Hypovirulence in the control of *Cryphonectria parasitica*

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Introduction

The American chestnut (*Castanea dentata*) was once prominent in the landscapes of eastern North American.[1] Fast-growing with a tall, broad trunk, this tree species was estimated to make up 25% of hardwood forests in its native range.[2] The regular mast crop produced by *C. dentata* was of substantial ecological value, supporting a wide variety of forest species throughout upland areas of the Eastern United States.[3] In the southern Appalachians, *C. dentata* made up 40-50% of forest canopy and 25% of lumber volume.[4] The valuable straight-grain timber of *C. dentata* was used to produce a variety of wood products.[4] Today, however, the American chestnut is nearly absent in mature form throughout its former native range.[5] The pathogenic fungus known as chestnut blight (*Cryphonectria parasitica*) was responsible for near elimination of the American chestnut tree in the United States.[5] *C. parasitica* was first observed in 1904 in New York City.[5] From this point, *C. parasitica* spread across American deciduous forests. American chestnut deaths due to *C. parasitica* were observed in the southern Appalachians by 1940.[6] American Chestnut trees may survive as saplings but are vulnerable to chestnut blight once reaching a sufficient size.[5] *C. dentata* has therefore been reduced to an understory shrub, sprouting and regrowing until trees are struck by blight-induced dieback.[5]

Cryphonectria parasitica is an ascomycete bark pathogen capable of reproducing sexually or asexually through spores.[7] In North America, *C. parasitica* ascospores are dispersed during rainfall in summer and autumn.[7] These spores germinate on bark and infect gaps or fissures in trunks and branches. *C. parasitica* infections result in necrotic lesions on the bark of susceptible hosts.[5] Sporulation on infected bark results in the development of visible fruiting bodies.[5] *C. parasitica* kills *C. dentata* when bark is girdled by cankers.[5] Cankers appear as sunken, orange-colored indentations in tree bark that slowly spread around the circumference of

the trunk or branch.[5] Cankers destroy the phloem of *C. dentata*, significantly reducing the transportation of water across the affected tissue.[8] When the bark is completely girdled by cankers, growth beyond the girdled area dies, having been deprived of water and nutrients.[9] *C. parasitica* is capable of causing less-lethal infections in other tree species, allowing the fungus to remain common even after the widespread extinction of *C. dentata.*[5] Trees in the genus *Quercus*, as well as other chestnut tree species, are capable of surviving infection by *C. parasitica*.[5] These trees may provide a reservoir for the continued presence and spread of *C. parasitica* even after the functional extinction of the American chestnut.[5] Limiting the harmful effects of *C. parasitica* therefore requires a more complex strategy. Attempts have been made to control the lethality of *C. parasitica* using hypovirulence, in which the infection of a fungal pathogen with a virus reduces the ability of the fungus to cause disease.[5] These attempts have had varying success and may be augmented in the future through the use of genetic engineering.

Control of *C. parasitica* using hypovirulence

The potential of hypovirulence to reduce the damaging effects of *C. parasitica* has been studied as a possible method of facilitating the return of the American chestnut.[10] Four major varieties of *Cryphonectria* hypovirus have been identified.[5] All are double-stranded RNA viruses.[10] These viruses are capable of causing hypovirulence in *C. parasitica*.[11] The hypovirulent fungal phenotype caused by *Cryphonectria* hypovirus is stable after initial infection.[11] Hypovirulence has been shown to effectively control *C. parasitica* on individual chestnut trees.[10] Hypoviruses are theoretically capable of being transmitted between fungi, making the intentional introduction of *Cryphonectria* hypovirus a plausible strategy for population-wide control of *C. parasitica.*[10.] Hypovirus infections can affect *C. parasitica* pathogenicity through a variety of mechanisms.[12] Hypovirus variant CHV-1 reduces fungal

reproduction and canker growth.[10] Hypovirus-infected *C. parasitica* isolates exhibit significantly reduced sporulation compared to uninfected isolates.[12] Overall canker size is also significantly reduced in these infected isolates.[12] Diminished canker size is a key variable when considering the survival of American chestnut trees infected by *C. parasitica.*[13] Hypovirus-infected *C. parasitica* tends to form superficial inactive cankers that stop expanding prior to girdling the tree.[13] Importantly, hypoviruses are transmitted into 95% of asexual spores, supporting the validity of vertical hypovirus transmission.[12]

The effects of hypovirus infection on *C. parasitica* involve multiple regulatory pathways.[14] Some phenotypic alterations can be attributed to changes to proteins involved in signaling pathways.[14] Hypovirus infection is shown to reduce levels of cpg-1 and lac-1 fungal proteins.[14] cpg-1 is a g-protein alpha-subunit.[14] Experimental deletion of *cpg*-1 has been shown to produce changes in fungal phenotype similar to those of hypovirus-infected fungi, including decreased virulence, decreased sporulation, and decreased *lac*-1 transcription.[15] These changes suggest that cpg-1 interacts with multiple signaling pathways involved with fungal virulence.[14] Reductions in cpg-1 are associated with elevated cAMP levels, suggesting a possible mechanism through which *Cryphonectria* hypovirus might alter host phenotypes.[14] Transcriptome analysis has revealed multiple virulence-related genes that have modified expression as a result of hypovirus infection.[16] The gene *crp1*, responsible for the protein hydrophobin cryparin, was found to be downregulated with hypovirus infection.[16] Hydrophobin cryparin is a cell-wall associated protein that may facilitate sporulation by assisting the formation of fruiting bodies.[16] Hypovirus infection also suppresses the gene for cutinase, a protein involved in fungal penetration of the host plant.[16] The altered expression of these genes may be responsible for reduced reproduction and lethality of hypovirus-infected *C. parasitica.*

Failure of hypovirulence in the control of *C. parasitica*

The introduction of *Cryphonectria* hypovirus has shown success at controlling individual cankers but has not achieved widespread success at a population level.[17] Fungal viruses lack an extracellular phase and are typically transmitted vertically through spores.[10] Horizontal transmission may occur through anastomosis, in which adjacent fungal colonies merge.[17] Horizontal transmission of *Cryphonectria* hypovirus is limited by vegetative incompatibility among *C. parasitica.*[18] Vegetative incompatibility occurs when alleles of at least one of six different vegetative incompatibility (*vic)* loci differ between two individual *C. parasitica* fungi.[17] Fungal strains with different alleles at *vic* loci show a decreased ability to fuse, reducing hypovirus transmission.[17] Adjacent growth of *vic* incompatible *C. parasitica* results in programmed cell death.[10] When this programmed cell death occurs, *Cryphonectria* hypovirus transmission is reduced.[10]

Cryphonectria hypovirus has been shown to be an effective method of reducing *C. parasitica* pathogenicity in areas with low *vic* gene diversity.[17] However, limited transmission among different vegetative compatibility types has led to the observed failure of attempts to use hypoviruses to control *C. parasitica* on a large scale.[17] North American *C. parasitica* populations display a high level of *vic* diversity.[17] Higher numbers of differing vegetative incompatibility alleles in a *C. parasitica* population are associated with decreased natural hypovirus transmission.[19] By contrast, European forests have a lower diversity of *vic* genotypes.[10] This lower *vic* diversity may explain the observed high incidence and low mortality of *C. parasitica* infections in Europe.[10] If lower *vic* diversity increases the effectiveness of hypoviruses in controlling the severity of *C. parasitica* infections, these areas would likely exhibit reduced mortality upon intentional introduction of *Cryphonectria* hypovirus.[10] While an examination of

vic diversity presents a convincing argument for the success of hypovirus control of *C. parasitica* in Europe and its corresponding failure in North America, it is possible that other factors are also at work. *C. parasitica* strains in Europe may not be as damaging as those in North America, while European chestnut stands may also be more resistant to blight.[10] Intentional treatment of trees with hypovirulence in areas of high *vic* diversity may be effective when managed at a small scale, but failure occurs when attempts are made to expand to population-level control.[10]

Attempts to enhance hypovirus transmission and effectiveness

Genetic engineering had been considered in order to increase the effectiveness of *Cryphonectria* hypovirus in the control of *C. parasitica*. Researchers have disrupted *vic* genes in *C. parasitica* responsible for restricting virus transmission in an attempt to overcome the barrier of vegetative incompatibility.[20] The disruption of multiple *vic* genes was accomplished through a sequence of excision and mating.[20] These mutant variants were capable of effectively transmitting hypovirus to strains with differing alleles at multiple *vic* loci.[20] The introduction of *C. parasitica* strains genetically engineered to transmit hypovirus with increased effectiveness could serve as a viable method of biological control. Hypovirulent strains of *C. parasitica* can also be directly engineered by integrating complementary DNA (cDNA) copies of hypovirus RNA into *C. parasitica* chromosomes.[21] This integrated chromosomal DNA is capable of producing viral double-stranded RNA and converting the modified strain to the hypovirulence phenotype.[21] The hypovirulence established through cDNA modification can then be spread through mating, bypassing the limitations imposed on hypovirus transmission by vegetative incompatibility.[21] When transgenic *C. parasitica* strains are mated, half of the resulting offspring contain the transgenic viral genome.[10] While the virus transmission potential of these transgenic strains is higher than non-transgenic strains as *vic* diversity increases, the inserted viral genome

is also selected against in the fungal population.[10] Hypovirulence reduces the fitness of the host fungus and is therefore usually greater when the horizontal transmission rate of the virus is also high.[10]

Discussion

The modification of *C. parasitica* to enhance hypovirus transmission offers a promising route to blunting the worst effects of chestnut blight in the United States. If these modified strains are successful in widely distributing a hypovirus that reduces the lethality of *C. parasitica*, it might be possible to begin the reintroduction of the American chestnut to eastern forests.[22] Other approaches to increasing the survivability of the American chestnut include alterations to the genes of *C. dentata* itself.[22] The significance of trees to forest ecosystems and the difficulty of controlling tree reproduction complicate proposals to limit the spread of transgenic trees in a forest environment while their environmental impact is being assessed.[22] The potential introduction of transgenic trees to American forests has raised public concern given that their ecological effects are not well studied.[22] Genetic modification of a fungus may therefore be more palatable to the public than the direct genetic engineering of a prominent tree species.

The possibility of using hypovirulence to restore viable American chestnut populations also requires consideration of the broader impacts of tree reintroduction. The long lifespan and fast growth of American chestnut trees suggest that the reintroduction of the American chestnut would increase plant biomass in eastern US forests.[23] American chestnut reintroduction could therefore moderately increase the carbon storage potential of eastern forests.[23] Ecological modeling has been used to predict that the reintroduction of *C. dentata* would proportionally displace other species in the landscape, rather than displacing a single or small number of species

exclusively.[23] The same qualities that made the American chestnut economically valuable prior to its decline also enhance its value in restoring damaged landscapes. The fast growth and regular nut production of *C. dentata* make it a promising candidate for use in rehabilitating former coal mines.[24] Reintroducing *C. dentata*'s mast crop might also increase the population density of forest consumers, including white-footed mice, eastern chipmunks, and gray squirrels.[25] The ecological impacts of returning a tree species to viability should be extensively studied before any attempts at reintroduction are made.

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