Reprogramming the expression of the double-stranded RNA mitovirus OnuMV1c from the mitochondria to the cytoplasm in the fungal pathogen *Ophiostoma novo-ulmi*

by

Erika Dort
Bachelor of Science, University of Victoria, 2011

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in the Department of Biology

Supervisory Committee

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Abstract

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Dutch elm disease (DED) is a debilitating wilt disease that has decimated elm populations globally. The current pandemic of this disease is caused by the ascomycete fungal pathogen *Ophiostoma novo-ulmi*. A number of strategies have been used to attempt to mitigate the effects of DED but none have met any sustainable success, and the disease continues to have severe ecological and economic impacts. Consequently, research focus has turned to the development of control strategies at the genetic level. One such genetic strategy is the use of naturally occurring fungal viruses (mycoviruses) to induce hypovirulence in their fungal hosts. Hypovirulence, or attenuation of fungal pathogenicity using mycoviruses, has been well studied in other systems but has yet to be developed for *O. novo-ulmi*.

A candidate virus, OnuMV1c, was found in an isolate of *O. novo-ulmi* (93-1224) at the western Canadian disease front and its genome was sequenced. OnuMV1c is a mitochondrial virus and has a 3.1 kb single-stranded positive RNA genome that encodes an RNA-dependent RNA polymerase (RdRp) involved in its replication as a double-stranded RNA molecule. It exists in *O. novo-ulmi* mitochondria in both its single-stranded and double-stranded forms.

Our research group identified OnuMV1c as a potential candidate for biological control of Dutch elm disease. Our long-term research goal is to use the virus as a means to activate the RNA interference pathway of *O. novo-ulmi*, leading to down-regulation of genes involved in pathogenicity. If OnuMV1c is engineered such that it carries an RNA interference cassette in addition to its own complement of genes, it could act as an enhanced hypovirus. RNA interference (RNAi) is a cytoplasmic process, and therefore in order to use OnuMV1c for RNAi the viral genome needed to be reprogrammed such that it could be expressed in the cytoplasm rather than the mitochondria. The objectives of my master's research were to 1) genetically engineer OnuMV1c to express in the cytoplasm using a cDNA reverse genetics approach, and 2) test the functionality of the re-engineered cDNA OnuMV1c virus (MV1cCyt).

The first objective was accomplished by modifying codons in the RdRp sequence of OnuMV1c such that the sequence could be translated in the cytoplasm. This genetically engineered cytoplasmic version of OnuMV1c, named MV1cCyt, was flanked with exogenous promoter and terminator sequences to drive its transcription. The entire construct was engineered as a cDNA molecule and was cloned into the fungal transformation vector pAN7-1, which was used to transform *O. novo-ulmi* protoplasts.

The second objective was achieved through the use of strand-specific RT-PCR, a technique that allowed the detection of both the positive and negative strands of MV1cCyt. Results indicated that while four individual cell lineages contained MV1cCyt cDNA stably integrated into the nuclear genome, only one transformant was able to produce double-stranded MV1cCyt RNA. These results have important implications for

the use of OnuMV1c as an engineered hypovirus and represent the first step towards the development of a biological control strategy for Dutch elm disease.

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List of Abbreviations

bp – base pairs

dH_2O – distilled water

ddH_2O – double de-ionized water (for RNA protocols)

DED – Dutch elm disease

DEPC – diethylpyrocarbonate

dsDNA – double-stranded DNA

dsRNA – double-stranded RNA

EDTA – ethylenediaminetetraacetic acid

kb – kilobase (1000 base pairs)

µL - microlitre

M – molar (moles/litre)

mL – millilitre

mM – millimolar (millimoles/litre)

MV1cCyt – re-engineered cytoplasmic version of OnuMV1c

OCM – *Ophiostoma* complete media

OMM – *Ophiostoma* minimal media (used to promote growth of the yeast-like morphology of *Ophiostoma novo-ulmi*)

OnuMV1c – *Ophiostoma novo-ulmi* mitovirus 1c

PCR – polymerase chain reaction

rpm – rotations per minute

RT-PCR – reverse transcription PCR

SDS – sodium dodecyl sulfate

ssDNA – single-stranded DNA

ssRNA – single-stranded RNA

STC – sorbitol; Tris-HCl; calcium chloride buffer

STE – sodium chloride; Tris-Cl; EDTA buffer

STE/15 – sodium chloride; Tris-Cl; EDTA buffer adjusted to 15 % ethanol

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Dedication

To my family – for your constant support and encouragement.

Chapter 1: General Introduction and Literature Review

1.1 The American Elm (*Ulmus americana*)

1.1.1 Elms

Elms (*Ulmus spp*.) represent a large genus of trees with approximately 45 recognized species that are distributed in North America, Europe, and Asia (Scheffer et al., 2008). The majority of elm species (25 to 28) are native to Asia with fewer species native to North America (9 species). The remaining species occur naturally in Great Britain and the rest of Europe (Scheffer et al., 2008). Ease of interspecific hybridization makes it difficult to differentiate certain species, which is why there is often debate over the taxonomic classification of many *Ulmus* species (Scheffer et al., 2008). This effortless interspecific hybridization makes breeding elm hybrids with varying levels of disease resistance very straightforward.

Elms have a long history of use by humans dating from the Neolithic Age when they were used for many purposes from building material to medicine (Smalley and Guries, 1993). Elms also had great cultural significance in ancient Greek and Roman civilizations and were regarded as sacred trees. They were used extensively by Romans as supports for the grape vines in vineyards. In fact, the use of elms in vineyards continued in Italy well into the 20th century at which point farmers were forced to replace the elms due to the devastation caused by Dutch elm disease (Fuentes-Utrilla et al., 2004). Their aesthetically pleasing form and tolerance to air pollution made them an extremely popular tree to plant during the urbanization of Europe, Asia, and North America (Smalley and Guries, 1993). The American elm (*Ulmus americana*) was

considered a particularly desirable species to plant in cities because of its characteristic umbrella-shaped crown that arches over streets and provides ample shade.

1.1.2 Ecology of American Elms

The American elm (*Ulmus americana*) is native to eastern North America. In Canada natural stands of the American elm range from Nova Scotia to southeastern Saskatchewan. In the United States of America (U.S.A.) it occurs as far west as Montana and as far south as Texas and Florida. In this natural range, the American elm is exposed to extremes of climates encountering temperatures between a maximum of forty one degrees celsius (southern U.S.A.) to a minimum of minus forty one degrees celsius (northern Canada) and tolerating a wide variety of precipitation, frost, and soil conditions. It is a major component in four forest types (Black Ash-American Elm-Red Maple, Silver Maple-American Elm, Sugarberry-American Elm-Green Ash, and Sycamore-Sweetgum-American Elm), and a minor component in 20 other forest types (Bey, 1990).

American elms are predominantly wind-pollinated and self-sterile. They begin to produce seed at 15 years of age (Gilman and Watson, 1994), however seed production does not usually occur in abundance until age 40, at which point it has been documented to continue until age 300 in some trees (Bey, 1990). The seeds are disseminated by wind and water, and are a food source for a wide variety of animals, including small rodents and birds (Bey, 1990).

1.1.3 Role of American Elms in Cityscapes

Today, American elms remain one of the most desirable species to plant in urban areas and have become an integral part of many cityscapes worldwide. As a result, they have been planted well out of their natural range and often at high densities, with trees planted in close proximity along city streets. They provide UV protection and shade, resulting in the cooling of city streets during warmer seasons. Their cooling role is crucial and contributes to energy conservation in city ecosystems (Hubbes, 1999). Additionally, American elms are tolerant of stressful environmental conditions and are able to survive periods of flooding and drought, high salt conditions (coastal populations), high winds, pollution, and compacted soil (Newhouse et al., 2007; Scheffer *et al*., 2008). All of these characteristics make American elms ideal trees for urban landscapes, and as such they are assigned a high economic value. For example, a mature elm in the city of Winnipeg, Manitoba has a calculated value of $3,600 using the tree appraisal formula developed by the International Society of Arboriculture (Westwood, 1991). Though this value was calculated for Winnipeg, the value would be similar, if not higher, for elms in other cities across North America (Westwood, 1991). With roughly 700,000 elms in Canadian cities that value adds up to over $2.5 billion (Hubbes, 1999), and for the 7.7 million elm trees in North American cities the value increases to over $27 billion.

In addition to their value in cityscapes, American elms have significant economic value as hardwood trees. The interlocking grain of the wood makes it highly resistant to splitting, and therefore desirable for use in flooring, construction, and furniture (Bey, 1990). These properties, combined with their roles in natural and urban ecosystems, make American elms a highly valuable species from both a cultural and ecological perspective.

1.1.4 American Elm Diseases

American elms contend with a number of diseases including phloem necrosis (elm yellows), *Verticillium* wilt, elm wetwood, twig blight and cankers. The trees are also attacked by hundreds of insect species such as bark beetles, borers, leaf rollers and twig girdlers (Bey, 1990). However, of all the pests and pathogens that American elms must contend with, Dutch elm disease has had the largest and most sustained impact. It has resulted in mass tree fatalities and the complete decimation of elm populations in many cities. With billions of dollars at stake in the American elms currently planted in North American cities, there is significant interest and investment in mitigating Dutch elm disease and preventing a continuation of this devastation.

1.2 Dutch Elm Disease (DED)

Dutch elm disease (DED) is a debilitating wilt disease that has resulted in one of the largest mass destructions of trees in modern history (Hubbes, 1999). It is caused by fungal pathogens in the genus *Ophiostoma*: *Ophiostoma ulmi*, *O. novo-ulmi*, and *O. himal-ulmi*. Dutch elm disease is so named because it was first recognized in the Netherlands by Bea Schwarz who originally isolated and characterized *Ophiostoma ulmi* in 1922 (Brasier, 1991). The disease has decimated elm populations globally, and continues to spread through remaining populations. The pathogenic fungi can infect all elm species with varying effects, but *U. americana* is the most susceptible. Unfortunately, the popularity of lining city streets with American elms resulted in large, densely populated monocultures of *U. americana* in cities that show no resistance once DED is introduced.

Dutch elm disease has spread in two major global pandemics. The first disease wave originated in northwest Europe in 1918, from which it spread southeast throughout the rest of Europe, and west to Britain and eventually North America in 1930 (Brasier, 1991; Hubbes, 1999). This first disease wave was caused by the less pathogenic species *Ophiostoma ulmi*, which had a minor effect on elm populations and declined by the 1940s. The second, and much more destructive, disease wave began in the 1950s and is thought to have had two points of origin: mid-western North America and Eastern Europe (Brasier, 1990). This second pandemic, which continues to spread today, is caused by the highly pathogenic species *Ophiostoma novo-ulmi*. *Ophiostoma novo-ulmi* is fatal to its elm hosts and has resulted in severe population declines. Southern England's 23 million elms were reduced by 17 million due to *O. novo-ulmi*, and in North America the 77 million elms in the United States were diminished to a population of 34 million by 1976. Canada was similarly affected with major cities such as Toronto losing up to 80 % of their elms (Huntley, 1982).

Ophiostoma himal-ulmi also causes disease symptoms but is restricted to Asia, and appears to be endemic to the Himalayas, acting as a natural endophyte to the elm species there. This has led to speculations that *O. novo-ulmi* may have evolved from *O. himal-ulmi* after introduction into Europe, acting as a pathogen towards naïve European elm species and spreading to other parts of Europe and North America (Brasier and Mehrotra, 1995).

The first report of *Ophiostoma novo-ulmi* in Canada was in Quebec in 1944. From there the pathogen spread east to the Maritime Provinces and west through Ontario and Manitoba (Hubbes, 1999). The current disease front has spread through Saskatchewan and is threatening elm populations in the eastern areas of Alberta. The city of Winnipeg has the largest elm population in Canada leading it to be known as the 'City of Elms' (Hubbes, 1999).

Approximately $2.7 million is spent by the City every year on sanitation and pruning of elms in efforts against DED. Even with this investment Winnipeg continues to experience losses of up to 5600 trees a year (Skerritt, 2012).

1.2.1 Disease Cycle

Dutch elm disease is spread through fungal spores that are vectored by elm bark beetles. In North America the two major vectors are the North American elm bark beetle (*Hylurgopinus rufipes*), a native species, and the smaller European elm bark beetle (*Scolytus multistriatus*), an introduced species. The banded elm bark beetle (*S. schevyrewi*), an Asian species recently introduced to North America, has also been confirmed to vector the DED pathogens (Jacobi et al., 2013). The DED fungal pathogens grow in recently dead or dying elm trees, which is where elm bark beetles lay their eggs. Once the beetle larvae hatch they create galleries under the bark, which is where they come into contact with the fungus, picking up the sticky fungal spores. When the beetles emerge from the galleries as adults they immediately fly to a healthy elm tree to feed, carrying thousands of spores with them. As the beetles feed on a healthy elm they create wounds through which the fungal spores enter, germinate, and spread throughout the tree via the xylem vessels. The presence of the fungus in the xylem induces the formation of gum plugs and tyloses (cellular outgrowths), both of which are a common defence employed by plants in response to infection. Typically the tyloses and gum plugs block the xylem vessels and cause the leaf wilting that is characteristic of DED. Subsequently, the fungus continues to spread throughout the tree, creating blockages that eventually prove fatal. In addition to the beetle vectors, the fungus can also enter neighbouring elms through root grafts formed between trees

(Hubbes, 1999). In this way, DED is able to spread rapidly wherever elms are planted in close proximity to one another.

1.2.2 The Phylogeny and Life History of *Ophiostoma novo-ulmi*

1.2.2.1 Fungi

As a fungal pathogen, *O. novo-ulmi* is a member of the supergroup Fungi, which was formerly classified as a kingdom. Fungi are heterotrophic, non-motile organisms that secrete digestive enzymes to obtain nutrients from their environments. Certain fungi can grow as unicellular yeasts, or as multicellular filamentous mycelia, and in some cases both. Mycelia are a collection of branching, walled, tubular filaments called hyphae, which can be separated into distinct cellular compartments by structures called septa (septate hyphae), or can be a multi-nucleate amoeboid mass with no cellular separations (non-septate hyphae). The cell walls of fungi are primarily composed of chitin and alpha-glucans, and can sometimes also contain cellulose (Blackwell et al., 2012).

Fungi generally exist in a haploid state: diploidy, if present, is usually only a small portion of the life cycle. Fungal reproduction can be asexual or sexual. Asexual reproduction occurs via budding in the yeast cells or via the production of asexual spores (conidia) in mycelia. Sexual reproduction occurs through the fusion of hyphae (anastomosis) followed by the production of sexual spores. After hyphal anastomosis there is a phase where the cells of the fused mycelium contain the nuclei from both parents; this phase is referred to as heterokaryosis. The term heterokaryon refers to an organism with cells containing two or more genetically different nuclei. Once heterokaryosis has occurred, the heterokaryon may continue to grow and persist vegetatively or it may go through the process of karyogamy where the two nuclei fuse to

form a zygote. The zygote usually immediately undergoes meiosis to form new haploid sexual spores that are then dispersed into the environment.

Fungi are not separated morphologically into genders, but instead employ a system of mating types, which correspond roughly to sexes in plants and animals. There is a diverse array of systems within fungi that maintain two or more mating types. These mating types are determined genetically by a mating-type (*mat*) locus in the nuclear genome. Therefore, during sexual reproduction one individual will act as the 'male' mating partner/type and the other individual will act as the 'female' mating partner/type. Prior to sexual reproduction two individuals communicate by pheromone signalling, thereby sharing important information for the mating process (Bölker and Kahmann, 1993). In relation to mating types, fungi can be heterothallic or homothallic. In heterothallic species the male and female reproductive structures are on separate individuals and sexual reproduction can only occur if individuals of different mating types meet (out-crossing). Homothallic species possess both mating types on one individual, and self-fertilize (Ni et al., 2011).

Fungi belong to supergroup Opisthokonta, which also includes animals (Metazoa), choanoflagellates, and other close protistan relatives (Simpson and Roger, 2004). The Fungi comprises an extremely large and diverse collection of organisms that exhibit a wide array of life histories. Historically, the classification of groups within the Fungi has been complex and controversial however the development of molecular phylogenetics has led to the current accepted classification of Fungi that breaks the group into seven major phyla: Microsporidia, Blastocladiomycota, Neocallimastigomycota, Chytridiomycota, Glomeromycota, Basidiomycota, and Ascomycota (Hibbett et al., 2007).

The Fungi contain a vast number of pathogenic species that affect both plants and animals (including humans) on a global scale (Fisher et al., 2012). Consequently, there is significant time and money being invested into research on these pathogens in order to improve our understanding of pathogen-host relationships and to develop potential mitigation strategies; *Ophiostoma novo-ulmi* is just one such fungal pathogen.

1.2.2.2 Ascomycota

Ophiostoma novo-ulmi belongs to the Ascomycota, a monophyletic phylum that contains close to 75 % of all described fungi, as well as a significant number of plant and animal pathogens (Taylor et al., 2006). The defining feature of fungi from this phylum is the formation of an ascus, a structure that holds and releases spores, called ascospores, during sexual reproduction. Within the Ascomycota, *O. novo-ulmi* belongs to the subphylum Pezizomycotina, class Sordariomycetes, subclass Sordariomycetidae, order Ophiostomatales, and family Ophiostomataceae (Blackwell et al., 2012).

Sexual reproduction in fungi is governed by the *mat* loci. In addition to the different mating types that all fungi possess, ascomycetes have a complex genetic system known as vegetative incompatibility (also called heterokaryon incompatibility) that regulates self and non-self recognition (Cortesi and Milgroom, 1998). Vegetative incompatibility governs the process of hyphal anastomosis and subsequent heterokaryosis outside of sexual reproduction: these processes can also occur as vegetative phenomena (Leslie, 1993). As a result of vegetative incompatibility, hyphal anastomosis and subsequent exchange of cytoplasmic and genetic material will only occur if two individuals are compatible. The system is governed by vegetative incompatibility (*vic*) genes; two individuals are compatible only if they share the same alleles at

all *vic* loci. This results in the creation of vegetative compatibility (VC) types/groups whereby hyphal anastomosis only occurs within VC groups (Milgroom and Hillman, 2011). Vegetative incompatibility can become extremely complex with up to 17 *vic* loci in some species (Cortesi and Milgroom, 1998). *Ophiostoma novo-ulmi* has a multi-locus vegetative compatibility system with an abundance of unique VC types. These VC types regulate the population structure of *O. novo-ulmi* and restrict transmission of cytoplasmic materials including viruses (Brasier, 1996). It has been observed that when DED is initially introduced to a new region the *Ophiostoma* population exhibits a low diversity of VC types due to the limited genetic variation inherent in a founding population. It was further observed that once the disease became established for many years the diversity of VC types tended to increase significantly though the driving forces for this diversification were initially unclear (Brasier, 1988).

1.2.2.3 *Ophiostoma novo-ulmi* Subspecies

When *Ophiostoma novo-ulmi* was first identified as the more aggressive pathogen responsible for the second DED pandemic, it was separated into two races based on genetic identity and geographic origin: a Eurasian or EAN race, and a North American or NAN race (Brasier, 1991). It was soon noted that the two races were partially reproductively isolated, displayed key morphological and genetic differences, and had distinct geographic ranges. They were subsequently separated into two subspecies with the EAN race assigned as *O. novo-ulmi* subspecies *novo-ulmi*, and the NAN race assigned as *O. novo-ulmi* subspecies *americana*. The two subspecies are distinguished by the form and dimensions of their sexual fruiting bodies, or perithecia (Brasier and Kirk, 2001).

1.2.2.4 Life Cycle

Ophiostoma novo-ulmi is a dimorphic fungus: it grows in both yeast and filamentous (mycelial) forms. This dimorphism contributes to its pathogenicity, as the mycelial form allows it to penetrate bark tissues, and the yeast form allows it to spread quickly through the xylem tissues and root grafts. It reproduces asexually via the production of conidia (asexual spores) that are released and germinate into new vegetative mycelia. Sexual reproduction involves the fusion of hyphae from different mating types, leading to the formation of a flask-shaped perithecium, which produces and releases ascospores (sexual spores). *Ophiostoma novo-ulmi* is heterothallic and has two mating types, 'A' and 'B' (Brasier, 1991). In nature it grows exclusively in elms (*Ulmus* spp.), overwintering under the bark until it can produce spores in the spring to be disseminated by elm bark beetles. Both conidia (asexual) and ascospores (sexual) are produced in sticky droplets that easily attach to the beetles. The production of conidia is triggered by a depletion of nutrients in the environment; the fungus senses the nutrient depletion and produces conidia that are dispersed to a new nutrient-rich habitat. The conidia can also multiply asexually through budding (Brasier, 1991).

1.2.2.5 *Ophiostoma novo-ulmi* in Canada

Populations of *O. novo-ulmi* in North America are generally less diverse than those in Europe. A survey of *O. novo-ulmi* populations across North America showed that they display significantly fewer VC types than their European counterparts (Brasier, 1996). Within Canada, a nine-year genetic survey of the *O. novo-ulmi* population at the disease front in Winnipeg, Manitoba showed genetic uniformity of VC types that did not change over time (Temple et al.,

2006). This low genetic diversity of the Canadian population of *O. novo-ulmi* makes it suitable for biological control as it presents a single uniform target (Temple et al., 2006).

1.2.3 Current Mitigation Strategies for Dutch Elm Disease

Control strategies for a disease can focus on the pathogen, the vector, or the host: mitigation of Dutch elm disease has involved targeting all three. A combination of strategies are required to have any effect. Control of the fungal pathogen has been restricted to use of chemical treatments, specifically benzimidazole-based fungicides and sterol biosynthesis inhibitors. These fungicides were first used in the 1970s after studies showed they were effective against *O. novo-ulmi*. However, the fungicides needed to be injected into the elms, causing wounds that the trees responded to using compartmentalization, which in turn resulted in reduced efficacy of the fungicides. Furthermore, the fungicides were costly to use and the fungal pathogens developed resistance. The fungicides also had unintended off-target effects on the elm hosts as well as the surrounding environment. All these factors created a barrier to large-scale application of a fungicide-based control of DED (Hubbes, 1999). Some fungicides such as Arbotect 20-S, a benzimidazole-based chemical, are still used today but only in very specific cases and only on a small scale. Chemical intervention has not been a long-term solution for elimination of DED (Scheffer et al., 2008).

Control of the bark beetle vectors of DED has also been explored as a mitigation strategy. This has mostly involved the use of insecticides in areas with large beetle populations. However, as with fungicides, the insecticides have off-target effects on the environment and are costly to use. They are only effective when used in specific situations and cannot be applied for large-scale control efforts (Hubbes, 1999). The use of a biological control agent against the elm bark

beetles, specifically entomopathogenic nematodes, was proposed by Tomalak et al. in 1989, but has not yet been further explored as a potential control strategy.

Control of DED has been the most extensively explored at the host level. Through the majority of the 20th century tree breeders experimented with selection and breeding of DED-resistant elms. Asian elm species exhibit the most natural disease resistance; subsequently they have been the focus of most breeding experiments. The American elm is so highly valued because of its aesthetic qualities but the Asian elm species do not display similar characteristics. In addition, despite the general ease of interspecific hybridization between most elm species, the American elm shows a strong incompatibility barrier with other elms. This makes it especially difficult for breeders trying to introduce DED-resistance from other species into the American elm, while maintaining the characteristic vase-shaped canopy that is so desired (Scheffer et al., 2008). Despite these difficulties, resistance breeding programs seem to provide the best long-term strategy for combating DED. Unfortunately, they do not provide a solution for the millions of remaining elms that are threatened by the disease (Hubbes, 1999).

Research into transgenics as a means of DED control has also been pursued at the host level. Newhouse et al. (2007) used *Agrobacterium* to transform leaves from American elm seedlings with a transgene encoding the antimicrobial peptide ESF39A. ESF39A has been shown to reduce the growth of a variety of fungal pathogens, and the American elms transformed with the peptide demonstrated increased resistance to *Ophiostoma novo-ulmi* (Newhouse et al., 2007). The study was the first to transform American elms with pathogen resistance genes and opened the possibility of using transgenics to create future populations of elms that can resist DED. Similarly to resistance breeding programs, transgenics show promise as a future strategy against DED but do not provide a current solution for elms threatened by the disease.

Currently, the most commonly used control strategy for DED is sanitation and pruning. This involves the pruning of infected tree parts from living elms, as well as the removal of dead elm tree material harbouring both the beetle and fungal populations (Hubbes, 1999). In Canada, it also involves the strict control of firewood transportation in order to prevent movement of potentially infected wood from province to province (Alberta Agriculture and Rural Development, 2012). Sanitation is a costly process and does not eliminate DED, but it is necessary for successful control of the disease (Hubbes, 1999).

Mitigating DED using a biological control approach has also been explored. The first attempts involved inoculating elms with minimally virulent strains of *O. ulmi* in order to induce resistance in the trees (Scheffer et al., 2008). Research done by Martin Hubbes and colleagues in the 1980s showed that elm trees inoculated with the less aggressive *O. ulmi* strains acquired resistance to *O. novo-ulmi* (Hubbes and Jeng, 1981). The inoculated trees still showed symptoms of DED, but were able to survive the disease. However, the long term results of these experiments showed that the success of induced resistance was dependent on tree genotype as well as tree health and local conditions (Hubbes, 2004). Other inoculation experiments have been performed on American elms using *Verticillium* species, which are fungal pathogens that cause vascular wilt diseases in other plant species. After inoculation with *Verticillium*, elms that were subsequently infected with *O. novo-ulmi* showed significantly reduced DED symptoms (Scheffer et al., 2008). Clearly, induced resistance is a promising control strategy for DED but it requires manual inoculation of individual elms and therefore cannot self-propagate through infected elm populations. It would thus be very difficult to use this induced resistance as a large-scale mitigation strategy for DED.

The advent of new molecular techniques allow for detailed genetic studies on both the fungal pathogen and elm host. Understanding both the virulence of the fungus and the defence response of the tree will lead to the creation of more informed approaches to control, specifically for transgenics. Breeding strategies and biological controls can be more effective if the specific genes involved in both *O. novo-ulmi* pathogenicity and *U. americana* resistance are known. In addition, approaches to disease control at the molecular level may result in highly specific and effective mitigation of DED. One such molecular approach is hypovirulence, a biological control approach that has been explored for other fungal pathogens.

1.3 Hypovirulence

Hypovirulence occurs when an infectious agent, such as a virus, reduces the virulence of its host pathogen. Fungal viruses, known as mycoviruses, are commonly found in all taxonomic groups of fungi (Nuss and Koltin, 1990). In some fungal pathogens hypoviruses have been discovered that reduce the ability of their fungal host to cause disease in plants. The effects of these hypoviruses on their fungal hosts include changes to morphology and phenotype, reduction of mycelial growth, inhibition of sexual and asexual reproduction, and changes to their mitochondrial function (Hillman et al., 1990; Nuss, 2005; Ghabrial and Suzuki, 2009; Pearson et al., 2009). The discovery of these hypoviruses has led to extensive research into hypovirulence as a biological control strategy for fungal pathogens.

The plant-pathogen system where hypovirulence has been studied most extensively is the chestnut blight system. *Cryphonectria parasitica* is the ascomycete fungus that causes chestnut blight, a severe disease that has destroyed populations of chestnut trees throughout North America and Europe. *Cryphonectria parasitica* normally infects chestnut trees through wounds

in the bark causing cankers that slowly girdle the trunk and kill the tree. In 1950, researchers in Italy made the first discovery of hypovirulence through their observations of abnormal, superficial cankers occurring in European chestnut trees (*Castanea sativa*) infected with *C. parasitica* (Biraghi, 1950). It was soon determined that these hypovirulence-associated healing cankers were caused by a naturally occurring cytoplasmic double-stranded RNA (dsRNA) mycovirus (Day et al., 1977; Choi and Nuss, 1992), which was later named *Cryphonectria hypovirus 1* (CHV-1). Since its discovery, CHV-1 has been studied extensively and three other species of hypoviruses have been described in *C. parasitica*: CHV-2 (Hillman et al., 1994), CHV-3 (Smart et al. 1999), and CHV-4 (Hillman et al., 2000). CHV-1 has been explored as a biological control for chestnut blight because the virus inhibits both asexual and sexual reproduction of *C. parasitica* and decreases canker growth on fungus-infected chestnut trees (Milgroom and Cortesi, 2004). To date, CHV-1 has been used as a biological control with mixed success in Europe and North America. In Europe it spreads naturally through *C. parasitica* populations and appears to control the disease front of chestnut blight, however in North America the effects of CHV-1 as a biological control have been less consistent (Milgroom and Cortesi, 2004).

The ease of transmission of CHV-1 between individuals via hyphal anastomoses and its ability to control a severe disease epidemic has led to research exploring the possibility of discovering or possibly genetically engineering other naturally occurring mycoviruses to induce hypovirulence in their fungal hosts. This method of virus-based biological control is very appealing as it presents the possibility to mitigate diseases in a highly specific manner. The key to unlocking the use of hypovirulence as a biological control strategy for fungal pathogens is a better understanding of mycoviruses.

1.4 Mycoviruses

Mycoviruses are present in all taxonomic groups of fungi. These viruses have only recently gained attention from the research community. Most mycoviruses have single-stranded or double-stranded RNA genomes. They are transmitted through hyphal fusion, and may be propagated through sexual and asexual reproduction of their fungal hosts. They generally have no extracellular route of transmission. The most studied mycoviruses are those that have economic significance such as the viruses that infect cultivated mushrooms, commercial yeasts, or plant-pathogenic fungi that affect crop plants (Milgroom and Hillman, 2011). Mycoviruses can have a number of different effects on their fungal hosts. Some increase fungal virulence (hypervirulence), others reduce virulence (hypovirulence), but most appear to have no effects on host fitness (Pearson et al., 2009). It was proposed by Milgroom and Hillman (2011) that the neutral effect of most mycoviruses is due to their restricted horizontal transmission imposed by vegetative incompatibility. Viruses that negatively impact host fitness will not spread easily through fungal populations (Milgroom and Hillman, 2011). This view is corroborated by findings that mycoviruses that cause severe debilitation in *C. parasitica* are found at low incidence in the fungal populations (Hillman and Suzuki, 2004). However, the asymptomatic mycoviruses have been proposed as the best candidates for the use of engineered biological control because though the viruses themselves have no harmful effects they can be modified to carry host genes that would result in reduced virulence (Pearson et al., 2009). The asymptomatic mycoviruses might also be the best candidates for dispersal because they do not immediately eliminate the newly infected host or trigger a natural defence response, which may increase the likelihood that the virus will spread to other individuals.

1.4.1 Mitoviruses

Narnaviridae is a large family of mycoviruses that do not have protein coats and, depending on their fungal host, can reside in the mitochondria or cytoplasm. *Narnaviridae* contains two genera: *Narnavirus* and *Mitovirus*. The mitochondrial viruses of this family are members of the genus *Mitovirus* and are found in filamentous ascomycetes and basidiomycetes (Milgroom and Hillman, 2011). These viruses have positive-sense (5' to 3') single-stranded RNA (ssRNA) genomes, but occur in their hosts as dsRNA molecules. The positive strand genome encodes one protein, an RNA-dependent RNA polymerase (RdRp) that is involved in replication of the virus. The RdRp acts on the positive strand to produce the double-stranded RNA (dsRNA) molecule that is the dominant form. In general, the viruses exist in the mitochondria as RNA-RdRp nucleoprotein complexes (Ghabrial and Suzuki, 2009). Both the ssRNA and dsRNA can be isolated from the fungal host tissue, with the genomic ssRNA being present in larger molar amounts than the dsRNA. These mitoviruses are not considered true positive-strand ssRNA viruses because they exist in the dsRNA form, but analysis of their RdRp genes has indicated that they are closely related to the positive-strand RNA viruses (Ghabrial and Suzuki, 2009).

Members of the *Mitovirus* genus have received particular interest from researchers because many of them have been shown to cause hypovirulence in their fungal hosts (Milgroom and Hillman, 2011). Such hypovirulent mitoviruses have been discovered in a number of European *O. novo-ulmi* isolates (Rogers et al., 1986, 1987; Cole et al., 1998) and have been shown to negatively impact the fungus by slowing growth, reducing asexual spores, and causing abnormal colony morphology (Brasier, 1983). Unfortunately, the high diversity of vegetative compatibility (VC) types within *O. novo-ulmi* populations in Europe, which inhibits cytoplasmic

fusion between competing individuals, has limited the development of these mitoviruses as biological controls (Brasier, 1983, 1988, 1996).

1.4.2 Discovery of Mitoviruses at the Disease Front in Winnipeg, Canada

From 1993 to 2002 Brad Temple and colleagues conducted a survey of the *O. novo-ulmi* (subspecies *novo-ulmi*) population in Winnipeg, Manitoba where DED continues to severely impact elm trees. From this survey they discovered two novel double-stranded RNA (dsRNA) viruses infecting *O. novo-ulmi* isolates 93-1224 and 02-0833 (Temple et al., 2006). The genomes of the viruses were sequenced using the single primer amplification technique (SPAT) and 5' Rapid Amplification of cDNA Ends (RACE). The results showed that the two viruses contained only one open reading frame coding for an RNA-dependent RNA polymerase (RdRp). The open reading frames were only present when the virus sequences were translated using the genetic code for mold, protozoan, coelenterate mitochondrial and mycoplasma. Consequently, the viruses were identified as members of the genus *Mitovirus*. The two mitoviruses were named OnuMV1c (*Ophiostoma novo-ulmi* Mitovirus 1c) and OnuMV7 (*Ophiostoma novo-ulmi* Mitovirus 7) based on their RdRp sequence similarities to other *Ophiostoma* mitoviruses. The genomes of OnuMV1c and OnuMV7 were determined to be 3.1 kb and 2.8 kb, respectively (Hintz et al., 2013). The viruses appear to be asymptomatic and preliminary experiments have shown that they are transmissible from virus-infected to virus-free individuals of *O. novo-ulmi* as well as between *O. novo-ulmi* and other DED-causing *Ophiostoma* species (Temple et al., 2006; Carneiro et al., 2013). The discovery of these viruses at the Canadian disease front presented the opportunity to explore them as candidates for engineered hypovirulence.

Of the two mitoviruses, OnuMV1c was deemed a more suitable candidate for engineered hypovirulence because it shared more similarities with other *Ophiostoma* mitoviruses than did OnuMV7, including recognizable panhandle and stem-loop structures, as well as higher RdRp sequence similarity (70% similarity for OnuMV1c versus 30% for OnuMV7) (Hintz et al., 2013). The appeal of using OnuMV1c over other fungal viruses is that it occurs naturally in the western Canadian isolates of *O. novo-ulmi* and appears to be benign, making it well suited for development as a biological control. Though other better-studied RNA viruses could be similarly engineered, using such viruses might increase the chance of inter-organism transfer. For example, the virus family *Partitiviridae* contains dsRNA viruses that infect fungi that could be potential biological control candidates but there are multiple instances of horizontal transfer of partitiviruses between fungi and plants (Ghabrial et al., 2008). Because fungi often have symbiotic relationships with plants and animals using a partitivirus as a biological control may not be ideal as there is a high likelihood of virus transfer between participating organisms. Indeed, the phylogenies of many fungal viruses suggest past transfer between organisms (Milgroom and Hillman, 2011). An effective biological control is one that is highly specific to its target and has minimal off-target effects. By using a virus that occurs naturally in *O. novo-ulmi* the likelihood of transmission to other organisms is more limited, thus minimizing off-target effects in the surrounding ecosystem.

Unlike other *Ophiostoma* mitoviruses, OnuMV1c appears to be asymptomatic. Therefore, it would have to be engineered to act as a hypovirus if it were to be used successfully as a biological control. One such approach to engineering hypovirulence is by the introduction of small inverted repeat sequences based on host genomic information that induce RNA interference in the fungal host.

1.5 RNA Interference

RNA interference (RNAi), or post-transcriptional gene silencing (PTGS), is a cellular gene regulation process in which small dsRNA molecules trigger the targeted degradation of specific messenger RNAs (mRNAs) after their transcription to prevent their translation into proteins. The process begins when dsRNAs in the cytosol are cleaved by the endonuclease DICER into small interfering RNAs (siRNAs) that are 21-25 nucleotides long. The siRNAs unwind and become associated as single-stranded RNAs with a nuclease complex termed the RNA-induced silencing complex (RISC). RISC uses a homology-dependent mechanism to degrade specific target mRNAs preventing their translation into proteins (Agrawal et al., 2003; Cottrell and Doering, 2003).

The first studies describing RNA silencing were on petunias in the 1990s and the process occurring in these plants was termed co-suppression by Richard Jorgensen (Napoli et al. 1990; van der Krol et al., 1990; Van Blokland et al., 1994). Subsequently, a number of studies throughout the 1990s discovered that RNA silencing occurred in a wide variety of eukaryotes. However, it was studies done by Andrew Fire and colleagues on *Caenorhabditis elegans* that first elucidated the mechanism behind RNAi whereby dsRNA molecules target specific mRNA sequences in order to silence certain genes (Fire et al., 1998; Fire 1999).

It has been proposed that the RNAi pathway may have evolved as a mechanism for the normal regulation of endogenous genes, as well as a defence against foreign RNA introduced by infectious agents such as viruses (Hannon, 2002). When plants become infected with viruses they activate an RNAi pathway that uses dsRNAs to target and degrade the invading virus RNA. This RNA-based virus defence can be exploited to target the plants own genes in a process

known as virus-induced gene silencing (VIGS) (Lu et al., 2003). VIGS takes place when a host gene is inserted into the viral genome thus triggering the host's RNAi pathway to target both the viral RNA as well as its own gene, down regulating the protein encoded by that gene. VIGS was first demonstrated with tobacco mosaic virus and potato virus X in *Nicotiana benthamiana* plants where the viruses were modified to carry inserts from the plant phytoene desaturase gene (Kumagai et al., 1995; Ruiz et al., 1998). Phytoene desaturase, a thylakoid membrane-bound enzyme, was chosen as the RNAi target in these studies because it is involved in the carotenoid biosynthesis pathway. Therefore, disruption of its synthesis lead to a distinct bleached phenotype in the *N. benthamiana* plants that made it easy to visually discern the efficacy of the RNAi experiments (Kumagai et al., 1995). Double-stranded RNAs have also been shown to induce gene silencing in fungi (Catalanotto et al., 2000, Agrawal et al., 2003), opening the possibility of using the VIGS approach to down-regulate endogenous genes in fungal pathogens. Thus, after the discovery of the mitovirus OnuMV1c the goal of our research became to engineer OnuMV1c with an RNAi-inducing gene cassette that would result in a form of VIGS that targets specific proteins involved in the pathogenicity of *O. novo-ulmi*.

Previous work done by Joyce Carneiro demonstrated that RNAi could be induced in *O. novo-ulmi* via the introduction of small inverted repeat sequences of genes. After they are transcribed, inverted repeat sequences fold into hairpin loops of RNA thus creating dsRNA. The hairpin dsRNAs then induce the RNAi pathway to activate and destroy the gene expressed in the dsRNA loop. Carneiro et al. (2010) targeted the endopolygalacturonase gene *epg*1, which while not central to the pathogenicity of *O. novo-ulmi* proved to be an easily assayable enzyme that allowed the functionality of the RNAi approach to be assessed. A construct was created that contained an inverted repeat sequence of the *epg*1 gene driven by a glyceraldehyde-3-phosphate

dehydrogenase (*gpd*) promoter and terminated by a *trpC* (tryptophan biosynthesis) transcription terminator from *Aspergillus nidulans*. This construct was cloned into a fungal transformation vector and was introduced into *O. novo-ulmi*. Results showed that the construct with the inverted repeat sequence of *epg*1 successfully induced the RNAi pathway in *O. novo-ulmi* and down regulated the expression of endopolygalacturonase. The successful use of RNAi to down regulate gene expression in *O. novo-ulmi* led to the hypothesis that the same method could be used as a means of engineered hypovirulence. If OnuMV1c was engineered to contain an RNAi cassette, it could act as a carrier molecule bringing the RNAi inducer into *O. novo-ulmi*. A schematic of this engineered hypovirulence process is shown in Figure 1. There is at least one major technological hurdle to be overcome for this process to be proven. OnuMV1c is a mitovirus that replicates in the mitochondria but RNAi occurs exclusively in the cytoplasm of cells. Therefore, in order for an engineered OnuMV1c to act as an RNAi-inducing hypovirus it needs to be present in the cytoplasm of *O. novo-ulmi*. This problem led to the research objectives explored in this master's project.

1.6 General Research Objectives

Currently there are no control strategies for Dutch elm disease that are able to effectively mitigate the disease as it infects existing American elms. Current mitigation strategies are not feasible on a large-scale due to their exorbitant costs and/or their toxicity to the surrounding environment. The overarching research objective for this project is to develop a control strategy for DED that is highly specific to *O. novo-ulmi* and has no unintended effects on the ecosystem. To that end, this project took the first steps to develop a biological control by engineering the dsRNA mitovirus OnuMV1c to act as a hypovirus using RNA interference. As RNAi is a

cytoplasmic process, and OnuMV1c is a mitochondrial virus that uses the mitochondrial codon pattern, the first step in accomplishing this goal was to re-engineer OnuMV1c to enable expression of the virus-encoded RdRp in the cytoplasm of *O. novo-ulmi*.

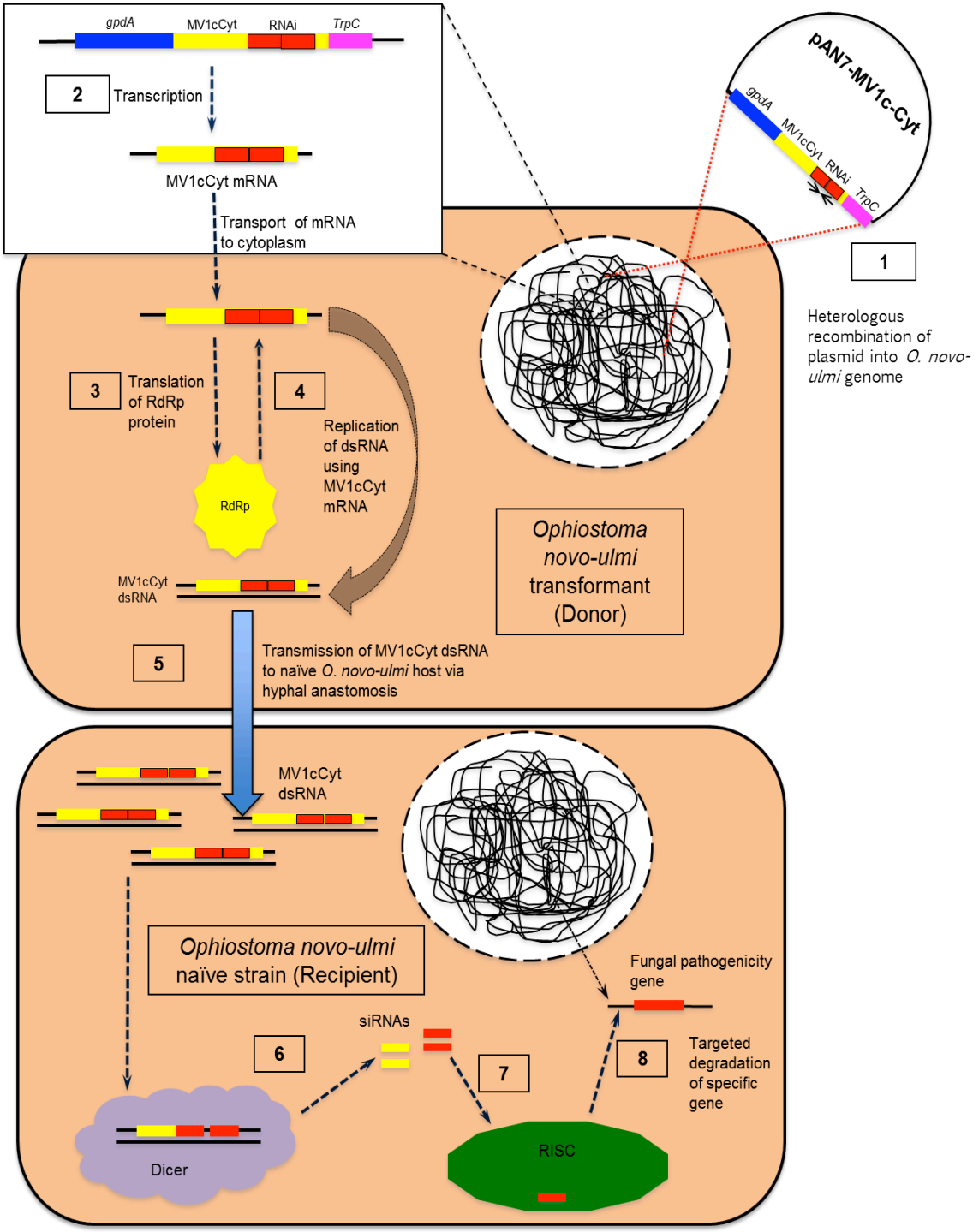
There is precedence for the permanent infection of fungal hosts with RNA viruses by the integration of cDNA versions of the viral genomes into the nuclear genome of the host. Choi and Nuss developed a reverse genetics system for the double-stranded RNA hypovirus L-dsRNA, which infects the chestnut blight pathogen *Cryphonectria parasitica*. They transformed a cDNA copy of the hypovirus into the *C. parasitica* genome and demonstrated that this genomic copy produced L-dsRNA mRNA that was translated into a functional RdRp protein, which subsequently created double-stranded RNA in the cytoplasm of the *C. parasitica* transformants. The chromosomal integration of the L-dsRNA virus in the *C. parasitica* transformants ensured that the virus was vertically transmitted to ascospore progeny. In addition, the research showed that this new cytoplasmic L-dsRNA was able to convert previously virulent strains of *C. parasitica* to hypovirulence through virus transmission via hyphal anastomosis (Choi and Nuss, 1992).

It is possible but unproven that OnuMV1c could be genetically engineered to express in the cytoplasm by modifying the specific codons that differ between the mitochondria and cytoplasm of *O. novo-ulmi* effectively reprogramming the site of expression of the mitovirus. Codons were modified such that the amino acid sequence of the RdRp would be conserved but the site of expression was altered. This process involved the creation of a genetic construct that contained the cytoplasmically engineered OnuMV1c sequence flanked with promoter and terminator sequences. This genetic construct was then introduced into *O. novo-ulmi* using a fungal transformation vector. In the mitochondrion the RdRp of OnuMV1c recognizes the

positive-strand sequence and acts on it to produce dsRNA. The goal was to test whether cytoplasmically engineered OnuMV1c would act in the same way, with the replicative cycle occurring in the cytoplasm instead of the mitochondrion. In order to redirect dsRNA replication to the cytoplasm the codon-modified OnuMV1c sequence would have to produce an RdRp protein that is functional in the cytoplasm. To assess whether the cytoplasmic RdRp was functional required testing for dsRNA: the presence of OnuMV1c dsRNA in the cytoplasm of transformed *O. novo-ulmi* would confirm that the re-engineered OnuMV1c virus was functional.

This research will determine if OnuMV1c can be used as a carrier for an RNAi cassette that will be used to down regulate genes involved in the pathogenicity of *O. novo-ulmi*. The concept is that OnuMV1c will carry the RNAi cassette in addition to its own complement of genes and spread naturally through *O. novo-ulmi* populations in Canada acting as an enhanced hypovirus. The results described here further the exploration of using OnuMV1c as a biological control for Dutch elm disease in Canada.

Figure 1. Schematic diagram showing the proposed process of OnuMV1c acting as an engineered hypovirus carrying an RNA interference (RNAi) cassette. 1) Recombination of the fungal transformation vector carrying the OnuMV1c cytoplasmic construct (MV1cCyt) and RNAi cassette into the *Ophiostoma novo-ulmi* genome. 2) Transcription of the cytoplasmic MV1cCyt virus and subsequent transfer of messenger RNA (mRNA) to the cytoplasm. 3) Translation of MV1cCyt positive-strand mRNA into the RdRp protein. 4) The RdRp acts on the positive-strand mRNA to produce MV1cCyt dsRNA with the RNAi cassette. 5) The MV1cCyt dsRNA is transmitted to a naïve *O. novo-ulmi* isolate at the disease front via hyphal anastomosis. 6) The presence of dsRNA in the cytoplasm of *O. novo-ulmi* induces the RNAi pathway to activate. DICER recognizes the dsRNA and cleaves it into small interfering RNAs (siRNAs). 7) The siRNAs unwind and are integrated into RISC as single-stranded pieces. 8) RISC uses the siRNA fragment in a homology-dependent mechanism to degrade target mRNAs and prevent their translation into proteins.


gpdA
MV1cCyt
RNAi
TrpC
2
Transcription
MV1cCyt mRNA
Transport of mRNA to cytoplasm
pAN7-MV1c-Cyt
1
Heterologous recombination of plasmid into O. novo-ulmi genome
3
Translation of RdRp protein
4
Replication of dsRNA using MV1cCyt mRNA
RdRp
MV1cCyt dsRNA
Ophiostoma novo-ulmi transformant (Donor)
5
Transmission of MV1cCyt dsRNA to naïve O. novo-ulmi host via hyphal anastomosis
MV1cCyt dsRNA
Ophiostoma novo-ulmi naïve strain (Recipient)
Fungal pathogenicity gene
siRNAs
6
7
8
Targeted degradation of specific gene
Dicer
RISC

Chapter 2: Materials and Methods

2.1 Fungal isolates and culture conditions

Stock cultures of *Ophiostoma novo-ulmi* subspecies *novo-ulmi* strain H327 and subspecies *americana* strain 93-1224 were stored in liquid nitrogen (-70 °C) in a 10 % glycerol (v/v) solution. Working cultures were prepared according to Bernier and Hubbes (1990) by growing isolates on solid *Ophiostoma* complete medium (OCM) agar plates for up to 7 days at 25 °C, after which cultures were maintained at 4 °C. Strain H327 was originally isolated from an infected elm in Bratislava, Czechoslovakia in 1979 by H. Jamnicky (Et-Touil et al. 1999). Strain 93-1224, a dsRNA-infected isolate (OnuMV1c and OnuMV7), was originally isolated from an infected elm in Winnipeg, Manitoba, Canada in 1993 (Temple et al., 2006). The sequence of mitovirus OnuMV1c was determined by Hintz et al (2013). In the context of this project, strain 93-1224 was used as a virus-positive control to which all experimentally manipulated H327 strains were compared.

2.2 Construction of OnuMV1c cytoplasmic version (MV1cCyt)

A DNA version of the dsRNA mitovirus was synthesized for subsequent expression in the nuclear genome. As the initial mRNA expressed from this construct would be located in the cytoplasm the codon usage was altered such that the encoded gene product, an RNA dependent RNA polymerase (RdRp), was consistent with expression in the cytoplasm. All codon modifications made to the cytoplasmic version of the OnuMV1c virus (MV1cCyt) accounted for codon bias as measured by the frequency at which they are used in the cytoplasm (values from

0.0 to 1.0). A codon usage table for *O. novo-ulmi subsp. novo-ulmi* constructed by Yasukazu Nakamura (Nakamura, NCBI-GenBank, 2007) was used to determine the best codon options.

All sequence modifications were made in Microsoft Word 2008 (Microsoft Corporation, Redmond, WA United States of America). SnapGene Viewer (GSL Biotech LLC, Chicago, IL United States of America) was used to predict the reading frame of the synthetic virus as expressed in the cytoplasm.

2.2.1 *gpdA*/*trpC* Promoter and Terminator Sequences

The *gpdA* (glyceraldehyde-3-phosphate dehydrogenase) promoter from *Aspergillus nidulans* was used to drive expression of the MV1cCyt construct. Transcription termination was achieved using the *trpC* (tryptophan biosynthesis) transcription terminator from *A. nidulans*. The *gpdA* promoter is constitutive and was chosen to ensure constant expression of the MV1cCyt construct. Two restriction enzyme sites were engineered into the promoter and terminator sequences to facilitate downstream cloning procedures: a *Bam*HI restriction site (5'-GGATCC-3') was added to the 5' end of the *gpdA* promoter, and a *Hin*dIII site (5'-AAGCTT-3') was added to the 3' end of the *trpC* terminator.

2.2.3 Codon Modifications of *Bam*HI/*Hin*dIII Sites

The cloning procedures relied on using *Bam*HI and *Hin*dIII to ligate the MV1cCyt construct into a pAN7-1 fungal transformation vector. Consequently, the entire *gpdA*-MV1cCyt-*trpC* construct was modified to remove any *Bam*HI and *Hin*dIII sites present. In this way ligation

of a single large fragment was facilitated. These sites were conservatively modified such that the expressed peptide remained unchanged.

2.2.3 Synthesis of Engineered MV1cCyt Construct

After all codon modifications were made to the *gpdA*-MV1cCyt-*trpC* construct, tails of seven base pairs were added to both the 5' end (5'-GGGGTTT-3') and 3' end (5'-AAACCCC-3') to act as identifying signatures. For cost efficient gene synthesis, only the last 85 base pairs of the 3' end of the *gpdA* promoter were used in the synthesized construct. The 5' end of this partial *gpdA* promoter segment began with a naturally occurring *Tth*111I restriction enzyme site (5'-GACAAGGTC-3'), which facilitated subsequent cloning procedures. The 85 base pairs of the *gpdA* promoter were used as a temporary linker for the synthesized construct until downstream cloning could be used to add the full *gpdA* promoter. The final codon-modified, signature-tailed *gpdA*(linker)-MV1cCyt-*trpC* design was sent to GeneArt Gene Synthesis (Life Technologies Incorporated, Burlington, ON Canada) where it was synthesized and cloned into a pMA-RQ vector having a bacterial origin of replication and a marker for ampicillin resistance.

2.3 Sub-cloning

The complete *gpd*A-MV1cCyt-*trp*C construct was inserted into a pAN7-1 fungal transformation vector to facilitate integration into the genome of *O. novo-ulmi*. The pAN7-1 vector developed by Punt et al. (1987) is a well-established fungal transformation vector that uses the hygromycin B phosphotransferase gene (*hph*) from *Escherichia coli* as a resistance marker to select putative transformants. Expression of the *hph* gene is driven by the *gpdA*

promoter and terminated by the *trpC* terminator of *A. nidulans*. The *trpC* terminator is flanked by a *Bam*HI site on the 5' end and a *Hin*dIII site on the 3' end. The pAN7-1 vector could not be used in the first cloning step because it contains multiple *Tth*111I restriction sites and *Tth*111I was the site required for sub-cloning the *gpdA*(linker)-MV1cCyt-*trpC* construct from the pMA-RQ vector. Therefore, a pGEM-T vector (Promega Corporation, Madison, WI United States of America) containing the 5' region of the *gpdA* promoter sequence was required as a cloning intermediate in order to obtain a complete *gpdA*-MV1cCyt-*trpC* construct with the full *gpdA* promoter. This complete construct was flanked by *Bam*HI and *Hin*dIII restriction sites, which were used to sub-clone the insert into the pAN7-1 vector.

2.3.1 Sub-cloning *gpdA*(linker)-MV1cCyt-*trpC* from pMA-RQ to pGEM-T

All restriction enzymes used were from New England Biolabs (New England Biolabs Incorporated, Ipswich, MA United States of America). Digests were performed according to the manufacturer's instructions: 10 units of enzyme were used to digest 1 μg of DNA in a 50 μl reaction with the appropriate 10X NEBuffer at an incubation temperature specific to the enzyme being used.

The *gpdA*(linker)-MV1cCyt-*trpC* construct flanked with *Tth*111I and *Hin*dIII sites was synthesized by GeneArt in a pMA-RQ vector (pMA-*gpdA*(linker)-MV1cCyt-*trpC*). A pGEM-T vector that contained the full *gpdA* promoter driving the expression of an RNAi cassette followed by a *trpC* terminator flanked by *Bam*HI and *Hin*dIII sites (p*gpd-epg*-409i) was retrieved from our archive (Carneiro et al., 2010). The old *epg*-409i construct including the *trpC* terminator was removed by digesting the vector with *Tth*111I and *Hin*dIII restriction enzymes (New England Biolabs Incorporated). This resulted in a pGEM-T vector containing only the leading region of

the *gpdA* promoter. The pMA-RQ vector containing the *gpdA*(linker)-MV1cCyt-*trpC* construct was also digested with *Tth*111I and *Hin*dIII enzymes. The resultant restriction fragments were separated by electrophoresis on a 1% agarose gel in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA (pH 8.0)) for 3 hours at 110 volts. Both the *gpdA*(linker)-MV1cCyt-*trpC* insert and the pGEM-T vector with the *gpdA* promoter were extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen Inc.). The *gpdA*(linker)-MV1cCyt-*trpC* insert was then ligated with the pGEM-T-*gpdA* vector using T4 DNA ligase (Promega Corporation, Madison, WI United States of America). A schematic of this sub-cloning procedure is shown in Figure 2. The ligated pGEM-*gpdA*-MV1cCyt-*trpC* vector was transformed into E. Cloni 10G Elite electrocompetent cells (Lucigen Corporation, Middleton, WI United States of America). Transformed cells were grown overnight at 37 °C on LB plates (lysogeny broth: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) supplemented with 100 μg/ml ampicillin (Sigma-Aldrich Canada Co., Oakville, ON Canada). Putative transformants were picked and transferred to 5 ml of liquid LB supplemented with 100 μg/ml ampicillin and grown overnight at 37°C on a shaker. Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen Inc., Mississauga, ON Canada). Six diagnostic restriction enzyme digests were performed on each of the plasmid DNA extractions to confirm the identity of the vector DNA they contained. A *Tth*111I/*Hin*dIII digest was performed as an initial screening of the transformants. Subsequently, the DNA from the transformants that passed the initial screening were subjected to five additional digests to further confirm vector identity: a *Bam*HI/*Hin*dIII digest, a *Bam*HI/*Tth*111I digest, a *Bam*HI/*Xho*I digest, a *Bam*HI-only digest, and a *Hin*dIII-only digest. All restriction enzyme digests were performed following the manufacturer's protocols (New England Biolabs Incorporated). To facilitate sequence confirmation the plasmid DNA from the final transformants

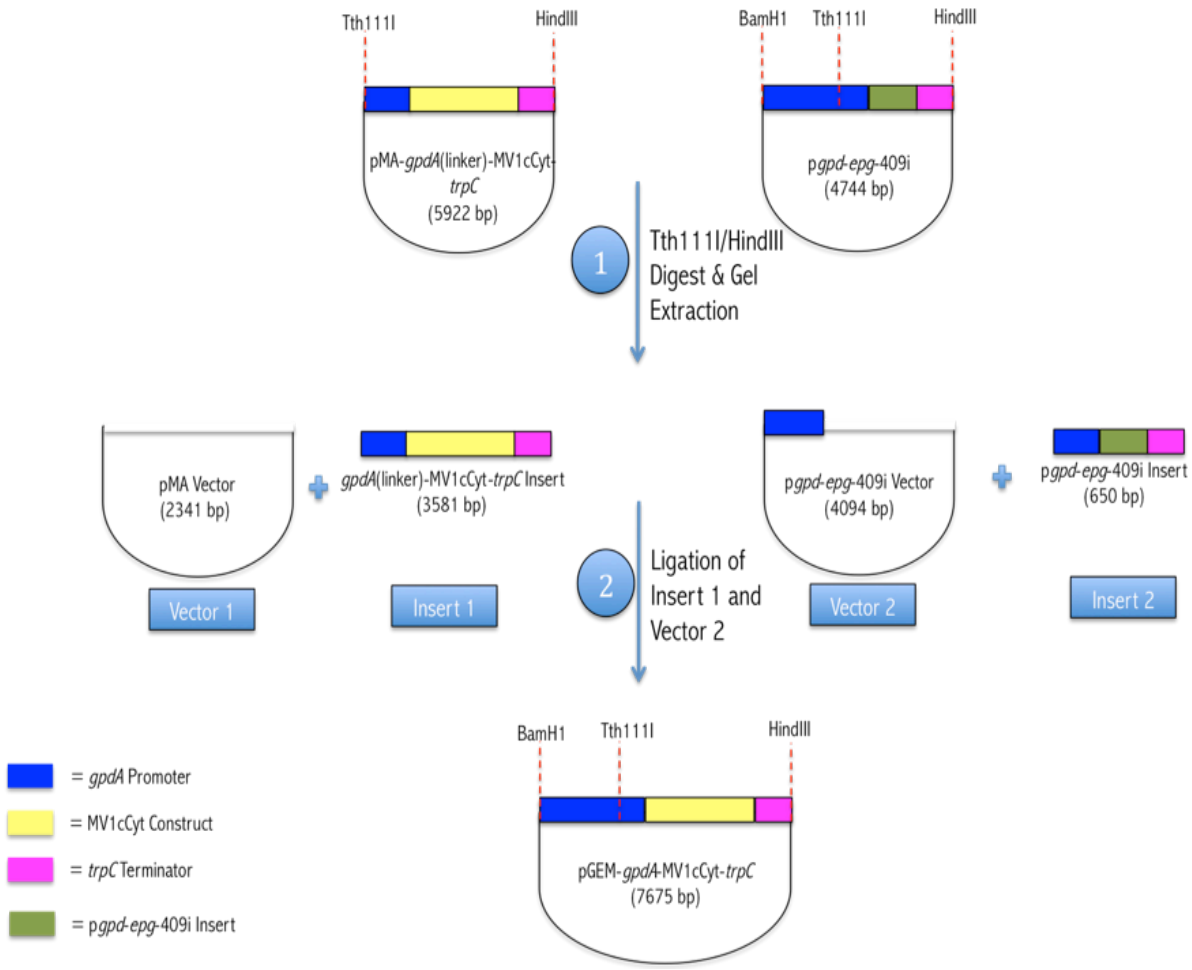


Figure 2. Schematic diagram showing the procedure of sub-cloning the *gpdA*(linker)-MV1cCyt-*trpC* construct from the pMA-RQ vector provided by GeneArt Gene Synthesis (Life Technologies Incorporated, Burlington, ON Canada) to a p*gpd-epg*-409i vector using *Tth*111I and *Hin*dIII restriction sites. (1) pMA-*gpdA*(linker)-MV1cCyt-*trpC* and p*gpd-epg*-409i were digested with *Tth*111I and *Hin*dIII restriction enzymes (New England Biolabs Incorporated) and run on a 1% agarose gel. The Insert 1 (*gpdA*(linker)-MV1cCyt-*trpC*) and Vector 2 (empty p*gpd-epg*-409i vector) bands were then excised from the gel and the DNA was extracted using the QIAquick® Gel Extraction Kit (Qiagen Inc.). (2) Insert 1 and Vector 2 were then ligated together using T4 DNA ligase (Promega Corporation) to form the pGEM-*gpdA*-MV1cCyt-*trpC* vector containing the full *gpdA* promoter.

was amplified using the M13 forward and reverse primers that flank the multiple cloning site (MCS) of pGEM-T (Table 1). The M13 PCR products were purified and the sequences were determined by Eurofins Genomics (Eurofins MWG Operon LLC, Hunstville, AL United States of America) to confirm that the plasmid DNA was pGEM-T containing the full *gpdA*-MV1cCyt-*trpC* construct (pGEM-*gpdA*-MV1cCyt-*trpC*).

2.3.2 Sub-cloning *gpdA*-MV1cCyt-*trpC* from pGEM-T to pAN7-1

Once the *gpdA*(linker)-MV1cCyt-*trpC* insert was ligated to the upstream *gpdA* promoter (pGEM-*gpdA*-MV1cCyt-*trpC*) the entire insert could be moved into the pAN7-1 fungal transformation vector via the terminal *Bam*HI and *Hin*dIII restriction sites. Both pGEM-*gpdA*-MV1cCyt-*trpC* and pAN7-1 were digested with *Bam*HI and *Hin*dIII restriction enzymes (New England Biolabs Incorporated). Both vectors were digested using a three-step process: 1) digestion with the first restriction enzyme, 2) dephosphorylation with Antarctic Phosphatase (New England Biolabs Incorporated) and, 3) digestion with the second restriction enzyme. The digested vectors were run on a 1% agarose gel for 3 hours at 110 volts. The *gpdA*-MV1cCyt-*trpC* insert DNA and empty pAN7-1 vector DNA were extracted from the gel, ligated together, and transformed using the exact same protocols described above (Section 2.3.1). A schematic of this sub-cloning procedure is shown in Figure 3. Plasmid DNA was extracted from the E. Cloni cells (Lucigen Corporation) using the QIAprep Spin Miniprep Kit (Qiagen Inc.) and underwent PCR using nested MV1cCyt sequence primers (Table 2). The resulting PCR amplicons were analyzed on an agarose gel, after which they were purified using the QIAquick PCR Purification Kit (Qiagen Inc.) and sequenced by Eurofins Genomics (Eurofins MWG Operon LLC) to confirm that the plasmid DNA was the pAN7-1 vector containing the *gpdA*-MV1cCyt-*trpC*

Table 1. M13 forward and reverse primers used for sequence confirmation of pGEM-*gpdA*-MV1cCyt-*trpC*. The M13 primers flank the multiple cloning site of pGEM-T.

Primer Name	Location in pGEM-T®	Primer Length (bp)	Primer Sequence
M13 Forward	2961-2977 bp	17	5'-GTAAAACGACGGCCAGT-3'
M13 Reverse	161-177 bp	17	5'-CAGGAAACAGCTATGAC-3'

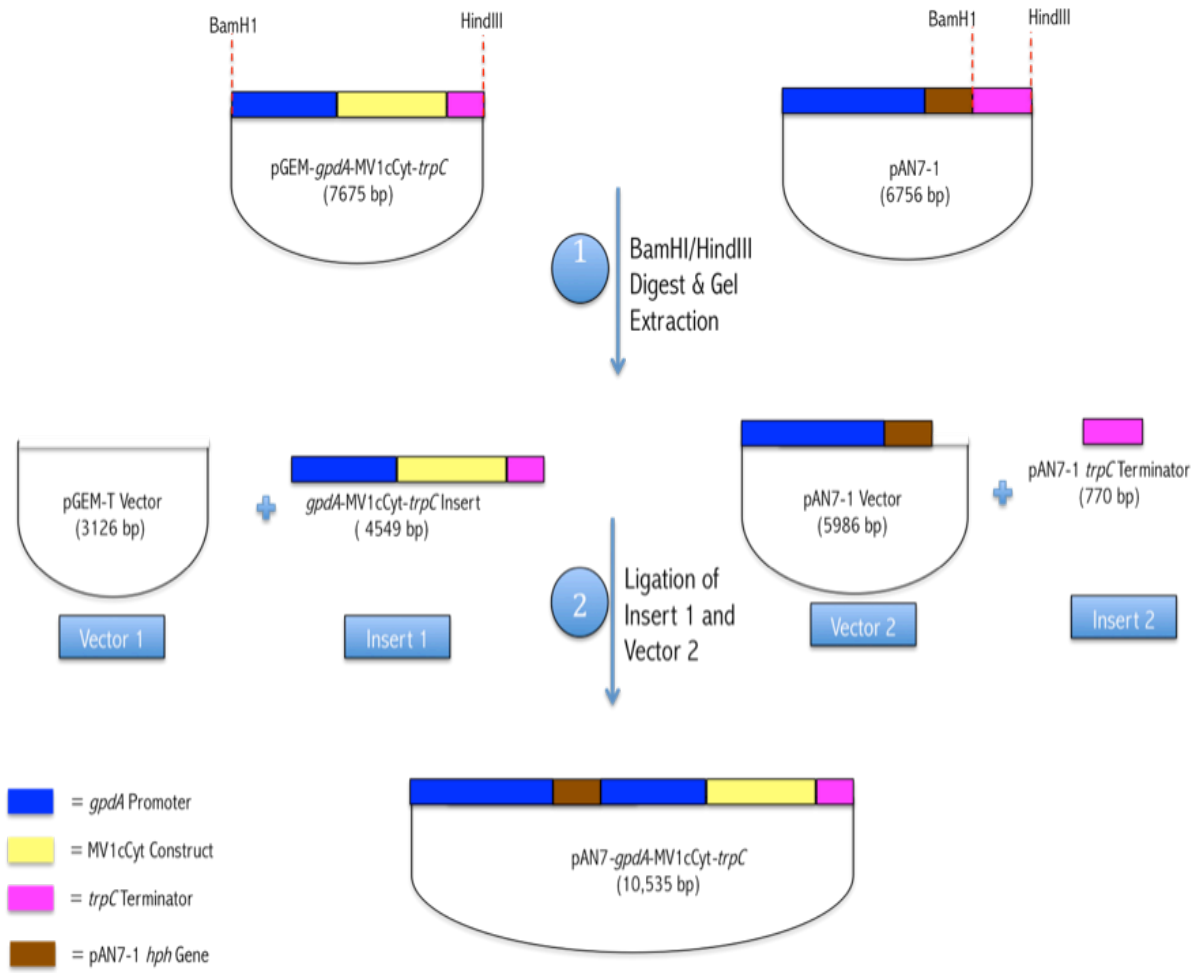


Figure 3. Schematic diagram showing the process of sub-cloning the *gpdA*-MV1cCyt-*trpC* construct from pGEM-T into the pAN7-1 fungal transformation vector using *Bam*HI and *Hin*dIII restriction sites. (1) pGEM-*gpdA*-MV1cCyt-*trpC* and pAN7-1 were digested with *Bam*HI and *Hin*dIII restriction enzymes (New England Biolabs Incorporated) and run on a 1 % agarose gel. The Insert 1 (*gpdA*-MV1cCyt-*trpC*) and Vector 2 (empty pAN7-1 vector) bands were then excised from the gel and the DNA was extracted using the QIAquick Gel Extraction Kit (Qiagen Inc.). (2) Insert 1 and Vector 2 were then ligated together using T4 DNA ligase (Promega Corporation) to form the final pAN7-*gpdA*-MV1cCyt-*trpC* plasmid ready for transformation into *Ophiostoma novo-ulmi* protoplasts.

Table 2. MV1cCyt nested sequencing primers used to confirm successful cloning of pGEM-*gpdA*-MV1cCyt-*trpC* and pAN7-*gpdA*-MV1cCyt-*trpC*. The primers were also used in diagnostic PCR as an additional confirmation of cloning success. (bp = base pairs)

Nested Primer Pair	Primer Name	Location in MV1cCyt Construct	Primer Length (bp)	Primer Sequence	Expected PCR Amplicon Size
1	MV1cCyt_Seq_Fwd_1	3657-3678 bp	22	5'-GGGTCCCTTGTGATGACTGACC-3'	3159 bp
1	MV1cCyt_Seq_Rev_1	519-539 bp	21	5'-AAGTGGAAAGGCTGGTGTGCC-3'	
2	MV1cCyt_Seq_Fwd_2	2954-2977 bp	24	5'-GACCCCTTGTAAAGGTCCCTCTGG-3'	1786 bp
2	MV1cCyt_Seq_Rev_2	1191-1214 bp	24	5'-GAGTGTCGTCCAAAGGCTGATTCC-3'	
3	MV1cCyt_Seq_Fwd_3	2346-2367 bp	22	5'-CAGTCCGCCATAGCGAATACCC-3'	560 bp
3	MV1cCyt_Seq_Rev_3	1807-1830 bp	24	5'-CGGTGGTCTACCGAGAATTCTTCC-3'	

construct (pAN7-*gpdA*-MV1cCyt-*trpC*). As above, multiple diagnostic restriction enzyme digests were performed on the plasmid DNA in addition to sequencing and PCR to confirm plasmid identity: a *Bam*HI/*Hin*dIII digest for initial screening of transformants followed by *Eco*RI, *Eco*RI/*Bam*HI, and *Eco*RI/*Hin*dIII digests for subsequent confirmation.

2.4 Preparation of *Ophiostoma novo-ulmi* Protoplasts

The protocol used to prepare fungal protoplasts was modified from Temple et al. (1997). A culture of *O. novo-ulmi* subsp. *novo-ulmi* strain H327 was grown in OCM liquid media made with L-proline (OCM/L-proline) as the nitrogen source in order to promote growth of the yeast-like phase of the fungus. After seven days of growth, two additional flasks containing 50 mL of OCM/L-proline were inoculated with 2 mL of this culture and were incubated overnight at room temperature (25 °C) on a shaker at 100 rpm. The fungal cells from these overnight cultures were collected onto a 0.45 μm filter using a Millipore All-Glass 47 mm vacuum filter system (EMD Millipore, Billerica, MA United States of America). The filtered cells were re-suspended in 100 mL of 0.7M KCl, re-collected with a 0.45 μm filter, and re-suspended again in 100 mL of 0.7M KCl. The cells were then vacuum-filtered for a third and final time through a 0.45 μm filter and re-suspended in 10 mL of 0.7M KCl containing 10 mg/mL of lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich Canada Co., Oakville, ON Canada) and 1 mg/mL of chitinase from *Streptomyces griseus* (Sigma-Aldrich Canada Co.). The cells were digested for one hour at 25 °C on a shaker at 60 rpm. In order to purify the protoplasts, the digested mixture was poured through a 70 μm filter and pelleted by centrifugation at 5000 rpm for 5 min. The pellet was resuspended in 10 ml STC and re-pelleted as before. This was repeated a second time whereupon the final pellet was re-suspended in 500 μL of STC. The protoplasts were then counted using a

haemocytometer and the suspension was diluted to a final protoplast concentration 10^7-10^8 cells/mL. The final protoplast solutions were made into 150 µl aliquots, stored on ice overnight, and transferred to -70 °C for long-term storage.

2.5 Transformation of *Ophiostoma novo-ulmi* Protoplasts

The H327 protoplasts were incubated on ice for 8-12 hours prior to transformation. After incubation, 4 µg of pAN7-MV1cCyt plasmid DNA was added to 150 µl of H327 protoplasts. The protoplast-DNA mixture was added to 250 µl of PEG-TC [polyethylene glycol 4000 (60 % w/v) in Tris–HCl (100 mM, pH 6.8) and $CaCl_2$ (10 mM)] and incubated at 25 °C for 30 minutes. The solution was then diluted with 5 ml of STC [sorbitol (1 M), Tris–HCl (100 mM, pH 6.8) and $CaCl_2$ (10 mM)] and centrifuged for 10 minutes at 3,000 rpm. The pellet was re-suspended in 1000 µl of OCM/0.6 M sucrose (OCM containing 0.6 M sucrose as an osmotic stabilizer). The transformed protoplasts were left overnight on ice before being plated on OCM/0.6 M sucrose with 100 µg/ml hygromycin B (Sigma-Aldrich Canada Co.). The plates were incubated at 25 °C for 7 days. Putative transformants were transferred twice to fresh OCM plates containing 400 µg/ml hygromycin B in order to ensure transformant stability and consistent expression of the hygromycin phosphotransferase gene.

2.6 RNA Extractions

2.6.1 Isolation of Total RNA

In preparation for extraction of total RNA, *O. novo-ulmi* cultures were grown in 50 ml of OCM liquid media at 25 °C for at least 7 days without shaking. Mycelia were harvested by

vacuum filtration onto Whatman 11 μm grade cellulose filter paper (GE Healthcare Life Sciences, Mississauga, ON Canada), flash frozen with liquid nitrogen, and mechanically ground in a mortar and pestle to obtain a fine powder. Total RNA was then extracted using the RNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON Canada), re-suspended in sterile, RNase-free water and stored at -20 °C for further use. All steps of the RNA extraction were carried out using RNase-free lab ware and were performed in a sterilized fume hood and countertops sterilized with RNase AWAY (Molecular Bio-Products Inc., San Diego, CA United States of America).

2.6.2 Isolation of dsRNA

Double-stranded RNA was extracted using a phenol-chloroform method based on the protocol developed by Morris and Dodds (1979), which relies on the adsorption of dsRNA to cellulose powder at ethanol concentrations of 15 %. The same precautions as stated above (Section 2.6.1) were taken to avoid RNase contamination. Cultures of *O. novo-ulmi* were grown and harvested as described for total RNA extraction. An extraction buffer containing 3 mL of 2X STE buffer (sodium chloride, Tris-Cl, EDTA) with 1 % SDS (sodium dodecyl sulfate), 0.5 mL of aqueous phenol, and 0.25 mL of a mixture of chloroform: isoamyl alcohol (24:1 v/v) was added to the mycelia powder. This emulsion was homogenized by vortexing at low speed for 10 minutes. The emulsion was then distributed into 1 mL aliquots in microcentrifuge tubes and centrifuged for 10 minutes at 14,000 rpm. The upper aqueous phase was transferred to a new microcentrifuge tube and adjusted to 15 % (v/v) ethanol. Whatman CF11 cellulose powder (GE Healthcare Life Sciences) was added to this aqueous phase, vortexed, and centrifuged for 5 minutes at 14,000 rpm to selectively bind the dsRNA. The cellulose pellet was then washed with STE/15 buffer (STE adjusted to 15 % ethanol) and centrifuged again for 5 minutes at 14,000

rpm. The dsRNA was eluted with STE and precipitated by adding 2 volumes of cold 95 % ethanol. After incubation at -20 °C for 30 minutes the dsRNA was pelleted by centrifugation for 10 minutes at 14,000 rpm. The pellet was washed with 1 mL of cold 70 % ethanol and air-dried under the fume hood. The dsRNA pellet was re-suspended in 60-200 µl of DEPC-treated water and stored at -20°C for further use.

In order to avoid contaminating genomic DNA, all RNA extractions were treated with DNase I (Thermo Fisher Scientific Inc.) before being used in any subsequent procedures. DNase I treatment was carried out according to the manufacturer's protocol (Thermo Fisher Scientific Inc.). Each DNase I treatment contained 2 µg of template RNA thereby standardizing the RNA concentrations. The purity and concentration of all RNA extractions were measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA United States of America).

2.7 Diagnostic One-Step Reverse Transcription PCR (RT-PCR)

RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen Inc.) with OnuMV1c diagnostic primers (Table 3). For this first diagnostic RT-PCR step the total RNA extractions were used (Section 2.6.2) from strain 93-1224 (positive control), untransformed H327 (negative control), and all H327 transformants. Prior to starting the RT-PCR cycle, an RNA denaturing step was performed by heating a mixture of the RNA template and primers to 90°C for 5 minutes. This step was added to ensure that any double-stranded RNA in the total RNA extraction was denatured into single-stranded RNA and ready for reverse transcription. The RT-PCR reaction had a total volume of 25 µl containing 5 µl of 5X OneStep RT-PCR Buffer (1X), 1 µl of dNTPs (400 µM of each), 1 µl of OneStep RT-PCR Enzyme Mix, 5 µl of 5X Q-Solution,

Table 3. OnuMV1c diagnostic primers used to amplify an 895 base pair sequence of the RNA-dependent RNA-polymerase (RdRp) region of both OnuMV1c and the MV1cCyt construct. Primers were used for both PCR and RT-PCR.

Primer Name	Location in MV1cCyt Construct	Primer Length	Primer Sequence	Expected PCR Amplicon Size
Diag_Fwd_MV1c	111-130 bp	20 bp	5'-GTGTCGTCCAAAGGCTGATT- 3'	895 bp
Diag_Rev_MV1c	988-1005 bp	18 bp	5'-TTGAGCCACTCGCTGATA-3'	

1.5 µl each of Diag_Fwd_MV1c and Diag_Rev_MV1c primers (400 µM), 5 µl of DNase I-treated RNA template (~0.5 µg), and 5 µl of RNase-free water. RT-PCR was conducted using one cycle of reverse transcription at 50 °C for 30 minutes, one cycle of initial PCR activation at 95 °C for 15 minutes, 35 cycles of PCR amplification using three-step cycling (denaturation at 94 °C, 30 seconds; annealing at 52 °C, 30 seconds; extension at 72 °C, 1 minute), and a final extension at 72 °C for 10 minutes. The RT-PCR products were visualized following electrophoresis on a 1.5 % agarose gel in 1X TAE buffer at 106 volts for 90 minutes.

2.8 Detection of dsRNA using Strand-Specific Primers with Two-Step RT-PCR

Two-step strand-specific RT-PCR was performed on both total RNA extractions (Section 2.6.2) and dsRNA extractions (Section 2.6.1) of strain 93-1224 (positive control), untransformed H327 (negative control), and all H327 transformants. Prior to reverse transcription a mixture of the dsRNA templates and RNase-free water were denatured at 65 °C for 5 minutes as per the manufacturers instructions (Qiagen Inc.). After denaturation, the first step of reverse transcription cDNA synthesis was carried out using the Omniscript Reverse Transcription Kit (Qiagen Inc.). Each strain being tested was assigned two experimental treatments: the first treatment contained only the negative-strand specific primer (Diag_Fwd_MV1c), and the second treatment contained only the positive-strand specific primer (Diag_Rev_MV1c). Each reverse transcription cDNA reaction had a total volume of 20 µl containing 2 µl of 10X Buffer RT (1X), 2 µl of dNTPs (0.5 mM each), 1 µl of Omniscript Reverse Transcriptase (4 units), 2 µl of either Diag_Fwd_MV1c or Diag_Rev_MV1c (1 µM), 5 µl of template RNA (~0.5 µg), and 8 µl of RNase-free water. Reverse transcription was conducted using one cycle at 37 °C for 60 minutes. Following reverse transcription, the cDNA product was used in a DreamTaq DNA Polymerase

(Thermo Fisher Scientific Inc.) PCR reaction. Each PCR reaction had a total volume of 25 µl containing 2.5 µl of 10X DreamTaq Buffer (1X final), 0.5 µl of dNTPs (0.2 mM of each), 1 µl each of Diag_Fwd_MV1c and Diag_Rev_MV1c primers (400 nM), 0.2 µl of DreamTaq DNA Polymerase (1 unit), 5 µl of cDNA template, and 14.8 µl of nuclease-free water. PCR was conducted using one cycle of initial denaturation at 94 °C for 1 minute, 30 cycles of PCR amplification using three-step cycling (denaturation at 94 °C, 30 seconds; annealing at 52 °C, 30 seconds; extension at 72 °C, 1 minute), and a final extension at 72 °C for 3 minutes. The final two-step RT-PCR products were visualized by electrophoresis on a 1.5 % agarose gel as before.

2.9 Diagnostic PCR: Testing for DNA Contamination

PCR was used to test for the presence of the transforming DNA to ensure that it did not contaminate the RNA extractions and was entirely degraded by DNase I treatment. The PCR was conducted using diagnostic primers (Table 3) designed to amplify an 895 base pair sequence of the RdRp region of OnuMV1c (and/or MV1cCyt). DreamTaq DNA Polymerase (Thermo Fisher Scientific Inc.) was used for all PCR reactions. The total reaction volume was 25 µl and contained 2.5 µl of 10X DreamTaq Buffer (1X), 0.5 µl of dNTPs (0.2 mM of each), 1 µl each of Diag_Fwd_MV1c and Diag_Rev_MV1c primers (400 nM), 0.2 µl of DreamTaq DNA Polymerase (1 unit), 2 µl of DNase I-treated RNA template, and 17.8 µl of nuclease-free water. PCR was conducted using one cycle of initial denaturation at 94 °C for 1 minute, 30 cycles of PCR amplification using three-step cycling (denaturation at 94 °C, 30 seconds; annealing at 52 °C, 30 seconds; extension at 72 °C, 1 minute), and a final extension at 72 °C for 3 minutes.

Chapter 3: Results

3.1 Construction of OnuMV1c cytoplasmic version (MV1cCyt)

3.1.1 Tryptophan Codon Modifications

OnuMV1c is a mitovirus; hence its RNA-dependent RNA-polymerase (RdRp) is normally translated in the mitochondria and therefore uses the mitochondrial codon pattern. In ascomycetes the codon UGA encodes tryptophan in the mitochondria, but is a stop codon in the cytoplasm. As a result, were a mitochondrial mRNA to be translated by cytosolic ribosomes it would create a truncated, non-functional protein as each tryptophan codon would be read as a stop codon. In order to correct for this, the OnuMV1c sequence was examined and any UGA codons (in the correct reading frame) were changed to UGG (cytoplasmic frequency = 1.0), which also encodes tryptophan. This substitution allowed the MV1cCyt construct to exhibit the same protein expression as OnuMV1c, while preventing any disruptions in the open reading frame of the modified virus during translation in the cytoplasm of *O. novo-ulmi*. In the entire MV1cCyt construct thirteen UGA codons were modified to UGG (Figure 4). The *gpdA* promoter and *trpC* terminator were not modified, as they are not included in the mRNA transcript.

3.1.2 Codon Modification of BamHI/HindIII Sites

To facilitate downstream cloning steps of the artificial construct one *Bam*HI site and one *Hin*dIII site were modified (Figure 4). The *Bam*HI site was changed from G*GAU*CC to G*GAC*CC, resulting in the aspartic acid codon GAU (frequency = 0.21) being changed to the synonymous codon GAC (cytoplasmic frequency = 0.79). The *Hin*dIII site was changed from

Figure 4. Engineered OnuMV1c sequence (MV1cCyt) with the 85 base pair *gpdA* promoter tail and 360 base pair *trpC* terminator that was sent to GeneArt Gene Synthesis (Life Technologies Incorporated). The *gpdA*(linker)-MV1cCyt-*trpC* construct was engineered with a *Tth*111I restriction enzyme site on the 5' end of the molecule (5'-GACAAGGTC-3') and a *Hin*dIII site on the 3' end of the molecule (5'-AAGCTT-3'), both of which are bolded in the figure. The different components of the synthesized construct sequence are colour-coded: the *gpdA* promoter region is highlighted in light blue with its transcription start site (TSS) highlighted in green, the MV1cCyt region is highlighted in yellow with its RNA-dependent RNA polymerase coding region highlighted in light grey, and the *trpC* transcription terminator region is highlighted in pink. All codons that were modified to make the cytoplasmic MV1cCyt virus are underlined and bolded with the altered nucleotide base highlighted with red. The entire modified construct was flanked with identifying signature sequences: a 5'-GGGGTTT-3' sequence at the 5' end of the construct and a 5'-AAACCCC-3' at the 3' end of the construct. The entire *gpdA*(linker)-MV1cCyt-*trpC* synthesized construct comprised 3582 base pairs.

Figure Legend			
NNNNNN	*gpdA* Promoter (85 bp Tail)	GGGGTTT	5’ Signature Tail
NNNNNN	Transcription Start Site	AAACCCC	3’ Signature Tail
NNNNNN	MV1cCyt	**GACAAGGTC**	5’ *Tth*111I Site
NNNNNN	MV1cCyt RdRp	**AAGCTT**	3’ *Hin*dIII Site
NNNNNN	*trpC* Terminator		
NNNNNN	Codon Changes		

***gpdA*-MV1cCyt-*trpC* Sequence**	**Base Pairs**
GGGGTTT**GACAAGGTC**GTTGCGTCAGTCCAACATTTGTTGCCATATTTTC	50
CTGCTCTCCCCACCAGCTGCTCTTTTCTTTTCTCTTTCTTTTCCCATCTT	100
CGGACCGTATGGGGTCGCTGACTTTCGCGAGTCAGAAACCTCCGTACGAT	150
GAGGAAGAGTCCTTCCTCTTATGCTTAATTGCATGTAATTCCTAATATAG	200
GTCGTGTAAGAGTGTCGTCCAAAGGCTGATTCCTTCTAATTAAAGTTAGT	250
TAGATCTTGTCTGTCGTTCCTCATCATAAGGGAGTTAAAAGTCTGGGGTT	300
TGCACCCTCTTTCTTTTAACAGACAGAACTTTCTACTAACCGAGATTAGT	350
CGGTGGGGCACAATGGAAAGCGATTAATTTCGTTGTTCTGTTGAACCCCA	400
AGGTTAACCATACCTGTTTAATCAAACTGCCTGCAGATTCCTCATTAGAG	450
GGAAAACTGAAGAAGGAAATGGTATTAAATTAATAATACTTGCAACTAAC	500
AACTTTTTACATAAATTATGATCATGTATCAACGTTGTTAGATGTACATT	550
TTCTTGGCACAAGGTCTAAAATCCCTTGACTTATTAGTC**TGG**CCACTTTT	600
ACAAGTGATAAGACTAGTAAGGGGTTGTTTAAGCCCAGATTTAATCAAAG	650
CAGTATTTGTTTTCGTGAAAAGATCAAGTTCTCTTCAGAAAACTGGAGGT	700

TTAAAGTTCGTTGCTTTATATTATA**AAGCGT**GTCATATCTACACTATGCA 750

ATTTGTTGCTAGTGGAGGTGTTAGACAATCCTTCATAACATCAACATGTT 800

ATGGAGTAAACGTATCTCTAACTTCCGGTGGTCTACCGAGAATTCTTCCT 850

ATTTATTTAAGAAGATTGGTTACTTCACAGAACAAAGAAGGTATCAAAAT 900

TGTTTTAACTTTATTTAACCTATATAGGGTCTTACCCTATCCAGGAAAAG 950

TAAAATTATCAACCATAACTGATAAA**TGG**GTCCGGATCATATCCCTTAGAT 1000

ATGATCTCATTCATTCCTAAGTTTTGGTTGCTTCTAAGATCCCAAGGAAA 1050

GATAGCTCCTTTTACCTTTATTCGATCACCATTCGCCATATCAGCGAGTG 1100

GCTCAATTACAGGTTTTGGGAAACATCTTTCCTCCATGTCTGGTTTCTTT 1150

AAAGCTCTTCTATTCCTAAGAAGAGAGGATTCTCTG**TGG**CAATCACTTCA 1200

A**TGG**TTTTTTACAGAGGCCCCTTTAAGAAGGGGA**TGG**GCTTCTAGAACTC 1250

TACGATGT**TGG**CAATCTATGGATTTTATTTCCAGATTGTTACTTGTTGCA 1300

GGTCAAAAAGTCGTATCTTCTCCTTTAGGAAAGTTAGCCTTTAAAGAGGA 1350

ACCGGGAAAAGTTAGGGTATTCGCTATGGCGGACTGTATAACTCAA**TGG**G 1400

TATTACATCCTCTCCACCAGTATTTATTTTCAATCTTGAAACAAATAAGC 1450

ATCGTTGATGCAACATTTGATCAAGAAGAAGGGGTAAGAACCCTTTCTCA 1500

GAAGATAAAATCTGGTAAGAAGATTGTATTCTCCTTGGATTTATCAGCCG 1550

CTACAGACAGATTACCACTAACAATTCAAGCACAGATCCTGAATCATATA 1600

GTTCCAAAGTTAGGGGACCACTGGGCCAATCTTCTGGTTAACAGAGATTA 1650

CTCAGTGCCAAACCATATTACTCTACCAGTTAATCCTGGTACTGTTAGAT 1700

ATGGAGCGGGCCAACCTATGGGAGCTTATAGTTCA**TGG**GCTATGCTTGCC 1750

CTTACACATCACTTCATAGTACAGTATTGTGCTTTCAAAGTCTATCATTC 1800

TAATTCATTCTTCAAAGATTATCTAATCTTAGGAGATGATTTATTATTGT 1850

TAGACGCGAGAGTTGCAAAACAGTACTTAAAAGTCATGAGCCAACTGGAT 1900

GTAGGAGTGAATCTAGCAAAATCCTTAATCTCTGTTAGAGGTTATGGAGA 1950

ATTTGCTAAACAATTCCTATCTCCAGAGGGACCTTTACAAGGGGTCAGTT 2000

TAAAAGAATTTTCTTCGTTAAAAGATGGAATGTCTAACATTCTATCCTTA 2050

TCGGTTAAAATGTCTCTTAAACCTTCCTTATTGTTAAGGCTGTTTGGGTT 2100

TGGTTCTAAATCAGTTGGTCATTCAACTTTACCCTTTTCTAGATACTCAT 2150

TGAGATCCAGTCTTGATCATCTATTCTTGAGTCCGCTTGCGAACTCTCTG 2200

AATAGT**TGG**CTAGATTATTTCTCGTGTATTAGACATTCAAAATTCTTATT 2250

GATTAGTAAGTCTTTATTGGCTTACTTTCTA**TGG**GGTTTTGAATTTATCA 2300

AATACAAA**TGG**GTTTTCAAGAAGGTTAGGGAGAATGAGCAACTGATGTTG 2350

CGACATGGAACACATCGCTACCATCAATTGGTAAGTGAGACTTTACTGAG 2400

TTAC**TGG**TGTCCTTCTCCATCAGAAGAGGTATTGAAATTACCAGAAGTTG 2450

CTGATTCATCAGCTAACTTCTTCTTTTCAACTCCTCCTCCGGTGAATAAG 2500

GCATCATTCAAGTTATTGATGATTAATCATATCAATATCTTGTTCTCATG 2550

TGGGTCTCTCTTTTCAGAATTGATGAATGGAGGTGCAATGAATCTAGTCC 2600

CTAAATTTTTAACATTTGAGTTGTCCTTTGAAGAGGGTTACTCAGATTTT 2650

GAGAAAGTGGAGAATGATCACTTA**TGG**TCAGTCATCACAAG**GGACCC**AAA 2700

AAGTTGGGTTAAACCCAACAATTATGGAGAAAACAATTTTGATATTGTTA 2750

AATTTATGAGCCAGCAAACATCTGTAGAGTTTCACAAACCAAGGACTCTT 2800

AAGGAAATTTCTTTAGCTTTACAACTAAAGAAGGATTTCTTTAATAGTAG 2850

TTTGGTAAATGGATCTCAAACCATACTAGATGTGCCTCTTGGTAAACCAA 2900

AAGGCTAGGTGACTCATTAGCAATCATAGTGATCTCTGGTTGAGAATACT	2950
CAGTTAAAGGTGATCGAGAATTATGAAGTTATTCATTTCTTTTCGAAAGA	3000
CCCTTTCGTGTAGTATTTATAACCAGTAGATCAGAGATCTATTATTGTTG	3050
CTAAATGTTTCATTCCTCATAAAGGAGATGTTCCAAAATAATCTGAGCTA	3100
TTTCCCCGTTAAGGGAAAACAACCTTGATCTTCGGCGATTGTAAAATCGT	3150
CCCATGGCTTGAAGGTGACTTGATCAAAGATAGTAAGGAGTCTAGCTCCT	3200
AACGGTCCCCATGTCAACAAGAATAAAACGCGTTTTCGGGTTTACCTCTT	3250
CCAGATACAGCTCATCTGCAATGCATTAATGCATTGACTGCAACCTAGTA	3300
ACGCCTTCAGGCTCCGGCGAAGAGAAGAATAGCTTAGCAGAGCTATTTTC	3350
ATTTTCGGGAGACGAGATCAAGCAGATCAACGGTCGTCAAGAGACCTACG	3400
AGACTGAGGAATCCGCTCTTGGCTCCACGCGACTATATATTTGTCTCTAA	3450
TTGTACTTTGACATGCTCCTCTTCTTTACTCTGATAGCTTGACTATGAAA	3500
ATTCCGTCACCAGCCCTGGGTTCGCAAAGATAATTGCATGTTTCTTCCTT	3550
GAACTCTCAAGCCTACAG**AAGCTT**AAACCCC	3582

AA*GCU*U to AA*GCG*U, resulting in the alanine codon GCU (cytoplasmic frequency = 0.21) being changed to the synonymous codon GCG (cytoplasmic frequency = 0.21).

3.1.3 Synthesis of Engineered MV1cCyt Construct

The final *gpdA*(linker)-MV1cCyt-*trpC* construct contained the tail end of the *gpdA* promoter starting with a naturally occurring *Tth*111I site, the codon-modified MV1cCyt cytoplasmic DNA, and 360 base pairs of the *trpC* terminator ending with a *Hin*dIII site (Figure 4). Due to the large size of the entire *gpdA* promoter, only the last 85 base pairs of the promoter were included in the synthesized construct. The remaining *gpdA* promoter was added to the construct in downstream cloning procedures. The final synthesized construct was 3581 base pairs in length and was named *gpdA*(linker)-MV1cCyt-*trpC*. GeneArt Gene Synthesis (Life Technologies Incorporated) synthesized the construct and packaged it into a pMA-RQ vector with ampicillin resistance (Figure 5). The correctness of this sequence was independently confirmed by sequence analysis of the construct.

3.2 Sub-cloning

3.2.1 Sub-cloning *gpdA*(linker)-MV1cCyt-*trpC* from pMA-RQ to pGEM-T

The synthesized *gpdA*(linker)-MV1cCyt-*trpC* insert from the pMA-RQ vector was ligated into the pGEM p*gpd*-*epg*-409i vector. After transformation of E. Cloni cells (Lucigen Corporation), 12 putative transformants grew in liquid LB supplemented with ampicillin. A diagnostic digest with *Tth*111I and *Hin*dIII restriction enzymes (New England Biolabs Incorporated) was performed on the plasmid DNA from these 12 putative transformants and run on an agarose gel (Figure 6). Transformants that contained the successfully ligated sections of

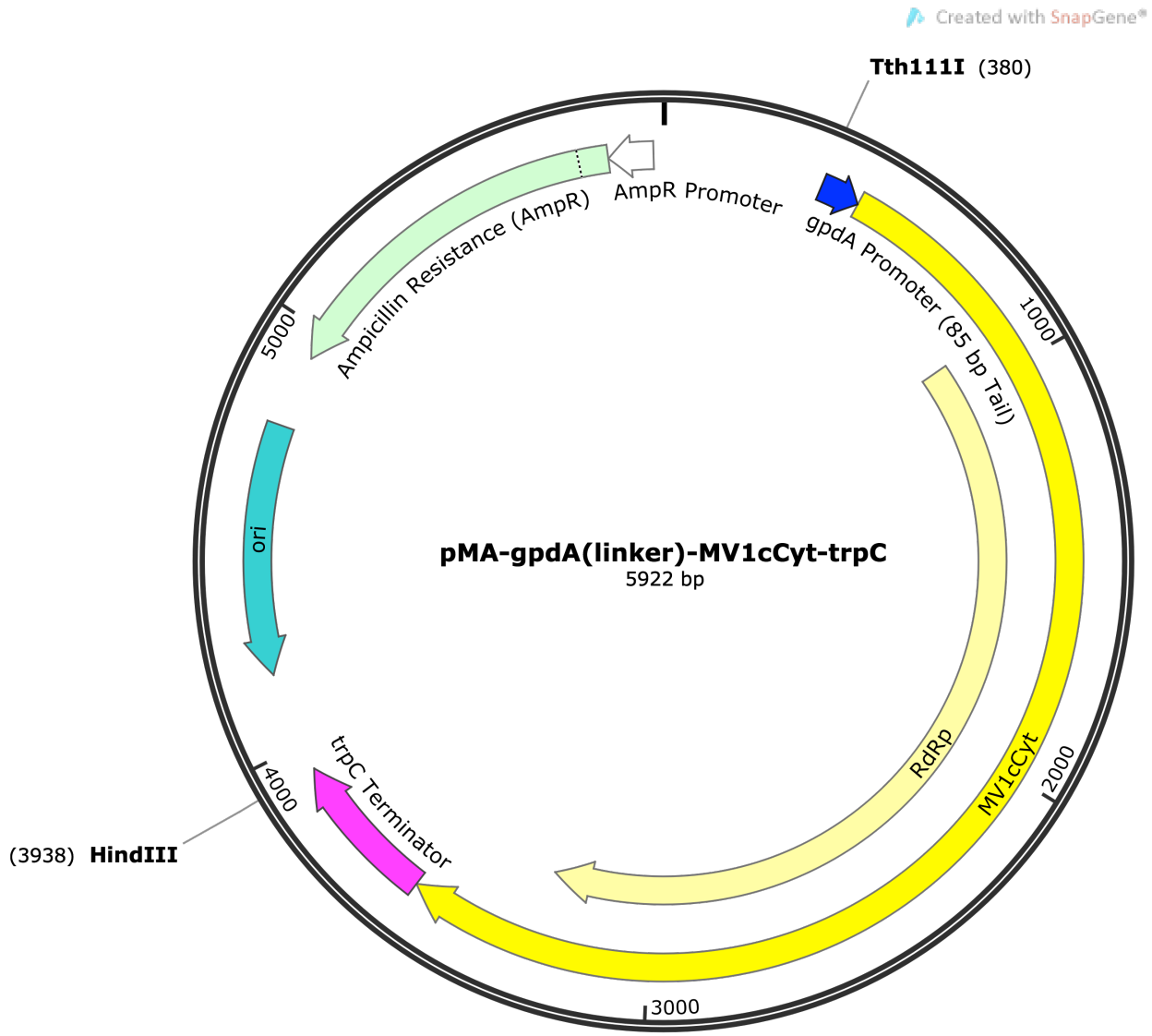


Figure 5. The *gpdA*(linker)-MV1cCyt-*trpC* construct synthesized by GeneArt Gene Synthesis (Life Technologies Incorporated, Burlington, ON Canada) was ligated into a pMA-RQ vector expressing ampicillin resistance as a selective marker. The entire vector with the construct comprised 5922 base pairs. The *Tth*111I and *Hin*dIII restriction enzyme sites used to sub-clone the *gpdA*-MV1cCyt-*trpC* construct are shown. Figure created in SnapGene Viewer (GSL Biotech LLC, Chicago, IL United States of America).

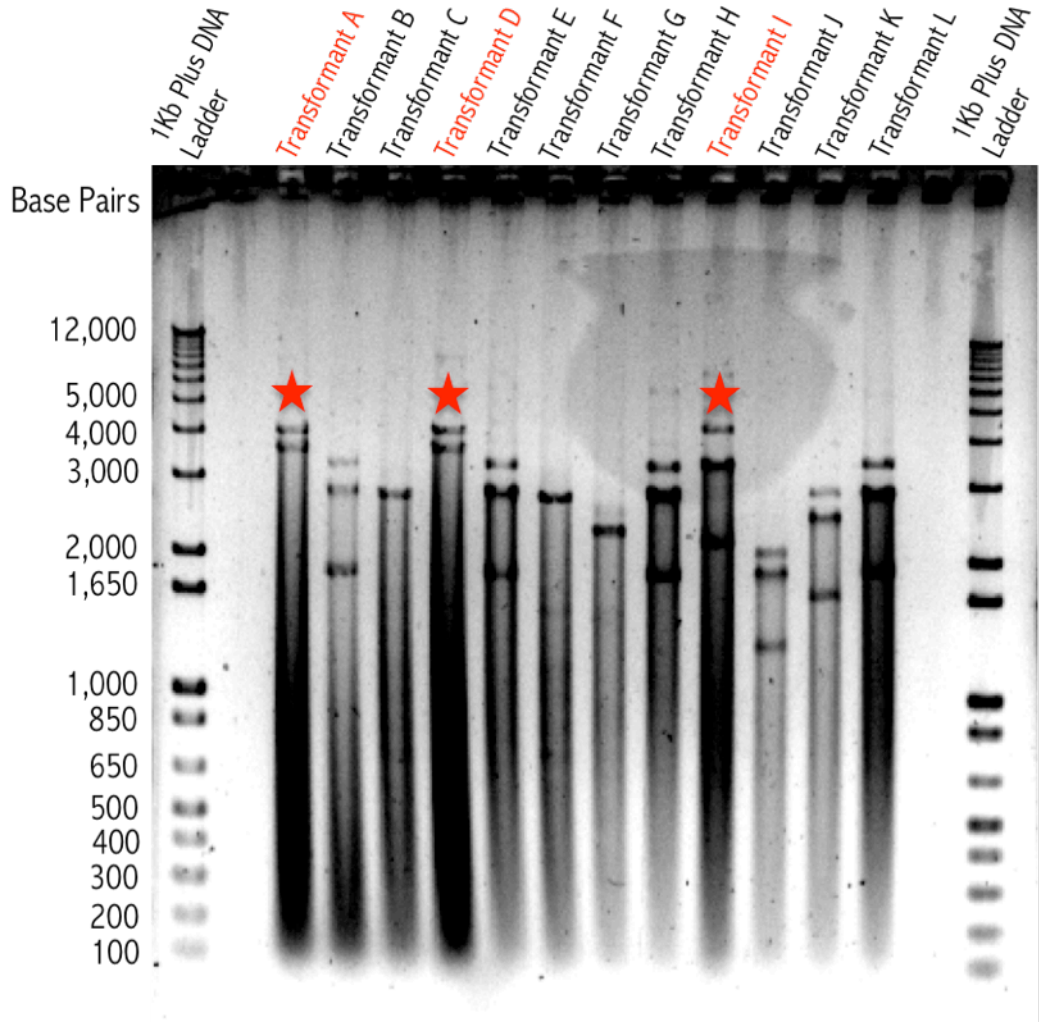


Figure 6. 1 % agarose gel showing the results of a *Tth*111I/*Bam*HI diagnostic restriction enzyme digest performed on 12 *E. coli* cultures (E. Cloni 10G Elite electrocompetent cells, Lucigen Corporation) transformed with pGEM-*gpdA*-MV1cCyt-*trpC* DNA. The gel was run for 2.5 hours in 1X TAE buffer at 90 volts with a 1 Kb Plus DNA Ladder (Life Technologies Incorporated). DNA from successful transformants yields two bands: a 3991 base pair band corresponding to the pGEM-T vector (3000 bp) with the first part of the *gpdA* promoter (991 bp), and a 3581 base pair band corresponding to the *gpdA*(linker)-MV1cCyt-*trpC* insert. The gel lanes containing the three putative successful transformants are marked with red stars (Transformants A, D, and I). These three transformants were selected for analysis with further diagnostic digests.

the final construct were expected to yield two bands from this diagnostic digest: a 3991 base pair band corresponding to the pGEM-T vector (3000 bp) with the first part of the *gpdA* promoter (991 bp), and a 3581 base pair band corresponding to the *gpdA*-MV1cCyt-*trpC* insert. Of the 12 putative transformants, three displayed these two bands. These three transformants were subjected to an additional five restriction enzyme digests to further confirm their identities: a *Bam*HI/*Hin*dIII digest (4572 bp and 3000 bp bands), a *Bam*HI/*Tth*111I digest (6581 bp and 991 bp bands), a *Bam*HI/*Xho*I digest (7572 bp band), a *Bam*HI only digest (7572 bp band), and a *Hin*dIII only digest (7572 bp band). Results from these additional digests revealed that only two of the three putative transformants showed the appropriate banding patterns (Figure 7): Transformant A and Transformant D. These two transformants were sent to Eurofins Genomics (Eurofins MWG Operon LLC) for sequence determination using the M13 forward and M13 reverse primers (Table 1). Sequence results confirmed that the *gpdA*(linker)-MV1cCyt-*trpC* construct had been successfully sub-cloned into the pGEM-T vector for both Transformant A and D (Figure 8). Therefore, both were used for the subsequent sub-cloning of the complete *gpdA*-MV1cCyt-*trpC* insert into the pAN7-1 fungal transformation vector.

3.2.2 Sub-cloning *gpdA*-MV1cCyt-*trpC* from pGEM-T to pAN7-1

After it was confirmed that the *gpdA*(linker)-MV1cCyt-*trpC* insert had been sub-cloned into pGEM-T, the insert with the complete *gpd*A promoter was moved into pAN7-1. The first several attempts to sub-clone the construct from pGEM-T into pAN7-1 were unsuccessful due to self-ligation of both vectors (re-circularization of vectors after restriction enzyme digestion). Therefore, a three-step dephosphorylation process was used in order to prevent self-ligation. After adopting this dephosphorylation protocol, the *gpdA*-MV1cCyt-*trpC* construct was

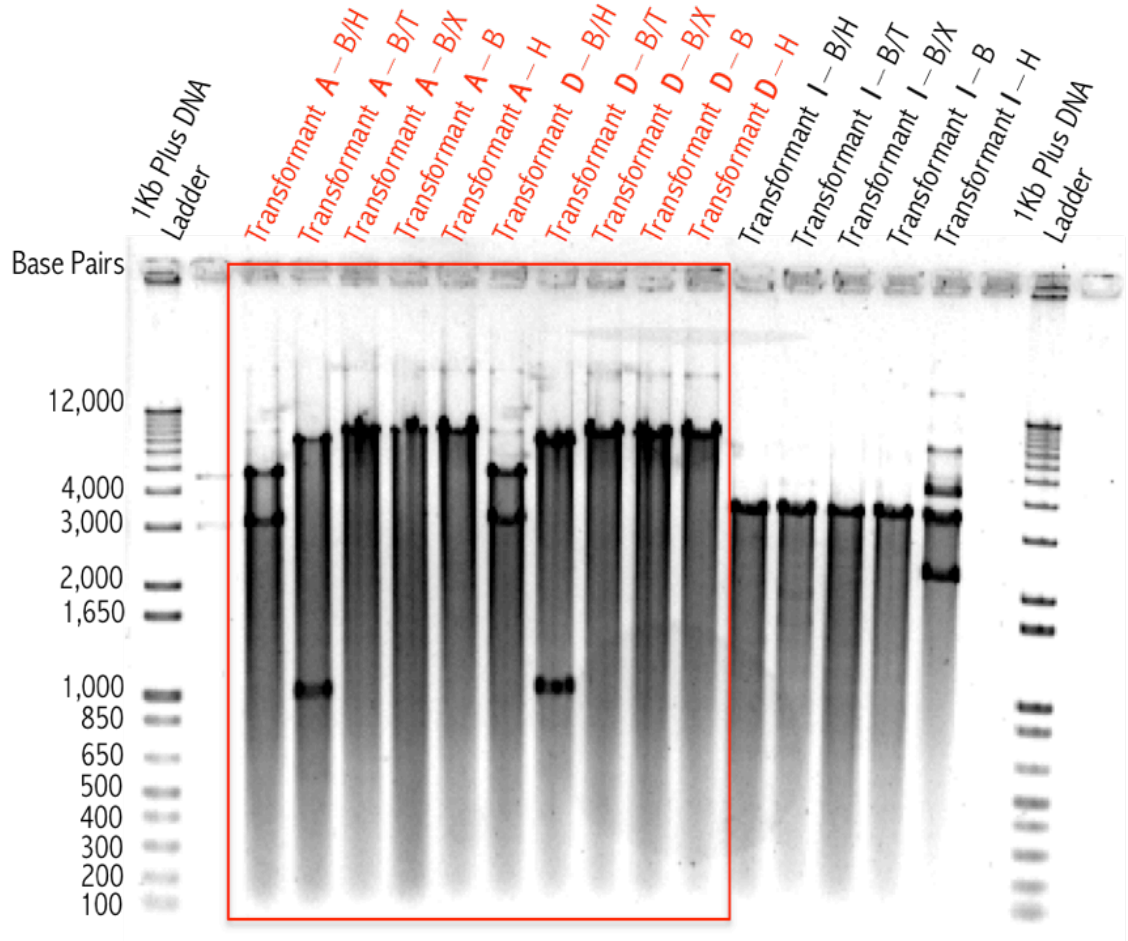


Figure 7. 1 % agarose gel showing the products of the five additional diagnostic restriction enzyme digests performed on putative Transformants A, D and I in order to confirm that they contained pGEM-*gpdA*-MV1cCyt-*trpC* DNA. The gel was run in 1X TAE buffer at 104 volts for 90 minutes with a 1 Kb Plus DNA Ladder (Life Technologies Incorporated). The five digests and their respective bands were: a *Bam*HI/*Hin*dIII (B/H) digest (4572 bp, 3000 bp), a *Bam*HI/*Tth*111I (B/T) digest (6581 bp, 991 bp), a *Bam*HI/*Xho*I (B/X) digest (7572 bp), a *Bam*HI (B) digest (7572 bp), and a *Hin*dIII (H) digest (7572 bp). From these five additional digests it was determined that only Transformant A and D (highlighted in red) displayed the correct banding patterns. Therefore, these two transformants were determined to be pGEM-*gpdA*-MV1cCyt-*trpC* and were selected for further analyses.

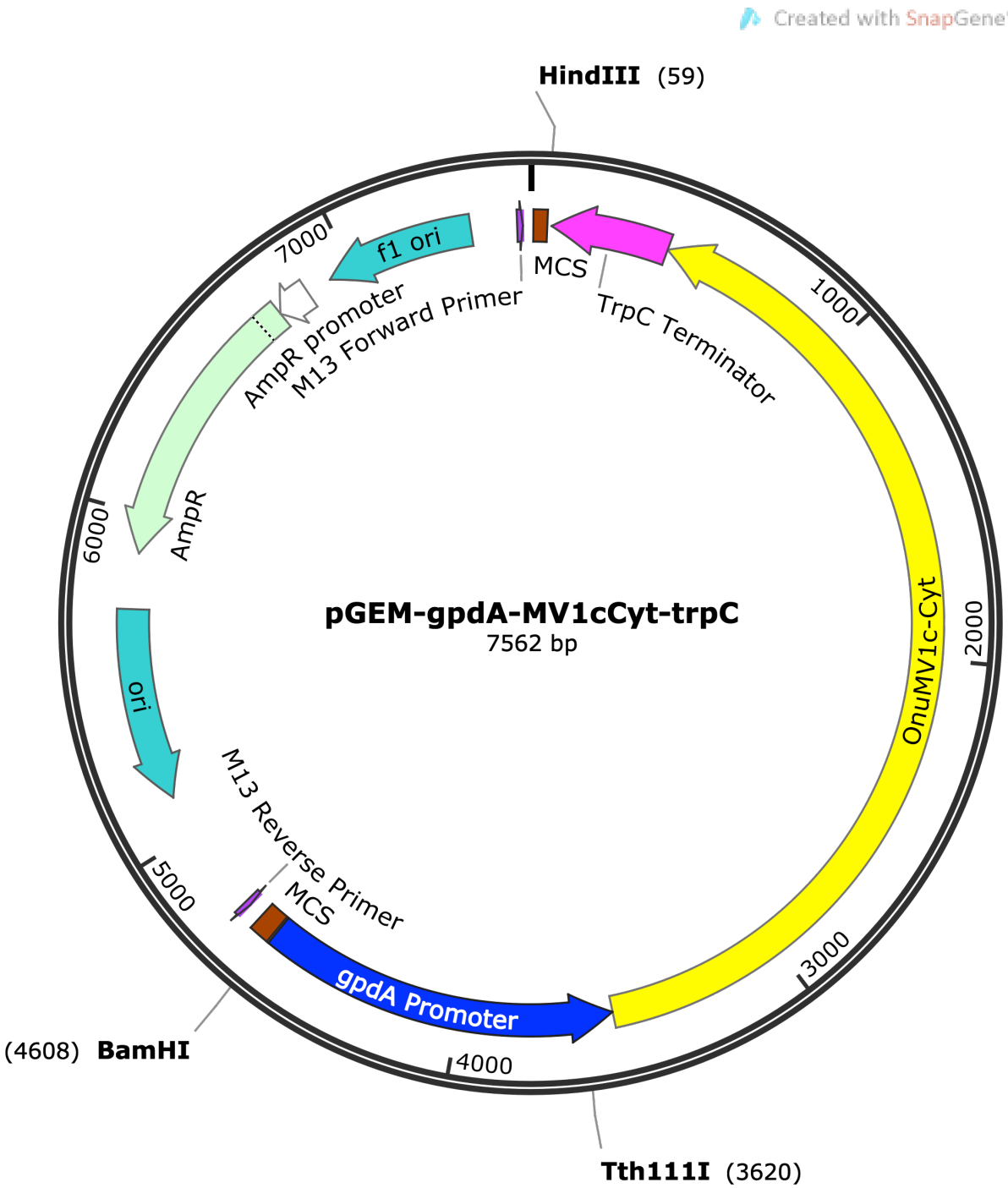


Figure 8. The *gpdA*-MV1cCyt-*trpC* construct with the full *gpdA* promoter cloned into the pGEM-T vector as confirmed by sequencing results from Eurofins Genomics (Eurofins MWG Operon LLC) using the M13 forward and reverse primers. The entire vector with the construct comprised 7562 base pairs. Figure created in SnapGene Viewer (GSL Biotech LLC, Chicago, IL United States of America).

successfully ligated into pAN7-1. After transformation of E. Cloni cells (Lucigen Corporation) only four putative transformants grew in liquid LB supplemented with ampicillin. The plasmid DNA extracted from these transformants was subjected to a diagnostic digest with *Bam*HI and *Hin*dIII restriction enzymes (New England Biolabs Incorporated) and was run on a 1 % agarose gel in 1X TAE buffer (Figure 9). Successful pAN7-*gpdA*-MV1cCyt-*trpC* transformants were expected to yield two bands: a 5980 base pair band corresponding to the pAN7-1 vector, and a 4549 base pair band corresponding to the *gpdA*-MV1cCyt-*trpC* construct. Of the four putative transformants, three displayed these two bands: Transformants A, B, and D (Figure 9). Transformant D was selected for three additional diagnostic digests to confirm its identity: an *Eco*RI digest (5370 bp, 2620 bp, and 2538 bp bands), an *Eco*RI/*Bam*HI digest (5370 bp, 2538 bp, 1820 bp, and 800 bp bands), and an *Eco*RI/*Hin*dIII digest (2729 bp, 2641 bp, 2620 bp, and 2538 bp bands). It showed the correct banding patterns for all three digests (Figure 10). The results of the PCR performed on Transformant D using the nested MV1cCyt sequence primers also showed bands corresponding to the correct amplicon size: a 3159 base pair band for the first primer pair, a 1786 base pair band for the second primer pair, and a 560 base pair band for the third primer pair (Figure 11). Finally, the sequencing results from Eurofins Genomics (Eurofins MWG Operon LLC) using the nested MV1cCyt sequence primers confirmed that the plasmid DNA extracted from Transformant D was pAN7-*gpdA*-MV1cCyt-*trpC* (Figure 12).

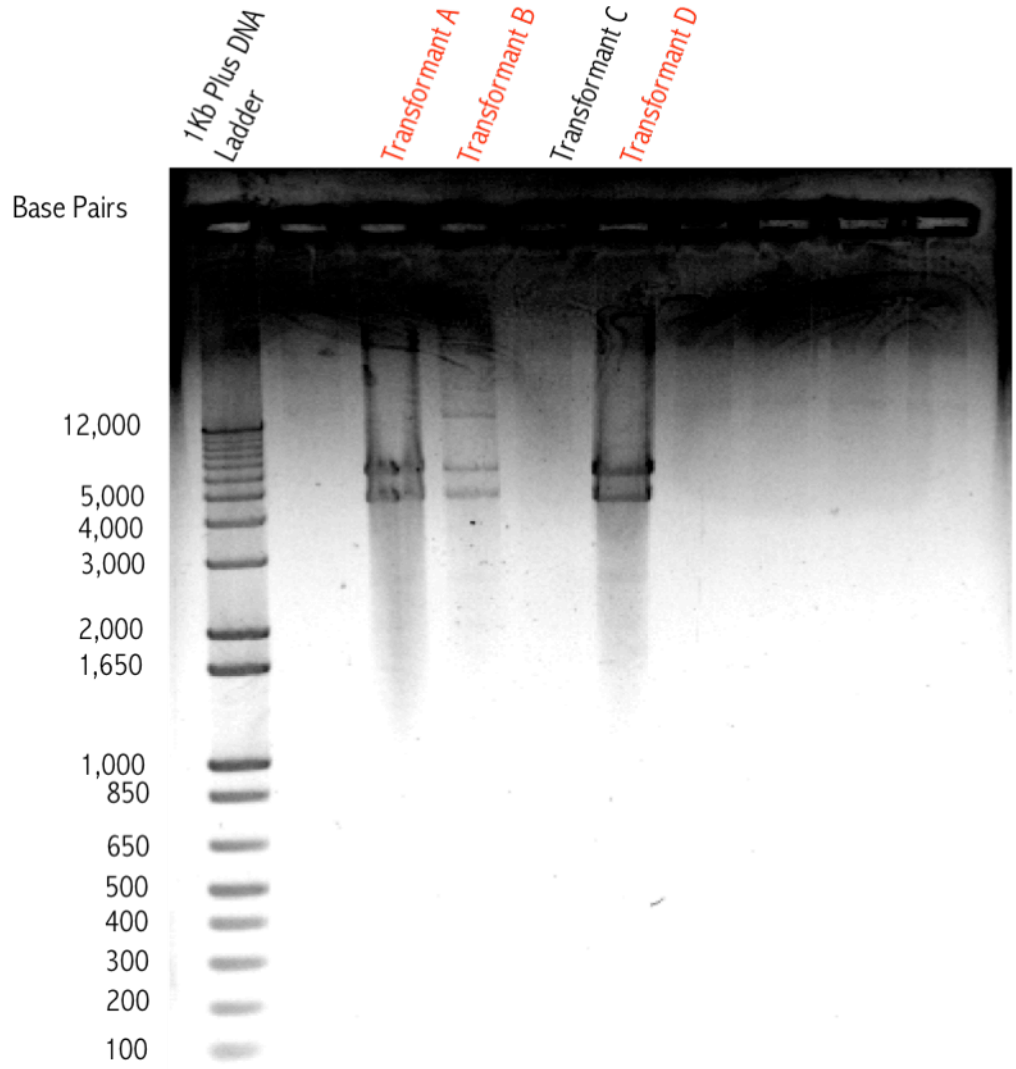


Figure 9. 1 % agarose gel showing the results of a *Bam*HI/*Hin*dIII diagnostic restriction enzyme digest performed on putative pAN7-*gpdA*-MV1cCyt-*trpC* transformants. The gel was run in 1X TAE buffer at 86 volts for 1.5 hours with a 1 Kb Plus DNA Ladder (Life Technologies Incorporated). Successful transformants were expected to yield two bands: a 5980 base pair band corresponding to the pAN7-1 vector, and a 4549 base pair band corresponding to the *gpdA*-MV1cCyt-*trpC* construct. Of the four putative transformants, three displayed these two bands: Transformants A, B, and D (highlighted in red).

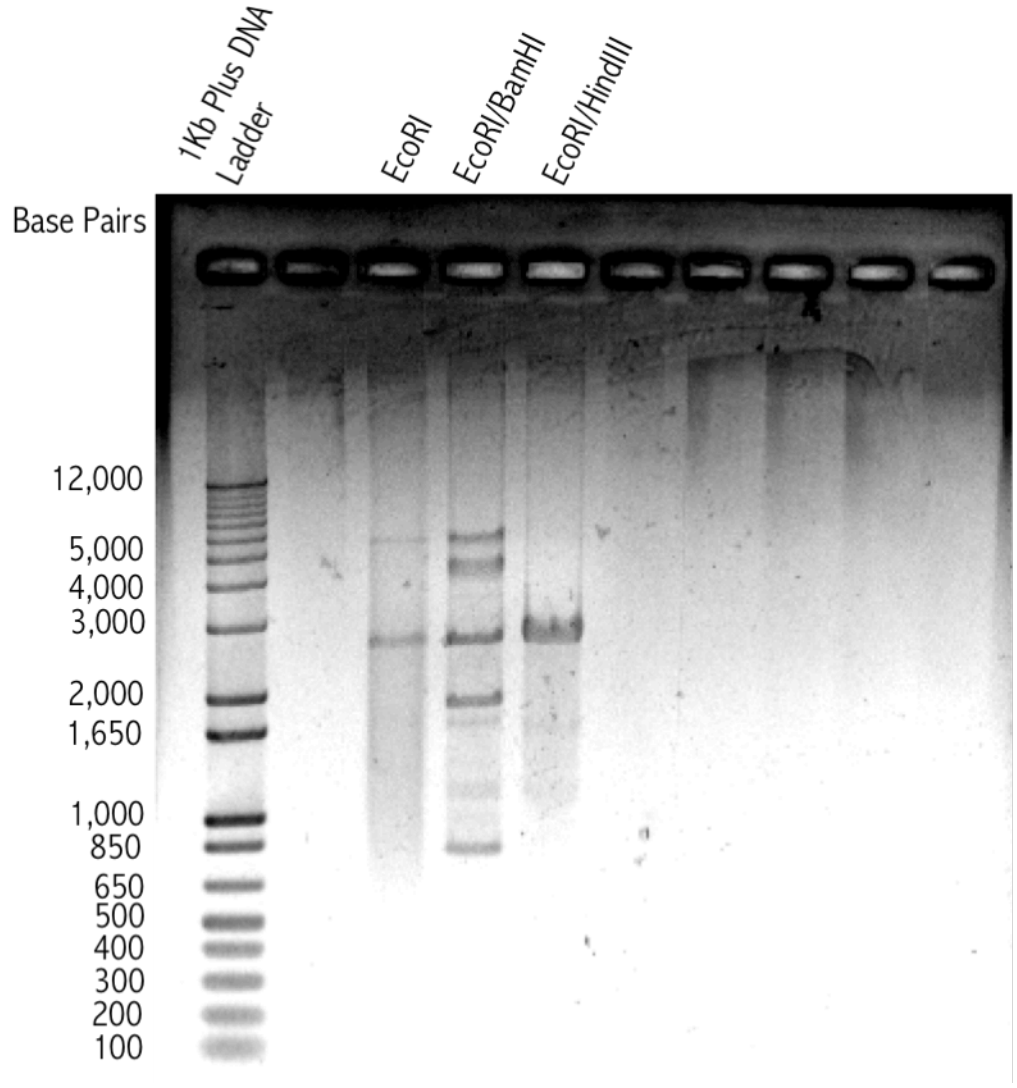


Figure 10. 1 % agarose gel showing the results of three diagnostic restriction enzyme digests on putative pAN7-*gpdA*-MV1cCyt-*trpC* Transformant D. The gel was run in 1X TAE buffer at 86 volts for 1.5 hours with a 1 Kb Plus DNA Ladder (Life Technologies Incorporated). The transformant showed the correct banding patterns for each digest. The *Eco*RI digest yielded 3 DNA fragments: an easily distinguishable 5370 bp band and two other bands 2620 and 2538 bp in size that were too close together to separate. The *Eco*RI/*Bam*HI digest yielded 4 DNA fragments: a 5370 bp band, a 2538 bp band, an 1820 bp band, and an 800 bp band. Finally, the *Eco*RI/*Hin*dIII digest yielded 4 DNA fragments all too close in size to distinguish: a 2729 bp band, a 2641 bp band, a 2620 bp band, and a 2538 bp band.

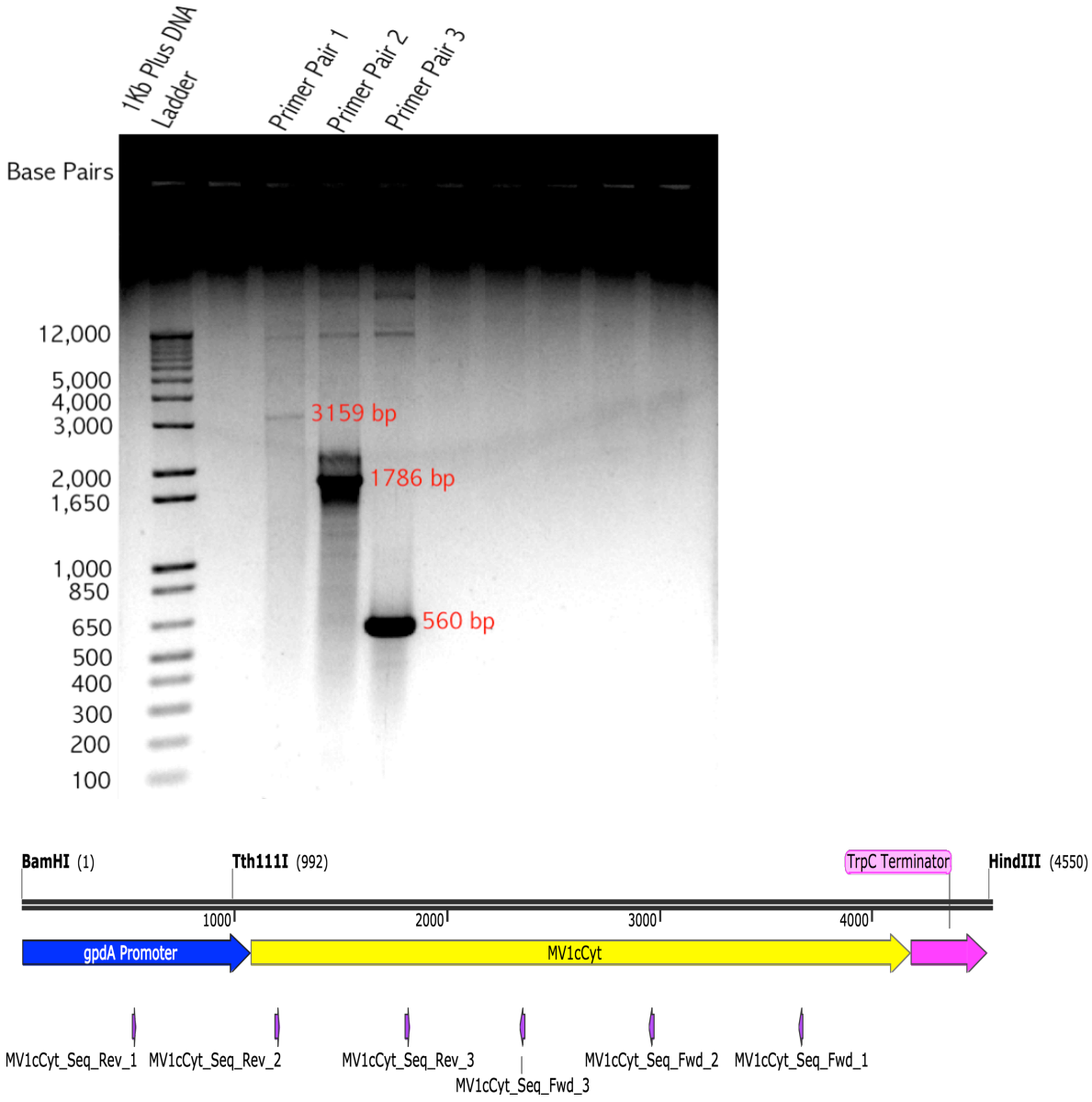


Figure 11. 1 % agarose gel showing the results from a PCR run on pAN7-*gpdA*-MV1cCyt-*trpC* putative Transformant D using the nested MV1cCyt sequence primers. The gel was run in 1X TAE buffer at 86 volts for 1.5 hours with a 1 Kb Plus DNA Ladder (Life Technologies Incorporated). Transformant D yielded the expected band sizes for all three nested MV1cCyt sequence primer pairs: a 3159 base pair amplicon from the first primer pair, a 1786 base pair amplicon from the second primer pair, and a 560 base pair amplicon from the third primer pair. Below the gel is a diagram of the *gpdA*-MV1cCyt-*trpC* construct showing how the MV1cCyt sequence primers are nested and where in the construct they bind and amplify. Diagram created using SnapGene Viewer (GSL Biotech LLC, Chicago, IL United States of America).

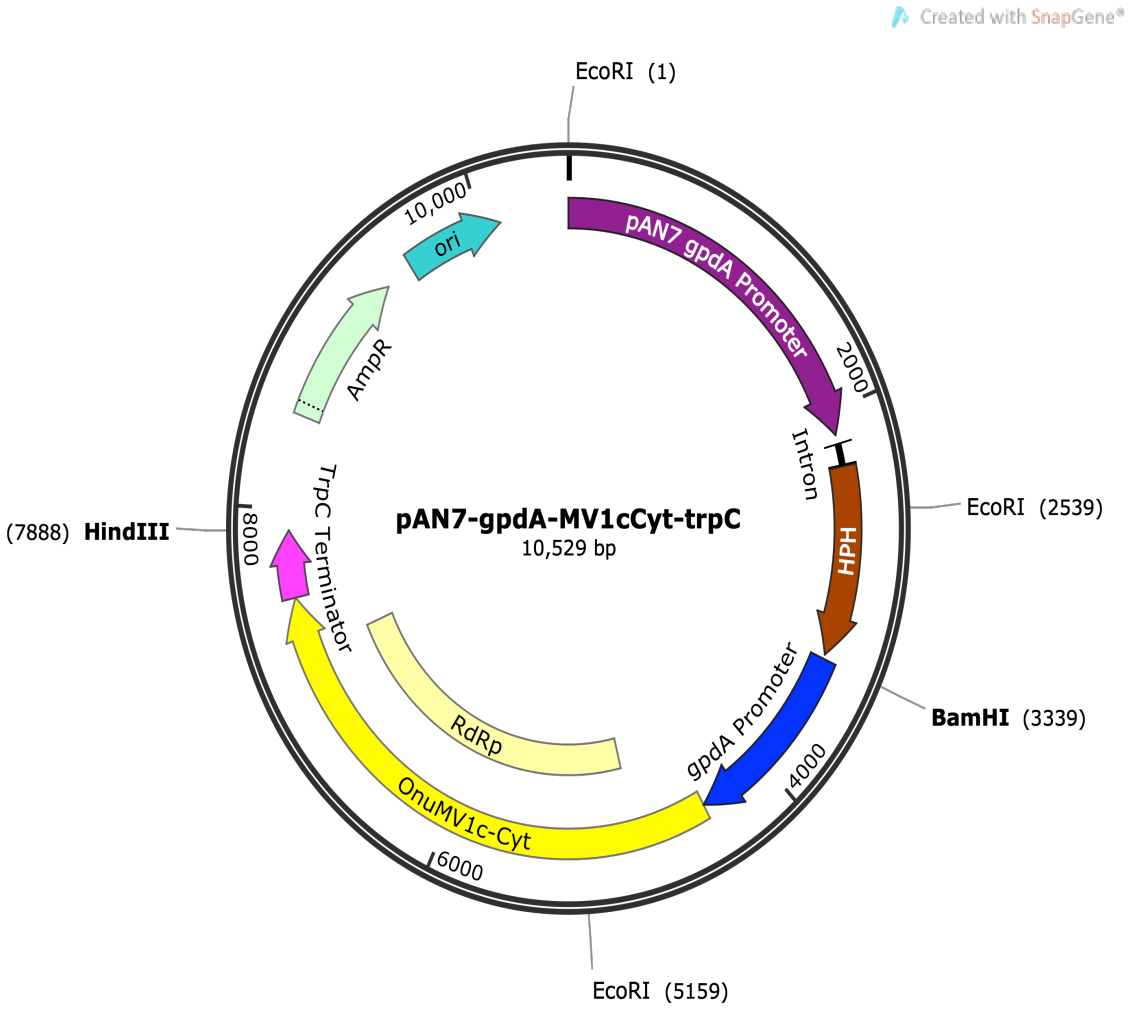


Figure 12. The pAN7-*gpdA*-MV1cCyt-*trpC* vector as confirmed by sequencing results from Eurofins Genomics (Eurofins MWG Operon LLC) using the nested MV1cCyt sequence primers. The entire vector comprised 10, 529 base pairs. The *Bam*HI, *Hin*dIII, and *Eco*RI restriction enzyme sites that were used for diagnostic digests are shown. Figure created in SnapGene Viewer (GSL Biotech LLC, Chicago, IL United States of America).

3.3 Preparation of *Ophiostoma novo-ulmi* Protoplasts

Ophiostoma novo-ulmi H327 protoplasts were prepared in two batches with concentrations of 1.13 x 10^7 protoplasts/ml and 6.75 x 10^7 protoplasts/ml, respectively. Prior to transformation, the viability of the protoplasts was confirmed by plating them on OCM medium supplemented with 0.6 M sucrose as an osmotic stabilizer. All protoplasts grew properly on the medium and were determined to be viable and ready for transformation.

3.4 Transformation of *Ophiostoma novo-ulmi* Protoplasts

Five putative H327 transformants grew on the OCM/0.6 M sucrose plates supplemented with 100 μg/ml hygromycin B. Of these five, only four subsequently grew on the OCM plates containing 400 μg/ml hygromycin B. These four stable putative transformants were named H327 + pAN7-*gpdA*-MV1cCyt-*trpC* Transformants 1 – 4 (abbreviated HT1 – HT4) and were used for all subsequent procedures.

3.5 RNA Extractions

3.5.1 Isolation of Total RNA

Total RNA was extracted from strain 93-1224, strain H327, and all four H327 transformants (HT1 – HT4) and treated with DNase I to remove any contaminating DNA. All total RNA extractions had purities that were within accepted ranges (A_{260}/A_{280} ratio of

1.9-2.1). These extractions were used for both the one-step RT-PCR and the strand-specific RT-PCR.

3.5.2 Isolation of dsRNA

Double-stranded RNA was extracted from strain 93-1224, strain H327, and all four H327 transformants (HT1 – HT4) and treated with DNase I. All dsRNA purities were within accepted ranges (A_{260}/A_{280} ratio of 1.9-2.1). The dsRNA extractions were visualized on an agarose gel (Figure 13), which clearly showed the two bands from strain 93-1224 corresponding to the OnuMV1c (3107 base pairs) and OnuMV7 (2804 base pairs) dsRNA viruses. The dsRNA extracted from untransformed H327 as well as transformed HT1, HT2, HT3, and HT4 did not show any bands. The virus dsRNA has to be present in relatively large amounts in order for it to be detected on a gel; therefore, the lack of a 3107 base pair MV1cCyt band in HT1 – HT4 did not confirm that it was not present in the transformed fungal cells. Consequently, as described previously, one-step RT-PCR and strand-specific RT-PCR were performed to further test for the presence of MV1cCyt dsRNA.

3.6 Diagnostic PCR: Testing for DNA Contamination

The H327 transformants contained MV1cCyt cDNA integrated into their genomes as well as MV1cCyt messenger RNA. The Diag_MV1c primers could anneal to both the DNA and RNA forms of MV1cCyt, so a diagnostic PCR was performed to ensure the signal from RT-PCR was from RNA only, and not from contaminating recombinant

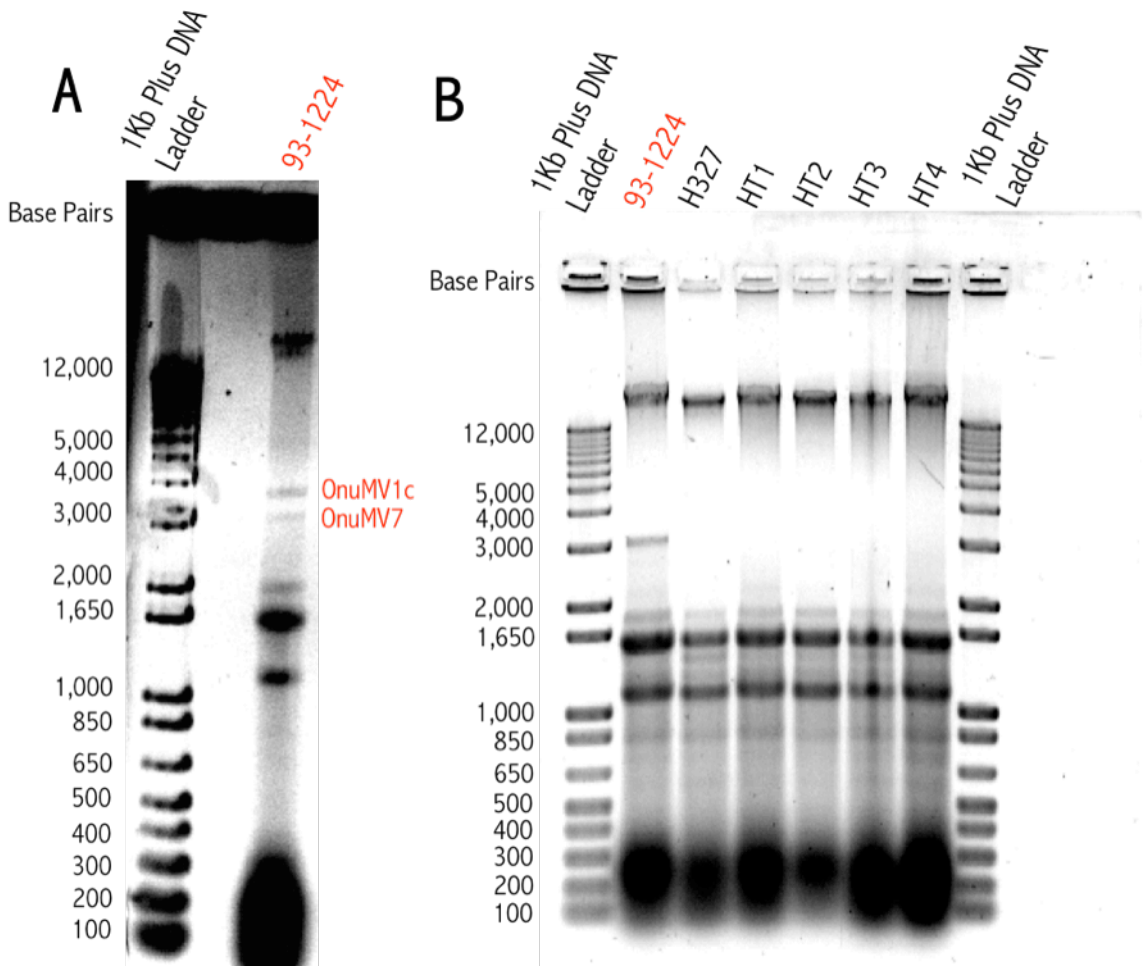


Figure 13. A) 1 % agarose gel showing a dsRNA extraction of *O. novo-ulmi* strain 93-1224. The gel was run in 1X TAE buffer at 80 volts for 2 hours and 20 minutes with a 1 Kb Plus DNA Ladder (Life Technologies Incorporated). The two bands corresponding to the dsRNA viruses OnuMV1c (3107 base pairs) and OnuMV7 (2804 base pairs) are labeled in red. B) 1 % agarose gel showing dsRNA extractions from *O. novo-ulmi* strains 93-1224, H327, and all four H327 transformants (HT1 – HT4). The gel was run in 1X TAE buffer at 86 volts for 1.5 hours with a 1 Kb Plus DNA Ladder. The gel was not run long enough for the OnuMV1c and OnuMV7 bands to separate, but they can be seen together in the 93-1224 lane (highlighted in red). There are no visible dsRNA virus bands in the untransformed H327 extraction or any of the H327 transformant extractions.

MV1cCyt cDNA. Therefore, PCR reactions using the Diag_MV1c primers were performed on the RNA extractions (total RNA and dsRNA) both before and after treatment with DNase I. The PCR products were run on agarose gels for analysis and results showed that prior to DNase I treatment the total RNA extractions were contaminated with recombinant MV1cCyt cDNA, but DNase I treatment effectively removed contamination (Figure 14).

3.7 Diagnostic One-Step Reverse Transcription PCR (RT-PCR)

H327 protoplasts that had stably integrated the pAN7-*gpdA*-MV1cCyt-*trpC* plasmid DNA were selected by their expression of the hygromycin phosphotransferase marker on OCM plates containing up to 400 ug/ml hygromycin. However, this process did not confirm whether the *gpdA* promoter in the construct was successfully driving the transcription of the MV1cCyt construct into mRNA. Therefore, the next step was to use reverse transcription PCR (RT-PCR) as a diagnostic tool to confirm that the H327 transformants were providing a primary transcript of MV1cCyt mRNA.

OneStep RT-PCR (Qiagen Inc.) using the Diag_MV1c primers (Table 3) was performed on the total RNA extractions from 93-1224 (virus-positive control), H327 (virus-negative control), and all four H327 transformants (HT1, HT2, HT3, and HT4). The Diag_MV1c primers were designed to amplify an 895 base pair region of the RdRp region of both OnuMV1c and MV1cCyt. Thus an amplicon should be present if either of those RNA species was present in a sample (Figure 15). The transformants (HT1 – HT4) were tested in triplicate: three RNA minipreps (MPs) of each were used. The RdRp amplicon was present as a strong band in 93-1224, HT1, HT3, and as a fainter band in

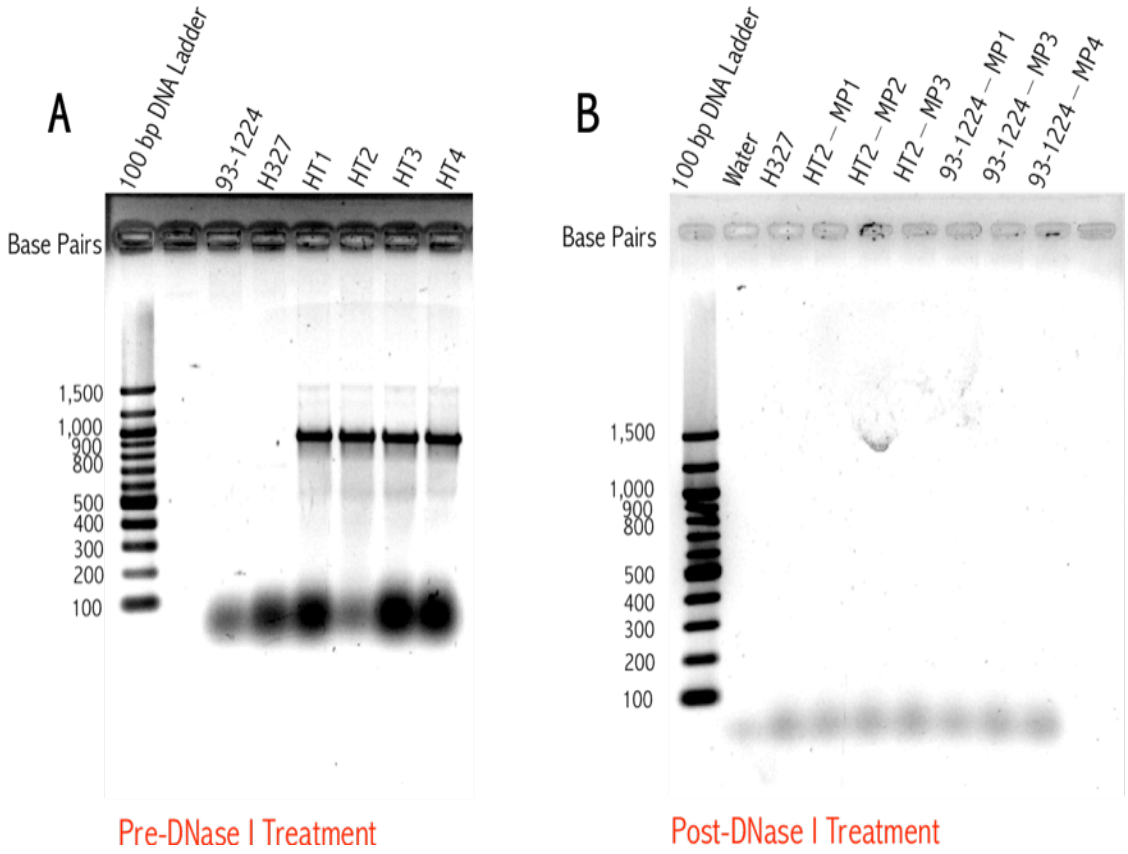


Figure 14. A) 1.5 % agarose gel showing the results of diagnostic PCR run with Diag_MV1c primers on RNA extractions of *O. novo-ulmi* strain 93-1224, untransformed H327, and H327 transformants (HT1 – HT4) *before* treatment with DNase I. The gel was run in 1X TAE buffer at 90 volts for 70 minutes with a 100 bp DNA Ladder (New England Biolabs Incorporated). The RNA for 93-1224 and H327 were dsRNA from phenol-chloroform extractions and the RNA from the H327 transformants were total RNA extractions from the RNeasy® Plant Mini Kit (Qiagen Inc.). The bands present in the H327 transformant lanes indicate that these extractions contained recombinant MV1cCyt cDNA. B) 1.5 % agarose gel showing the results of diagnostic PCR run with Diag_MV1c primers on RNA extractions of *O. novo-ulmi* strain 93-1224 (3 minipreps), untransformed H327 (one miniprep), and H327 Transformant 2 (3 minipreps) *after* treatment with DNase I. The gel was run in 1X TAE buffer at 86 volts for 90 minutes with a 100 bp DNA Ladder (New England Biolabs Incorporated). All samples were from total RNA extracted using the RNeasy® Plant Mini Kit (Qiagen Inc.). The lack of bands indicates that there was no genomic DNA contamination.

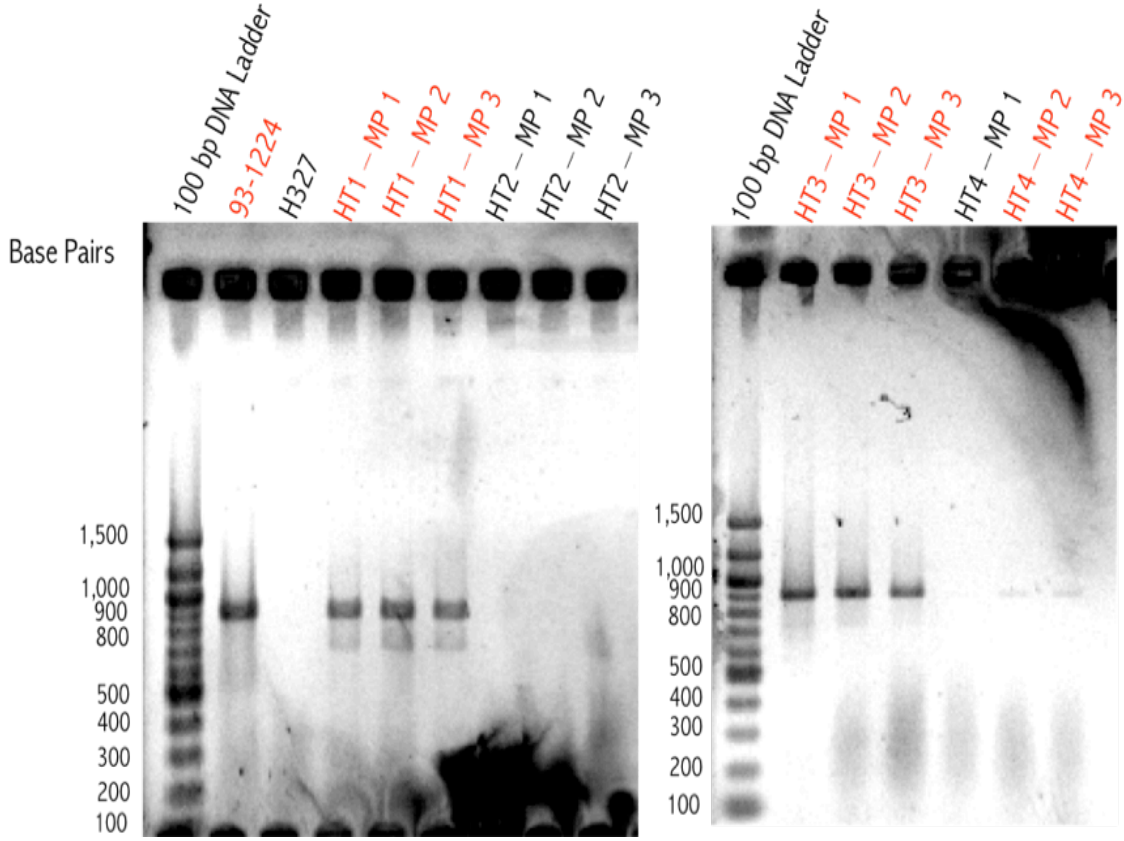


Figure 15. 1.5 % agarose gel showing results from OneStep RT-PCR (Qiagen Inc.) performed on DNase I-treated total RNA extractions from *O. novo-ulmi* strain 93-1224 (dsRNA virus-positive control), untransformed H327 (virus-negative control), and four H327 strains transformed with pAN7-*gpdA*-MV1cCyt-*trpC* DNA (HT1, HT2, HT3, and HT4 – three RNA minipreps of each). The gel was run in 1X TAE buffer at 106 volts for 90 minutes with a 100 bp DNA Ladder (New England Biolabs Incorporated). The RT-PCR was performed with the Diag_MV1c forward and reverse primers that amplify an 895 base pair sequence of the OnuMV1c and MV1cCyt RNA-dependent RNA polymerase coding region. The RdRp amplicon was present as a strong band in 93-1224, HT1, HT3, and as a weaker band in HT4. The untransformed H327 and HT2 transformant did not display the amplicon.

HT4, but was not present in the untransformed H327 sample or the HT2 transformant. These results confirmed the presence of at least MV1cCyt mRNA in the HT1, HT3, and HT4 transformants.

3.8 Detection of dsRNA using Strand-Specific Primers with Two-Step RT-PCR

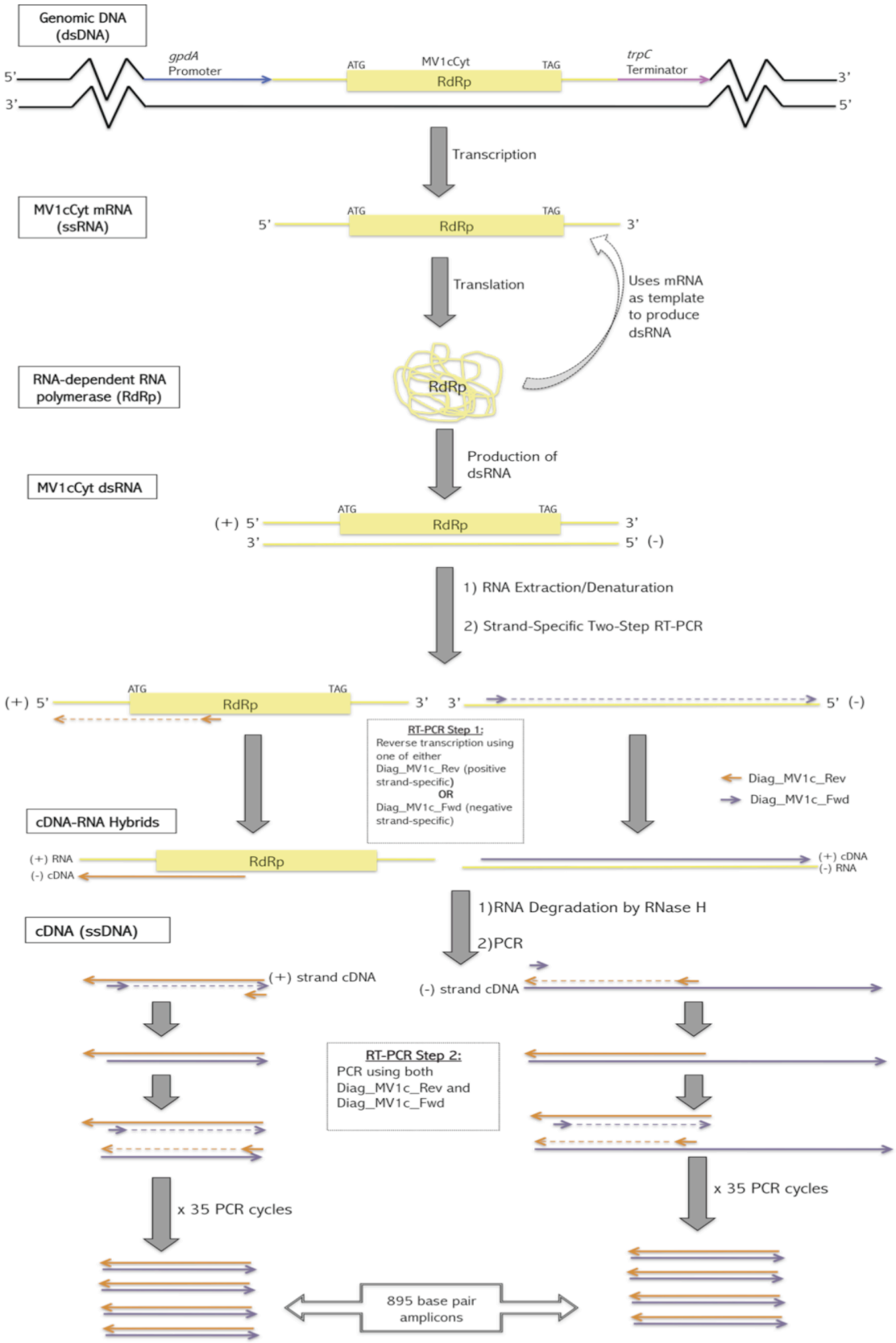
The OneStep RT-PCR was used to determine if the synthesized MV1cCyt construct was being successfully driven by the *gpdA* promoter and thus producing an mRNA transcript. In order to assess if the MV1cCyt mRNA transcript was then being translated into a functional RdRp that was producing a complementary RNA to the primary transcript (i.e. dsRNA), two-step RT-PCR using strand-specific primers was employed based on the protocol first described by Lin et al. (2002). Using only one of either the negative strand-specific primer (Diag_Fwd_MV1c) or positive strand-specific primer (Diag_Rev_MV1c) in the first reverse transcription step ensured that cDNA would only be produced if the respective strand was present. Therefore, it was expected that the subsequent PCR step using both primers should only produce an amplicon if the specific strand was present in the original RNA extraction. Both the total RNA and dsRNA extractions of 93-1224 (dsRNA virus-positive control), H327 (virus-negative control), and all four H327 transformants (HT1, HT2, HT3, and HT4) were tested. A schematic diagram showing the process of strand-specific RT-PCR is shown in Figure 16.

The results from the strand-specific RT-PCR performed on the total RNA extractions are shown in Figure 17. The RdRp 895 base pair PCR amplicon was produced

for both the positive and negative strand-specific primers in 93-1224, HT1, HT3, and HT4 total RNA extractions, suggesting that dsRNA corresponding to the inserted MV1cCyt DNA was present in the three transformants.

The results from the strand-specific RT-PCR performed on the dsRNA extractions showed a different pattern (Figure 18). The RdRp 895 base pair PCR amplicon was produced for both the positive and negative strand-specific primers in 93-1224 and HT1, but the amplicon only appeared for the negative strand-specific primer in HT3 and was not present for either strand in HT4.

Figure 16. Schematic diagram showing the process of strand-specific RT-PCR used in this study to detect double-stranded RNA from MV1cCyt. In an H327 transformant the genomic MV1cCyt cDNA was transcribed into a positive-sense (+) single-stranded messenger RNA transcript. This MV1cCyt mRNA was translated into the RNA-dependent RNA polymerase (RdRp) which then used the positive-sense mRNA as its template to make the negative-sense (-) RNA strand of MV1cCyt. The positive strand and negative strand RNAs formed a double-stranded MV1cCyt molecule. The dsRNA was extracted from the fungal cells and was denatured into single-stranded RNAs in preparation for strand-specific RT-PCR. Reverse transcription of the denature RNA was carried out as two separate reactions: each reaction contained one of either Diag_MV1c_Rev (positive-strand specific primer) or Diag_MV1c_Fwd (negative-strand specific primer). The concept was that the primers would only bind and produce cDNA if their respective RNA strand was present. After reverse transcription, the original RNA template was degraded by RNase H, and the cDNA was used in a PCR reaction containing both Diag_MV1c_Rev and Diag_MV1c_Fwd. The purpose of the PCR reaction with both primers was to amplify the signal so that it could be detected using gel electrophoresis. The presence of a signal from both reverse transcription reactions would indicate that the MV1cCyt RdRp protein was functional and producing double-stranded RNA.


Genomic DNA (dsDNA)
gpdA Promoter
ATG
MV1cCyt RdRp
TAG
trpC Terminator
5'
3'
3'
5'
Transcription
MV1cCyt mRNA (ssRNA)
ATG
RdRp
TAG
5'
3'
Translation
Uses mRNA as template to produce dsRNA
RNA-dependent RNA polymerase (RdRp)
RdRp
Production of dsRNA
MV1cCyt dsRNA
(+) 5'
ATG
RdRp
TAG
3'
3'
5' (-)
1) RNA Extraction/Denaturation
2) Strand-Specific Two-Step RT-PCR
(+) 5'
ATG
RdRp
TAG
3'
3'
5' (-)
RT-PCR Step 1:
Reverse transcription using one of either
Diag_MV1c_Rev (positive strand-specific)
OR
Diag_MV1c_Fwd (negative strand-specific)
Diag_MV1c_Rev
Diag_MV1c_Fwd
cDNA-RNA Hybrids
(+) RNA
(-) cDNA
RdRp
(+) cDNA
(-) RNA
1)RNA Degradation by RNase H
2)PCR
cDNA (ssDNA)
(+) strand cDNA
(-) strand cDNA
RT-PCR Step 2:
PCR using both Diag_MV1c_Rev and Diag_MV1c_Fwd
x 35 PCR cycles
x 35 PCR cycles
895 base pair amplicons

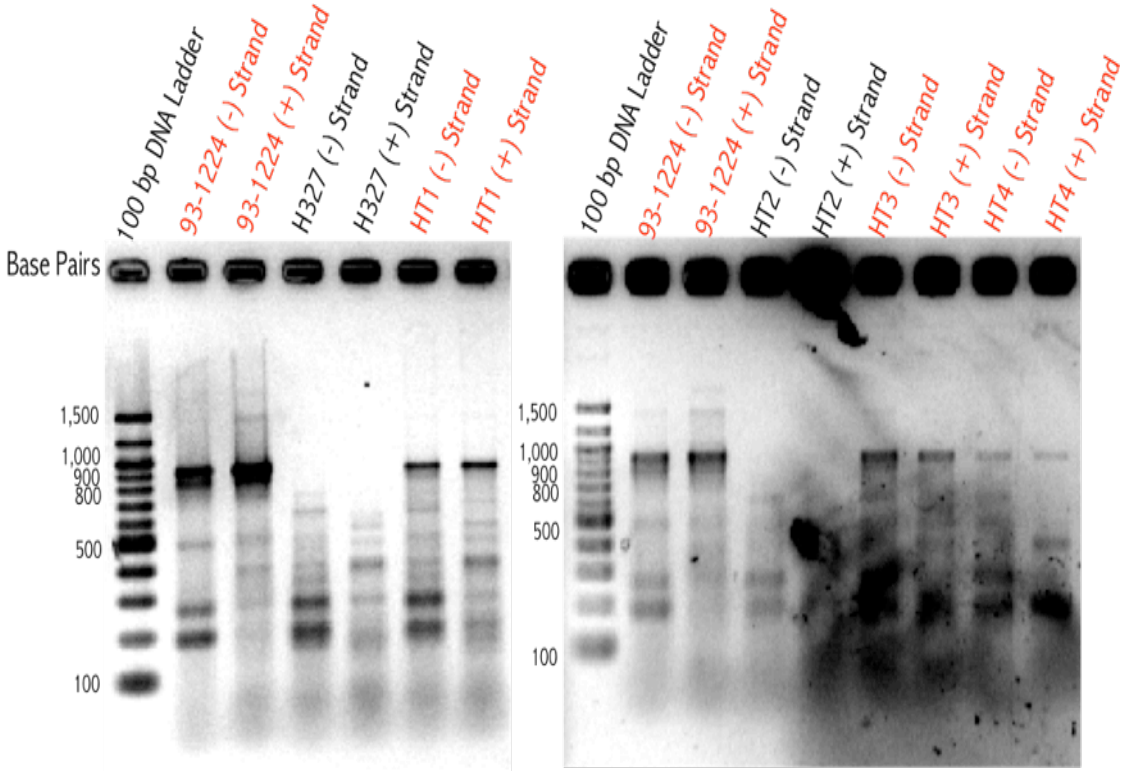


Figure 17. 1.5 % agarose gel showing the results from strand-specific two-step RT-PCR performed on DNase I-treated total RNA extractions from *O. novo-ulmi* strain 93-1224 (dsRNA virus-positive control), untransformed H327 (virus-negative control), and four H327 strains transformed with pAN7-*gpdA*-MV1cCyt-*trpC* DNA (HT1, HT2, HT3, and HT4). Gel was run in 1X TAE buffer at 106 volts for 70 minutes with a 100 bp DNA Ladder (New England Biolabs Incorporated). Each lane in the gel represents an RNA extraction run with only one of either a negative strand-specific primer (Diag_Fwd_MV1c) or a positive strand-specific primer (Diag_Rev_MV1c) in the reverse transcription phase of the RT-PCR (creation of cDNA).

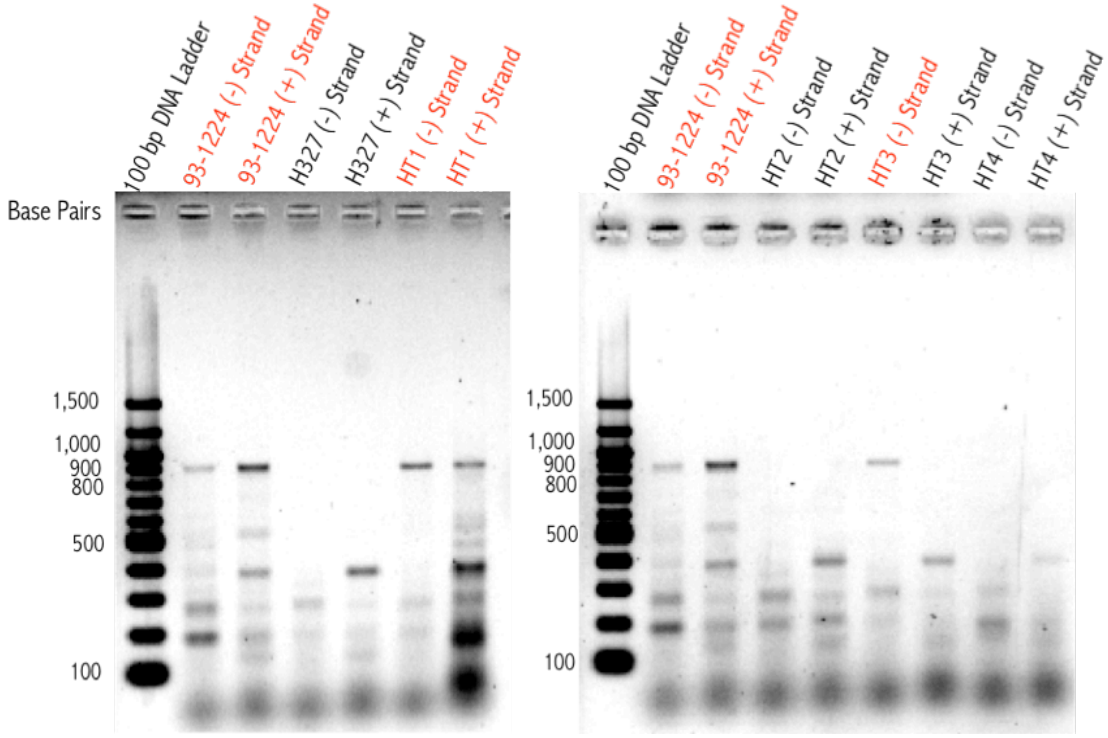


Figure 18. 1.5 % agarose gel showing the results from strand-specific two-step RT-PCR performed on DNase I-treated double-stranded RNA (dsRNA) extractions from *O. novo-ulmi* strain 93-1224 (dsRNA virus-positive control), untransformed H327 (virus-negative control), and four H327 strains transformed with pAN7-*gpdA*-MV1cCyt-*trpC* DNA (HT1, HT2, HT3, and HT4). Gel was run in 1X TAE buffer at 106 volts for 70 minutes with a 100 bp DNA Ladder (New England Biolabs Incorporated).

Chapter 4: Discussion

Mycoviruses have been researched extensively for their potential to be used as biological controls for fungal pathogens. A number of double-stranded RNA (dsRNA) mitoviruses were discovered in European populations of the Dutch elm disease (DED) pathogen *Ophiostoma novo-ulmi*, but to date none have been used for biological control due to the high diversity of vegetative compatibility types in the fungal populations in Europe. The discovery of two novel dsRNA mitoviruses (OnuMV1c and OnuMV7) in the relatively clonal Canadian populations of *O. novo-ulmi* led to the hypothesis that the virus OnuMV1c could be engineered to carry an RNA interference (RNAi) cassette that would down-regulate a gene involved in fungal pathogenicity. One approach for studying both single and double-stranded RNA mycoviruses is the construction of cDNA cassettes, which upon integration into the genomic DNA of the host are transcribed by the host machinery into functional viral RNAs.

The goals of the research described here were to 1) genetically engineer OnuMV1c, a mitochondrial virus, to express in the cytoplasm of *O. novo-ulmi* and, 2) assess whether this cytoplasmic version of OnuMV1c (MV1cCyt) was successfully replicating viral dsRNA. By changing the UGA codons (mitochondrial tryptophan codon, cytoplasmic stop codon) in the RNA-dependent RNA-polymerase (RdRp) sequence of OnuMV1c to UGG codons (mitochondrial and cytoplasmic tryptophan codon) the amino acid sequence of the RdRp was conserved. At the same time, any stop codons that would result in the translation of a truncated protein in the cytoplasm were removed. This cytoplasmic version of OnuMV1c (MV1cCyt) was constructed as a cDNA sequence flanked by a *gpdA* gene promoter driving expression and *trpC* transcription terminator

and was engineered into the pAN7-1 fungal transformation vector. Results from one-step reverse transcription PCR (RT-PCR) showed that H327 isolates transformed with MV1cCyt cDNA were producing MV1cCyt messenger RNA (mRNA) transcripts. Subsequent two-step strand-specific RT-PCR results indicated that the mRNA was translated into a functional RdRp protein that was able to produce MV1cCyt dsRNA in at least one of the H327 transformants, HT1. These results have established a proof of concept for the movement of a functioning mitochondrial virus to the cytoplasm and suggest that OnuMV1c can be employed as a cytoplasmic virus potentially capable of carrying an RNAi gene cassette that will disrupt the pathogenicity of *O. novo-ulmi*.

4.1 Engineered Hypovirulence

This is not the first study that has explored genetic engineering of a mycovirus for use as a biological control. The model system for engineered hypovirulence is the chestnut blight fungus *Cryphonectria parasitica*. Though the pathogenicity of *C. parasitica* is attenuated by naturally hypovirulent dsRNA mycoviruses, there has still been extensive research into developing cDNA-based reverse genetics systems for these viruses. Hypoviruses are normally transmitted through either hyphal anastomosis or asexual conidia. However, vegetative compatibility types limit transmission through hyphal anastomosis, and transmission through conidia is extremely inconsistent with anywhere from 10 to 90 % of conidia losing the hypovirus from the parent. In addition, naturally occurring hypoviruses are generally not transmitted through sexual reproduction to the ascospores. Consequently, the appeal of a cDNA-based hypovirulence system is

that it integrates a copy of the hypovirus into the host genome, ensuring that hypovirulence is passed to both conidia and ascospore progeny (Nuss et al., 2002). Choi and Nuss (1992) engineered a full-length cDNA copy of the *C. parasitica* hypovirus CHV1-EP712 and fused it to a *C. parasitica* gene promoter and transcription terminator. Using the fungal transformation vector pXH9 (with hygromycin resistance) they integrated the viral cDNA into the genome of virus-free *C. parasitica* strains and found that the symptoms of the transformed strains were indistinguishable from naturally infected strains. Subsequently, a study performed by Chen et al. (1994) showed that the cDNA-infected strains were not only producing mRNA transcripts and trimming them of non-viral vector nucleotides, but were also successfully replicating viral dsRNA. These findings lend support to the results found here that a cDNA-based reverse genetics system can successfully produce viral dsRNA in the cytoplasm of fungal transformants.

Even within the *Narnaviridae* family, reverse genetics systems have been developed. In the yeast *Saccharomyces cerevisiae* there are two cytoplasmic positive-strand RNA viruses in the genus *Narnavirus*. Like OnuMV1c, these viruses are unencapsidated and encode only a single RdRp protein (p104) involved in viral replication of dsRNA. They are transmitted both vertically and horizontally through yeast populations and have no obvious effects on host fitness (Esteban and Fujimura, 2003). In their 2003 study, Esteban and Fujimura developed a reverse genetics system in order to study the replication process of one of the yeast viruses, ScNV-23S. They engineered a cDNA copy of the virus sequence into the yeast expression vector pRE637. They modified the p104 RdRp protein sequence of ScNV-23S with two silent mutations that did not change the amino acid sequence of the protein, but acted as tags for the cDNA

virus to distinguish it from the natural virus. They then transformed virus-free yeast strains with the cDNA vector and monitored replication of the virus using strand-specific probes in Northern blotting. The results showed that the cDNA copy of the virus was able to produce positive strand mRNA transcripts that were translated into the p104 RNA polymerase. The p104 polymerase then acted back on the positive strand mRNA to produce the negative strand of the virus and ultimately produced dsRNA. In addition, the cDNA-derived dsRNA virus was able to autonomously replicate in the transformant cells, and this replication was maintained over at least 100 generations (Esteban and Fujimura, 2003). These findings from ScNV-23S firmly support the current study with OnuMV1c, as there are direct similarities between the two studies. The modification of the p104 RdRp with silent mutations and the subsequent ability of the protein to synthesize dsRNA indicates that codon modifications can be tolerated by these viral proteins. However, the modification of a mitochondrial protein to express in the cytoplasm remains unprecedented.

4.2 Strand-Specific RT-PCR as a Means of Detecting dsRNA

In order to assess if the MV1cCyt mRNA transcript was being translated into a functional RdRp that was producing dsRNA, two-step RT-PCR using strand-specific primers was employed based on the protocol first described by Lin et al. (2002). This method involves splitting the RT-PCR process into two phases. The first phase, reverse transcription, is the creation of cDNA from a dsRNA template using only one of either a negative strand-specific primer (forward primer) or a positive strand-specific primer (reverse primer). The use of only one primer in this phase ensures that cDNA will only be

created if the strand that the primer is specific to is present. The second stage, PCR, involves the amplification of the cDNA created in the first step using both the forward and reverse primers in the reaction. There are numerous studies that have used this method to detect dsRNA in a variety of contexts. The original study by Lin et al. (2002) used strand-specific primers in RT-PCR to detect the negative-strand RNA of hepatitis C virus (HCV) from total RNA extractions of HCV patients' livers. Total RNA was denatured and converted to first-strand cDNA by reverse transcription using only a negative-strand-specific primer. The cDNA created from this single-primer reverse transcription was then amplified using PCR with both the positive strand and negative strand-specific primers. Their results showed that this strand-specific RT-PCR method was successful in detecting the negative strand HCV RNA (Lin et al., 2002). A study done by McBeath et al. (2013) also used strand-specific RT-PCR to distinguish between 3 different species of infectious salmon anaemia virus (ISAV): negative-sense viral RNA, positive-sense complementary RNA, and positive-sense messenger RNA. Similarly, Purcell et al. (2006) distinguished between negative-sense genomic viral RNA and positive-sense mRNA in a fish rhabdovirus using strand-specific primers in quantitative RT-PCR.

In the current study, strand-specific RT-PCR was performed on both total RNA extractions and dsRNA extractions but yielded different results for each. The total RNA extractions from transformants HT1, HT3, and HT4 all gave a signal for both strands of MV1cCyt. However, when the dsRNA extractions were tested only HT1 gave a signal for both strands, and HT3 gave a signal for only the negative strand of MV1cCyt. In their study Lin et al. (2002) did find that a false negative-strand signal was obtained from

positive-strand HCV RNA when it was present in amounts greater than 10^{10} copies/μg of tissue tested. The authors attributed this phenomenon to non-specific priming occurring at high concentrations of positive-strand RNA. This could explain why in our study a signal was obtained in transformants HT3 and HT4 from the total RNA extractions and was then absent when the RT-PCR was repeated on the dsRNA extractions. If positive-strand MV1cCyt RNA was present in large concentrations in the total RNA extractions, it is possible that it gave a false signal from the negative strand-specific primer.

The presence of only a negative-strand signal in the HT3 dsRNA extraction was another unexpected result. The signal could mean that the dsRNA extraction contained some residual single-stranded RNA. The phenol-chloroform dsRNA extraction method used in this study relies on the adsorption of dsRNA molecules to cellulose at an ethanol concentration of 15 %. At this concentration of ethanol, only dsRNA should be bound to the cellulose; single-stranded RNA (ssRNA) is favored to bind cellulose at ethanol concentrations above 20 % (Morris and Dodds, 1979). However, in their original study Morris and Dodds (1979) found that ssRNA was not effectively eliminated from the RNA extractions until the ethanol concentration was lowered to 10 %. Therefore, it is possible that at 15 % ethanol single-stranded negative sense MV1cCyt RNA was present in HT3 extraction.

Another possible reason why transformant HT3 gave a signal for only the negative strand MV1cCyt RNA lies in antisense promoters and transcripts. The existence of antisense transcripts was discovered in bacteria in the 1990s, and has since been discovered in many eukaryotes including fungi. Antisense transcripts, or non-coding RNAs (ncRNAs), can arise from independent antisense promoters, bidirectional

promoters, or cryptic promoters. Though ubiquitous, antisense transcripts are present in organisms in amounts 10 times lower than that of sense transcripts (Pelechano and Steinmetz, 2013). Thus, even though it occurs rarely, it is possible that pAN7-1 could integrate into the *O. novo-ulmi* genome next to an antisense promoter and be processed as an antisense transcript. This could be one possible explanation for the apparent presence of only negative-strand MV1cCyt RNA in transformant HT3. However, the likelihood that in this transformant the pAN7-1 vector only integrated once into the genome and only behind an antisense promoter is very low.

As with most transformation plasmids, pAN7-1 can integrate into the host genome anywhere from one time at one locus to multiple times at various loci. Generally, the transformants with multiple copies of pAN7-1 exhibit higher hygromycin resistance. The site of integration can affect hygromycin resistance as well; certain locations in the genome are more amenable to higher expression of pAN7-1 (Punt et al., 1987). These factors would also have an effect on how MV1cCyt mRNA was expressed in the *O. novo-ulmi* transformants of the current study. In the original study that developed the pAN7-1 transformation plasmid, Punt et al. (1987) used Southern blot analyses to determine the DNA copy number in each of their pAN7-1 transformants. In the current study, no such quantitative test was used for the transformants so a conclusion cannot be made in regards to the relative expression of MV1cCyt between the transformants. However, it is possible that transformant HT1 had multiple copies of the pAN7-*gpdA*-MV1cCyt-*trpC* plasmid and was therefore expressing higher levels of MV1cCyt mRNA transcripts. This would translate into higher concentrations of RdRp protein. One of the many changes from the mitochondrion to the cytoplasm is the significant increase in area.

The RdRp normally finds its single-stranded RNA target in the small space of the mitochondrion; searching for the same target in the much larger area of the cytoplasm could prove more difficult. Consequently, a higher concentration of RdRp in the cytoplasm would result in an increased chance of the RdRp finding its MV1cCyt mRNA target. Therefore, if pAN7-*gpdA*-MV1cCyt-*trpC* did in fact integrate at multiple locations in the genome of transformant HT1, it could have resulted in an increased chance of forming MV1cCyt dsRNA.

4.3 Codon Usages and Bias: Mitochondria vs. Cytoplasm

Amongst all living organisms there are 64 codons that are responsible for the translation of the 20 essential amino acids used in the synthesis of proteins. Each of the 64 codons is assigned the same amino acid in a universal genetic code. There are some exceptions to this universal code, one of which is the translation of mitochondrial genes. Mitochondria are unique organelles in that they have their own genetic system distinct from nuclear genome. The presence of this distinct genome is due to the evolutionary origin of mitochondria as endosymbiotic bacteria. Mitochondrial genomes are usually circular DNA and are much smaller than their nuclear counterparts. They generally encode a small subset of genes that vary little amongst organisms. Fungal mitochondrial genomes tend to encode for mitochondrial rRNAs and tRNAs, as well as proteins involved in the respiratory chain (Sethuraman et al., 2009). In general however, the majority of proteins required by mitochondria are encoded by the nuclear genome and are imported from the cytoplasm (Dudek et al., 2013). One aspect of the distinct mitochondrial genetic system is the use of alternate codons in mitochondrial protein

translation. This difference in codon usage between the mitochondria and cytoplasm can vary depending on the organism; however there are certain conserved features that have remained fixed for a long period of time (Cooper, 2000). This has effectively created a barrier for the translation of mitochondrial encoded genes in the cytoplasm and has served to fix the subset of genes, which are encoded by and expressed exclusively in the mitochondrion. There are, inevitably, some exceptions to this rule.

In *Ophiostoma novo-ulmi* the codon UGA codes for the amino acid tryptophan in the mitochondria, but is a stop codon in the cytoplasm. Consequently, if messenger RNA from a mitochondrion were to be translated in the cytoplasm of *O. novo-ulmi* it would result in a truncated protein because every UGA codon encountered would stop translation. This codon discrepancy is why the engineering of MV1cCyt involved modifying all UGA codons in the RdRp protein sequence of OnuMV1c to UGG codons. The codon UGG also encodes tryptophan in the mitochondria of *O. novo-ulmi*, therefore the amino acid sequence of the codon-modified RdRp would remain the same whether it was translated in the cytoplasm or the mitochondrion. Though UGA is the only codon discrepancy in *O. novo-ulmi*, codon usage bias between the mitochondria and cytoplasm are different. Codon bias is the frequency at which synonymous codons are used in the translation of genes both within and among species (Behura and Severson, 2013). For example, in *O. novo-ulmi* the codons UUU and UUC both encode phenylalanine, but the frequencies with which these codons are utilized differs between the cytoplasm and mitochondria. This type of codon preference is usually related to the general availability of amino-acyl tRNAs for each codon and a low-availability may be rate-limiting for optimal gene expression. In the cytoplasm the frequencies of UUU and UUC are 0.22 and

0.78, respectively, but in the mitochondrion the frequencies are 0.96 and 0.04 (Nakamura, 2007). In the current study, optimization of codon usage in the expression of the RdRp of OnuMV1c was trumped by the desire to maintain the sequence of the modified mitovirus sequence as close to the original as possible. Only the most conservative changes were included. Though all the modified codons of MV1cCyt accounted for codon bias, the rest of the viral sequence was not adjusted. This could have lead to issues in translation of the RdRp in *O. novo-ulmi* H327 transformants. However, the putative production of MV1cCyt dsRNA in transformant HT1 indicates that differences in codon bias did not affect the translation of the RdRp protein.

4.4 Mitochondrial vs. Cytoplasmic Proteins

The challenges presented by modifying a mitochondrial virus to express in the cytoplasm are many-fold. There are no published studies where a similar experiment has been attempted, making this research unique and exciting. Engineered hypovirulence using mycoviruses has to date only been applied to cytoplasmic viruses (Nuss et al., 2002; Milgroom and Hillman, 2011).

There are proteins that reportedly function in both the cytoplasm and mitochondria. Bandlow et al. (1988) discovered a yeast adenylate kinase, AKY2, which was active in both mitochondria and the cytoplasm. Their results indicated that a single nuclear gene encoded the two forms of AKY2 and that minimal processing of the protein occurred before its transport to the mitochondria. At the time of the study, only three other enzymes had been found to be active in both the cytoplasm and mitochondria, but all three required extensive processing before import to the mitochondria. The ability of

AKY2 to be active in both the mitochondria and cytoplasm in essentially the same form indicates that it is possible for some proteins to function in the two drastically different cellular environments.

Another example of the ease of exchange of genetic information between the mitochondrial and nuclear genomes is the *rps3* gene in filamentous ascomycetes. The *rps3* gene encodes the mitochondrial ribosome protein S3 and within fungi the location and organization of this gene is highly variable. In some fungal species the gene is encoded by the mitochondrial genome, but in many other species *rps3* has been transferred to the nuclear genome (Sethuraman et al., 2009). In addition to the variation in genome location, the *rps3* gene varies greatly in its organization with some versions inserted into introns, and other versions having gained large gene insertions or homing endonucleases. However, despite all this variability *rps3* still encodes the same ribosomal protein (Sethuraman et al., 2009).

These examples lend support to the idea that the RdRp of OnuMV1c could be genetically modified to survive and be functionally relevant in a new cellular environment. There has historically been a significant amount of genetic exchange between mitochondrial and nuclear genomes, with the expression of proteins changing throughout evolution. The boundaries between the mitochondrial and nuclear genomes may not be entirely fixed and there may still be opportunity for the exchange of genetic information between these two systems. Codon usage does present one such barrier but this can be circumvented by selective engineering. It could be that the codon modifications performed on OnuMV1c in this study could be viewed as a form of assisted evolution, allowing the viral RdRp to be translated normally and function in the

cytoplasm. Alternately, it may be that the apparent barrier between the nuclear and mitochondrial genomes can be overcome more easily than was once thought.

4.5 Transport of Nuclear mRNA to Mitochondria

Recent research in human sperm cells has led to the discovery that nuclear encoded mRNA can be translated by mitochondrial ribosomes (Gur and Breitbart, 2006; 2008). From fairly recent studies it appears that mRNA can be imported into the mitochondria for translation, and that the synthesized proteins are then sometimes exported back to the cytoplasm where they function (Gur and Breitbart, 2008). These authors suggest a model whereby a nuclear-encoded mRNA molecule can be translated in both the cytoplasm and the mitochondria to create the same protein product. The exact mechanisms by which the mRNA is imported to the mitochondria and the protein product is exported to the cytoplasm have not yet been elucidated (Gur and Breitbart, 2008).

At some point in the evolution of mitochondria, the genes encoding proteins involved in replication, transcription, and translation of the mitochondrial genome were acquired by the nuclear genome. Gur and Breitbart (2008) suggest that the ability of mitochondrial ribosomes to translate nuclear mRNA is due to this evolutionary history between the mitochondrial and nuclear genomes and that this phenomenon may occur in all living cells. They further indicate that there is likely a great deal of communication between the cytoplasm and mitochondria, with more sharing of genetic materials than was previously thought (Gur and Breitbart, 2008). These studies indicate the close relationship between the mitochondrial and nuclear genomes and the genes that they encode. Clearly, transfer of genes from the mitochondria to the nucleus has occurred

multiple times in the evolution of cells leaving a small subset of essential gene subunits remaining in mitochondrial genomes. This occurred very early in the evolution of all modern lineages and has remained relatively fixed since. Therefore, change in expression of the mitochondrial protein of OnuMV1c to the cytoplasm described in this study may not be such a dramatic leap. The study done by Gur and Breitbart (2008) is additionally significant because it showed that nuclear-encoded mRNA could be imported to mitochondria to be translated. This opens the possibility that in *O. novo-ulmi* transformant HT1 the MV1cCyt mRNA could be transported to the mitochondria where the RdRp would be translated. The UGG codon encodes tryptophan in both the mitochondria and cytoplasm, so translation of the modified RdRp sequence would not be affected in the mitochondria. After translation the MV1cCyt RdRp could be exported to the cytoplasm to form dsRNA or it could remain in the mitochondria to form dsRNA. The dsRNA extractions performed in this study were from the entire cellular contents of *O. novo-ulmi*, so it is not possible to determine the location of the MV1cCyt dsRNA product.

4.6 Potential Gene Targets for RNAi Biological Control

Development of MV1cCyt as a biological control using RNA interference (RNAi) requires an *O. novo-ulmi* gene target. For preliminary experiments testing the ability of MV1cCyt to carry an RNAi cassette it would be useful to use the *epg*1 RNAi cassette developed by Carneiro et al (2010). Because this gene cassette is proven to activate the RNAi pathway in *O. novo-ulmi*, using it with MV1cCyt would provide a definitive test of the virus' ability to act as an RNAi activator. In addition, endopolygalacturonase is an

easy enzyme to assay, providing a good indication of the functionality of the MV1cCyt-RNAi system.

If RNAi could be established using MV1cCyt with an *epg*1 cassette, then future work should focus on finding a gene in *O. novo-ulmi* that when targeted, reduces the pathogenicity of the fungus. As the ultimate goal is transmissible hypovirulence, it is important to choose a gene that is not imperative to survival of the fungus: killing the host before it can pass on the hypovirus would make it impossible for the virus to spread through the disease front. A study done by Anagnostakis et al. (1998) released a transgenic *C. parasitica* strain containing hypovirus cDNA into a forest site in the United States and monitored the spread of the transgenic strain over time. Their results showed that the cDNA-derived hypovirus RNA was able to spread through the *C. parasitica* population through both cytoplasmic mixing (hyphal anastomosis) and transmission to ascospore progeny. However, the transgenic hypovirulent strain was unable to persist in the population beyond two years (Anagnostakis et al., 1998) indicating that the hypovirus reduced the ecological fitness of its fungal host to such an extent that the virus could not find another naïve host in time for efficient propagation. This experiment highlighted the need to balance the level of virulence attenuation of the fungal host with its ecological fitness when designing a hypovirus-based biological control (Nuss et al., 2002).

In order to achieve this balance with the proposed RNAi approach using MV1cCyt, it will be necessary to find a gene in *O. novo-ulmi* that is involved in its pathogenicity, but will not be fatal to the fungus if its expression is reduced. A recent study by Comeau et al (2015) annotated the entire *O. novo-ulmi* strain H327 genome with the goal of elucidating the genes involved in phytopathogenicity. They found a number of

different gene groups potentially involved in H327 virulence. The first group was a series of peptidases that the authors suggested are involved in the destruction of antifungal peptides released by elms as a defence response. The second group of genes was those encoding for plant-cell-wall-degrading enzymes (CAZymes) involved in elm-host invasion. The third gene group included a number of different cytochrome P450 (CYP450) enzymes involved in fungal toxin production: Comeau et al. (2015) suggested the fungus might use these toxins to attack its elm host. One example of a CYP450, CYP52P6, was of particular interest to the authors because it seemed to be involved in modifying elm terpene defences. Terpenes are volatile organic compounds (VOCs) that plants use for a wide variety of functions including chemical defence against herbivores, pests, and pathogens. Recent studies have shown the involvement of certain terpenes in plant resistance to fungal infection (Rodríguez et al., 2014). CYP52P6 was also implicated in the pathway that produces verbanol, an aggregation pheromone for bark beetles that is secreted by many beetle-associated fungal phytopathogens, including *Ophiostoma* species, which increases the efficiency of spore dissemination. Of all the phytopathogenicity genes uncovered by Comeau et al. (2015) CYP52P6 seems like the most promising potential gene target for RNAi. The severity of DED as a pathogen lies in its ability to overcome elm defences and spread throughout the tree, eventually killing it. Interfering with the ability of *O. novo-ulmi* to modify elm defences would allow the tree to more effectively combat the fungus before it is able to spread too rapidly. This approach would be ideal because it would not directly kill the fungus in itself, but instead would allow the elm host to better defend itself. The reproduction of *O. novo-ulmi* would not be affected thus allowing the CYP52P6 RNAi cassette to disseminate through the

disease front. However, further studies exploring the effects of reduced CYP52P6 expression on *O. novo-ulmi* pathogenicity would have to be conducted first.

Another group of genes that could serve as potential targets for RNAi are those involved in the transition between the yeast and mycelial growth forms of *O. novo-ulmi*. As previously discussed, *O. novo-ulmi* exhibits a yeast-mycelial dimorphism. The mycelial phase is the invasive form, penetrating into the xylem tissues of the elm and growing radially, while the yeast phase is responsible for the dispersal of the fungus throughout the tree by traveling through the vascular system, and ultimately to other trees via root grafts (Berrocal *et al.*, 2012; D'Arcy, 2012). It is possible that this transition from mycelia to yeast is imperative for propagation and virulence of DED in populations of elms. Though this dimorphism is well documented, it has not yet been studied at the molecular level so the specific genes involved have not been elucidated (Berrocal *et al.*, 2012).

4.7 Experimental Limitations and Future Directions

The relationships between fungal viruses and their hosts are complex, and this complexity needs to be considered when altering the relationship. We have demonstrated that it is possible to move the primary transcription of a mitovirus to the nuclear genome and that functional RdRp can transcribe the second strand of the dsRNA. Whether this second stage occurs in the cytoplasm or in the mitochondrion is not yet known. Though the results indicate that the expression of OnuMV1c has been successfully changed from the mitochondria to the cytoplasm, there are other factors that may be involved.

The results of Gur and Breitbart (2008) showed that nuclear-encoded mRNA could be imported to the mitochondria for translation. Though their research was on sperm cells Gur and Breitbart did suggest that this process could occur in other organisms. Therefore, it could be possible that in *O. novo-ulmi* transformant HT1 the MV1cCyt mRNA was imported to the mitochondria for translation and stayed there to produce dsRNA. Future experiments should use sub cellular fractionation prior to RNA extraction in order to determine where in the cell the MV1cCyt dsRNA is residing.

Though strand-specific RT-PCR is commonly used for the detection of dsRNA, there are limitations to this method. The results from the study performed by Lin et al. (2002) on Hepatitis C Virus showed that in the presence of high concentrations of positive strand virus, non-specific priming could create false-positive signals for the negative virus strand. Though the use of dsRNA-specific extraction method in the current study should rule out the presence of a false signal from single-stranded RNA, it would be useful to corroborate the findings with another analysis. One option for further analysis is to design nested primers to be used in the second phase of strand-specific RT-PCR. In the first phase of reverse transcription the DiagMV1c primers would still be used in a strand specific fashion. However, in the second phase of PCR a second set of primers nested within the DiagMV1c primers would be used to test for the presence of a smaller MV1cCyt amplicon. Another option for further analysis of MV1cCyt dsRNA is Northern hybridization, which employs strand-specific probes to detect RNA. This method was used for OnuMV1c to originally detect its dsRNA form (Hintz et al., 2013). The probes used in Northern hybridization are more specific than those used in RT-PCR as they are derived from a larger number of nucleotides.

Another issue that was not addressed in this study is that of transformant stability. If MV1cCyt cDNA is not stable in the genomes of the transformants then it will not serve as an effective biological control. It needs to persist in *O. novo-ulmi* populations in order to spread effectively. Future work should monitor the stability of HT1 to ensure that it continues to produce double-stranded MV1cCyt RNA. The addition of an RNA interference gene cassette to the MV1cCyt cDNA system may help with long-term stability. The pAN7-1 fungal transformation vector allows ectopic expression of MV1cCyt as well as any gene insert it carries. In addition, transformation efficiency and mitotic/meiotic stability increase across generations when pAN7-1 contains a homologous sequence from the host (Ball et al., 1991). A study done on *Trichoderma* species showed that isolates transformed with pAN7-1 containing no homologous sequences to the host were unable to maintain hygromycin resistance beyond a few generations. However, isolates transformed with pAN7-1 containing a 2.4 kilobase host gene fragment exhibited phenotypic and mitotic stability over multiple generations (Herrera-Estrella et al., 1990). Therefore, the insertion of an RNA interference cassette containing an *O. novo-ulmi* gene sequence into MV1cCyt in future experiments may improve the stability of transformants, thus increasing the chances that the whole vector-construct system will be passed both vertically and horizontally to subsequent generations.

In addition to transformant stability, it is also important to determine whether the MV1cCyt dsRNA is both self-replicating and transmissible. In their study in which a modified cDNA version of the yeast virus ScNV-23S was engineered, Esteban and Fujimura (2003) found that the cDNA-derived dsRNA could replicate autonomously

without the plasmid DNA for more than 100 generations. It would be informative to test the same process in MV1cCyt transformants because autonomous replication in the cytoplasm of *O. novo-ulmi* would increase the chances of horizontal transmission. Currently, the development of MV1cCyt as a biological control relies solely on vertical transmission because it depends on the RNA interference pathway of *O. novo-ulmi* to degrade MV1cCyt dsRNA. If the dsRNA is able to self-replicate and produce multiple copies, it could exist in large quantities in the cytoplasm eventually being transferred via hyphal anastomosis to other individuals. To be an effective biocontrol an engineered version of the MV1cCyt dsRNA would need to self-propagate from an initial fungal donor to the extant populations of resident mycelia in infected elm trees.

Finally, quantitative strand-specific RT-PCR could be performed to determine the relative amounts of single-stranded and double-stranded MV1cCyt mRNA in the transformants. This analysis would determine the ratio of positive-strand RNA to negative-strand RNA. The resultant information would reveal how the modified virus exists in the cell and from there comparisons could be made to how OnuMV1c normally behaves.

4.8 MV1cCyt as a Functional Biological Control

In order for MV1cCyt to be considered as a viable biological control, it needs to be highly specific to its host. Studies have shown that fungal viruses can be transmitted between species (Milgroom and Hillman, 2011), and these events, albeit rare, present a huge risk for a virus acting as a biological control. A study done by Deng et al. (2003) concluded that interspecies' transmission of a mitovirus must have occurred between

Sclerotinia homoeocarpa and *O. novo-ulmi* because the nucleotide and amino acid sequences of the two viruses studied were significantly more related than their respective fungal hosts. Therefore, extensive pairing experiments need to be performed with MV1cCyt to determine its ease of transmission. Ideally, it would transmit easily to other *O. novo-ulmi* strains of both subspecies, but would not transmit to other *Ophiostoma* species. As an added layer of protection, the gene target used for RNAi should be specific to the DED pathogen. That way, even if MV1cCyt were horizontally transmitted to another *Ophiostoma* species, it would not have detrimental effects. An effective biological control is one that does not have unintended effects on other organisms in the ecosystem. Fortunately vegetative incompatibility is a major barrier to horizontal transmission of mycoviruses in fungi. As the goal in designing MV1cCyt is for use as a biological control at the DED disease front in Canada, where there is low genetic diversity, it is likely that horizontal transmission of the virus would be high between the strains causing disease (low vegetative compatibility diversity) but low between other closely related fungal species (high vegetative compatibility diversity). However, future research with OnuMV1c would need to further explore its natural occurrence and the extent of its horizontal transmission both within *O. novo-ulmi* strains and between *O. novo-ulmi* and other *Ophiostoma* species. This knowledge will be required for the safe deployment of OnuMV1c as a biological control.

4.9 Conclusion

The results given here lay the groundwork for the development of the dsRNA mitovirus OnuMV1c as a biological control agent for Dutch elm disease. A cytoplasmic

version of the virus, MV1cCyt, was successfully engineered through modifications to mitochondrial tryptophan codons and transformed into the OnuMV1c-naïve *Ophiostoma novo-ulmi* strain H327. Results from strand-specific reverse transcription PCR indicated that at least one of the transformants contained positive-sense MV1cCyt mRNA transcripts that were being translated into a functional RdRp protein producing dsRNA. However, these results are by no means comprehensive, and further research is required to corroborate these findings.

Though the development of OnuMV1c as a biological control pertains to *Ophiostoma novo-ulmi* specifically, the theory and science behind the project can potentially be applied to other fungal species. Ophiostomatoid fungi cause a number of diseases in different plant species as well as in humans (Wingfield et al., 1993). In fact, the Fungi as a whole group are increasingly responsible for emerging infectious diseases affecting the health of animals, plants and whole ecosystems (Fisher et al., 2012). These issues are inextricably linked with human populations as human health relies on the well being of the environment. In addition, there are a growing number of fungal pathogens directly interacting with humans, and in some cases causing life-threatening mycoses (Pfaller and Diekema, 2004). It is understandable that with this increasing threat from fungal pathogens there is great interest in developing highly effective, pathogen-specific control strategies. The ubiquitous nature of mycoviruses presents a unique opportunity to enhance the natural phenomenon of hypovirulence. The development of a highly specific biological control strategy using OnuMV1c will not only potentially help mitigate one of the most devastating plant diseases seen to date, but will also contribute to a larger body

of science working towards control of the many other fungal pathogens wreaking havoc in global ecosystems.

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