (B) indicates canker areas measured for three trees per virus-infected strain two months post inoculation. Data of the measurements made at different time points are shown in Table 2.

FIG. 4. Virus transmission in chestnut trees. As shown in Fig. 2, Three-year-old chestnut trees were first inoculated with a virus free virulent fungal strain, PC7. EP155 strains each infected by the respective viruses were inoculated at the lengthwise growing edge of the canker two weeks post the first inoculation. Eighteen bark plugs were taken and placed onto water agar containing streptomycin to isolate fungal strains. After a few days, mycelia were transferred to PDA plates. Fungal isolates obtained 3 weeks post challenge inoculation from cankers treated with RnPV6-W113-, CHV2-NB58-, CHV1-EP713- and CHV3-GH2a-infected EP155 were cultured on PDA for one week on the bench top and photographed (left panels). RT-PCR analysis of the fungal isolates recovered from bark samples. The direct colony RT-PCR with toothpicks was employed to examine recovered fungal isolates for virus infection. Amplified cDNA fragments were electrophoresed in 1.2% agarose gel in the 1X TBE buffer system (89 mM Tris-borate, 89 mM boric acid, 2.5 mM EDTA, pH 8.3). GeneRuler 1 kb Plus DNA ladders (Thermo Fisher Scientific) were used as size standards. Virus transmission rates are summarized in Table 3.

FIG. 5. Schematic representation of bark sampling sites for virus-free isolates and isolates with a mixture of the EP155 and PC7 genomes. Incomplete spread of MyRV1-9B21 (**A**) and RnPV1-W113 (**B**) and detection of the EP155 nuclear genome in the CHV3-GH2a spread assay (**C**). As shown in Fig. 1, the central blue and bottom yellow filled circles refer to the sites of the primary and challenge inoculations. Grey circles indicate the sites where the respective virus was detected and white circles where it was not detected. Panel A corresponds to the results of Experiment I "6 weeks post challenge inoculation," while panel B shows the results of Experiment II "6 weeks post challenge inoculation" (see Table 3). Genotyping revealed a mixture of the EP155 and PC7 nuclear genomes in a few isolates obtained from the CHV3-GH2a spread Experiments I and II (see Table 3). From the four sampling sites with an orange symbol, isolates with the mixed genotype were recovered. Grey symbols denote the sites where only the PC7 genotype was present.

FIG. 6. Biological control effects of different viruses. Three-year-old European chestnut trees were first inoculated with a virus free virulent fungal strain, PC7 at one site per tree (see Fig. 2). After two weeks, the trees (five per fungal strain) were challenged by EP155 infected with different viruses (Table 1) were inoculated to 8 sites per tree at the periphery of growing canker. Resulting cankers induced by the first and second challenge inoculations were photographed 8 weeks post the first inoculation. Representative cankers are shown in panel (**A**). The viruses used for the challenge inoculation are shown on the bottom of the panel. Cankers induced by the challenge inoculation with

765 agar as a negative control in parallel is also shown. The original canker area induced by PC7 at the
766 time point of the challenge inoculation is shown by a white bracket, while the canker area expanded
767 6 weeks post challenge inoculation is denoted by a yellow bracket. Canker areas were measured six
768 weeks post challenge inoculation. Mean canker expansions and standard deviations, calculated by
769 five biological replicates, are shown graphically (**B**). Biocontrol effects of viruses on the fungal
770 pathogen are greater as canker expansions are smaller.
771
772 **FIG. 7. Chestnut trees surviving and dying of inoculation by PC7.** Two representative trees,
773 inoculated with the virulent strain PC7, are shown. The trees were challenged by the
774 CHV1-Δp69-infected fungus (right) or mock-inoculated (Agar) (left) and photographed 15 weeks
775 post challenge inoculation.

776 **Table 1 Fungal and viral strains used in this study.**

VIRAL						
Strain	**Description**		**Original host**		**Accession no**	**Reference**
MyRV1-9B21	Exemplar strain of *Mycoreovirus 1* within the genus *Mycoreovirus*		*Cryphonectria parasitica* strain 9B21		AY277888–AY277890 & AB179636–AB179643	(30, 49)
CHV1-EP713	Prototype of the family *Hypoviridae*		*Cryphonectria parasitica* strain EP713		M57938	(62)
CHV1-Δp69	ORF-A deletion mutant of CHV1-EP713 lacking the p29 and p40 coding domains		Genetically engineered		M57938	(32)
CHV2-NB58	Exemplar strain of the species *Cryphonectria hypovirus 2*		*Cryphonectria parasitica* strain NB58		L29010	(51)
CHV3-GH2a	Exemplar strain of the species *Cryphonectria hypovirus 3*		*Cryphonectria parasitica* strain GH2		AF188515	(29)
CpMV1-NB631	Exemplar strain of the species *Cryphonectria mitovirus 1*		*Cryphonectria parasitica* strain NB631		L31849	(26)
RnPV6-W113	Exemplar strain of *Rosellinia necatrix partitivirus 6*		*Rosellinia necatrix* strain W113		LC010952, LC010953	(22)
FUNGAL						
Strain	**Description**	**VC type (*vic* genotype[a])**	**Mating type**	**Isolation site & year**	**Accession no**	**Reference**
EP155	Standard strain of *Cryphonectria parasitica* (virus free)	EU5 (2211-22)	MAT1-2	Bethany, Connecticut, USA, 1977	ATCC 38755	(55)
PC7	Field collected isolate (virus free)	EU5 (2211-22)	MAT1-2	Bergamo, Italy, 1993	M1334	(53)

777 [a]*vic* genotype is expressed according to Choi et al. (7).

778

779 **Table 2 Mean canker areas induced by virus-infected and virus-free EP155 fungal colonies**

Infecting virus	Canker area (cm^2)[a]				VDR[b]
	2 wks (Oct 23)	4 wks (Nov 7)	6 wks (Nov 21)	2 months (Dec 9)	
CHV1-EP713	0.99±0.41	1.26±0.39	1.09±0.38	1.13±0.28	94.4% (17/18)
CHV1-Δp69	0.64±0.12	0.69±0.16	0.68±0.11	0.76±0.11	96.7% (30/31)
CHV2-NB58	0.50 ±0	0.52±0.04	52.3±0.04	0.52±0.04	93.3% (14/15)
CHV3-GH2a	2.29±0.31	4.41±0.20	12.93±7.42	22.30±10.65	93.3% (14/15)
MyRV1-9B21	0.50±0	0.55±0.08	0.55±0.08	0.55±0.08	66.7% (22/33)
RnPV6-W113	1.16±0.38	3.06±1.57	9.99±3.66	18.28±6.18	73.3% (11/15)
CpMV1-NB631	0.50±0	2.33±1.40	2.46±1.50	2.72±1.50	94.4% (17/18)
Virus-free (EP155)	4.14±1.39	9.57±1.49	20.31±3.77	29.10±3.39	0% (0/10)

780 [a]Averages and standard deviations calculated from three biologic replicates are shown.

781 [b]VDR, virus detection rates of fungal isolates recovered on December 20. The number of fungal isolates recovered from bark cankers varied.

782

783 **Table 3 Summary of virus spread assay**

Virus strain	Experiment I										Experiment II									
	3 weeks post challenge inoculation					6 weeks post challenge inoculation					3 weeks post challenge inoculation					6 weeks post challenge inoculation				
	VDR[a]	Nuclear genotype			MH	VDR[a]	Nuclear genotype			MH	VDR[a]	Nuclear genotype			MH	VDR[a]	Nuclear genotype			MH
		PC7	EP155	PC7+ EP155			PC7	EP155	PC7+ EP155			PC7	EP155	PC7+ EP155			PC7	EP155	PC7+ EP155	
CHV1-EP713	18/18	18	0	0	NT	16/17	17	0	0	NT	12/13	13	0	0	NT	17/18	18	0	0	NT
CHV1-Δp69	16/16	16	0	0	NT	14/15	15	0	0	NT	12/15	15	0	0	NT	16/16	16	0	0	NT
CHV2-NB58	14/15	15	0	0	NT	16/16	16	0	0	NT	14/16	16	0	0	NT	14/15	15	0	0	NT
CHV3-GH2a	18/18	18	0	0	NT	17/17	14	0	3[d]	NT	15/18	18	0	0	NT	14/15	14	0	1[d]	NT
MyRV1-9B21	11/15	15	0	0	NT	11/18[b]	18	0	0	NT	12/16	16	0	0	NT	11/15	15	0	0	NT
RnPV6-W113	6/18	18	0	0	NT	17/17	17	0	0	NT	16/16	16	0	0	NT	11/15[c]	15	0	0	NT
CpMV1-NB631	16/17	17	0	0	PC7	14/17	17	0	0	PC7	16/16	16	0	0	PC7	17/18	17	0	1	PC7

784 [a]VDR, virus detection rate; MH, mitochondrial haplotype; NT, not tested; NA, not applicable.

785 [b,c,d] Sampling sites are shown in Fig. 5A, 5C, and 5C, respectively.

786

787 **Table 4 Growth of canker area after challenge inoculations with virus-infected fungal carrier EP155**

Infecting virus	Canker area (cm^2)[a]					Canker expansion 8 wks after biocontrol treatment[c]
	0 wks (Aug 12)[b]	2 wks (Aug 27)[b]	4 wks (Sept 10)[b]	6 wks (Sept 23)[b]	8 wks (Oct 7)[b]	
CHV1-EP713	15.5±5.2	15.4±5.1	16.4±5.0	17.7±6.8	16.7±5.6	1.2±1.8 c
CHV1-Δp69	13.7±3.5	14.3±5.5	14.9±4.6	14.8±4.5	15.8±5.5	2.0±2.1 c
CHV2-NB58	13.9±2.9	13.2±1.6	14.5±1.5	14.5±2.5	14.9±2.2	1.1±3.4 c
CHV3-GH2a	16.6±2.8	23.0±6.5	34.4±6.4	50.3±7.0	67.5±12.7	50.9±11.9 ab
MyRV1-9B21	17.7±3.6	21.6±3.2	36.0±9.2	42.9±18.8	48.0±20.9	30.3±21.2 b
RnPV6-W113	13.6±2.8	20.2±4.4	34.5±11.2	64.4±15.3	52.8±9.8	50.8±13.0 ab
CpMV1-NB631	18.5±7.8	25.0±5.7	32.0±10.6	39.9±12.2	46.1±14.7	27.6±17.1 b
Agar	16.3±3.7	22.4±5.4	40.0±7.8	66.7±16.5	80.0±10.4	63.7±8.5 a

788 [a]Averages and standard deviations calculated from five biological replicates are shown.

789 [b]Canker area induced by the inoculated fungal strain PC7 at the time point of biological control treatment

790 [c]Means followed by different letters are significantly different ($p < 0.05$)

791

792 **Table 5 Nuclear genotype and virus detection rate of fungal isolates recovered in**
793 **the biocontrol assay**

Virus strain	Inside of the cankers				Periphery of the cankers			
	VDR	Nuclear genotype			VDR	Nuclear genotype		
		PC7	EP155	PC7+EP155		PC7	EP155	PC7+EP155
CHV1-EP713	15/16	11[a]	0	0	NA			
CHV1-Δp69	16/16	16	0	0	NA			
CHV2-NB58	17/17	14[a]	0	0	NA			
CHV3-GH2a	16/16	14	0	2	18/18	9	6	3
MyRV1-9B21	7/16	16	0	0	12/18	18	0	0
RnPV6-W113	11/19	15	0	0	15/19	15	1	3
CpMV1-NB631	19/20	20	0	0	14/14	14	0	0
Agar	NA	19	0	0	NA	17	0	0

794
795 NA, not applicable; VDR, virus detection rate.
796 [a]A few fungal isolates were omitted from genotyping experiments.
797

798

799 **Table 6 Biological properties of the seven viruses infecting *C. parasitica*.**

	CHV1-EP713	CHV1-Δp69	CHV2-NB58	CpMV1-NB631	MyRV1-9B21	RnPV6-W113	CHV3-GH2a
Hypovirulence	+++	+++	+++	++	+++	(+)	NO
Efficient spread	YES	YES	YES	YES	NO	NO	YES
Biocontrol	+++	+++	+++	+	+	NO	NO

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801
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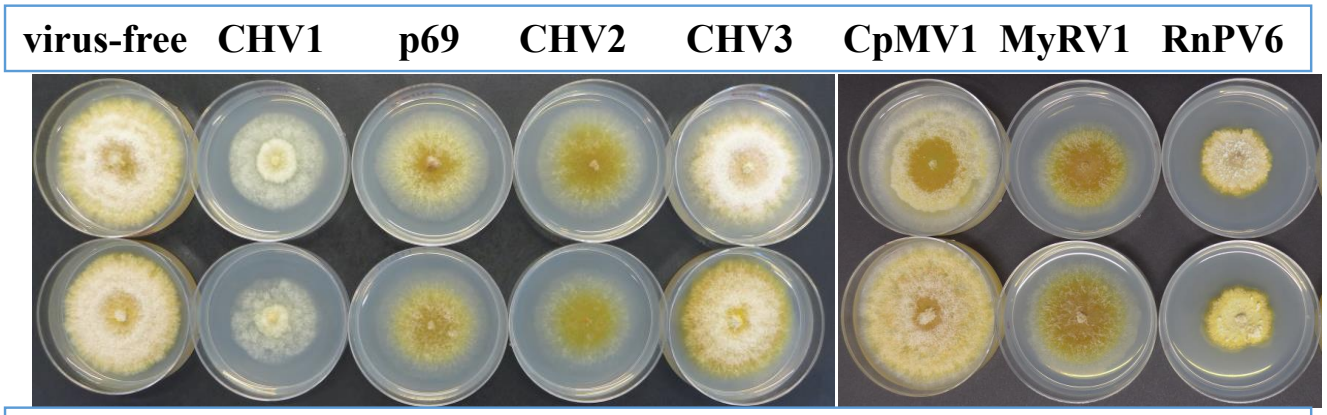
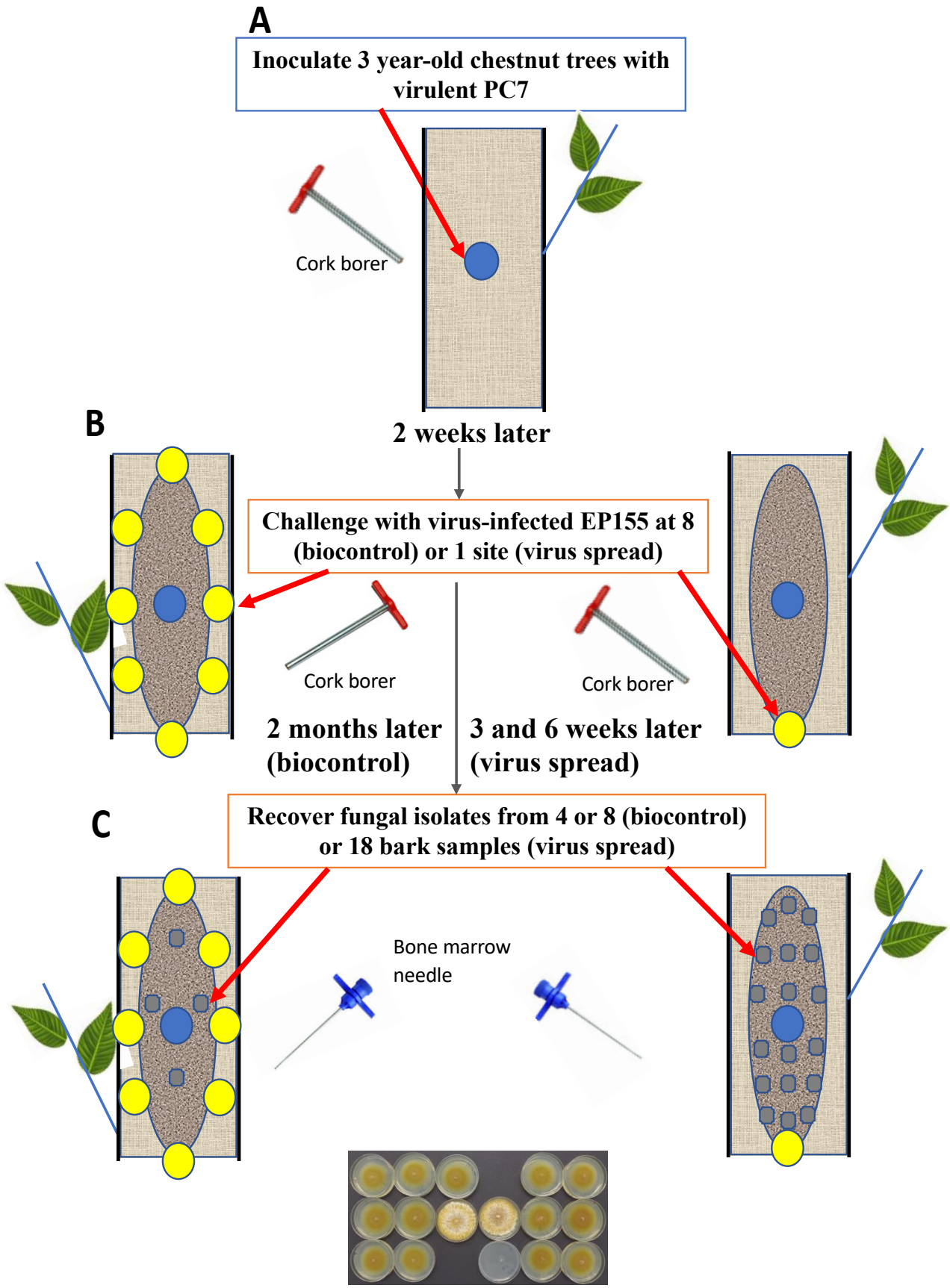
EP155 of US origin



PC7 (Italian strain)



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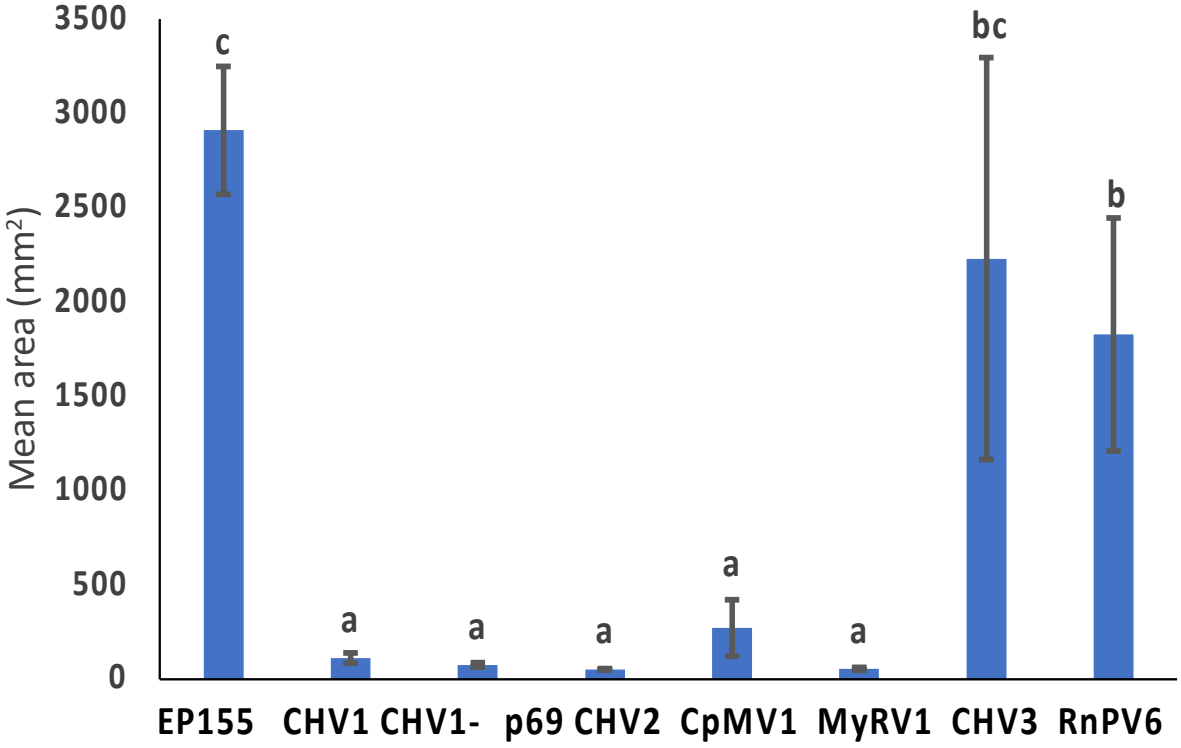
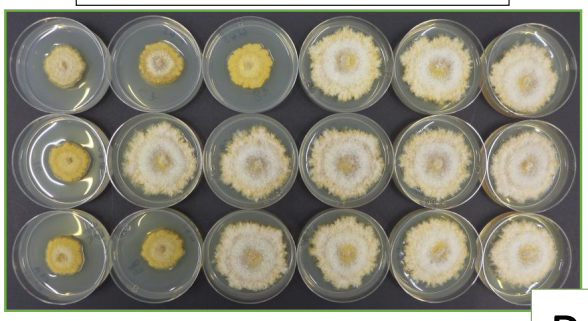


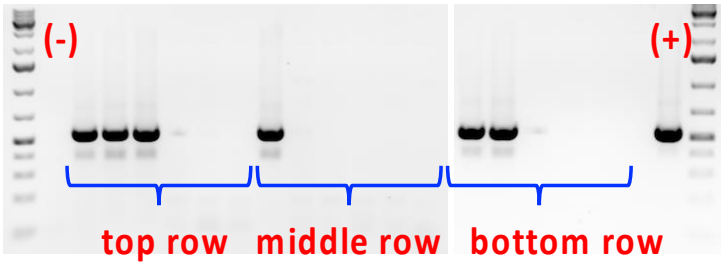
B

Virus infecting EP155	Canker area (mm²)*		
	Two months post inoculation		
EP155 (virus-free)	2669.0	3297.0	2762.4
CHV1-EP713	141.3	86.4	109.9
CHV1- p69	63.6	78.5	86.4
CHV2-NB58	50.2	56.5	50.2
CpMV1-NB631	293.6	113.0	409.8
MyRV1-9B21	50.2	63.6	50.2
CHV3-GH2a	1923.3	3414.8	1351.8
RnPV6-W113	2013.5	1138.3	2331.5

Colony morphology

Virus detection by RT-PCR





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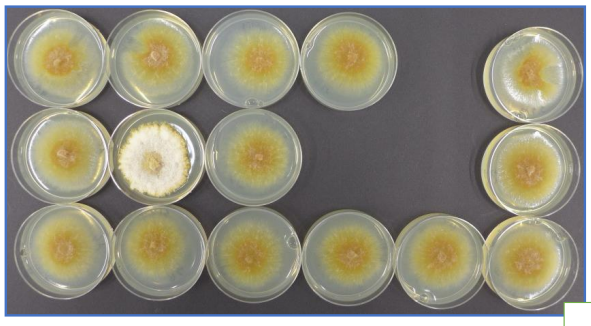

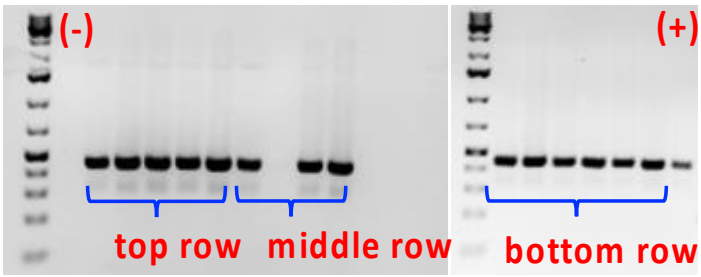

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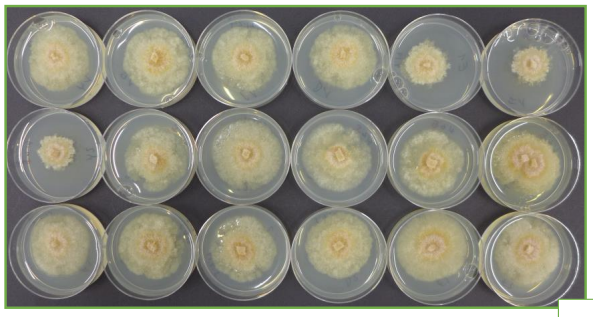

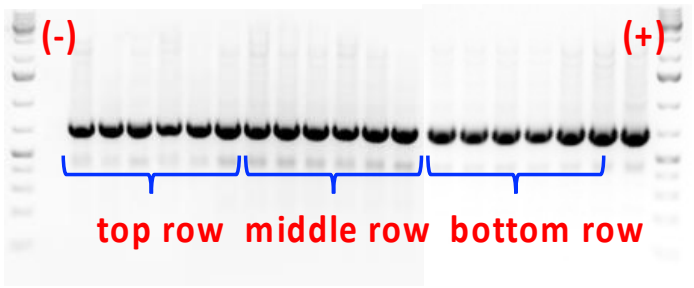

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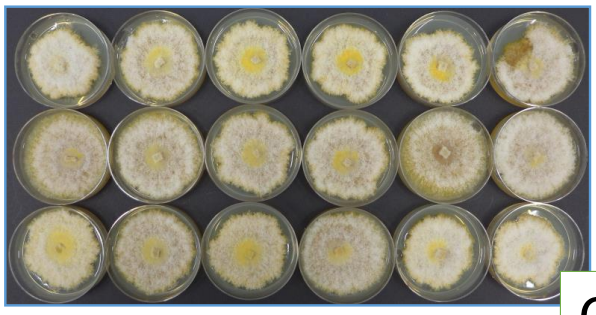

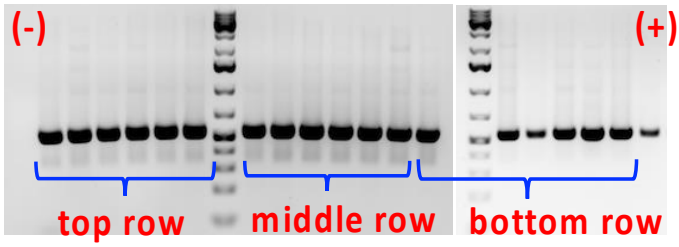

CHV3-GH2a