



Enhanced hypovirus transmission by engineered super donor strains of the chestnut blight fungus, *Cryphonectria parasitica*, into a natural population of strains exhibiting diverse vegetative compatibility genotypes[☆]

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ABSTRACT

Horizontal transmission of virulence attenuating hypoviruses of *Cryphonectria parasitica* is restricted by an allorecognition system termed vegetative incompatibility (*vic*). A super donor formulation of two engineered *C. parasitica* strains (SD328/SD82) with gene disruptions at four of six *vic* loci transmitted hypovirus to strains in the laboratory independent of *vic* genotype. We now report the transmission of hypovirus by the SD328/82 formulation to a diverse, natural *C. parasitica* population infecting American chestnut in a forest setting. Hypovirulent (HV) isolates were recovered from 94% of cankers treated with the hypovirus-infected SD328/82 formulation compared to 51% of cankers treated with a hypovirus-infected EU5/6 formulation (strains having the same *vic* genotypes as SD strains but lacking *vic* gene disruptions). Overall, the SD328/82 formulation transmitted hypovirus into more divergent *vic* genotypes compared to the EU5/6 formulation. These results demonstrate the SD328/82 formulation can serve as an enhanced hypovirus vector for highly divergent *C. parasitica* populations.

1. Introduction

The development of strains of the chestnut blight fungus, *Cryphonectria parasitica* (Murr.) Barr, with enhanced ability to transmit virulence-attenuating hypoviruses was reported by Zhang and Nuss (2016). This was accomplished by the systematic disruption of genes that regulate the vegetative incompatibility (*vic*) fungal allorecognition system to remove restrictions to cytoplasmic mycovirus transmission. With few exceptions (Yu et al., 2013), mycoviruses have evolved exclusive intracellular replication cycles (Buck, 1986), limiting their transmission to vertical transmission through asexual spores and horizontal transmission through anastomosis (fusion of hyphae). The *vic* systems restrict horizontal mycovirus transmission due to the incompatible reactions triggered when genetically distinct, *vic* incompatible individuals of the same species interact. This reaction results in localized programmed cell death along the zone of contact (Glass et al., 2000; Jacobson et al., 1998; Saupe, 2000), thereby restricting transmission of cytoplasmic elements, including viruses (Biella et al., 2002; Boland, 2004; Caten, 1972; Hall et al., 2010; Newhouse

and MacDonald, 1991).

The *vic* system in *C. parasitica* is controlled by at least six loci that have been identified at the genetic level (Cortesi and Milgroom, 1998) and characterized at the molecular level (Choi et al., 2012; Zhang et al., 2014). Gene disruption analyses (Choi et al., 2012; Zhang et al., 2014) provided formal confirmation that five of the six loci contribute to restriction of virus transmission. Allelic differences at the *vic4* locus have been shown not to restrict virus transmission (Anagnostakis et al., 1986). Zhang and Nuss (2016) reported the disruption of multiple *C. parasitica* *vic* genes to engineer super hypovirus donor (SD328 and SD82) strains. This involved the systematic disruption of four of the five virus-restricting *vic* gene alleles, *vic1a-2*, *vic3b-1*, *pix6-2* and *vic7a-2*, in *C. parasitica* strain EP155 using an adapted Cre-loxP recombination system. Since the *vic2-2* locus in EP155 was refractory to donor-promoting gene disruption, the quadruple *vic* gene mutant was crossed with the *vic2-1*-containing strain EP146 and progeny containing the four *vic* gene disruptions and either *vic2-1* or *vic2-2* were selected. The combination of these two mutant strains (SD328/82 formulation) was able to transmit hypoviruses to virus-free strains that were heteroallelic

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at one-to-five of the virus-restricting *vic* loci under laboratory conditions.

The effective use of hypoviruses for biological control of chestnut blight requires efficient transmission to virulent (virus-free) strains (Dawe and Nuss, 2013; Milgroom and Cortesi, 2004). There is general agreement, based on extensive field studies in Europe and North America, that a negative correlation exists between *vic* diversity and hypovirus transmission and thus also, biological control efficacy (Anagnostakis et al., 1986; Heiniger and Rigling, 1994; Milgroom and Cortesi, 2004; Robin et al., 2000, 2009). The results of laboratory transmission studies (Zhang and Nuss, 2016) predict that the SD328/82 formulation could circumvent *vic*-imposed restrictions to virus transmission by serving as an effective vector to introduce hypovirus into natural field strains with widely diverse *vic* genotypic combinations of the six defined diallelic *vic* loci. This prediction was tested by comparing hypovirus transmission by the hypovirus CHV1-EP713-infected SD328/82 formulation and a corresponding hypovirus CHV1-EP713-infected formulation (EU5/6) lacking *vic* gene disruptions into a divergent native *C. parasitica* population typically found in North American forest settings.

2. Results

2.1. Pre-treatment survey for *vic* genotype diversity and pre-existing hypovirulent isolates

Pre-existing cankers infected with *C. parasitica* were sampled to characterize naturally occurring *vic* genotype diversity (Short et al., 2015) and to screen for isolates exhibiting the characteristic CHV-1-induced hypovirulence (virus-containing) phenotype (Hillman et al., 1990) prior to treatment. Analysis of natural isolates from 119 cankers revealed only virulent *C. parasitica* isolates; there was no evidence of the CHV-1 hypovirulent phenotype. These virulent isolates exhibited a high level of *vic* diversity, with 28 of the possible 64 *vic* genotypes (Cortesi and Milgroom, 1998) present (Fig. 1A). In total, the number of virulent *vic* genotypes characterized in each plot prior to treatment was 21 for the SD328/82 plot and 16 for the EU5/6 plot (Fig. 1A).

2.2. SD328/82 formulation exhibits enhanced hypovirus transmission

All 119 cankers representing 28 *vic* genotypes were subjected to a one-time treatment following pre-treatment sampling. This included 70 cankers treated with a combination of CHV1-EP713-infected super donor strains SD328 and SD82 and 49 cankers treated with a

combination of CHV1-EP713-infected donor strains EU5 and EU6, that have the same *vic* genotype as SD328 and SD82 without the gene disruptions (see Section 4 for canker details). Plot designations were SD328/82 and EU5/6.

All cankers were sampled to assess hypovirus transmission efficacy within 12-months post-treatment. The four tissue samples collected from each canker yielded various combinations of virulent and hypovirulent *C. parasitica* and other fungi (Fig. 2). The percentage of hypovirulent (HV) isolates from the SD328/82 tissue samples (38%) was double that recovered from EU5/6 tissue samples (19%) (Fig. 2A). Overall, at least one hypovirulent isolate was recovered from 94.3% (66 of 70) and 51.0% (25 of 49) of SD328/82 and EU5/6 treated cankers, respectively (Fig. 2B&C).

2.3. Hypovirus characterization of treatment inoculum and recovered HV isolates

Hypovirus characterization was conducted for 14 HV isolates recovered post-treatment from the SD328/82 plot and 15 HV isolates recovered post-treatment from the EU5/6 plot (Table S1). In addition, SD328/82 and EU5/6 treatment strains also were assessed. Sequences were successfully generated for a polymorphic region of the ORF A and compared to CHV1-EP713 NCBI Genbank accession: M57938. All HV isolates and treatment strains yielded sequences with $\geq 99\%$ sequence identity with $\geq 99\%$ sequence coverage for CHV1-EP713 hypovirus, thus confirming the successful transmission of the CHV1-EP713 hypovirus from treatment strains to resident virulent strains.

2.4. Hypovirus transmission by SD328/82 formulation unimpeded by *vic* diversity

Since the SD328/82 formulation was designed as a vector to overcome vegetative incompatibility barriers to hypovirus transmission, the *vic* genotypes of HV isolates recovered post-treatment were determined to confirm hypovirus transmission to natural strains exhibiting divergent *vic* genotypes. Fig. 1 presents the *vic* genotypes of virulent and HV isolates recovered from cankers in each plot. The *vic* genotypes are ordered according to similarity to the *vic* genotypes of the SD328/82 and corresponding EU5/6 treatment donor strains with the number of allelic differences increasing from left to right. Since the increasing number of allelic differences from treatment strains' *vic* genotypes decreases the likelihood of hypovirus transmission, *vic* genotypes that exhibited allelic differences at three or more *vic* loci were considered "highly divergent". Of the *vic* genotypes present in each plot, the

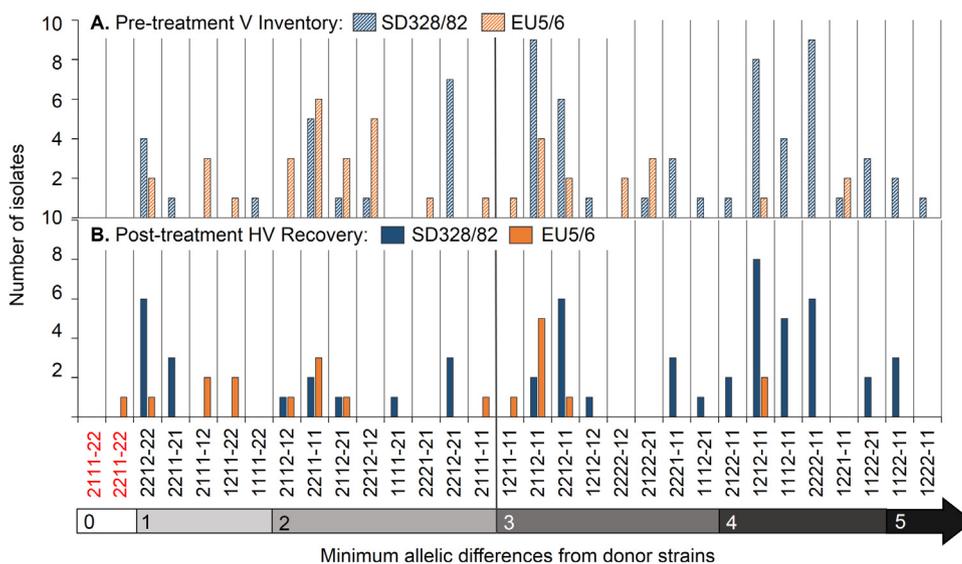


Fig. 1. *Vic* genotypes of *C. parasitica* isolates recovered pre-treatment (A) and post-treatment (B). *Vic* genotypes are ordered by increasing allelic differences from treatment strains; *vic* genotypes with 3 or more allelic differences from treatment strains are considered "highly divergent". The numerical *vic* genotype presented here specifies which allele, designated 1 or 2, is present at the six defined diallelic *vic* genetic loci. For example, the *vic* genotype for the *C. parasitica* reference strain EP155 is *vic*1-2, *vic*2-2, *vic*3-1, *vic*4-1, *vic*6-2 and *vic*7-2 (abbreviated 2211-22). The *vic* genotypes for strains EU5 and EU6 (included in red) are respectively 2211-22 and 2111-22, while the *vic* genotypes for SD strains SD328 and SD82 are respectively 21- and 11-, where the strikes indicate a gene disruption at the respective *vic* loci.

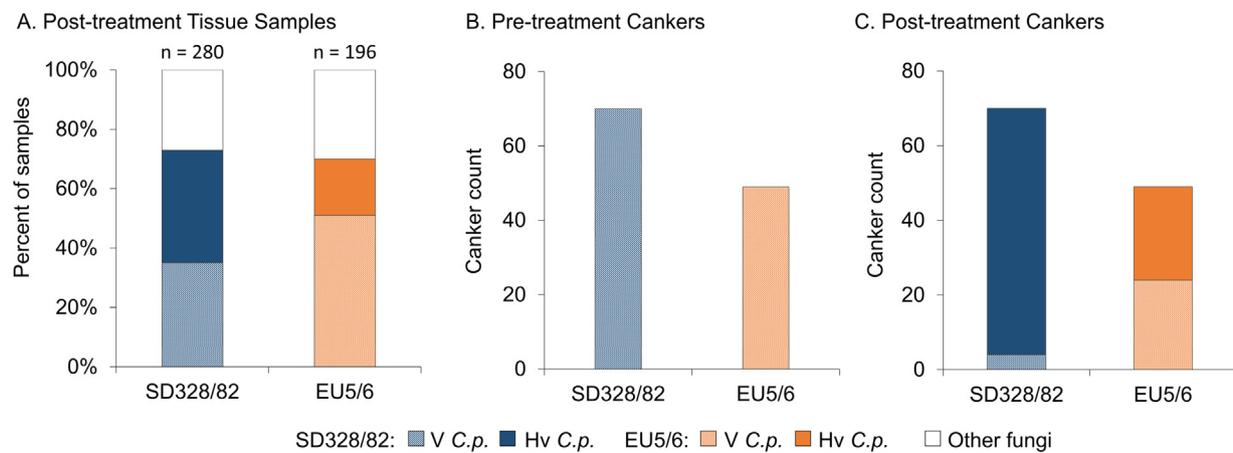


Fig. 2. Pre- and post-treatment sampling of study cankers. A) Percentages of canker fungi (virulent and hypovirulent *C. parasitica* and non-*C. parasitica*) yielded from four tissue samples taken from each SD328/82 and EU5/6 treated canker. B) Number of pre-treatment cankers yielding virulent *C. parasitica* isolates by plot. C) Number of post-treatment virulent and hypovirulent cankers. Cankers were considered “virulent” if no hypovirulent isolates were recovered and “hypovirulent” if at least one hypovirulent isolate was recovered.

number of highly divergent genotypes represented by virulent isolates prior to treatment was 14 in the SD328/82 plot and 7 in the EU5/6 plot.

The 56 HV isolates recovered from SD328/82 treated cankers encompassed 18 *vic* genotypes of which 11 *vic* genotypes (70%) were highly divergent (Fig. 1B). In contrast, of the 21 HV isolates recovered in the EU5/6 plot encompassing 12 *vic* genotypes, only 4 *vic* genotypes (43%) were highly divergent.

2.5. Comparison of in vitro hypovirus transmission efficacy to field-collected *C. parasitica* strains

Since HV transmission frequencies from EU5/6-treated cankers were higher than predicted based on previous laboratory results (Zhang and Nuss, 2016), hypovirus transmission to field-collected virulent isolates by the hypovirus-infected SD328/82 and EU5/6 strains was compared under laboratory conditions. A set of 33 *C. parasitica* isolates recovered across the two test plots and representing 19 distinct *vic* genotypes were paired under laboratory conditions with hypovirus-infected SD328 or SD82 and hypovirus-infected EU5 or EU6, based on a matching *vic2* allele. All 33 isolates exhibited successful transmission by SD328 or SD82 with high transmission regardless of *vic* gene differences. For example, average transmission decreased only slightly from 100% for zero *vic* gene differences to 81% for 1–2 *vic* gene differences and did not drop below 66% with increasing allelic differences (Table 1, Table S2). Eight of the 33 isolates representing eight genotypes exhibited successful transmission by either EU5 or EU6 under lab conditions (Table S2). Overall, percent transmission was lower for EU5 and EU6 pairings and decreased with increasing *vic* gene differences (Table 1). For example, average transmission decreased sharply from

Table 1

In vitro hypovirus transmission to field-collected virulent isolates by CHV1-EP713-infected SD328 (21-), SD82 (11-), EU5 (2211-22), and EU6 (2111-22) when paired (10 times) on PDA in the laboratory, organized by increasing allelic differences from the hypovirulent treatment strains.

Heteroallelic loci (#)	Recipient isolates (#)	Hypovirus transmission	
		EU5/6	SD328/82
0	1	9/10 (90%)	10/10 (100%)
1	8	5/80 (6%)	65/80 (81%)
2	11	5/110 (5%)	86/110 (78%)
3	7	2/70 (3%)	46/70 (66%)
4	5	0/50 (0%)	39/50 (78%)
5	1	0/10 (0%)	9/10 (90%)

90% for zero *vic* gene differences to 6% for 1–2 *vic* gene differences and remained below 5% with increasing allelic differences. Thus, the *vic*-imposed restrictions to hypovirus transmission by the hypovirus-infected EU5/6 strains was much greater under laboratory conditions than under field conditions, while these restrictions on transmission by the SD328/82 strains were mitigated under both conditions.

3. Discussion

High *vic* genotypic diversity of *C. parasitica*, which impedes hypovirus transmission, has been demonstrated for North America and areas in Europe (Mlinarec et al., 2017; Short et al., 2015). The development of super hypovirus donor strains of *C. parasitica* (Zhang and Nuss, 2016) demonstrated the feasibility of enhancing hypovirus transmission by systematic disruption of fungal-host vegetative incompatibility genes. The results reported here confirmed the prediction that the SD328/82 formulation can serve as an effective vector for introducing hypoviruses into a natural, *vic* genotypically diverse *C. parasitica* population.

Vic genotyping of the HV isolates recovered from nearly 95% of SD328/82-treated cankers confirmed hypovirus transmission from the SD328/82 formulation into new resident *vic* genotypes. These included 16 of the 21 *vic* genotypes identified in the pre-treatment survey and two *vic* genotypes not identified in that survey. Notably, the five *vic* genotypes in the SD328/82 plot for which HV isolates were not recovered were highly receptive to hypovirus infection when paired in the laboratory with one or the other of the SD328/82 strains (Table S1). In contrast, laboratory transmission from hypovirus-infected EU5 or EU6 to all included highly divergent *vic* genotypes failed or was very inefficient (Table 1, Table S2). Therefore, the failure to recover HV isolates for all pre-treatment *vic* genotypes is more likely the result of undersampling and/or canker complexity, in which individual cankers harbor multiple *vic* genotypes that failed to receive hypovirus in part due to inadequate application of donor inoculum. Such canker complexity was observed in this study as individual cankers yielded virulent and hypovirulent isolates that differed from each other and the donor strains (Table S3).

Canker complexity was further demonstrated as many cankers yielded both virulent and hypovirulent *C. parasitica* and non-*C. parasitica* isolates. Such diversity has been verified from other studies of chestnut blight cankers (Double et al., 2018; Kolp et al., 2018; Ćurković-Perica et al., 2015). The complexity of cankers, comprised of multiple *vic* genotypes and mixtures of virulent, hypovirulent, and non-*C. parasitica* isolates may partially explain the incomplete acquisition of hypovirus by all residing *C. parasitica* strains. Additionally, the limited

inoculum application as described here may have been sub-optimal for maximizing contact between donor strains and all virulent *C. parasitica* strains residing within these complex cankers.

The recovery rate of HV isolates from EU5/6-treated cankers (51%) exceeded expectations (Zhang and Nuss, 2016) but was half that of the SD328/82-treated cankers (94%). Moreover, the recovered HV isolates represented 12 of the 16 *vic* genotypes in the EU5/6 plot, four of which differed from the treatment strains at three or more *vic* loci (Fig. 1). However, these results are consistent with several reports that *vic*-mediated resistance to hypovirus transmission is lower in infected chestnut tissue than in laboratory transmission assays (Brusini and Robin, 2015; Carbone et al., 2004; Ding et al., 2007; Hogan and Griffin, 2002; Milgroom and Cortesi, 2004; Robin et al., 2010) and were confirmed for virulent field isolates recovered from test plots in this study (Table 1, Table S2). This higher rate of transmission under natural conditions may be mediated by host anatomical features (i.e. bark thickness) and/or other biological interactions (Griffin et al., 2009; Yaegashi et al., 2013; Yaegashi and Kanematsu, 2016; Velasco et al., 2018). Additionally, over half of the *vic* genotypes in the EU5/6 plot differed from that of the treatment strains at only two or fewer *vic* loci, likely contributing to the higher than expected hypovirus transmission rates.

Similar sequence identity between the donor virus (CHV1-EP713) and viruses present in 26 recipient strains of varying *vic* genotypes provides molecular confirmation of successful transmission of the CHV1-EP713 virus from the treatment strains to recipient virulent strains. The HV strains tested represented 22 *vic* genotypes that differed from the treatment strains by 1–5 genes. While further refinements in formulation, application method, and hypovirus composition are warranted, the results of this initial field study clearly show that the SD328/82 formulation can serve as a highly effective vector for introducing hypovirus into natural, genetically diverse *C. parasitica* populations. These results further highlight the potential for the SD328/82 formulation to serve as an integral resource for biological control of natural cankers for effective woodland restoration of the American chestnut.

4. Materials and methods

4.1. Site description

Two plots were established in the Savage River State Forest near Grantsville, MD (APHIS permit #16-133-102r) within a seven-year-old clearcut stand containing an abundance of diseased American chestnut (*Castanea dentata* (Marsh.) Borkh.) basal sprouts symptomatic for chestnut blight. Stems with *C. parasitica* cankers, none girdling more than 75% of the stem's circumference, were selected within each plot. Initially, plots 1 and 2 had 19 trees/49 cankers and 17 trees/33 cankers, respectively. New cankers arising on these same trees over the course of this study were included for a total of 70 and 49 cankers in plots 1 and 2, respectively.

4.2. Pre-treatment *vic* genotype diversity survey

To characterize the natural *vic* genotype diversity of the *C. parasitica* population prior to treatment, four 2 mm-diameter bark samples were excised from canker margin tissues at 0°, 90°, 180°, and 270° using bone marrow biopsy instruments (Lee-Lok, Plainsboro, NJ) and placed into 96-well microtiter plates (Nalge Nunc International, Rochester, NY) (Fig. 3). Bark samples were secured in microtiter plates with tape and stored in a refrigerated cooler until returned to the laboratory where they were stored at –22 °C until processed. To culture *C. parasitica* isolates, bark samples were thawed, soaked for 15 min in a 1:10 commercial bleach-sterile water solution for surface disinfection, and then transferred onto glucose yeast-extract (GYE) agar amended with streptomycin-sulfate and tetracycline-hydrochloride antibiotics, as



Fig. 3. Canker sampling and treatment procedure. Four 2 mm-diameter tissue samples (designated as red circles) were excised from canker margin tissues at 0°, 90°, 180°, and 270° using bone marrow biopsy instruments. One centimeter circular reservoirs for treatment inoculum were made every 2-cm around the margin of each canker excluding 0°, 90°, 180°, and 270°, which were not punched to permit subsequent sampling distant from treatment reservoirs. The designated treatment inoculum was applied to the reservoirs, and then the reservoirs were covered with masking tape to minimize desiccation of the treatment inoculum.

described by Short et al. (2015). A multilocus PCR (Short et al., 2015) protocol resolved the *vic* genotype of one recovered virulent *C. parasitica* isolate per canker to characterize the *vic* diversity of study cankers.

4.3. Fungal strains and growth conditions

C. parasitica strains used in this study included *vic* genotype tester strains (Cortesi and Milgroom, 1998) EU5 (ATCC MYA-1048) and EU6 (ATCC MYA-1049) and the super mycovirus donor strains SD328 and SD82 (Zhang and Nuss, 2016). The EU5/6 and SD328/82 strains were infected with hypovirus CHV1-EP713 (Chen et al., 1994). Hypovirus CHV1-EP713 infection results in morphological changes that include reduced growth rate and reduced pigment production resulting in white colonies (Hillman et al., 1990) with a central area that is orange- or brown-pigmented depending on the pigmentation characteristics of the corresponding virus-free strain: orange for EU5 and EU6 strains and brown for SD328 and SD82 strains (Zhang and Nuss, 2016). Strains EU5 and EU6 used as non-engineered controls in this study have the same *vic* genotype as the engineered SD strains SD328 and SD82, respectively, without *vic* gene disruptions. True isogenic non-engineered strains of SD328 and SD82 do not exist because the SD strains were selected from progeny of a mating step between strain EP155 containing disruptions of *vic* gene alleles *vic1a-2*, *vic3b-1*, *pix6-2* and *vic7a-2* with brown-pigmented strain EP146 (Zhang and Nuss, 2016).

Each *C. parasitica* strain was grown on potato dextrose agar (PDA; Difco, Detroit, Michigan) at 20 °C with a 16 h/8 h light/dark cycle under cool fluorescent lamps for 14 days. A 4 L Waring® commercial-grade blender was used to create a slurry of each treatment inoculum. The SD328/82 inoculum consisted of five colonized 10 cm-diameter PDA plates each of CHV1-EP713-infected strains SD328 and SD82. The EU5/6 inoculum consisted of five colonized plates each of CHV1-

EP713-infected strains EU5 and EU6. Each treatment inoculum was blended with 750 mL sterile dH₂O and 500 mL 2.5% solidified water agar. Each inoculum was added to a 500 mL sterile squirt bottle (autoclavable polypropylene) and kept at 4 °C prior to treatment application.

4.4. Basic study design and treatments

This one-year study was conducted between July 2016 and July 2017. Cankers in Plot 1 (SD328/82 plot) were treated with a combination of CHV1-EP713-infected strains SD328 (21-) and SD82 (11-). Plot 2 (EU5/6 plot) cankers were treated with the combination of CHV1-EP713-infected strains EU5 (2211-22) and EU6 (2111-22). A 1 cm-diameter steel punch and hammer were used to create reservoirs (through the bark to the vascular cambium) for treatment inoculum around the margin of each canker. Reservoirs were placed every 2 cm excluding 0°, 90°, 180°, and 270°, which were not punched to permit subsequent sampling distant from treatment reservoirs (Fig. 3). The designated treatment inoculum was applied to the wounds, after which reservoirs were covered with masking tape to minimize desiccation of the treatment inoculum. New cankers arising on test trees were sampled and treated similarly when discovered.

4.5. Post-treatment sampling and vic genotyping

Four tissue samples were taken from each canker away from treatment reservoirs (at 0°, 90°, 180°, and 270°) within twelve months post-treatment to recover *C. parasitica* isolates (Fig. 3). The recovered *C. parasitica* isolates were examined for white colony morphology characteristic of CHV1-EP713 infection (Heiniger and Rigling, 1994). The vic genotyping protocol served to resolve the vic genotype of one representative hypovirulent isolate recovered from each individual canker. A hypovirulent isolate with a vic genotype different than the treatment strains was considered indicative of successful hypovirus transmission.

4.6. In vitro virus transmission assay

Selected virulent *C. parasitica* isolates recovered from cankers within the study area and representing vic genotypes with 0- to 5-gene differences were paired ten times on PDA (with 0.4 mL Tween 20/L) with hypovirus-infected SD328 or SD82 and hypovirus-infected EU5 or EU6, based on a matching vic 2 allele (Table S1). Mycelial plugs (~5 × 7 mm) of 7-day-old cultures of virulent and hypovirulent isolates were paired adjacent to one another and incubated at 20 °C under cool white fluorescent lamps for 12–14 days. Successful virus transmission was indicated by a change in morphology of the virulent isolate to the white phenotype characteristic of CHV-1 infection (Hillman et al., 1990; Zhang and Nuss, 2016).

4.7. dsRNA isolation, cDNA synthesis, PCR amplification, and sequencing

A dsRNA isolation protocol previously described by Morris and Dodds (1979) (see Method 1) was used with some modifications to confirm the presence of dsRNA associated with treatment strains and HV isolates recovered from both treatment plots. Modifications included grinding samples in liquid nitrogen and suspension of extracted dsRNA in 20 µl of 22 mM Tris, 22 mM boric acid, 250 µM Na₂EDTA buffer. Extracted samples were separated in 1.5% w/v agarose gels made with 0.5% TBE buffer containing 4 µl of 1X SYBR gold (Invitrogen, Grand Island, NY, USA) at 90 V for 1.5 h. First strand cDNA was synthesized using GoScript Reverse Transcription System (Promega Corporation, Madison, USA) using random primers according to the manufacturer's directions with 4 µl of purified dsRNA as described by Mlinarec et al. (2017). PCR was performed using a primer set specific for ORF A of the CHV-1 hypovirus (EP713-5 and R2280) (Allemann

et al., 1999). Two sequencing primers (hvep1 and hvep2) located at conserved regions surrounding a variable region of ORF A (Gobbin et al., 2003) were selected for Sanger sequencing (Eurofins, Louisville, KY, USA). Sequences of 26 HV isolates (14 SD328/82 and 12 EU5/6 isolates) were compared to the CHV1-EP713 genome (GenBank Accession: M57938) using pairwise blastn searches.

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Conflict of interests

The authors declare that they have not competing financial interests.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2018.12.007.

References

- Allemann, C., Hoegger, P., Heiniger, U., Rigling, D., 1999. Genetic variation of *Cryphonectria* hypoviruses (CHV1) in Europe, assessed using restriction fragment length polymorphisms (RFLP) markers. *Mol. Ecol.* 8, 843–854.
- Anagnostakis, S.L., Hau, B., Kranz, J., 1986. Diversity of vegetative compatibility groups of *Cryphonectria parasitica* in Connecticut and Europe. *Plant Dis.* 70, 536–538.
- Biella, S., Smith, M.L., Aist, J.R., Cortesi, P., Milgroom, M.G., 2002. Programmed cell death correlates with virus transmission in a filamentous fungus. *Proc. Biol. Sci.* 269, 2269–2276.
- Boland, G.J., 2004. Fungal viruses, hypovirulence and biological control of *Sclerotinia* species. *Can. J. Plant Pathol.* 26, 6–18.
- Brusini, J., Robin, C., 2015. Mycovirus transmission revisited by in situ pairings of vegetatively incompatible isolates of *Cryphonectria parasitica*. *J. Virol. Methods* 187, 435–442.
- Buck, K.W., 1986. Fungal virology—an overview. In: Buck, K.W. (Ed.), *Fungal Virology*. CRC Press, Boca Raton, FL, pp. 2–84.
- Carbone, I., Liu, Y.-C., Hillman, B.I., Milgroom, M.G., 2004. Recombination and migration of *Cryphonectria hypovirus 1* as inferred from gene genealogies and the coalescent. *Genetics* 166, 1611–1629.
- Caten, C.E., 1972. Vegetative incompatibility and cytoplasmic infections in fungi. *J. Gen. Microbiol.* 72, 221–229.
- Chen, B., Choi, G.H., Nuss, D.L., 1994. Attenuation of fungal virulence by synthetic infectious hypovirus transcripts. *Science* 264, 1762–1764.
- Choi, G.H., Dawe, A.L., Churbanov, A., Smith, M.L., Milgroom, M.G., Nuss, D.L., 2012. Molecular characterization of vegetative incompatibility genes that restrict hypovirus transmission in the chestnut blight fungus *Cryphonectria parasitica*. *Genetics* 190, 113–127.
- Cortesi, P., Milgroom, M.G., 1998. Genetics of vegetative incompatibility in *Cryphonectria parasitica*. *Appl. Environ. Microbiol.* 64, 2988–2994.
- Čurković-Perica, M., Ježić, M., Karin, V., Idžojtić, M., Sotirovski, K., Risteski, M., Rigling, D., Prospero, S., Kolp, M., Double, M., 2015. Biological Control of Chestnut Blight: Persistence of Biocontrol Agent *Cryphonectria parasitica* Hypovirus 1 in Healed Chestnut Cankers 24–27. *International Plant Protection Congress, Berlin, Germany*, pp. 661.
- Dawe, A.L., Nuss, D.L., 2013. Hypovirus molecular biology: from Koch's postulates to host self-recognition genes that restrict virus transmission. *Adv. Virus Res.* 86, 109–147.
- Ding, P., Liu, F.-X., Xu, C.-X., Wang, K.-R., 2007. Transmission of *Cryphonectria hypovirus 1* to protect chestnut trees from chestnut blight. *Biol. Control* 40, 9–14.
- Double, M.L., Jarosz, A.M., Fulbright, D.F., Davelos Baines, A., MacDonald, W.L., 2018. Evaluation of two decades of *Cryphonectria parasitica* hypovirus introduction in an American chestnut stand in Wisconsin. *Phytopathology* 108, 702–710.
- Glass, N.L., Jacobson, D.J., Shiu, P.K., 2000. The genetics of hyphal fusion and vegetative

- incompatibility in filamentous ascomycete fungi. *Annu. Rev. Genet.* 34, 165–186.
- Gobbin, D., Hoegger, P.J., Heiniger, U., Rigling, D., 2003. Sequence variation and evolution of *Cryphonectria hypovirus 1* (CHV-1) in Europe. *Virus Res.* 97, 39–46.
- Griffin, G.J., Eisenback, J.D., Yancey, M.M., Templeton, J., 2009. *Aphelenchoides hylurgi* as a carrier of white, hypovirulent *Cryphonectria parasitica* and its possible role in hypovirulence spread on blight-controlled American chestnut trees. *J. Nematol.* 41, 267–273.
- Hall, C.J., Welch, J., Kowbel, D.J., Glass, N.L., 2010. Evolution and diversity of a fungal self/non-self recognition locus. *PLoS One* 5, e14055.
- Heiniger, U., Rigling, D., 1994. Biological control of chestnut blight in Europe. *Annu. Rev. Phytopathol.* 32, 581–599.
- Hillman, B.I., Shapira, R., Nuss, D.L., 1990. Hypovirulence-associated suppression of host functions in *Cryphonectria parasitica* can be partially relieved by high light intensity. *Phytopathology* 80, 950–956.
- Hogan, E.P., Griffin, G.J., 2002. Spread of *Cryphonectria hypovirus 1* into 45 vegetative compatibility types of *Cryphonectria parasitica* on grafted American chestnut trees. *For. Pathol.* 32, 73–85.
- Jacobson, D.J., Beurkens, K., Klomparens, K.L., 1998. Microscopic and ultrastructural examination of vegetative incompatibility in partial diploids heterozygous at *het* loci in *Neurospora crassa*. *Fungal Genet. Biol.* 23, 45–56.
- Kolp, M., Fulbright, D.W., Jarosz, A.M., 2018. Inhibition of virulent and hypovirulent *Cryphonectria parasitica* growth in dual culture by fungi commonly isolated from chestnut blight cankers. *Fungal Biol.*
- Milgroom, M.G., Cortesi, P., 2004. Biological control of chestnut blight with hypovirulence: a critical analysis. *Ann. Rev. Phytopathol.* 42, 311–338.
- Mlinarec, J., Ježić, M., Čosić, J., Čurković-Perica, M., 2017. Multilocus PCR assay reveals high diversity of vegetative compatibility types in populations of *Cryphonectria parasitica* in Croatia. *Plant Pathol.* <https://doi.org/10.1111/ppa.12751>.
- Morris, J.T., Dodds, J.A., 1979. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. *Phytopathology* 69, 854–858.
- Newhouse, J.R., MacDonald, W.L., 1991. The ultrastructure of hyphal anastomoses between vegetatively compatible and incompatible virulent and hypovirulent strains of *Cryphonectria parasitica*. *Can. J. Bot.* 69, 602–614.
- Robin, C., Anziani, C., Cortesi, P., 2000. Relationship between biological control, incidence of hypovirulence and diversity of vegetative incompatibility types of *Cryphonectria parasitica* in France. *Phytopathology* 90, 730–737.
- Robin, C., Capdeville, X., Martin, M., Traver, C., Colinas, C., 2009. *Cryphonectria parasitica* vegetative compatibility type analysis of populations in south-western France and northern Spain. *Plant Pathol.* 58, 527–535.
- Robin, C., Lanz, S., Soutrenon, A., Rigling, D., 2010. Dominance of natural over released biological control agents of the chestnut blight fungus *Cryphonectria parasitica* in south-eastern France is associated with fitness-related traits. *Biol. Control* 53, 55–61.
- Saupe, S.J., 2000. Molecular genetics of heterokaryon incompatibility in filamentous ascomycetes. *Microbiol. Mol. Biol. Rev.* 64, 489–502.
- Short, D.P.G., Double, M.L., Nuss, D.L., Stauder, C.M., MacDonald, W.L., Kasson, M.T., 2015. Multilocus PCR assays elucidate vegetative incompatibility gene profiles of *Cryphonectria parasitica* in the United States. *Appl. Environ. Microbiol.* 81, 5736–5742.
- Velasco, L., Arjona-Girona, I., Ariza-Fernández, M.T., Cretazzo, E., López-Herrera, 2018. A novel hypovirus species from Xylariaceae fungi infecting avocado. *Front. Microbiol.* 9, 1–14.
- Yaegashi, H., Kanematsu, S., 2016. Natural infection of the soil-borne fungus *Rosellinia necatrix* with novel mycoviruses under greenhouse conditions. *Virus Res.* 219, 83–91.
- Yaegashi, H., Nakamura, H., Sawahata, T., Sasaki, A., Iwanami, Y., Ito, T., Kanematsu, S., 2013. Appearance of mycovirus-like double-stranded RNAs in the white root rot fungus, *Rosellinia necatrix*, in an apple orchard. *FEMS Microbiol. Ecol.* 83, 49–52.
- Yu, X., Li, B., Fu, Y., Xie, Y., Cheng, J., Ghobrial, S.A., Li, G., Yi, X., Jiang, D., 2013. Extracellular transmission of a DNA mycovirus and its use as a natural fungicide. *Proc. Natl. Acad. Sci. USA* 110, 1442–1457.
- Zhang, D.X., Nuss, D.L., 2016. Engineering super mycovirus donor strains of chestnut blight fungus by systematic disruption of multilocus *vic* genes. *Proc. Natl. Acad. Sci. USA* 113, 2062–2067.
- Zhang, D.X., Spiering, M.J., Dawe, A.L., Nuss, D.L., 2014. Vegetative incompatibility loci with dedicated roles in allorecognition restrict mycovirus transmission the chestnut blight fungus. *Genetics* 197, 701–714.