Molecular aspects of virulence in the causal agent of Dutch elm disease, *Ophiostoma novo-ulmi*

by

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ABSTRACT

Dutch elm disease has been one of the most economically destructive plant diseases of the last 100 years. The American elm (*Ulmus americana)* has great value in the urban landscape but has been decimated by *Ophiostoma ulmi* (*sensu lato*) across North America since the early 1920's with mortality in some areas as high as 95%. The highly aggressive elm pathogen *Ophiostoma novo-ulmi* Brasier has potential as a model system for other destructive *Ophiostoma* species and other fungal plant pathogens. Thus, deciphering the genetic basis for virulence in this fungus is an important step in determining the best methods of controlling Dutch elm disease and other destructive plant diseases. This work describes the cloning and characterization of the gene encoding the cell wall-degrading enzyme, polygalacturonase, and characterization of glycosylation mutants of *O. novo*-ulmi. The genetic variation, vegetative compatibility and dsRNA virus infection in the *O. novo-ulmi* population in Winnipeg, Manitoba, Canada is also detailed.

Cell wall-degrading enzymes (CWDEs), and polygalacturonase in particular, have important roles in fungal virulence and host/pathogen recognition and initiation of host defense responses. Precisely how polygalacturonases act in the Dutch elm

disease system is unknown. The work presented in this thesis showed that disruption of the polygalacturonase gene in *O. novo-ulmi* reduced the expression of wilt symptoms in host trees and likely had a role in contributing to the overall aggressiveness and parasitic fitness of the fungus.

Glycosylation enzymes are likely to affect virulence and host/pathogen interactions in the pathology of Dutch elm disease. Callus tissue of clonal *Ulmus americana* was used to determine if *O. novo-ulmi*, treated by random insertional mutagenesis followed by enrichment for glycosylation mutants, would show variation in interaction with callus tissue relative to the wild type. The activity of the secreted pectinolytic enzyme polygalacturonase was assayed in the mutants to determine whether interference with glycosylation machinery would have a deleterious effect on the secretion or activity of glycoproteins produced by this pathogen. Several of the putative glycosylation mutants demonstrated a lack of pectinolytic activity without a corresponding decrease in mRNA expression of polygalacturonse. Our results suggest that glycosylation mutants appeared to interact differently with callus tissue when compared to the wild type. Glycosylation mutants produced a profusion of synnemata, while the wild type control did not produce any synnemata. A reverse genetics technique was applied to characterize a gene from *O. novo-ulmi* that segregated with other fungal glycosyltransferases. The link between N-glycan status, glycoprotein secretion and virulence is detailed.

In North America, the population dynamics of *O. novo-ulmi* have been largely unknown. By using Randomly Amplified Polymorphic DNA (RAPD) markers, in conjunction with vegetative compatibility analysis, the data presented here suggest that the North American population of the pathogen was highly clonal, and almost completely free of dsRNA viruses, and had remained so for at least a 9-year duration. As some dsRNA viruses isolated from *O. novo-ulmi* in Europe have been shown to dramatically affect the parasitic fitness of the infected fungus, the possibility that these dsRNAs may be used for control becomes a possibility. Thus, the continued genetic uniformity of the population and the almost complete absence of dsRNA in the Winnipeg pathogen population are two highly favorable indicators in control strategies for this disease.

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This thesis is dedicated to the memory of John and Helen Ritchie.

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CHAPTER ONE

Molecular Analysis of Fungal Pathogenesis in Forest Pathogens

Introduction

While the large majority of fungi are not pathogens of plants, the roughly 8% that are phytopathogens have had significant impact on forest management and health in North America (Schäfer 1994). Despite this low percentage, the damage caused by forest pathogens and impact on the economy is considerable. An amount of timber equal to an estimated 42% of the annual timber harvest in Canada is lost to forest pathogens each year (Manion 1991). Additionally, forest epidemics such as Dutch elm disease, chestnut blight and white pine blister rust have caused tremendous damage to individual tree species. Pathogens can have a profound effect on both host and pathogen species distribution and serve a vital role in the health of an undisturbed ecosystem by elimination of less competitive genotypes and recycling of nutrients (Castello et al. 1995). Forest diseases only become truly destructive when human activity plays a role. Forest diseases are caused by an interaction between pathogen and host in conjunction with a variety of factors that interact to predispose hosts in a given area to disease (Manion 1991). The majority of earlier analyses of virulence at a molecular level have focused on agricultural model systems, mostly due to a paucity of data available for forest systems. The molecular basis of pathogenesis and virulence in fungi is reviewed here to provide insight into host-pathogen interactions, and to relate agricultural models of disease to forest disease systems.

Definition of Terms

Because of the general flexibility of terms such as pathogenicity and virulence, it is important to define these terms. Pathogenicity is the ability of the organism to cause disease. While genes essential for survival or propagation of the organism could be considered integral to pathogenicity, since the organism could not cause disease without them, their inclusion would render the definition too broad to be meaningful. Limiting the definition of pathogenicity genes to be those exclusively involved in producing gene products involved in pathogenicity under natural conditions is a more meaningful definition (Schäfer 1994). Virulence is defined as a measure of disease, and thus a virulence factor is a genetically encoded entity that has an effect on the level of disease caused by a given pathogen on a given host. Although suggested to be incorrect (Shaner et al. 1992), avirulence will be retained as used in the literature as a descriptor for avirulent pathogens (Andrivon, 1993). Given the likely multifaceted nature of pathogenicity in forest pathogens, it becomes difficult to accurately classify genes. Many genes implicated in virulence or pathogenicity could be more accurately called parasitic fitness factors (Shaner et al. 1992), which are genes that contribute to the overall parasitic fitness of the organism, but not necessarily directly to the virulence of the organism.

Stages of Infection

Infection of a host by a pathogen usually proceeds in well-defined stages, including attachment, penetration, germination, and colonization. Attachment is the first stage in a successful infection of a host by a fungus (Knogge 1998). Adhesion in different pathogens is likely to vary widely, given the variety of surface structures and molecules that have been implicated in interactions between host and pathogen (Schäfer 1994). Adhesion may depend partly on enzymatic activity, as many spores carry a variety of enzymes that alter the plant cuticle and cell wall and may help alter the surface of the host such that the pathogen can attach (Schäfer 1994). Wound pathogens, such as *Ophiostoma novo-ulmi* Brasier and *O. ulmi* (Buisman) Nannf., the causal agents of Dutch elm disease, largely circumvent the first stages of infection but require a pre-existing wound in order to infect a host. A combination of signals acts at the molecular level to signal germination in an infectious spore. Topographical signals can trigger morphogenesis and pathogenesis. For example, the rust fungus *Uromyces appendiculatus* (Pers.) Unger could be induced to differentiate penetration structures by specific morphological features of the host cuticle or even by ridges on a plastic substrate. Chemical surface signals can also affect differentiation. Purified avocado (*Persea americana* Mill) wax induced germination and appressoria development in *Colletotrichum gloeosporioides* Penz., while wax from a nonhost species had no inducing properties (Podila et al. 1993).

The cuticle of a host plant, including pectin and cutin layers, presents a barrier that must be breached or avoided by a potential pathogen. Penetration of the host can occur in two ways: enzymatic or mechanical, either singly or in combination. Presented with the cuticle, fungi secrete a battery of enzymes designed to aid penetration, including cutinases, cellulases, pectinases and proteases (Knogge 1998). Although saprophytic fungi also secrete these enzymes, suggesting that they may have a limited role in pathogenicity, these enzymes may be developmentally regulated in pathogens to adjust to specific host-pathogen interactions. For some fungi, melanin has been proven to be an important factor in penetration. When present in fungal cell walls, melanin allows the build-up of hydrostatic pressure within the appressorium that aids formation of a penetration peg (Mendgen and Deising 1993). *Magnaporthe grisea* (Hebert) Barr, the causal agent of rice blast, was shown to use turgor pressure as part of the mechanism for penetration of the host cuticle (Howard et al. 1991). Additional molecular evidence for the role of melanin in buildup of hydrostatic pressure comes from analysis of albino mutants in other fungi. Although such mutants in *Colletotrichum lagenarium* (Pass.) Ellis and Halsted were able to form appressoria, the appressoria were not melanized and had little penetrating ability. Transformation of these mutants with the melanin gene fully restored the penetrative ability of the appressoria (Kubo et al. 1991). It appears likely that most fungi are highly adapted to penetrate their particular hosts in ways that most logically coincide with conditions found at the host cuticle (Mendgen and Deising 1993). Successful penetration of the host cuticle does not necessarily lead to a

successful infection, however, as many infections are stopped in later stages beyond the cuticle. It is in this last stage, colonization, in which the fungal pathogen is in most complete contact with its host and the success or failure of the infection is truly determined.

The Plant Defense Response

Basic or non-host resistance mechanisms account for plants being resistant to most potential fungal pathogens. Basic resistance is composed of responses that can be preformed or actively triggered, and are not specific to any given pathogen (Heath 1981). Most plant species have an array of non-specific defense mechanisms, including the preformed physical barrier of the cuticle and its components, as well as chemical barriers that exist within the cuticle (Kolattukudy et al. 1995). A common actively triggered plant response to invasion is termed the hypersensitive reaction (HR). The HR is a localized necrosis at the site of infection that can be incited by specific elicitors of fungal pathogens (Keen 1982). Concurrent with the HR are such processes as ion flux, phosphorylation state changes and generation of oxygen radicals. Oxygen radicals have been identified as important in initiating the HR. They are also diffusible signals that initiate later defense responses in nearby cells (Tenhaken et al. 1995; Tzeng and DeVay 1993).

Later defense responses include expression of pathogenesis-related genes and strengthening of the cell wall to restrict pathogen movement. Phytoalexins, which

are fungicidal secondary plant metabolites, can also be induced locally during a host defense response and accumulate after pathogen invasion (Keen 1982). Elicitors, produced either by the fungal pathogen or by the host plant, induce plant defense responses. Exogenous elicitors are those that originate from the pathogen, while endogenous elicitors are those that come from the host and are activated as a result of pathogen interaction (Ebel and Cosio 1994). Fungal elicitors need to be effective molecules by which a pathogen can be identified, and as such are commonly components of the fungal cell wall.

A reaction of plants to infection is induced resistance or physiological acquired immunity, which has been more lately called systemic acquired resistance (SAR). This is a broad spectrum, long-lasting systemic resistance to subsequent infection. Although there is some disagreement about its role, salicylic acid has been suggested to act as a long-range signal in initiating SAR (Cameron 2000). Genes induced in the SAR include antifungals such as chitinases, β-1,3-glucanases, and the membrane-disrupting permatins (Ryals et al. 1994). Another class of genes implicated in resistance that may be part of the SAR is the polygalacturonase inhibiting proteins (PGIPs). These inhibitors have been isolated from a variety of plants. Host PGIPs may to function by slowing the activity of the fungal cell wall-degrading enzyme, polygalacturonase. The PGIPs increase the biological life span of hydrolytic fragments released from the host cell wall by fungal polygalacturonases. Normally, polygalacturonases rapidly reduce the fragments to smaller biologically inactive fragments that would not be recognized by the

host. Larger hydrolytic fragments can act as endogenous elicitors to induce host defense reactions, thus PGIPs may therefore help the host recognize invasion by a fungal pathogen (Cook et al. 1999). An example of SAR can be observed in Dutch elm disease: inoculation of American elm (*Ulmus americana* L.) with the less aggressive *O. ulmi*, or with elicitors derived from *O. ulmi*, can give some protection from subsequent infection by the normally more aggressive *O. novo-ulmi.* However, the genes that are being expressed in this SAR are currently unidentified (Hubbes 1999). Similarly, inoculation of the susceptible *U. hollandica* Mill. with *O. ulmi* decreased symptoms caused by *O. novo-ulmi* (Scheffer et al. 1980). It is possible that mansonones, phytoalexins secreted by elms in response to pathogen attack, could be partly responsible for the induced resistance seen in elm (Hubbes, 1999). Phenylalanine ammonia-lyase activity and production of a hydroxycoumarin scopoletin in suspension cultures of elm have also been correlated with disease resistance in elm (Corchete et al. 1993; Valle et al. 1997). In particular, scopoletin was shown to have antifungal activity and may be important in elm defense reactions (Valle et al. 1997).

The Gene-for-Gene Hypothesis

If a pathogen can circumvent or accommodate the defenses of the host species, it can establish basic compatibility and cause disease on the host (Heath 1981). To establish basic compatibility on a host, a pathogen requires, among other things, the ability to secrete pectinolytic enzymes and toxins, production of molecules to shield elicitors from detection and production of hypersensitive response

suppressors (Keen 1982). It should be noted that determinants of pathogenicity are not necessarily limited to the production of toxins, but could include a variety of processes that may have other integral roles in establishing basic compatibility (Scheffer and Livingston 1984). It has been known that protection from host defenses has been shown to mediate basic compatibility. The cereal root pathogen *Gaeumannomyces graminis* (Sacc.) von Arx and Olivier secretes the saponin-detoxifying enzyme, avenacinase; without secretion of avenacinase, *G. graminis* is unable to infect oat species that produce avenacin A-1 (Bowyer et al. 1995). Different pathogens are likely to require different mechanisms to overcome the basic resistance mechanisms of the host (Keen 1982).

Even when basic compatibility has been established, a given cultivar of the host species can become resistant to a pathogen (Heath 1991). Host-pathogen interactions on a cultivar level could include specific recognition of the pathogen by the host, or elimination of pathogen processes that induce susceptibility (Heath 1991). Cultivar resistance requires complementary gene expression in pathogen and host. Cultivars of a normally susceptible host species that show resistance to fungal pathogens are hypothesized to express this resistance under control of a single gene. The avirulent phenotype of the pathogen on this host would thus be described as having an *avr* gene. The *avr* gene of the pathogen is suggested to encode an elicitor that interacts with the gene product produced by the resistance gene (R gene) in the host. This interaction allows recognition of the pathogen by the host and expression of resistance (Heath 1991). Plant R genes have been

suggested to encode receptors for *avr*-encoded gene products (Baker et al. 1997; Bent et al. 1994; Staskawicz et al. 1995). Thus, an *avr* gene gives a pathogen an avirulent phenotype on a plant expressing the corresponding R gene. Plant R genes may also have multiple specificity as a way of numerically reducing R genes required for a plant to maintain broad resistance (Grant et al. 1995). It is likely that *avr*-encoded elicitors induce a cascade of responses leading to initiation of plant defense responses. Thus, this scenario of interacting *avr* genes in the pathogen and R genes in the host gives rise to the gene-for-gene hypothesis (Heath 1981). After cultivar resistance has been established, the pathogen can overcome this resistance by a single step mechanism (i.e. by a mutation in the gene encoding the elicitor specifically recognized by the host).

Given that *avr* genes exist and appear to be physiologically dispensable (Gabriel 1999), their presence in the genome of pathogens is a mystery, especially if absence of an *avr* gene is sufficient for a pathogen to regain virulence. It is possible that supernumerary chromosome transfer may have transferred *avr* genes between fungal pathogens, particularly if *avr* genes had or currently have an important role in pathogenicity on other potential hosts (Gabriel 1999). Thus, an *avr* gene may be part of establishing basic compatibility on a given host at the cost of decreased virulence on another host (Staskawicz et al. 1995). Maintenance of *avr* genes in a pathogen population suggests that in the absence of resistance genes in the host, *avr* genes confer selective advantage to the pathogen

but would function to decrease biological fitness in the presence of the resistance allele (Keen 1982).

One possible role for avirulence genes is that they may code for specific glycosyltransferases. While glycosylation genes have been suggested to have important roles in maintaining fungal virulence in human pathogens (Buurman et al. 1998; Fernandes et al. 1999; Southard et al. 1999), the ubiquitous presence of these genes in fungi and their prominent role in modifying the cell surface also makes them candidates for *avr* genes in plant pathogens. Glycosylation genes could function to create elicitors by addition or deletion of key sugar residues. Therefore, it could be envisioned that a wild type glycosylation gene could be an *avr* gene in some fungal pathogens, while virulent isolates may be deficient in glycosylation functions. In the absence of resistance genes in the host, pathogens with deficient glycosylation genes would be expected to be of lower parasitic fitness. If the selective pressures of resistance genes in the host population arise, then glycosylation-deficient strains could be expected to have increased fitness (Keen 1982). There have been correlations between glycosylation and virulence, as well as evidence that glycoproteins can act as elicitors. Invertases of *Phytophthora megasperma* Drechs. var. *sojae* A.A. Hildebrand (Pms) from three differentially virulent races showed different carbohydrate structures, suggesting that glycosylation patterns may have an effect on specificity in host-pathogen interactions (Ziegler and Albersheim 1977). Analysis of yeast glycoproteins illustrated that glycoproteins act as elicitors of the defense response in tomato

(Basse et al. 1992). A glycoprotein capable of eliciting mansonone (phytoalexin) production in elm has been isolated from *O. ulmi* (Yang et al. 1994) and a glycoprotein in the cell wall of the wheat pathogen *Puccinia graminis* f. sp. *tritici* Eriks. and Henn. is an elicitor (Kogel et al. 1988), which suggests an important role for glycosylation patterns in host-pathogen recognition.

Cell-Wall-Degrading Enzymes and Virulence

Cell-wall-degrading enzymes (CWDEs) have been suggested to be important virulence factors in some fungal plant pathogens (Collmer and Keen 1986). Pectin polymers, a major component of the cell wall, are attacked by a variety of pectinolytic enzymes including polygalacturonase. Cutin, another structurally important part of the cell wall, is attacked by cutinase. To be effective, the CWDEs must be secreted so that they are in contact with the correct target tissue in the host at the correct stage in pathogenesis. Secreted CWDEs enhance invasion of the host and spread of the pathogen within the host tissue. However, it is difficult to discern if pectinolytic enzymes are important virulence factors, since these enzymes are produced both by non-pathogenic and pathogenic fungi (Collmer and Keen 1986). Disruption of genes encoding hydrolytic enzymes involved in cell wall degradation or degradation of the host cuticle has been inconclusive in providing much insight into the role of these enzymes in pathogenesis. Complicating determination of the role of pectinolytic enzymes in virulence is the production of isozymes that can function to increase the biological flexibility of the pathogen, and by temporal changes in expression of genes

encoding CWDEs during infection (Annis and Goodwin 1997). Studies using mutagenic chemicals or UV irradiation to create CWDE-deficient strains have been replaced by more precise molecular studies, but problems still remain. Enzyme assays are not always sensitive enough to detect low levels of enzymatic activity in isolates with deficient CWDE production, and wound inoculation, used in some virulence assays, destroys any chance of detecting the role of CWDEs in early infection stages (Annis and Goodwin 1997). Additionally, genetic disruption of the genes encoding these enzymes followed by a lack of appreciable reduction in virulence can be related to other mechanisms compensating for the lack of the given enzyme. Expression of genes encoding CWDEs in closely related species is a good alternative to disruption, and has proven effective in suggesting possible roles in virulence for these enzymes (Dickman et al. 1989; Yakoby et al. 2000).

Pectinolytic Enzymes and Pathogenesis

Pectinolytic enzymes have been suggested to have an important role in pathogenesis in plant pathogens; pectinolytic enzymes attack the structurally important cell walls of plants and disrupt osmotic capabilities (Collmer and Keen 1986). Pectinolytic enzymes are produced early in the infection process and in a purified form are capable of macerating and killing plant cells, causing symptoms characteristic of the diseases caused by necrotrophic pathogens (Annis and Goodwin 1997). Thus, the pectinolytic enzymes appear to be among the most significant virulence factors of the CWDEs. Other CWDEs have not appeared as

capable of causing as much destruction as pectinolytic enzymes, suggesting the importance of pectinolytic enzymes, particularly those that have an endo-activity (Collmer and Keen 1986).

To increase parasitic fitness, some fungal pathogens produce isozymes of pectinolytic enzymes. The pectinolytic enzyme polygalacturonase (PG) was shown to be a single copy gene in *Fusarium moniliforme* Sheldon, yet four differentially glycosylated isozymes were derived from this gene, possibly permitting alternate activities or regulation, and perhaps allowing enzymes to avoid triggering host defenses (Caprari et al. 1993). Pectinolytic enzymes may also act to facilitate spread of the pathogen within the host tissue (Collmer and Keen 1986). The activities of pectinolytic enzymes appear to cause accumulation of phytoalexins in some systems. It is hypothesized that pectinolytic enzymes may be involved in host recognition of pathogens by uncovering receptors for elicitors in the host cell wall, or by releasing biologically active cell wall fragments or other degradation products (West 1981).

Polygalacturonase has been suggested to be an important virulence factor in fungal pathogens. The functions of PG genes in virulence have been analyzed in several fungal systems. Targeted disruption of one of the *Botrytis cinerea* Persoon: Fries PG genes showed that this fungus requires this gene for full virulence (ten Have et al. 1998). *Aspergillus flavus* Link:Fries also requires endoPG for spread within its host; over-expression of endoPG in *A. flavus*

increased isolate aggressiveness, while targeted elimination of the gene diminished the aggressiveness of the transformants (Shieh et al. 1997). Although a correlation of virulence to endoPG secretion can be identified in some fungi, abolition of endoPG gene function in other fungi has not been correlated to a diminished virulence. Disruption of the endoPG gene in *Cochliobolus carbonum* R. R. Nelson did not affect pathogenicity in transformants when tested on maize (Scott-Craig et al. 1990). Disruption of *enpg-1*, a polygalacturonase gene of *Cryphonectria parasitica* (Murr.) Barr, did not reduce virulence of *enpg-1* disrupted isolates on chestnut; however, polygalacturonase levels *in vivo* were indistinguishable between wild types and disruptants, suggesting that undetected genes are also involved in production of the enzyme (Gao et al. 1996). Over-expression of endoPG in other fungi also has not led to an increase in virulence. Over-expression of endoPG in three endoPG-deficient isolates of *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hans. did not increase virulence in transformants when tested on muskmelon, suggesting that endoPG is not a virulence factor in naturally occurring PG-deficient isolates of *F. oxysporum* (Di Pietro and Roncero 1998).

Ophiostoma novo-ulmi has been shown to have a superior set of CWDE abilities compared to the less aggressive *O. ulmi* (Binz and Canevascini 1996a; Scheffer and Elgersma 1982), and has been shown to be a more effective colonizer of bark (Webber and Hedger 1986), but there has been no direct correlation between polygalacturonase production and virulence in *O. novo-ulmi* (Elgersma 1976).

Other CWDEs produced by *O. novo-ulmi* also have not been individually correlated to virulence; no difference in xylanase or cellulase activity was observed in *O. novo-ulmi* when compared to *O. ulmi* (Binz and Canevascini 1996b; Elgersma 1976). Disruption of the endoPG gene in *O. novo-ulmi* eliminated the ability of the fungus to grow on media with pectin as a sole carbon source; however, the assay used suggested the presence of multiple pectinolytic activities that will likely complicate determination of the exact role of polygalacturonase in virulence. The cloning and disruption of polygalacturonase (*epg1*) in *O. novo-ulmi* is the first reported targeted gene disruption of a CWDE enzyme in this fungus; the effects of the disruption of *epg1* on pathogenicity of *O. novo-ulmi* on elm is examined in the following chapter.

Cutin-degrading Enzymes and Pathogenesis

Cutin is a major structural component of plant cuticles, and is an obstacle to pathogens seeking entry to a potential host; enzymatic penetration is likely of high importance in pathogens that must penetrate a thick host cuticle (Gevens and Nicholson 2000). Cutinase has been implicated as a major virulence factor in some fungi, including *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (F.R. Jones) W.C. Snyder and H.N. Hans. Inhibition of cutinase using antibodies to cutinase, or chemical inhibitors of cutinase prevented infection of pea by *F. solani* f. sp. *pisi* (Maiti and Kolattukudy 1979). Both cutinase-deficient mutants and isolates with a targeted disruption of the cutinase gene were found to have dramatically reduced ability to infect pea. Adding exogenous cutinase could restore the

pathogenicity of *F. solani* f. sp. *pisi* isolates with a disrupted cutinase gene (Kolattukudy et al. 1995). Insertion of the *F. solani* cutinase gene into the wound pathogen *Mycosphaerella* allowed this fungus to infect intact papaya, directly through the cuticle (Dickman et al. 1989). Spores of *F. solani* carry cutinase, which releases cutin monomers that induce expression of the cutinase gene, suggesting that production of cutinase is important in early stages of infection and penetration (Kolattukudy et al. 1995). When cutinase-deficient mutants of *Colletotrichum gloeosporioides* were isolated by UV and chemical mutagenesis, these mutants were unable to infect papaya fruit unless the cuticle had been mechanically wounded, suggesting that cutinase is also a vital enzyme in penetration of the host cuticle by *C. gloeosporioides* (Dickman and Patil 1986).

Other Virulence Factors in Plant Pathogens

Toxins are potentially virulence factors in forest pathogens. Phytopathogens produce two types of toxins. Nonspecific toxins are capable of exhibiting toxicity to a wide range of plants whether the plant is a host or not (Scheffer and Livingston 1984). Host-selective toxins (HSTs) are toxic to specific host species, and are generally produced by fungi specialized to a restricted range of hosts. Toxins have been generally defined as molecules that cause damage to host tissues and are involved in disease development, but are not related to recognition of pathogens by a host (Scheffer and Livingston 1984). Likewise, elicitors have been classified as molecules that are signals, but not primary physiological effectors (Ebel and Cosio 1994). However, it is difficult to distinguish toxins

from elicitors given an overlap in function, especially given the relative speed at which molecules governing recognition between a host and pathogen can act. A putative toxin that is fast acting might be toxic on one host, but may act as an elicitor in a host where it exhibits a slower action. In particular, toxins that cause cell death may cause the release of endogenous elicitors from a plant. Accuracy in defining a molecule as an elicitor or toxin is also complicated by using cell death as an indicator of function as cell death alone may not give a fully accurate picture of the overall role of the molecule in pathogenicity (Walton and Panaccione 1993).

Given that basic compatibility has been established, HSTs can be responsible for determining host specificity. Host selective toxins have been directly linked to expression of virulence, while resistances to HSTs have been correlated to resistance in the host (Scheffer and Livingston 1984; Walton and Panaccione 1993). While production of toxins is potentially a multistep process, it is possible that toxin production can still fit with a gene-for-gene model. For example, HC-toxin production by the maize pathogen *Cochliobolus carbonum* appears to be controlled by a single locus encoding a multifunctional enzyme, which may also be the case with other pathogens (Panaccione et al. 1992). Toxins have been identified in forest pathogens, however their role in pathogenesis remains unclear. Dothistromin toxin, produced by *Dothistroma pini* Hulbary (teleomorph *Mycosphaerella pini* E. Rostrup) the causal agent of Dothistroma needle blight, is a possible virulence factor; purified, it can cause toxicity when injected into

needles, but does not directly elicit host defense response (Bradshaw et al. 2000). A variety of non-specific toxins with a phenolic nature have been identified that are produced in culture by the causal agent of red oak leaf spot, *Tubakia dryina* (Sacc.) B. Sutton (Venkatasubbaiah and Chilton 1992). *Ophiostoma novo-ulmi* and *O. ulmi* also produce an array of putative toxins, including phenolics, hydrophobins, polysaccharides and glycopeptides, none of which have been conclusively identified as key virulence factors (Claydon et al. 1980; Richards 1993; Scheffer et al. 1987; Takai 1974; Van Alfen and Turner 1975). *Cryphonectria parasitica* has been shown to produce toxic polysaccharides (Corsaro et al. 1998).

Other secreted molecules may also have important roles in establishing an infection, and may have particular importance in allowing pathogens to avoid or delay host defenses. Delay of defense reactions of host plants may be a consequence of the host being infected by a pathogen that is able to suppress plant defense responses. Plants can be conditioned towards increased susceptibility by infection with a pathogen, suggesting that suppressor molecules are produced by the pathogen to delay the host defense response, possibly by inhibiting elicitor binding (Doke et al. 1980; Yamada et al. 1989; Yoshioka et al. 1990). There is increasing evidence that glycans play an important role in the suppression of host defences. Analysis of interactions between potato tubers and *Phytophthora infestans* (Montagne) de Bary showed that water-soluble glycans were responsible for suppression of the host hypersensitive response. Quantitative differences in

glycan production were not noted between virulent and avirulent strains, which suggest qualitative reasons for the suppressor activity of the glycans (Doke et al. 1980). Further analysis of fungal elicitors in tomato showed that glycoproteins are important elicitors of the defense response in plants; however, once a glycan was cleaved from a protein, the glycan was capable of suppressing host defense responses (Basse et al. 1992).

By broadly affecting a variety of genes in *C. parasitica*, virulence of the chestnut blight fungus can be lowered, suggesting the involvement of many genes in pathogenesis. Infection of *C. parasitica* with a mycovirus can down-regulate secretion of hydrolytic enzymes, putatively involved in pathogenesis, and can induce a phenomenon known as hypovirulence (Wang and Nuss 1995). Hypovirulence associated viruses appear to be able to disrupt signal transduction pathways, which can account for the broad effects of the mycoviruses on the phenotype of the pathogen (Choi et al. 1995; Larson et al. 1992). This suggests that these pathways are important in maintaining virulence of an organism (Gao and Nuss 1998). Hypovirulent strains of *C. parasitica* can also initiate in the host a much more pronounced and rapid defense response compared to wild type strains, and hypovirulent strains appear to be more susceptible to chitinases produced by the infected host (Vannini et al. 1999). Hypovirulent strains of *Ophiostoma novo-ulmi* have been found that contain virus-like double stranded RNA viruses (Brasier 1983; Webber 1993). The role of the dsRNA viruses in affecting virulence in *Ophiostoma* has not been fully examined, but could be

related to transcriptional down-regulation, especially in the mitochondria (Charter et al. 1993).

One class of molecules possibly involved in pathogenesis is the hydrophobins, which are hydrophobic proteins that are common on the cell surface of some fungi. Hydrophobins, in addition to a role in fungal morphogenesis, may be adapted to a variety of parasitic functions in phytopathogens (Temple et al. 1997; Temple and Horgen 2000). Hydrophobins have been shown to mediate fungal virulence. The ascomycete *Magnaporthe grisea*, causal agent of rice blast, produces a hydrophobin known as MPG1. In this case, disruption of the *mpg1* gene led to a reduced virulence on rice (Talbot et al. 1993). Cerato-ulmin (CU), a hydrophobin produced by the Dutch elm disease pathogens *O. novo-ulmi* and *O. ulmi*, was initially thought to be a wilt toxin involved in expression of virulence of *O. novo-ulmi* (Bowden et al. 1994; Takai, 1974). However, neither genetic disruption nor over-expression of the *cu* gene altered virulence as measured by trials using three-year old elm seedlings (Bowden et al. 1996; Temple et al. 1997). Naturally occurring isolates of *O. novo-ulmi* that do not produce CU also showed high levels of virulence (Brasier et al. 1995). However, expression of *cu* in *Ophiostoma quercus* Georgevitch, normally non-pathogenic on elm, enabled the fungus to cause wilt symptoms in elm similar to those caused by the elm pathogens *O. novo-ulmi* and *O. ulmi*. Virulence was intermediate between the less virulent *O. ulmi* and the highly virulent *O. novo-ulmi* in one transformant (Del Sorbo et al. 2000). The evidence that CU is a wilt toxin or virulence factor

of any kind in *O. ulmi* or *O. novo-ulmi* is weak and it appears the biological role of CU may have more of an effect on the parasitic fitness of the pathogens (Temple et al. 1997). However, the expression of *cu* in *O. quercus* seems to allow the fungus to establish basic compatibility on elm. It is possible that *O. quercus* has at least some of the necessary genes for pathogenicity on elm, especially given that the Dutch elm disease fungi are closely related to *O. quercus* (Harrington et al. 2001); however, exactly how CU is functioning to allow a non-pathogen to become a pathogen is unknown. *Ophiostoma quercus* may normally be expressing gene products that induce elm defenses and prevent it from being pathogenic on elm, or the cell wall of the fungus might be susceptible to preformed elm defenses. Cerato-ulmin functions to coat hyphae and is a prominent cell wall protein (Temple et al. 1997) and may coat the cell surfaces of transformed CU producing isolates of *O. quercus*, masking elicitors or protecting the cell wall and releasing the pathogen such that disease on elm can be established. Cerato-ulmin may also suppress host defense responses, again allowing establishment of disease.

The correlation of pathogenicity to the expression of a single gene in *O. quercus* suggests a similarity to the concepts of basic compatibility found in the gene-for-gene hypothesis. Normally *O. quercus*, a non-pathogen on elm would initiate or be stopped by host defenses, but with the expression of *cu*, plant defenses are somehow circumvented and allow establishment of disease. *Ophiostoma novo-ulmi* and *O. ulmi* differ in ability to elicit host defenses as demonstrated by

analysis of mansonone (phytoalexin) elicitation in several species of elm. Elms are able to produce a variety of mansonones that show a range of inhibitory effects on the two elm pathogens (Dumas et al. 1986). The level of mansonones produced depends on host species and on the virulence of the pathogen. Highly resistant species such as *Ulmus pumila* L. show rapid accumulation of mansonones in response to challenge by both pathogens (Duchesne et al. 1986); challenge of susceptible American elm with either of the two pathogens shows that mansonones accumulate to a higher concentration in response to the less aggressive *O. ulmi* (Duchesne et al. 1985). However, it seems resistance to mansonones by *O. novo-ulmi* is unlikely to account for differences in virulence between the two pathogens (Proctor et al. 1994). It appears that *O. novo-ulmi* is capable of avoiding the elicitation of inhibitory accumulations of mansonones at critical times during infection (Proctor et al. 1994), as well as being able to suppress mansonone production in the host (Duchesne et al. 1985).

Additional evidence that the Dutch elm disease pathogens mimic the agricultural gene-for-gene hypothesis comes from recent work that explores inheritance of pathogenicity loci (Et-Touil et al. 1999). A single major gene appears to have an important role in pathogenicity of *O. novo-ulmi*. Crosses with a moderately aggressive strain and a highly aggressive strain led to a 1:1 ratio of high to moderate aggressiveness. The gene or locus responsible for the diminished aggressiveness is suggested, through use of RAPD markers, to be introgressed into *O. novo-ulmi* from the less aggressive *O. ulmi* (Et-Touil et al. 1999). It is

apparent that additional genes still appear to have a role in pathogenesis, but the association of a single gene that appears to have a significant effect on aggressiveness has implications for assessing Dutch elm disease from the perspective of the gene-for-gene hypothesis. The presence of a single locus that appears to function to decrease aggressiveness could be functioning as *avr* gene and suggests that pathogenesis in Dutch elm disease may be theoretically similar to the gene-for-gene model of virulence. Even a single base pair change in an *avr* gene can be sufficient to restore pathogenicity to avirulent pathogens (Joosten et al. 1994). The destruction of secondary or tertiary structure in the *avr* gene product could possibly prevent binding to receptors in the plant, enhance enzymatic degradation of the *avr* product by the host or damage secretion, all of which could ameliorate the function of an *avr* gene (De Wit 1995). It does appear from the data that an *avr* gene is present in the less aggressive *O. ulmi*, while a non-functional allele, or complete absence of that allele could be a contributing factor to the difference in aggressiveness between *O. novo-ulmi* and *O. ulmi.*

Other forest disease systems possibly emulate a gene-for-gene model as well. A possible elicitor with putative glycosylation sites has been isolated from *Cronartium ribicola* J. C. Fisch., the causal agent of white pine blister rust. Expression of this protein may be related to expression of pathogenesis related proteins in the host, white pine (Ekramoddoullah et al. 1999). Sugar pine (*Pinus lambertiana* Dougl.) was shown to have a single dominant gene for resistance to

C. ribicola; the R gene, once cloned, has potential use in development of resistant pines (Harkins et al. 1998).

It seems certain that a multitude of genes are involved in establishing infection. Interestingly, recent evidence suggests that genes involved in pathogenicity have possibly been passed among fungal pathogens via horizontal gene transfer of supernumerary chromosomes (Covert 1998). It is possible that multigenic disease resistance does occur in many disease systems, and that resistance is determined by a gene-for-gene type interaction in other systems. By determining mechanisms of plant defense, there exists the possibility that these mechanisms may be exploited for use in control strategies. Evidence continues to support the hypothesis that single genes control host species specificity in many plant disease systems (Heath 1991; Staskawicz et al. 1984). Some forest disease systems appear to mimic the gene-for-gene hypothesis of agricultural models but for the most part, analogy to the gene-for-gene hypothesis in most forest pathogens has not been examined due largely to a lack of data. How fungi become pathogens, and maintain themselves as pathogens, is the critical question. Fungal pathogenicity may have evolved from saprophytic fungi that have adapted their life cycle to development in host plants. Molecular analysis of host-pathogen interactions in forest pathogens can be instructive in determining a viable control strategy. Of particular interest is the re-interpretation of some of the knowledge available for forest pathogens in order to assess these fungi in light of the gene-

for-gene hypothesis, which has its origins in studies of agriculturally significant pathogens.

Dutch elm disease has decimated elms in the Western Hemisphere for over a century. The American elm (*Ulmus americana)* has been widely planted in the urban landscape and consequently control of Dutch elm disease would have considerable economic benefit and horticultural value. This study details the cloning of the gene encoding the cell wall-degrading enzyme, polygalacturonase, the characterization of glycosylation mutants of *O. novo-ulmi* and a survey of the population structure, vegetative compatibility and the occurrence of dsRNA virus infection in the pathogen population in Winnipeg, Manitoba, Canada. Research to date has determined that interactions between host and pathogen are controlled, or at least broadly affected by one or a few genes. The basis of pathogenicity in *O. novo-ulmi* appears to be a function of numerous genes, such as CWDEs, acting in an additive manner. Despite the multitude of genes that are involved in pathogenicity, host-pathogen recognition may be ultimately controlling virulence and gene-for-gene interactions predict recognition may be under the control of a single gene, or very few genes. Genes encoding glycosyltransferases represent one possible family of genes that may have a significant impact on recognition and thus on virulence and other host-pathogen interactions. A valuable step in determining a worthwhile approach to development of viable control strategies for Dutch elm disease is to fully understand the host-pathogen interactions, from the

population dynamics of *O. novo-ulmi*, to the molecular basis of disease in the host.

CHAPTER TWO

A single gene in the Dutch elm disease pathogen *Ophiostoma novo-ulmi* encodes polygalacturonase

Chapter summary

Ophiostoma novo-ulmi, responsible for decimation of elms in the Northern Hemisphere during the twentieth and twenty-first century, is the causal agent of Dutch elm disease. This chapter describes the cloning and characterization of a single PG gene from *O. novo-ulmi*. This gene segregated with *endo*-acting PG's of other fungi, but unlike many other fungi, only a single copy of the gene was found in *O. novo-ulmi*. Genetic disruption of the gene was not lethal to the organism and led to reduction of pectinolytic activity *in vitro*. There was one potential N-glycosylation site in the *O. novo-ulmi* predicted PG protein. The data presented here suggest that PG may have an important role in fungal virulence and parasitic fitness in the life history of both the Dutch elm pathogen and other *Ophiostoma* species.

Introduction

Ophiostoma novo-ulmi Brasier and *O. ulmi* (Buisman) Nannf. have been responsible for two separate epidemics of Dutch elm disease and together have caused the virtual elimination of elms (*Ulmus* species) from the landscape of North America and Europe. The two pathogen species have been separated on the basis of aggressiveness and phenotypic characteristics, with *O. novo-ulmi* being the much more aggressive of the two species (Brasier 1991). This increased aggressiveness is likely due to the interaction of several genetic factors, each contributing singly and in concert to virulence. It has been speculated that pectinolytic enzymes are important in virulence of phytopathogens (Annis and Goodwin 1997). The pectinolytic cell wall degrading enzyme polygalacturonase (PG) may have a role in host-pathogen interactions between *Ophiostoma* and *Ulmus* yet the role of PG, and other pectinolytic enzymes in *Ophiostoma,* has yet to be demonstrated.

The contribution of PG genes to virulence has been analyzed in other fungi through gene disruption and over-expression. The targeted disruption of *Botrytis cinerea Bcpg1* showed that this fungus required this gene for full virulence, with expression of *Bcpg1* seeming to have a particularly vital role during infection and host tissue colonization (ten Have et al. 1998). Deletion of *Acpg1* in *Alternaria citri* led to a phenotype that exhibited reduced virulence (Isshiki et al. 2001). Overexpression of *pecA* in *Aspergillus flavus* was shown to correlate with increased isolate aggressiveness in cotton bolls (Shieh et al. 1997). Although PG

secretion can be correlated to virulence for certain fungi, alteration of *pg* gene function does not necessarily impact upon virulence or pathogenicity. Expression of *epg1* in three naturally occurring endoPG deficient isolates of *Fusarium oxysporum* did not increase virulence in transformants (Di Pietro and Roncero 1998). Similarly, disruption of *pgn1* in *Cochliobolus carbonum* (Scott-Craig et al. 1990), *Aapg1* of *Alternaria alternata* (Isshiki et al. 2001) and *pg5* of *Fusarium oxysporum* (García-Maceira et al. 2001) did not reduce virulence in any of these fungi.

The main function of PG in opportunistic pathogens like *O. novo-ulmi* may be to increase survival during the saprophytic life cycle and promote the release of pathogenic isolates from the xylem to vector-breeding galleries in the outer layers of the tree. As part of the disease cycle of *O. novo-ulmi*, the fungus must spread through the phloem and into the breeding galleries of the disease vector (bark beetles belonging to the genera *Scolytus* and *Hylurogopinus*). Once the fungus is present in the breeding galleries, emerging beetles become coated with fungal spores and transmit the disease to healthy trees. Competition between *O. novo-ulmi* isolates likely occurs as a variety of pathogen genotypes may be present in the outer layers of the tree due to colonization of the outer layers by the pathogenic isolate and by saprophytic isolates (Webber et al. 1987). Polygalacturonase, and other pectinolytic enzymes may be important fitness factors at this stage by enhancing nutrient mobilization and enabling spread of the fungus through maceration of the host tissue.

To assess the role of PG in parasitic fitness and virulence, this study details the disruption of the polygalacturonase gene, *epg1*, from *O. novo-ulmi*. The cloning and disruption of *epg1* represented the first step in determination of the role of *epg1* in the life history of the Dutch elm pathogen and permitted a determination of the possible roles for this gene in the life history of *O. novo-ulmi*.

Materials and methods

Isolates and culture conditions

Cultures of *O. novo-ulmi* VA30 (isolated by L. Schreiber and A. Townsend, Virginia) and MH75 (isolated by M. Hubbes, Toronto) were maintained for long-term storage in 10 % glycerol at -70 °C. An inoculum source on solid *Ophiostoma* complete medium (OCM) (Bernier and Hubbes 1990) was kept at 4 °C. Cultures were grown on OCM or minimal pectin medium at 21 °C for 7 d. Minimal pectin medium was prepared using 1 % agar supplemented with 1 % w/v pectin.

Nucleic acid extraction

Cultures for DNA extraction were grown in stationary liquid cultures for 7 d in 5 ml OCM at 23 °C. Mycelia were harvested, freeze-dried and ground into a fine powder. Two hundred mg of mycelia were re-suspended in 0.5 ml of 50 mM EDTA, 0.2 % SDS, mixed and incubated for 20 minutes at 65 °C. Samples were centrifuged to remove cell debris and protein was precipitated on ice with 1/6 volume 3.0 M potassium acetate, 5.0 M acetic acid. After removal of the supernatant to a fresh tube, an equal volume of isopropanol was used to precipitate the DNA. The DNA was then re-suspended in 500 µl of 10 mM Tris-HCl (pH 7.5), 5.0 mM EDTA (TE buffer), treated for 30 min with ribonuclease (100 µg per sample), extracted once with an equal volume TE-saturated phenol and extracted twice with equal volumes of 24:1 chloroform:isoamyl alcohol. The

DNA was then precipitated from the aqueous phase with 1/10 volume of 3 M sodium acetate (pH 5.3) and 2 volumes of 95 % ethanol and washed once with 70% ethanol. The DNA pellet was then air-dried and re-suspended in 100 µl water.

Derivation of a polygalacturonase specific DNA probe

Oligonucleotide primers were designed according to conserved regions deduced from sequence alignments of polygalacturonase genes from other ascomycetes. A codon frequency chart derived by compilation of highly expressed genes from *Aspergillus nidulans* was used to predict the expected sequence in *O. novo-ulmi* and reduce the redundancy of the primers. Two primers were designed that corresponded to amino acids GARWWDGK (base pairs 370-393) OUEPGF and NQDDCVAVNS (base pairs 641-668) OUEPGR. The sequence of the primers used was OUEPGF: 5' GGCGCTCGCTGGTGGGACGGCAAGGG 3' and OUEPGR: 5' GGAGTTAATGGCGAGGCAGTCGTCCTGGTT 3'. The target genomic DNA (10-50 ng) was amplified using 200 pmol of OUEPGF and OUEPGR, 2 units of Taq polymerase (Pharmacia; Uppsala, Sweden) and 0.1 µM each of dATP, dCTP, dTTP, and dGTP in a 50 µl final volume containing the Pharmacia Taq polymerase buffer at 1X concentration. A total of 30 cycles of PCR were used for amplification. All denaturing steps took place at 94 °C for 60 s; all primer extensions were for 120 s at 72 °C. The first 5 cycles of primer annealing took place at 50 °C for 90 s followed by 25 additional cycles at 60 °C. The amplification products were separated on a 1.0 % agarose gel in 1 X TAE

(0.04 M Tris-acetate; 1mM EDTA). The amplification products were excised from the gel, purified using the Wizard PCR prep kit (Promega; Madison, WI, USA), ligated into pGEM using the TA cloning system (Promega) and cloned into *Escherichia coli* DH5α. DNA sequencing identified the amplification products corresponding to fungal polygalacturonase genes.

Genomic library screening and subcloning epg1

Ophiostoma novo-ulmi MH75 genomic DNA was digested with *Mbo* I and ligated in bacteriophage lambda EMBL3. *Escherichia coli* VCS257 (Stratagene, La Jolla, CA, USA) was transfected with genomic library as previously described (Bowden et al. 1994). Hybridization analysis of the plaques was as per Sambrook and Russell (2001) using a ^{32}P-dCTP Easytides (Perkin Elmer, Wellesley, MA, USA) PCR labeled *epg* fragment derived using oligonucleotide primers OUEPGF and OUEPGR that incorporated approximately 15 μCi of activity. Positive plaques were re-screened a second time and phage DNA was isolated using PEG precipitation (Sambrook and Russell 2001). The position of *epg1* was determined by restriction mapping of the isolated recombinant phage followed by hybridization analysis (Southern 1975) (results not shown). A single 3.5 KB *Sal* I fragment containing *epg1* was subcloned into pUC18 and the entire gene sequence determined by automated fluorescent sequencing using the LI-COR model 4200 (LI-COR; Lincoln, NE, USA).

Sequence analysis and multiple sequence alignment

Potential active sites and N-glycosylation sites were predicted using Generunner (Ver. 3.05 Hastings Software, Inc; Hastings-on-Hudson, NY, USA). Nucleotide sequence alignments were carried out using GenBank data from http://www.ncbi.nlm.nih.gov/. The predicted protein sequence of the *O. novo-ulmi* PG (genebank accession # AF052061) was analyzed in alignment with selected fungal *endo*PG genes using neighbor joining analysis as produced by the web based ClustalW WWW Service at the European Bioinformatics Institute found at http://www2.ebi.ac.uk/clustalw (Higgins et al. 1994).

PGA plate assay of EPG activity

To determine the level of EPG1 secreted, *O. novo-ulmi* was inoculated into 5 ml liquid OCM and grown in stationary culture at 23 °C for 7 d. Assay plates were prepared containing 50 mM potassium acetate, 5mM EDTA, 0.1 % polygalacturonic acid and 1 % agarose, pH 4.5 (Bussink et al. 1992). After solidification, a cork borer (5 mm diameter) was used to remove agarose plugs and create wells. One ml of culture filtrate was lyophilized overnight. The resultant pellet was resuspended in 0.1 ml 50 mM potassium acetate, 5.0 mM EDTA at pH 4.5 and applied to the well on assay plates. After 24 h of incubation at room temperature, the undigested polygalacturonic acid remaining in the plates was stained for 20 min using 0.05 % Ruthenium red (Sigma-Aldrich; Oakville, ON, Canada) and washed with three changes of distilled water. The activity of

EPG1 was characterized by visually assessing the amount of staining in the halo surrounding the wells.

Disruption of epg1 in O. novo-ulmi

The *O. novo-ulmi epg1* gene locus was disrupted via the homologous recombination at the authentic locus by a gene cassette containing the hygromycin phosphotransferase gene (*hph*) (Punt et al. 1987) flanked by authentic *epg1* sequence to provide targeting. Protoplast formation and transformation of *O. novo-ulmi* was as previously detailed in Temple et al. (1997). This strategy took advantage of four unique restriction sites within the *hph* gene to introduce flanking DNA derived from, and homologous to *O. novo-ulmi epg1*. Flanking DNA, representing the authentic *epg1* locus, was amplified with the following two primer sets OUHPH F1 5' CATCACAAGATCTGGCATGGGCAGTTC 3' (tailed with *Bgl*II) and OUHPH R1 5' TTGAAGAGGTCACCCGACGCGC 3' (tailed with *Bst*Ell) or OUHPH F2 5' GTCAAGGGCACCGTAGGATCCACC 3' (tailed with *Bam*HI) and OUHPH R2 5' TAACCGCAAAATAAGCTTTGACACTGAAC 3' (tailed with *Hin*dIII). These primers were tailed to provide restriction sites corresponding to sites within the *hph* gene. Using *Bgl*II, *Bst*EII, *Bam*H1 and *Hin*dIII these flanking sequences were inserted into the *hph* gene and the construct was used for homologous gene targeting of *epg1*. Putative disruptants were screened using diagnostic PCR with a forward primer site located upstream of the insertion site, and a reverse primer seated within the *hph* gene. The sequence for each primer was as follows

*epg*DIAG 5' TCCCATATGGTCGACTGCCTCCTC 3' and *hph*DIAG 5' CCAACGCAGGTGCCCCAAGC 3'.

Virulence trials on Ulmus parvifolia x U. americana

Yeast-like cells of *O. novo-ulmi* VA30 or *epg1*$^-$ were isolated from 3-day-old OCM shake cultures at 110 revolutions per minute were kept at room temperature. Mycelial fragments were removed by filtering culture media through several layers of sterile cheesecloth. Yeast-like cells were washed once in sterile water and re-suspended in sterile water to a concentration of 2 million spores/ml using an improved Neubauer Haemocytometer (Fison Scientific Equipment, Loughborough, UK). Seedlings (2 yr old) of clonally propagated *Ulmus parvifolia* x *U. americana* F1 hybrid clone 2245 (Smalley and Guries 1993) grown in a 25% perlite, 75% peat moss substrate were inoculated with 100 µl of spore suspension or, in the case of the controls, injected with water through a 1 cm vertical slit made 5 cm above the root crown with a sterile scalpel. The inoculation wound was then wrapped in parafilm and trees were distributed in eight randomly distributed blocks, with one treatment of each inoculation per block. Inoculated trees were incubated in a growth cabinet maintained at 24°C during the day and 18 °C at night with a 16 h photoperiod. The percent defoliation was determined for each treatment by counting leaves at the beginning of the treatment and comparing this total to the number of healthy leaves 22 d post inoculation. A non-parametric STP test based on the Wilcoxon-Mann-

Whitney statistic was used to statistically compare the mean percent defoliations between each treatment.

Results

Structural analysis of the epg1 gene

Degenerate primers, designed according to conserved regions of endoPG genes from other fungi, were used to amplify a 298 bp fragment of the *O. novo-ulmi epg1* gene. Of 15 clones sequenced, there was only a single unique sequence discovered, suggesting the presence of a single *epg* gene in *O. novo-ulmi*. This amplification product was used to retrieve the full-length gene from a lambda library. Hybridization of the gene fragment to restriction enzyme digests of total genomic DNA indicated that the *epg1* gene corresponding to the amplified DNA fragment in *O. novo-ulmi* exists as a single copy. The DNA sequences of the entire 1140 base pair coding region of the *epg1* gene locus plus 370 base pairs of upstream sequence putatively identified as the promoter, and 244 base pairs of downstream sequence, were determined (Genebank accession AF052061). The amino acid sequence corresponding to the major uninterrupted reading frame was predicted by the primary nucleotide sequence (Fig. 1). The putative promoter sequence shared characteristics with many other fungal promoters. Filamentous fungi show less of a requirement than mammalian genes for the CAAT and TATA boxes, and generally exhibit more variation in the position of elements involved in initiation of transcription (Ballance 1986). The endoPG sequence of *O. novo-ulmi* showed a putative TATA box at –30. Areas of pyrimidine-rich sequence characteristic of fungal promoters were also found in the upstream region. The coding sequence started at position 1 with the characteristic ATG codon and ended at 1140. There were no frame-shifts in the sequence and

consensus splice sites characteristic of fungal introns were not found. The TCCAAA**ATG**CTG sequence immediately surrounding the proposed start site was similar to the TCA[C/A][A/C]**ATG**[G/T]C consensus for filamentous fungi proposed by Balance, 1986. The predicted protein sequence was 390 amino acids long. The putative active site was at 231 to 241 and the peptide sequence of CXGGHGXSIGSVG at position 239-251 reported by Reymond et al., (1994) to be characteristic of polygalacturonase was in 100% agreement with the *O. novo-ulmi* predicted peptide sequence. Multiple sequence alignment showed close segregation with other fungal endoPG proteins, with the closest relation to the endoPG of the fungal phytopathogen *Alternaria citri* (Fig. 2).

Targeted disruption of the epg1 gene

Disruption of *epg1* in *O. novo-ulmi* was accomplished by homologous recombination at the authentic *epg1* locus with a DNA construct incorporating a selectable marker into the central portion of the fungal gene. Recombination at the authentic site would lead to the insertion of a total of 3588 bp comprised of the hygromycin phosphotransferase gene *(hph)* driven by the *A. nidulans gpd* promoter (Punt et al. 1987). Incorporation of the disruption cassette at the authentic *epg1* locus would simultaneously provide a dominant selectable marker and prohibit translation of complete EPG1 by gene interference. Construction of a disruption vector based on *hph* with flanking DNA homologous to *epg1* was accomplished by amplification from the *epg1* gene with primers tailed to correspond to unique sites flanking *hph* (Fig. 3). Disruptions were confirmed

using a PCR-based strategy for which a PCR product resulted only in the presence of the correctly integrated disruption vector (Fig. 4). Neither the disruption vector, nor the authentic gene would produce an amplification product. The amplification product produced from the *epg1*⁻ strain was recovered, cloned and sequenced to verify accurate disruption, which showed disruption occurred at the expected locations within *epg1*⁻. Targeted disruption of *epg1* led to nearly complete reduction of pectinase activity *in vitro* as shown by pectinase assays followed by Ruthenium red staining (Fig. 4). Disruption prevented growth of *epg1*⁻ on media containing pectin as the sole carbon source.

Virulence trials on Ulmus parvifolia x U. americana

Inoculations of two-year old seedlings of clonally propagated *Ulmus parvifolia* x *U. americana* F1 hybrid clone 2245 with wild type VA30 and *epg1*⁻ mutant showed that the onset of wilt symptoms in the *epg1*⁻ strain was slower and less severe compared to VA30, which showed rapid onset of symptoms and wilting by six d post inoculation (data not shown). Trees were assessed for the extent of defoliation at 22 d post inoculation and *epg1*⁻ was found to produce a lower mean percent defoliation ± standard deviation (54.5 ± 38.5) compared to that of the wild type control VA30 (81.9 ± 34.5). Water controls demonstrated no wilt symptoms at six d post inoculation and showed a mean percent defoliation of 13.1 ± 11.3 at 22 d post inoculation, which is within the expected normal range for water controls. In order to statistically compare the mean percent defoliations, a non-parametric STP test based on the Wilcoxon-Mann-Whitney statistic was used and

the analysis found that VA30 showed significantly higher levels of mean defoliation relative to the water control. However, *epg1*$^{-}$ showed no significant differences between the water control and the wild type VA30 suggesting that *epg1*$^{-}$ produces an intermediate phenotype on the elm clone tested (Fig. 5).

Discussion

The activities of cell wall degrading enzymes secreted by phytopathogenic fungi have an effect on the nature of host-pathogen interactions. The activity of endoPG within the host tissue may be very important to the initiation of host defenses (Boudart et al. 1995). Responses can include the release of phytoalexins or other elicitors and instigation of the hypersensitive response. The specific activity of EPG1 produced by *O. novo-ulmi* may, in turn, also be influenced by elicitation of elm defenses. Polygalacturonases that differ in their ability to elicit phytoalexin accumulation have been suggested to have a role in limiting excessive stimulation of host defenses (Favaron et al. 1993). A highly active enzyme will instigate host response at a critical stage of infection, while a more slowly acting enzyme may delay onset of host responses long enough to provide a better chance of host colonization. Diversification of function may result from post-translational modification to a single gene product. For example, glycosylation is related to biological function of PG and to avoidance of host defense elicitation in *Fusarium moniliforme* (Caprari et al. 1993). The *pg* gene was demonstrated to be present in a single copy in *F. moniliforme* but four different PG proteins were derived from this gene. Each of these forms appeared to be glycosylated differently, possibly accounting for the differences in molecular mass. The secretion of endoPG by *F. oxysporum* was abolished by application of the glycosylation inhibitor tunicamycin, suggesting N-glycosylation is a requirement for secretion in this fungus. A single potential N-linked glycosylation site exists at position 155-159 of the predicted protein sequence

derived from the *O. novo-ulmi epg1*. The potential glycosylation site in the *O. novo-ulmi* EPG1 may be related to biological function and secretion of the protein. Glycosylation may have an important role in secretion of the enzyme, and in modulating the relative activity of EPG1 in the host tissues; additionally, the activity of *O. novo-ulmi* EPG1 in host tissues may relate to recognition in host-pathogen interactions. This could be determined by altering expression of glycosylation genes in the pathogen and correlating N-glycan status of the EPG1 to virulence.

Pectinolytic enzymes appear to function as virulence factors in *Ophiostoma*, as the disruption of *epg1* in *O. novo ulmi* gave rise to a phenotype with an intermediate level of virulence. Thus, while there may be a direct role for EPG1 in contributing to wilt symptoms in infected elms, expression of *epg1* may also be linked to host invasion. It is likely that infection of *Ulmus* species by *O. novo-ulmi* requires degradation of cell wall polymers through secretion of several cell wall degrading enzymes (Svaldi and Elgersma 1982). The dimorphic *O. novo-ulmi* exists primarily as a budding yeast-like cell in xylem tissue and it appears that the fungus must switch to mycelial growth for pit membrane penetration. The expansion of the fungus into xylem vessels may rely on pectinolytic activity. Virulence of *O. novo-ulmi* isolates has been correlated to glycosidase and exo-glycanase activities. When compared to the less aggressive *O. ulmi*, highly virulent isolates of *O. novo-ulmi* released more cell wall degradation byproducts from the cell walls of elm wood (Svaldi and Elgersma 1982). Electron

microscope analysis of elm infected by *O. novo-ulmi* showed that highly virulent isolates produced significant cell wall erosion. This erosion was not limited to the area directly underneath fungal hyphae or conidia and was possibly due to diffusion of secreted pectinolytic enzymes (Scheffer and Elgersma 1982). Disruption of *epg1* may also affect recognition between the host and pathogen. Normally, polygalacturonases rapidly reduce pectin fragments to smaller biologically inactive fragments that would not be recognized by the host, but without the action of EPG1, these fragments may persist longer and elicit defense response in the host leading to the observed phenotype of *epg1*$^-$ in the virulence trials presented in this study. Similarly, polygalacturonase inhibiting proteins have been isolated from a variety of plants and have been suggested to function by slowing the activity of polygalacturonases thus giving the host a greater chance to recognize elicitors (Cook et al. 1999).

Although no correlation was observed between PG production *in vitro* and virulence in four strains of *O. ulmi* (Elgersma 1976), other studies have reported aggressive isolates (eventually reclassified as *O. novo-ulmi*) had increased capacity to destroy the cell wall of host plants (Svaldi and Elgersma 1982). There appeared to be only one functional copy of the e*pg1* gene present in *O. novo-ulmi,* but other pectinase activities may be present. Isolates with a disrupted *epg1* gene were not able to grow on pectin-based media but the *epg1*$^-$ strain still showed some limited pectinolytic activity on assay plates. Disruption of the *enpg-1* gene of *Cryphonectria parasitica* revealed PG activity *in planta* previously undetected

in vitro (Gao et al. 1996), so the presence of additional pectinolytic activity in *O. novo-ulmi* is not particularly surprising. Pectinolytic genes in other fungi have been disrupted with varying results during *in vitro* growth. For example, disruption of the *pgn1* gene of *C. carbonum* (Scott-Craig et al. 1990), the *Bcpg1* gene in *B. cinerea* (ten Have et al. 1998), and the *Aapg1* gene of *A. alternata* (Isshiki et al. 2001) did not halt growth of these fungi on media in which pectin was the only carbon source. Disruption of a polygalacturonase gene has in one case led to a phenotype of reduced growth on pectin supplemented media. Disruption of the *Acpg1* gene in *A. citri* led to the pathogen being almost totally unable to grow on media where pectin was the sole carbon source (Isshiki et al. 2001).

The activity of cell wall degrading enzymes may play an important role in invasion of the host and therefore *epg1* in *O. novo-ulmi* may contribute the parasitic fitness of the fungus. Polygalacturonase may also function to increase the fitness of other fungi. For example, isolates of *F. oxysporum* over-expressing endoPG were more competitive *in vitro* on pectin media compared to PG deficient (wild type) isolates. Additionally, *A. flavus* has been shown to require the pectinolytic enzyme PC2 for effective spread within cotton bolls (Brown et al. 1992). Because pectinolytic activity can lead to recognition of pathogens by host plants, the temporal and spatial expression of these enzymes in pathogens must be carefully balanced. When comparing the parasitic fitness of *O. novo-ulmi* to *O. ulmi*, it is important to note that *O. ulmi* caused a significant epidemic in North

American elm populations and cannot really be considered a weak pathogen. However, relative to *O. novo-ulmi*, which appears to be replacing *O. ulmi* in natural populations (Hintz et al. 1993), *O. ulmi* appears to have less parasitic fitness (Temple et al. 1997). In this regard, production of EPG1 and other pectinolytic enzymes may have relevance as not only as virulence factors, but also as parasitic fitness factors. According to the definitions of Shaner et al. (1992) and Andrivon (1993), parasitic fitness is the ability of an organism to survive and reproduce within a pathogen population. *Ophiostoma novo-ulmi* shows superior bark colonization relative to *O. ulmi* (Webber and Hedger 1986). Survival of aggressive isolates has been shown to be better than survival of non-aggressive isolates in both resistant and susceptible elm cultivars (Scheffer and Elgersma 1982). As disruption of *epg1* led to a delay in onset of wilt symptoms, our results suggest that *epg1* is involved in establishing infection in elm. Thus, the increased fitness of *O. novo-ulmi* relative to *O. ulmi* may include a more vigorous selection of cell wall degrading enzymes, such as PG.

There has been a correlation between cell wall degradation and virulence in the Dutch elm disease pathogens (Scheffer and Elgersma 1982; Svaldi and Elgersma 1982) however the precise role of individual enzymes has not been determined. Disruption of *epg1* in *O. novo-ulmi* permitted testing of the role of the enzyme *in planta*. Of the phytopathogens shown in Fig. 2, the predicited protein sequence of *O. novo-ulmi* is most closely related to *A. citri*, the causal agent of *Alternaria* black rot. Disruption of *O. novo-ulmi epg1* suggested a role for this enzyme in

pathogenicity on elm; similarly, disruption of *Acpg1* in *A. citri* significantly reduced black rot symptoms on citrus (Isshiki et al. 2001). Results from *in planta* studies must be interpreted cautiously as the effects of disruption of a single gene and determination of the subsequent effects on virulence in a fungus such as *O. novo-ulmi* is difficult to predict due to the almost certain polygenic nature of pathogenicity. However, disruption of *epg1* led to a phenotype intermediate in virulence between VA30 and water controls, and the *epg1*$^{-}$ strain showed a delay in onset of symptoms relative to VA30. This suggests that polygalacturonase has an important role in virulence and parasitic fitness in the Dutch elm disease pathogen

Figure 1. The genetic organization of *epg1* of *O. novo-ulmi*. The 379 amino acid predicted protein sequence is shown above the 1137 bp coding sequence. The two highly conserved areas of the protein sequence starting at 123 and 214 used for the design of degenerate primers are underlined. The active site, located at position 231 of the predicted protein sequence is underlined in bold. A putative glycosylation site at protein position 155 is shown in bold. The putative CAAT, TATA and consensus start sequence are underlined at -114, –30 and –4, respectively.

```
          -342   TCCCATATGGTCGACTGCCTCCTCATCTTCGATGGATACAGA -300
CTAGAAAAATTCCATCGCATCACAAAATCTGGCATGGGCAGTTCTTCGCTGGTGCAGCTG
TAGAAAGTATAAAGACGGCGGTCTTGGCCTTCGTCTTGGAAAGAAAAGAGAAGAGAGCAG
ACACAGCTCAGTGCACCGTCATACACATTCCTGTTCACTTCCAGTCGTTTCTGTTGATAT
TGATTCAATCACTTGCTATTCGTTTGTAAATACATTTCTTTTCATCACTATTTTATATTT
AATCTTTTACAAGTCTCTTTTCTTCATCTTTATATCTACAAGCCCTTTCGTTCTTCCAAA   -1

 M  L  G  I  T  T  L  L  L  T  L  A  V  A  A  N  A  S  P  V
ATGCTGGGCATCACTACTCTGCTACTGACCTTGGCGGTTGCCGCCAATGCTTCCCCTGTG   60

 V  A  P  A  N  T  P  T  P  S  P  V  L  A  E  R  A  T  S  C
GTAGCTCCCGCCAATACTCCGACGCCCTCCCCGGTCCTGGCTGAGCGTGCCACTAGCTGC  120

 T  F  S  G  A  N  G  A  S  L  A  I  Q  S  Q  A  A  C  A  T
ACCTTTTCTGGCGCCAACGGTGCGTCCTTGGCGATCCAGTCCCAGGCGGCCTGTGCTACT  180

 I  V  L  N  N  V  A  V  P  S  G  T  T  L  D  L  S  K  L  A
ATTGTCTTGAACAATGTTGCCGTGCCGTCAGGCACCACTCTGGATCTGTCCAAGCTCGCC  240

 D  G  A  T  V  I  F  E  G  E  T  T  W  G  Y  K  E  W  A  G
GATGGCGCGACTGTCATCTTTGAGGGCGAGACCACCTGGGGCTACAAGGAATGGGCGGGA  300

 P  L  L  Q  I  K  G  N  G  I  T  V  Q  G  A  S  G  A  V  F
CCCCTGCTGCAGATCAAGGGCAACGGTATCACCGTCCAGGGCGCGTCGGGTGCCGTCTTC  360

 N  A  N  G  A  R  W  W  D  G  K  G  S  N  S  G  K  T  K  P
AACGCCAACGGTGCTCGCTGGTGGGACGGCAAGGGCAGCAACAGTGGCAAGACCAAGCCC  420

 K  F  F  Y  A  H  G  L  T  N  S  K  I  L  N  L  S  I  K  N
AAGTTCTTCTATGCCCATGGCCTGACCAACTCCAAGATTCTCAACCTCAGCATCAAGAAC  480

 T  P  I  Q  A  V  S  I  N  G  C  D  G  L  T  I  T  D  M  T
ACACCCATCCAGGCTGTCAGCATAAACGGCTGCGATGGCCTCACCATCACAGACATGACC  540

 I  D  S  S  A  G  D  S  A  G  G  H  N  T  D  G  F  D  I  G
ATCGACAGCTCGGCCGGTGACAGCGCTGGCGGCCATAACACGGACGGCTTTGACATTGGC  600

 T  S  K  N  I  I  I  D  G  A  K  V  Y  N  Q  D  D  C  V  A
ACCAGCAAGAACATTATCATTGACGGTGCCAAGGTCTACAACCAGGATGACTGTGTGGCT  660

 V  N  S  G  T  K  I  T  F  Q  N  G  L  C  S  G  G  H  G  L
GTCAACTCAGGAACTAAAATCACCTTTCAGAACGGTCTCTGCTCGGGCGGTCACGGCCTC  720

 S  I  G  S  V  G  G  R  S  D  N  T  V  D  T  V  T  F  Y  N
TCCATTGGCTCTGTCGGTGGTCGCTCCGACAACACAGTCGATACCGTCACCTTTTACAAC  780

 S  Q  I  K  N  S  V  N  G  I  R  V  K  G  T  V  G  T  T  G
AGCCAGATCAAGAACTCCGTCAACGGTATCCGCGTCAAGGGCACCGTAGGCACCACCGGC  840

 T  I  K  G  V  T  Y  N  K  I  T  L  S  G  I  T  N  Y  G  V
ACCATTAAGGGTGTGACGTACAACAAGATCACGCTCTCTGGCATTACTAACTACGGTGTC  900

 L  I  E  Q  N  Y  N  G  G  D  L  K  G  T  A  T  S  G  V  P
TTGATCGAGCAGAACTACAACGGCGGCGACCTCAAGGGCACGGCGACCTCCGGTGTTCCT  960

 I  T  G  L  T  I  K  D  I  S  G  T  K  A  I  A  S  S  G  Y
ATCACGGGCCTCACCATTAAGGACATCTCAGGAACCAAGGCCATCGCGAGCTCCGGTTAC 1020

 N  V  V  I  T  C  G  S  G  A  C  S  G  W  T  W  S  G  V  S
AATGTCGTTATCACCTGCGGCAGTGGTGCTTGCTCTGGCTGGACTTGGAGCGGTGTTTCG 1080

 V  T  G  G  K  S  Y  D  K  C  T  N  V  P  N  G  I  S  C  #
GTGACGGGTGGCAAGTCCTACGACAAGTGCACCAATGTGCCTAACGGAATTTCCTGCTAA 1140

ATGCACACATACACACACATCCCCGAACCCGGACCAACCTAAAGGATATGTCTACCACTT 1200
CTTGTTGATCTTGTTTATGTTGCAATCGACAAATGTGTCATTGCATTTATATCGGCATTA
GTTAATTAATATAAATATAGACTATTTATCCTCATCTTGATCTCCTATTGCCAATGTTCA
GTGTCAACGCTTATTTTGCGGTTAATAGTTTTCTCTGGATTGGCAACGTCTTGATATTAA
TATAATAA                                                      1388
```

Figure 2. The predicted protein sequence of *O. novo-ulmi* EPG1 in alignment with selected fungal phytopathogen endoPG genes. The *O. novo-ulmi* gene segregated closely with *endo*-acting polygalacturonase genes of other fungi, suggesting a similar activity. Genebank accession numbers are shown in brackets.

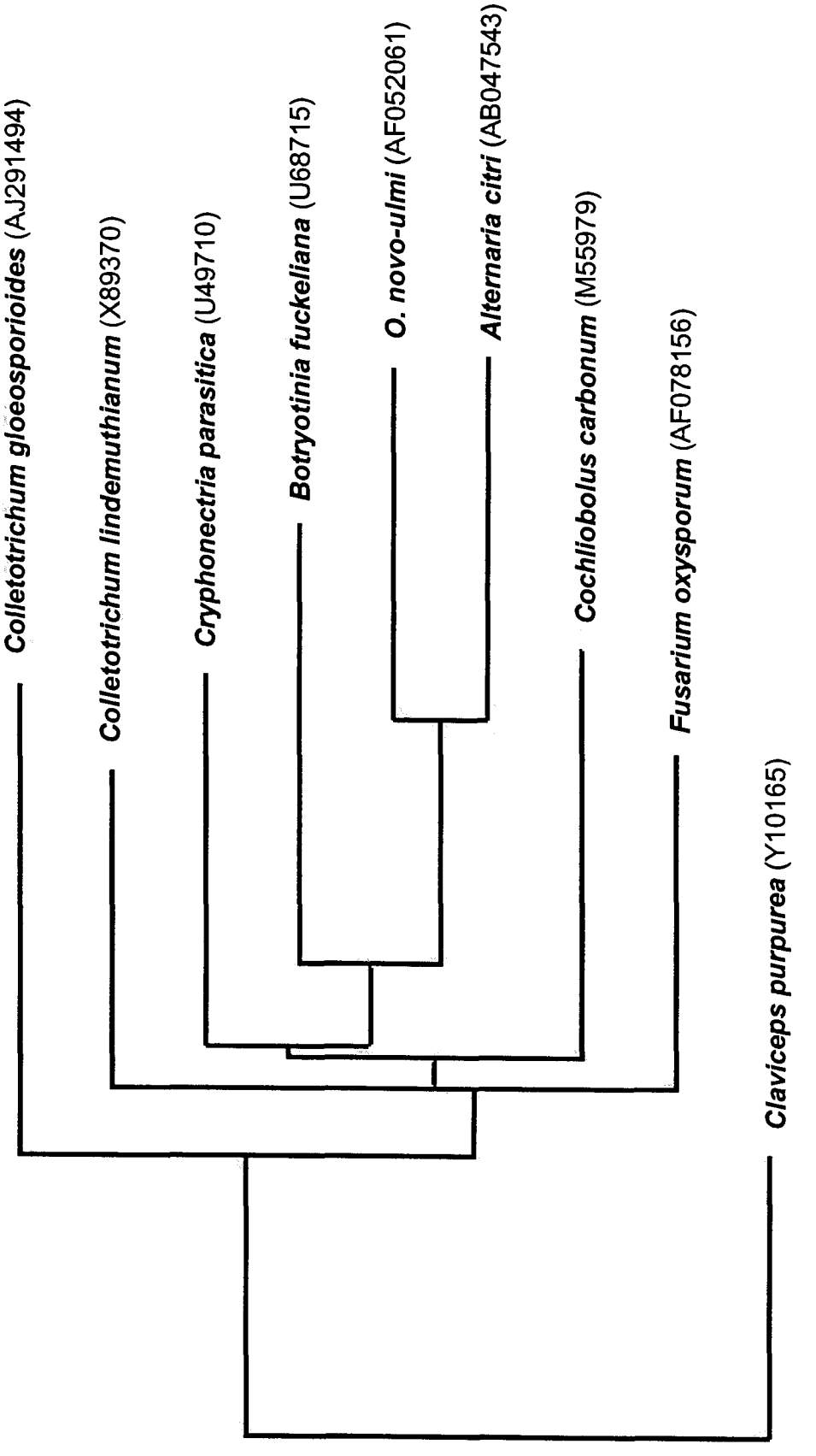

Colletotrichum gloeosporioides (AJ291494)
Colletotrichum lindemuthianum (X89370)
Cryphonectria parasitica (U49710)
Botryotinia fuckeliana (U68715)
O. novo-ulmi (AF052061)
Alternaria citri (AB047543)
Cochliobolus carbonum (M55979)
Fusarium oxysporum (AF078156)
Claviceps purpurea (Y10165)

Figure 3. Targeted disruption of *epg1* in *O. novo-ulmi*. Expression of the *O. novo-ulmi epg1* gene was disrupted by the insertion of the selectable marker for hygromycin resistance (*hph*) at the *epg1* locus. The *hph* marker was flanked with DNA amplified from the authentic *O. novo-ulmi epg1* locus and inserted into the disruption cassette. Gene disruption was achieved by the homologous recombination at the *epg1* locus. Putative *epg1* disruptants were screened using diagnostic PCR with a forward primer site located upstream of the disruption vector insertion site (*epg*DIAG), and a reverse primer seated within the *hph* gene disruption vector (*hph*DIAG).

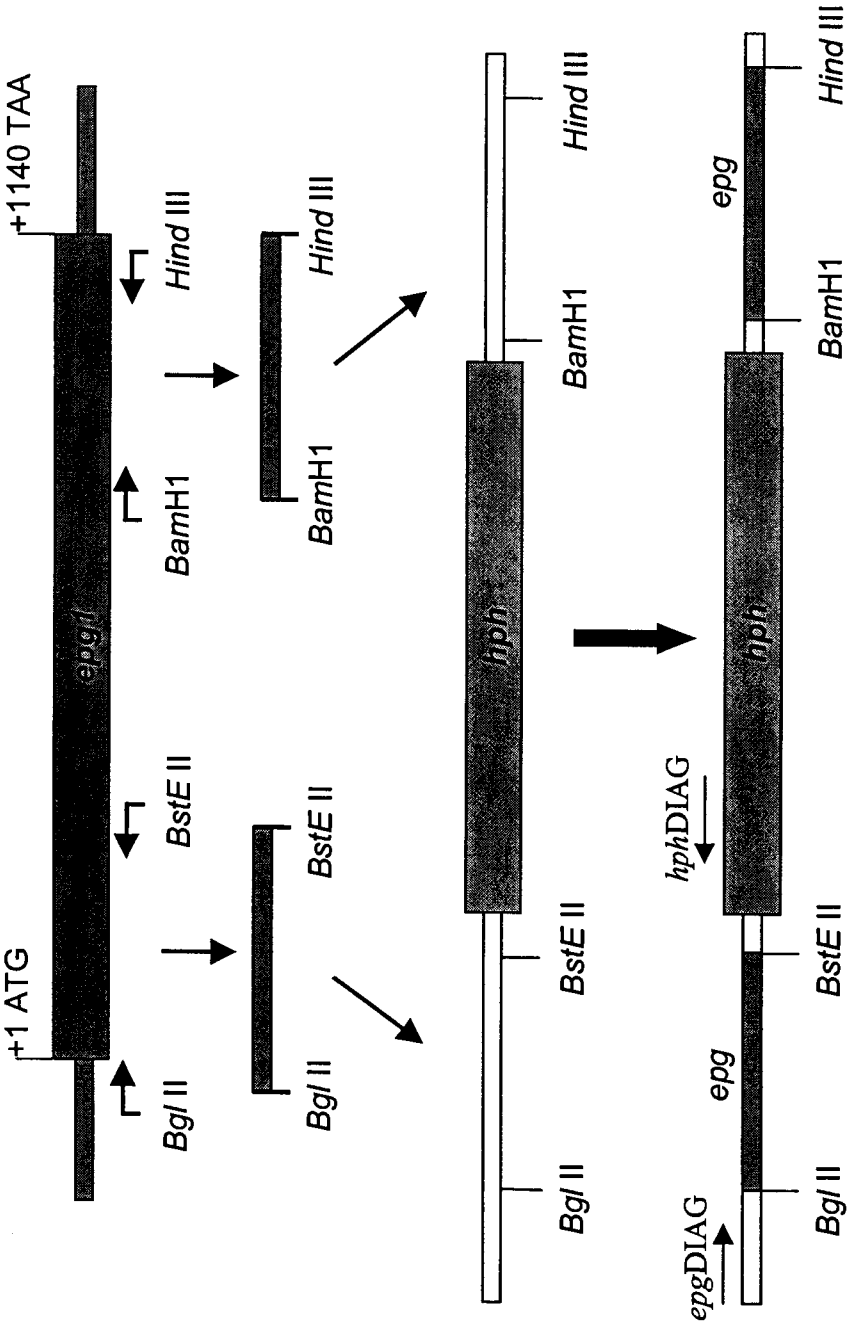

+1140 TAA
+1 ATG
epgI
Hind III
BamH1
BstE II
Bgl II
hph
epg
hphDIAG
epgDIAG

Figure 4. Characterization of EPG activity in *O. novo-ulmi* VA30 and *epg1*$^{-}$. Putative *epg1* disruptants were screened initially using diagnostic PCR with a forward primer site located upstream of the insertion site, and a reverse primer seated within the *hph* gene disruption vector. It was expected that this primer set would produce an amplification product only from disrupted genes. The product, separated by 1.0 % TAE gel electrophoresis, indicated by an arrow in the disruptant, *epg1*$^{-}$, was sequenced to confirm targeted disruption of *epg1* (A). Activity assays were used to confirm reduction of EPG1 activity in *epg1*$^{-}$. These activity assays were performed using 1 ml of cell free culture filtrate concentrated by freeze-drying. The resultant pellet was re-suspended in buffer and applied to a well in the assay plates, which were stained after 24 h using Ruthenium red (B).

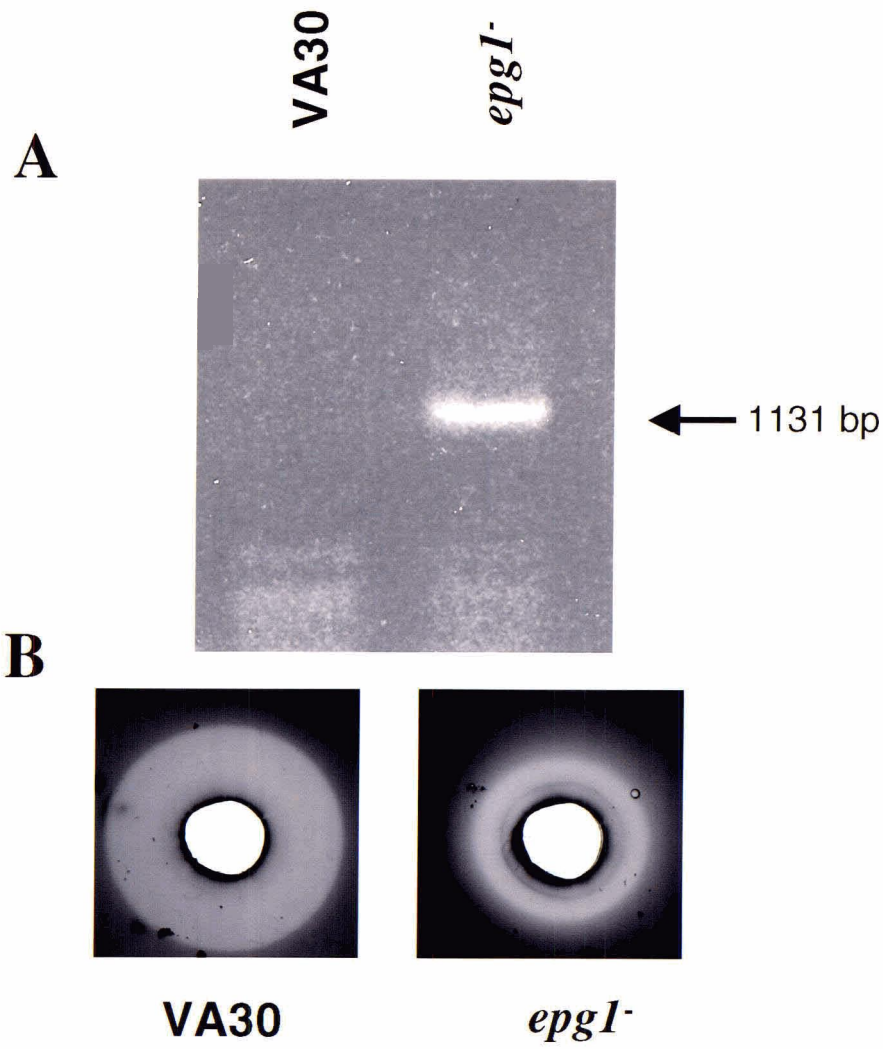
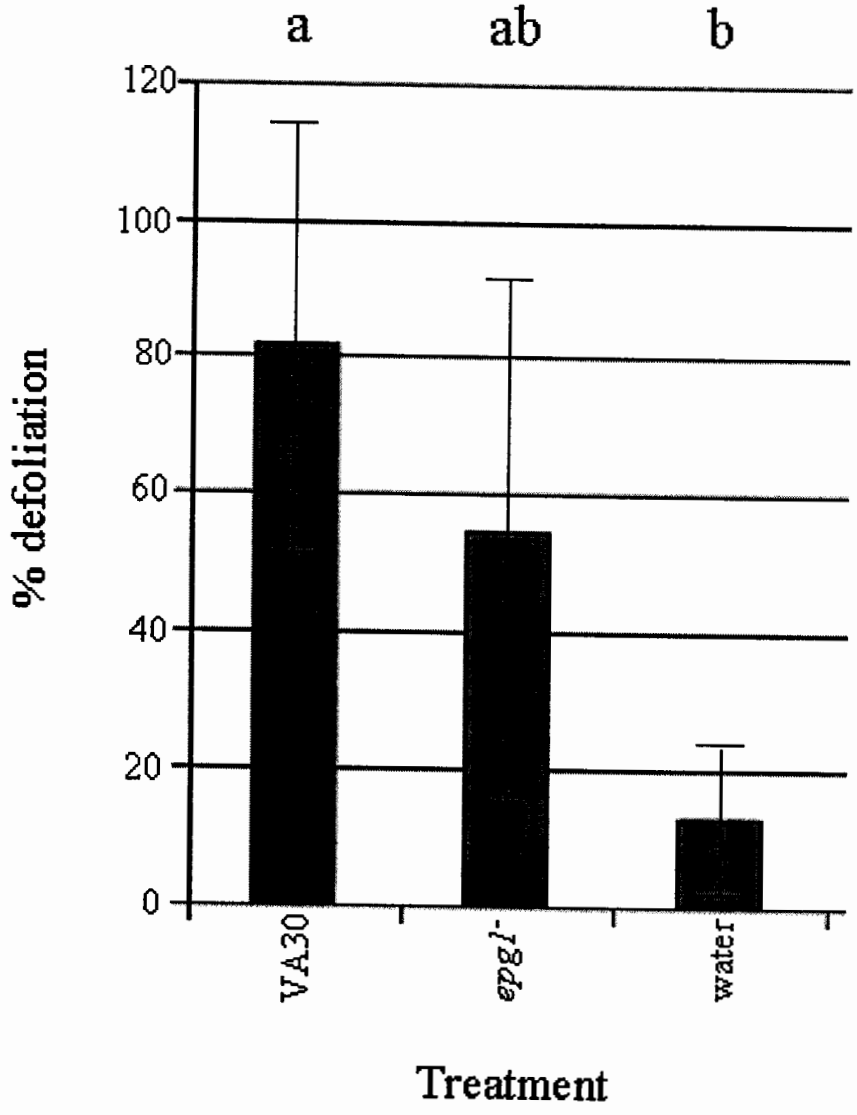

VA30
epg1-
A
1131 bp
B
VA30
epg1-

Figure 5. Percent defoliation in two-year old seedlings of clonally propagated *Ulmus parvifolia* x *U. americana* F1 hybrid clone 2245, 22 d post inoculation with *O. novo-ulmi* VA30, *epg1*$^{-}$ and water control. Trees were distributed in 8 randomly distributed blocks, with one treatment of each inoculation per block (n=8). Statistically different treatments, as determined by a non-parametric STP test based on the Wilcoxon-Mann-Whitney statistic, are designated by different letters at the top of the bars when differences are significant and by the same letter when no difference is detectable.


a
ab
b
120
100
80
60
40
20
0
% defoliation
VA30
epg1-
water
Treatment

CHAPTER THREE

Partial characterization of glycosylation mutants in *Ophiostoma novo-ulmi*, the causal agent of Dutch elm disease

Chapter summary

The prominent role of glycosylation genes in cell surface modifications makes these enzymes candidates for involvement in fungal pathogenesis in the causal agent of Dutch elm disease, *Ophiostoma novo-ulmi*. Two approaches to finding glycosylation genes in the pathogen were utilized. Random mutants of *O. novo-ulmi* were generated by insertional mutagenesis. Enrichment of mutants specific to glycosylation processes was accomplished by tritium ([^{3}H]) mannose suicide selection, followed by further screening for orthovanadate resistance and Alcian blue staining. The activity of the secreted pectinolytic enzyme polygalacturonase was assayed in the mutants to determine whether interference with glycosylation machinery would have a deleterious effect on the secretion or activity of glycoproteins produced by this pathogen. Several of the putative glycosylation mutants demonstrated a lack of pectinolytic activity without a corresponding decrease in mRNA expression of polygalacturonse. These mutants showed differences in their interaction with American elm callus tissue, suggesting that glycosylation is a factor in both normal cellular function and host-pathogen interactions in *O. novo-ulmi*. A second approach used reverse genetics techniques to characterize a gene from *O. novo-ulmi* that segregated with other fungal glycosyltransferases. The link between N-glycan status, glycoprotein secretion and virulence is detailed.

Introduction

Ophiostoma novo-ulmi Brasier and *O. ulmi* (Buisman) Nannf., the causal agents of Dutch elm disease, have destroyed much of the American elm stands in North America over the last century. *Ophiostoma novo-ulmi* is the more aggressive of the two pathogens and is replacing the less aggressive *O. ulmi* in natural populations (Hintz et al. 1993). Molecular examination of host-pathogen interactions between *O. novo-ulmi* and elm have been focused on finding specific virulence factors that modulate the relationship between plant and pathogen. An alternate approach was undertaken in this study by searching for associations between glycosylation, virulence and recognition between host and pathogen, with particular focus on fungal glycosyltransferases and their effects.

It has been proposed that glycosylation genes have important roles in the virulence of human pathogenic fungi (Fernandes et al. 1999; Southard et al. 1999; Buurman et al. 1998); however, their role in phytopathogens has not yet been explored. Given that all fungi express glycosyltransferases, and the prominent role glycosyltransferases have in modifying the cell surface, it is reasonable to suggest a role for these enzymes in host-pathogen interactions in plant diseases. There are several possible ways that glycosyltransferases may serve to regulate these interactions. Glycans may be involved directly in the suppression of the host plant defense responses (Basse et al. 1992). Glycosyltransferases could function to directly create or mask elicitors by the addition of sugar residues, or

act indirectly by modification of sites recognized by other glycosyltransferases (Keen 1982). The activity of glycosyltransferases in the pathogen may also influence the secretion and activity of enzymes and other molecules that mediate interactions with the host.

The possibility that glycosylation may be integral to virulence has prompted us to search for glycosyltransferases in the Dutch elm disease pathogen, *O. novo-ulmi*. Given the unusual presence of cellulose in the cell walls of *O. novo-ulmi* (Benhamou 1988), it is possible that glycosyltransferases not usually found in fungi may exist. Thus, the identification of glycosyltransferases in *O. novo-ulmi* by insertional mutagenesis appeared to be the most appropriate approach to identification of these genes in the pathogen. Glycosyltransferase genes in *Ophiostoma* would be of considerable interest, as mutations in these genes may impair *in vivo* function to an extent that would permit the role of glycosylation in virulence to be determined. We have isolated several putative glycosylation mutants using a [^{3}H] mannose suicide technique. These mutants showed differences in cell surface qualities, impaired polygalacturonase secretion and interacted differently with elm callus tissue when compared to wild type isolates. Cloning of specific glycosyltransferases, specifically those homologous to other fungal mannosyltranferases, can now be undertaken to detail the role of specific enzymes in host-pathogen interactions. The relevance of these results in relation to virulence and parasitic fitness is discussed.

Materials and Methods

Isolates, culture conditions and tissue culture of Ulmus americana

Working cultures of *O. novo-ulmi* VA30 (isolated by L. Schreiber and A. Townsend, Virginia) were grown on solid *Ophiostoma* complete media (OCM) (Bernier and Hubbes 1990), or in liquid OCM in flasks (either as static cultures or with agitation at 150 rpm) at 22 °C. Cultures were maintained at 4 °C for short-term storage. Fungal stock cultures were kept at –196 °C or colder in liquid nitrogen, following infusion with either 15 % glycerol or 10 % DMSO. Clonal *Ulmus americana* LA-1 elm tissue cultures were derived from 5 mm wide sections of newly flushed leaves, that included a piece of main vein, after surface sterilization for 20 min in 1.0 % bleach. Explants were maintained on solid Murashige and Skoog (MS) media (1962) to which was added, per litre, 30 g sucrose, 2 mg thiamine HCl, 100 mg myo-inositol and 6 g agar adjusted to pH 5.6 and supplemented with 0.1 μM thidiazuron (Bolyard et al. 1991). Once formed, callus tissue was maintained on the same media, and subcultured every six weeks until use in virulence assays. For virulence assays, callus tissue that had been in culture for one year was used.

Nucleic acid extraction

Cultures for DNA extraction were grown in stationary cultures for 7 d in 5 ml of liquid OCM at 23 °C. Mycelia were harvested, freeze-dried and ground into a fine powder. Two hundred mg of mycelia were re-suspended in 0.5 ml of 50 mM EDTA, 0.2 % SDS, mixed and incubated for 20 min at 65 °C. Samples were

centrifuged to remove cell debris and protein was precipitated on ice with 1/6 volume 3.0 M potassium acetate, 5.0 M acetic acid. After removal of the supernatant to a fresh tube, an equal volume of isopropanol was used to precipitate the DNA. The DNA was then re-suspended in 500 µl of 10 mM Tris-HCl (pH 7.5), 5.0 mM EDTA (TE), treated for 30 min with ribonuclease (100 µg per sample), extracted once with an equal volume TE-saturated phenol and extracted twice with equal volumes of chloroform: isoamyl alcohol (24:1). The DNA was then precipitated from the aqueous phase with 1/10 volume of 3 M sodium acetate (pH 5.3) and 2 volumes of 95 % ethanol and washed once with 70 % ethanol. The DNA pellet was then air-dried and re-suspended in 100 µl water. Total RNA for dot blot analysis and cDNA synthesis was extracted from 200 mg of mycelia using Trizol reagent (Gibco Industries, Inc., Langley, OK, USA) as per manufacturer's instructions.

Insertional mutagenesis of O. novo-ulmi and selection of mutants

The *O. novo-ulmi* gene loci were disrupted via random ectopic recombination at the gene locus by pAN7-1, a vector that contains the hygromycin phosphotransferase gene (*hph*) marker (Punt et al. 1987). Protocols for protoplast formation and transformation of *O. novo-ulmi* were as previously detailed in Temple et al. (1997). [^{3}H]-mannose suicide enrichment was used to enrich for mutants with impaired glycosylation in a procedure adapted from Newman and Ferro-Novick (1987). Mutants were released from hygromycin selection plates (200 µg hygromycin per ml) by flooding with liquid OCM and then grown for 48

h in 50 ml OCM at 22 °C with shaking. Cells were sedimented by centrifuging at 2000g for 5 min and re-suspended in 0.1 ml of OCM with 0.1 % sucrose and 1 mCi of [^{3}H] mannose for 5 h. After incubation, the cells were washed 4 times with 5 ml cold, sterile distilled water followed by re-suspension in 2 ml of STC buffer and frozen at –70 °C for 90 d. After 90 d, glycosylation mutants were selected from the pooled mutants by plating the cells on OCM supplemented with 10 mM sodium orthovanadate and selecting resistant isolates. These isolates were further screened by culturing on OCM agar for 3 d, followed by flooding the plates for 20 min with 0.05 % Alcian blue stain which preferentially binds to cells with high mannose structures (Sigma-Aldrich; Oakville, ON, Canada). The stained plates were washed over a one-hour period by flooding with three changes of sterile water. Three colonies that stained less intensely with Alcian blue stain, relative to the wild type, were chosen for further study. The three mutants selected for further analysis were examined using hybridization analysis (Southern 1975) of total genomic DNA to determine copy number of the pAN7-1 integrative plasmid.

Analysis of epg1 activity and cerato-ulmin production in glycosylation mutants

To determine the level of EPG1 secreted in wild type and mutant isolates, *O. novo-ulmi* was inoculated into 5 ml liquid OCM and grown as static cultures at 22 °C for 7 d. Assay plates were prepared containing 50 mM potassium acetate, 5 mM EDTA, 0.1 % polygalacturonic acid and 1 % agarose. After solidification, a cork bore (5 mm diameter) was used to remove agarose plugs and create wells.

One ml of culture filtrate was lyophilized overnight. The resultant pellet was re-suspended in 0.1 ml 50 mM potassium acetate, 5.0 mM EDTA (pH 4.5) and applied to the well on assay plates. After 24 h of incubation at room temperature, the undigested polygalacturonic acid remaining in the plates was stained for 20 min using 0.05 % Ruthenium red (Sigma) and washed with three changes of distilled water. The activity of EPG1 was characterized by visually assessing the staining, or lack thereof, in the halo surrounding the wells. Dot blot analysis of total RNA from glycosylation mutants and the wild type VA30 was carried out as per Sambrook and Russel (2001). For hybridization analysis of mRNA, a ^{32}P-dCTP (Easytides, Perkin Elmer, Wellesley, MA, USA) PCR labeled *epg1* fragment derived using oligonucleotide primers OUEPGF and OUEPGR that incorporated approximately 15 μCi of activity was used. The sequence of the primers used was: OUEPGF: 5' GGCGCTCGCTGGTGGGACGGCAAGGG 3' and OUEPGR: 5' GGAGTTAATGGCGAGGCAGTCGTCCTGGTT 3'. To further characterize mutant isolates, cultures were assayed for the production of cerato-ulmin, a feature common to all *O. novo-ulmi* isolates, except for two NAN isolates discovered in Europe (Takai, 1974; Brasier et al. 1995). Cultures for cerato-ulmin isolation were grown in as static cultures in 5 ml of liquid OCM at 22 °C for 7 d. One ml of culture filtrate was then sedimented by microcentrifuge for 3 min to remove cells and cell debris. The clear filtrate was then shaken in 15 ml test tubes at 200 rpm for 30 min and assessed for turbidity characteristic of cerato-ulmin production (Bowden et al. 1996).

Virulence trials with U. americana callus tissue

A one-year old line of clonal *U. americana* LA-1 callus tissue was used in virulence trials that included wild type control VA30 and three *O. novo-ulmi* glycosylation mutants derived from [^{3}H] suicide enrichment, orthovanadate selection and Alcian blue screening. Six replicates were used in each treatment, all grown on solid MS supplemented as per Bolyard et al. (1991). One cm diameter callus tissue was inoculated with 5 µl of cell suspensions of *O. novo-ulmi* yeast-like cells. Yeast-like cells were obtained by growing *O. novo-ulmi* cells in 5 ml OCM with shaking at 150 rpm of for one week, followed by sedimentation of cells by microcentrifuge, and washing twice with sterile distilled water. Yeast-like cells were then re-suspended to 1000 cells per µl and then used to inoculate callus tissue. Inoculated and control tissue cultures were maintained at 22 °C and assessed after 3 weeks for growth of the fungus and for unusual fungal and tissue culture morphology.

Derivation of an mnn9 specific probe

Oligonucleotide primers for polymerase chain reaction (PCR) were designed according to conserved regions deduced from sequence alignments of *mnn9* genes from other ascomycetes. A codon frequency chart, derived by the compilation of highly expressed genes from *O. novo-ulmi*, was used to predict the expected sequence in *O. novo-ulmi* and reduce the redundancy of the primers. Two primers were designed corresponding to the highly conserved amino acids DEILLEGY for the forward primer OUMNT1F and ETEGFAKM for reverse primer OUMNT1R.

The sequence of the primers used were: 5' GA(C/T)GAGATCATCGT(C/G)GAGGG(A/T/C)TA(C/T)GC 3' (OUMNT1F) and: 5' GCCATCTTCGCGAA(A/T/G)CCCTC(A/C/G)GT(C/T)TC 3' (OUMNT1R). The target genomic DNA (10-50 ng) was amplified using 200 pmol each of the primers OUMNT1F and OUMNT1R, 2 units of Taq polymerase (Pharmacia) and 0.1 μM each of dATP, dCTP, dTTP, and dGTP in a 50 μl final volume containing the Pharmacia Taq polymerase buffer (Pharmacia, Uppsala, Sweden) at a 1X concentration. A total of 30 cycles of PCR were used for amplification reactions. All denaturing steps took place at 94 °C for 60 s; all primer extensions were for 120 s at 72 °C. The first 5 cycles of primer annealing took place at 50 °C for 90 s followed by 25 additional cycles at 60 °C. The amplification products were separated on a 1.0% agarose gel in 1 X TAE (0.04 M Tris-acetate; 1mM EDTA). The amplification products were excised from the gel, purified using the Wizard PCR prep kit (Promega, Madison, WI, USA), ligated into pGEM using the TA cloning system (Promega) and the resultant vector was transformed into *Escherichia coli* DH5α. DNA sequencing of the cloned PCR products identified the amplification products corresponding to fungal mannosyltransfersase (*mnt*) genes.

Genomic library screening and subcloning mnt1

Ophiostoma novo-ulmi MH75 genomic DNA was digested with *Mbo* I and ligated into bacteriophage lambda EMBL3 (Stratagene, La Jolla, CA, USA) as per manufacturer's instructions. *Escherichia coli* VCS257 (Stratagene) was

transfected with the resulting genomic library as described by Bowden et al. (1994). Hybridization analysis of the plaques was performed as per Sambrook and Russell (2001) using the 216 bp ^{32}P-dCTP (Easytides, Perkin Elmer, Wellesley, MA, USA) labeled *mnt1* fragment derived by PCR amplification using the oligonucleotide primers OUEPGF and OUEPGR that incorporated approximately 15 μCi of activity. Positive plaques were re-screened a second time and phage DNA was isolated using polyethylene glycol precipitation (Sambrook and Russell 2001). The position of *mnt1* was determined by restriction mapping of the isolated recombinant phage followed by hybridization analysis (Southern 1975). A single 4.0 KB *Sal* I fragment containing *mnt1* was subcloned into pUC18 and a 800 base pair fragment of the 3' end of the gene sequence was determined by automated fluorescent sequencing, using the LI-COR model 4200. The remaining sequence was obtained by constructing a genome-walking library (Clontech, Franklin Lakes, NJ, USA) as per manufacturer's instructions. Amplification and re-amplification of *mnt1* from the genome walking library were performed in two rounds as per manufacturer's instructions using two nested primers complementary the *O. novo-ulmi mnt1* gene, MNT1NEST1 (5' GGCGTACTCGGCGTAACCCTCAACAATGAC 3') and MNT1NEST2 (5' GGGTCTTCGTTCAGGGTATCGGCAAGGGC). An 1800 bp fragment was amplified and separated on a 1.0% agarose gel in 1 X TAE (0.04 M Tris-acetate; 1mM EDTA). The 1800 bp amplification product was excised from the gel, purified using the Wizard PCR prep kit (Promega), ligated into pGEM using the TA cloning system (Promega) and transformed into *E. coli*

DH5α for sequencing. The translation start site, coding sequence and potential introns of the gene were verified by synthesizing cDNA (Stratagene) as per manufacturer's instructions and using PCR primers complementary to the *mnt1* sequence to amplify and sequence transcript.

Sequence analysis and multiple sequence alignment

Nucleotide sequence alignments were carried out with GenBank data from http://www.ncbi.nlm.nih.gov/ using Generunner software (Ver. 3.05 Hastings Software, Inc.). The predicted protein sequence of the *O. novo-ulmi mnt1* was analyzed in alignment with selected fungal mannosyltransferase genes using neighbor joining analysis as provided by the web based ClustalW WWW Service (http://www2.ebi.ac.uk/clustalw) of the European Bioinformatics Institute (Higgins et al. 1994).

Results

A [^{3}H]-mannose suicide approach was used to enrich for glycosylation mutants of *O. novo-ulmi*. When grown in the presence of [^{3}H]-mannose, *O. novo-ulmi* incorporates tritium into the cell wall in amounts that can be potentially lethal over a long term. Isolates binding less [^{3}H]-mannose to the cell wall had higher survival rates during incubation, thus selecting isolates with reduced glycosylation. These mutants were demonstrated to have been resistant to orthovanadate and exhibit reduced Alcian blue staining, characteristics common in glycosylation mutants due to the altered cell wall structure (Ballou et al. 1991). Hybridization analysis of probable glycosyltransferase mutants suggested a single integration event in the three mutants chosen (data not shown). The pectinolytic enzyme, polygalacturonase (EPG1) was selected as a reporter for secretion and enzymatic activity in the putative glycosylation mutants. These mutants were deficient in secretion of the reporter protein EPG1 (Figure 1) yet dot blot analysis of total RNA showed that these mutants were producing similar amounts of *epg1* RNA relative to the wild type VA30, suggesting the N-glycosylation status affects the secretion or activity of EPG1. A second reporter protein, cerato-ulmin (CU), a hydrophobin produced by *O. novo-ulmi*, was secreted in normal amounts by all isolates. Interestingly, CU is not glycosylated, while EPG1 has one possible glycosylation site. These results combined indicate that isolated mutants were defective in glycosylation, rather than secretion. Similarly, proper secretion of polygalacturonase required correct glycosylation in *Fusarium moniliforme* (Caprari et al. 1993). In addition, yeast mutants with defects in glycosylation

showed secretion irregularities and instability in the biological life of proteins (Nagasu et al. 1992). It appears likely that protein secretion and stability in *O. novo-ulmi* has a similar dependence on glycosylation.

Three mutants were chosen for further study, however, it is uncertain which genes have been disrupted in these glycosylation mutants, only that glycosylation in these mutants is somehow impaired. On tissue cultures of clonally propagated *U. americana* LA-1, the three glycosylation mutants showed differences in interaction with callus tissue when compared to wild type VA30, the parent strain from which these mutants were derived. On callus tissue, the mutants were able to grow a profusion of synnemata (Figure 2), while such structures were never seen in the wild type control during the virulence trials. None of the isolates, including VA30, appeared to have inhibited growth on callus tissue, and all were able to grow to the point where the callus tissue was completely covered.

In many fungi, glycan structures are of a high-mannose type, composed of numerous, highly branched mannose polymers. By using sequence alignment of conventional yeast mannosyltransferases, which create α-linkages, we were able to isolate a single gene, *mnt1,* from *O. novo-ulmi* (Figure 3), which had homology to fungal mannosyltransferases, with the closest homology to the *mnn9* gene of *Candida albicans* (Figure 4). Degenerate primers, designed according to the conserved regions of *mnn9* genes from other fungi, were used to amplify a 216 bp fragment of the *O. novo-ulmi mnt1* gene. This fragment was used to recover the

downstream and coding sequence of the gene from a genomic library. A genome walking procedure was used to sequence the promoter. The DNA sequence of the entire 744 base pair coding region of the *mnt1* gene locus plus 1236 base pairs of upstream sequence putatively identified as the promoter, and 568 base pairs of downstream sequence, were determined. The 248 amino acid sequence corresponding to the major uninterrupted reading frame was predicted by the primary nucleotide sequence and confirmed by rtPCR. The putative promoter sequence shared characteristics with many other fungal promoters. Filamentous fungi show less of a requirement than mammalian genes for the CAAT and TATA boxes, and generally exhibit more variation in the position of elements involved in initiation of transcription (Ballance 1986). The *mnt1* sequence of *O. novo-ulmi* had a putative CAAT sequence at –88 but no TATA sequence was identified. In the upstream region of the putative promoter were areas of pyrimidine rich sequence characteristic of fungal promoters. The coding sequence started at position 1 with the characteristic ATG codon and ended at 783. There were no frame shifts in the sequence and one intron was found. The TGCAC**ATG**TT sequence immediately surrounding the proposed start site was similar to the TCA[C/A][A/C]**ATG**[G/T]C consensus sequence for filamentous fungi proposed by Balance (1986).

Discussion

There is increasing evidence that glycans have an important role in the suppression of host defenses (Doke et al. 1980; Yamada et al. 1989; Yoshioka et al. 1990). Analysis of interactions between potato tubers and *Phytophthora infestans* showed that water-soluble glycans were responsible for the suppression of the host hypersensitive response. Quantitative differences in glycan production were not noted between virulent and avirulent strains, which suggest qualitative reasons for the suppressor activity of the glycans produced by the virulent isolates (Doke et al. 1980). Analysis of elicitors on tomato showed that glycoproteins are important elicitors of the defense response in plants; however, once a glycan was cleaved from a protein, the glycan was capable of suppressing host defense responses (Basse et al. 1992). Thus, the possibility exists that glycans of *O. novo-ulmi* could function in the suppression of defense responses of the host.

The possibility that virulence and host-pathogen interactions may be governed by glycosylation can be related to the effect these processes have on the overall health of the organism. Glycosylation has a wide range of functions in the cell, and includes proper protein targeting, secretion and function. Glycosylation also influences the biological life and stability of proteins. Despite the fact that such functions may not directly contribute to virulence and thus should not be considered virulence factors, glycosyltransferases could still have an effect on pathogenicity, especially given the multitude of deleterious effects that may arise from the disruption of glycosylation pathways. These effects could include the

alteration of the cell wall, thus affecting recognition between *O. novo-ulmi* and the elm host. Additionally, the disruption of glycosylation pathways in *O. novo-ulmi* may well affect secretion and function of molecules that are directly involved in host-pathogen interactions, such as cell wall-degrading enzymes. The mutants characterized in this paper showed deficiency in the secretion of EPG1, a polygalacturonase secreted by *O. novo-ulmi*, and the enzyme chosen as a reporter for the mutant isolates characterized here. Previous work has suggested that polygalacturonase is an important virulence factor in *O. novo-ulmi* (Chapter 2). Thus, the disabling of the secretion mechanism of this enzyme might therefore have accounted for an overall effect on the virulence of *O. novo-ulmi*. In addition to the observed elimination of pectinolytic activity, a disruption of the glycosylation pathway in *O. novo-ulmi* could possibly have had broad effects that would in turn have had an impact upon other virulence factors.

Glycans may function indirectly to mask or create elicitors in the pathogen and therefore may affect the defense response of the host (Keen 1982). Differential glycosylation may correlate to differences in virulence in *O. ulmi sensu lato*. Due to the broad ranging effects of glycosylation in the cell, the genes encoding glycosyltransferases become candidates for avirulence genes in the pathogen. Mutations in mannosyltransferases of *Saccharomyces cerevisiae* and *Candida albicans* have resulted in the reduction of the number of mannosyl residues on attached N-glycans (Nagasu et al. 1992; Nakanishi-Shindo et al. 1993; Nakayama et al. 1992; Yip et al. 1994; Suzuki et al. 1996; Wang et al. 1997) and similar

effects would be expected in glycosylation mutants of *O. novo-ulmi*. Cloning of the *mnt1* gene of *O. novo-ulmi* will now permit determination of the role of this gene in virulence by using targeted gene disruption. Previous experiments have set some precedence for the specific involvement of glycosyltransferases in virulence. Invertases of *Phytophthora megasperma* from three differentially virulent races showed different carbohydrate structures suggesting that glycosylation patterns may have an effect on host specificity in host-pathogen interactions (Ziegler and Albersheim 1977). A glycoprotein in the cell wall of the wheat pathogen *Puccinia graminis* f. sp. *tritici* was found to be an elicitor (Kogel et al. 1988) and again suggests a possible role for glycosylation patterns affecting host-pathogen recognition. The profusion of synnemata produced by glycosylation mutants of *O. novo-ulmi,* as compared to the complete absence of such structures in the wild type control suggested that modification of glycosylation in the pathogen had affected host-pathogen interactions.

Although the correlation between how *O. novo-ulmi* acts on callus tissue as compared to whole trees is inexact in some cases, previous studies suggested that some interactions may be predicted using a tissue culture-based system (Corchete et al. 1993; Valle et al. 1997). Our results suggested that glycosylation mutants of *O. novo-ulmi* appeared to interact differently with American elm callus tissue when compared to a wild type isolate of the pathogen. Although synnemata production has been correlated with virulence in comparisons between aggressive and less aggressive isolates of *O. ulmi sensu lato* (Brasier 1991), it cannot be

conclusively said that altering glycosylation enzymes makes an isolate more or less virulent. Glycoproteins secreted by *O. ulmi* have been implicated as elictors of the elm defense response, again suggesting the possibility that glycosyltransferase genes may function as avirulence genes in the pathogen (Yang et al. 1994). The results presented in the current study demonstrate that host-pathogen interactions have changed as a result of the modification of glycosylation pathways in *O. novo-ulmi*.

Figure 1. Alcian staining, RNA dot blot analysis of *epg1* expression and EPG1 activity in VA30 wild type control and three glycosylation mutants (GM1-3) of *O. novo-ulmi*.

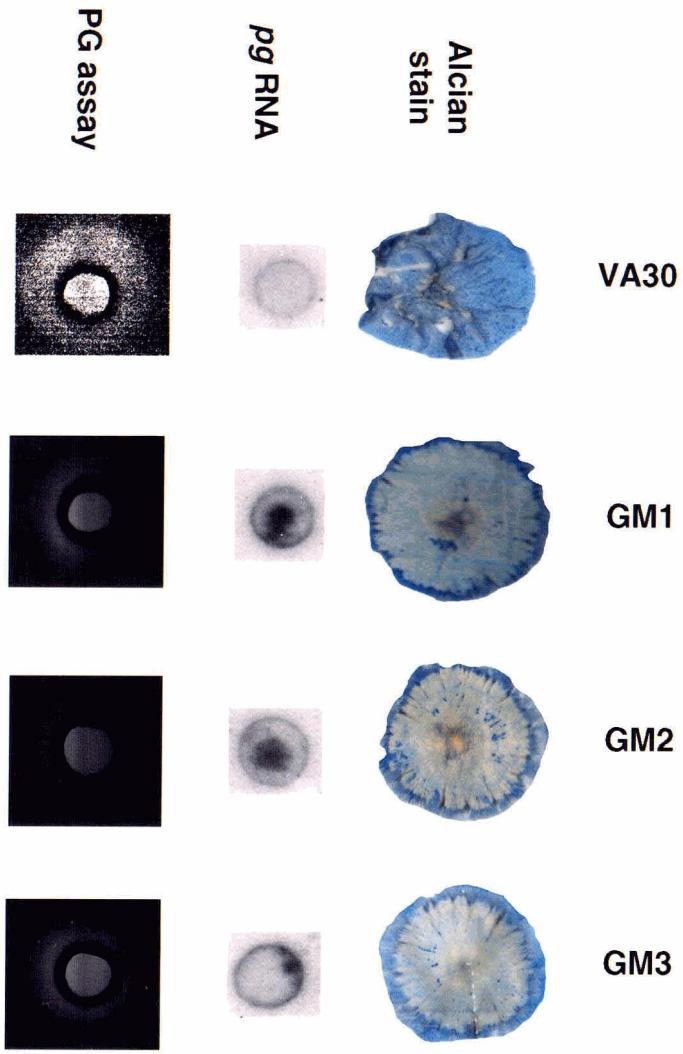
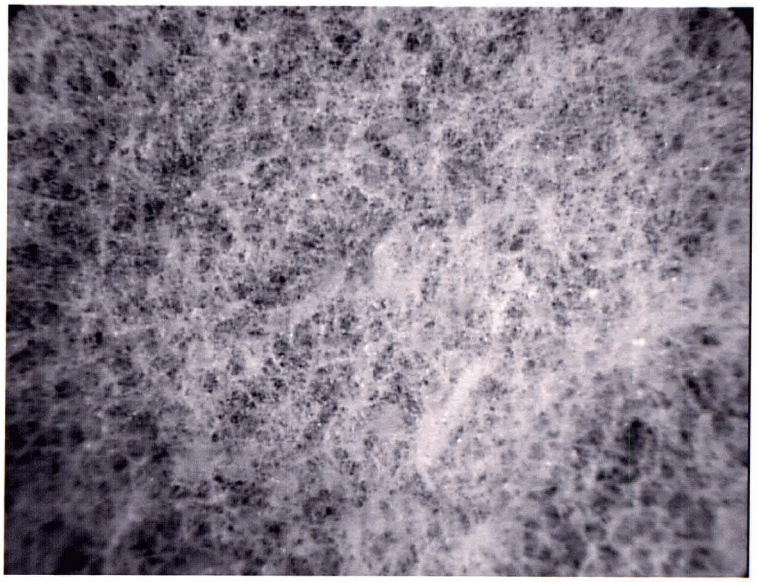

PG assay
pg RNA
Alcian stain
VA30
GM1
GM2
GM3

Figure 2. Analysis of the interactions between a one-year old line of clonal *U. americana* LA-1 callus tissue and glycosylation mutants of *O. novo-ulmi* after 3 weeks at 100X magnification. Typical results are shown with an arrow pointing to a group of synnemata produced by the glycosylation mutant.

VA30



Glycosylation mutant

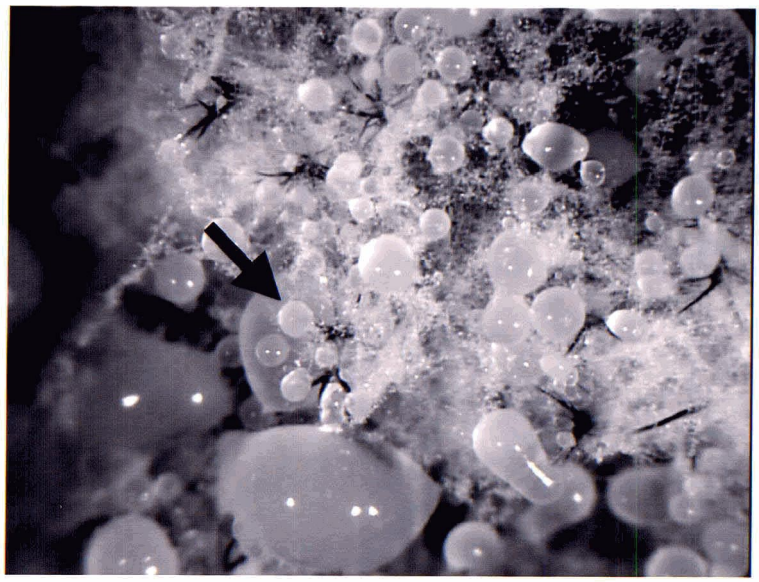
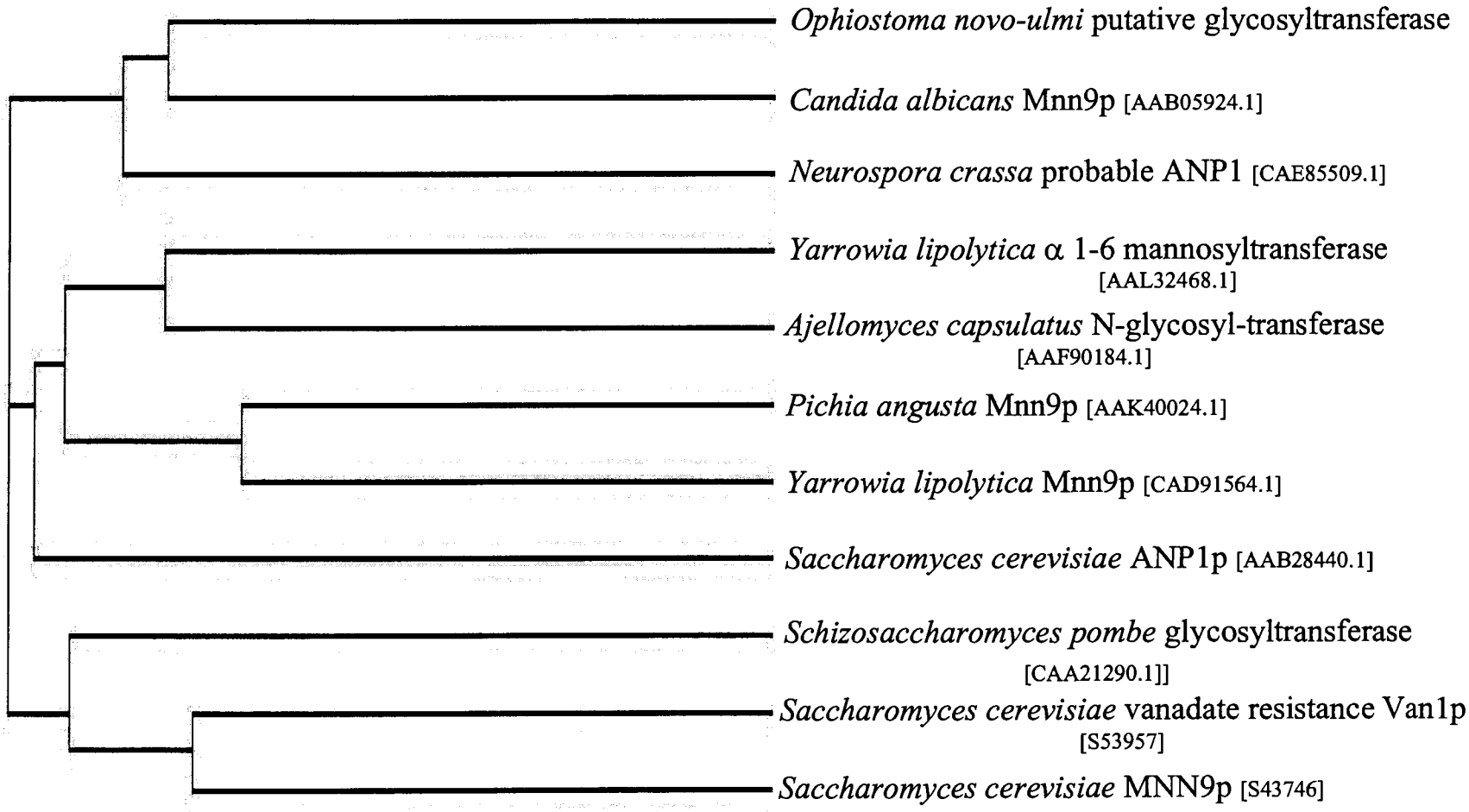


Figure 3: The genetic organization of the *mnt1* gene *of O. novo-ulmi*. The 248 amino acid predicted protein sequence is shown above the 744 bp coding sequence. The two highly conserved areas of the protein sequence used for the design of degenerate primers are underlined starting at position 184 and 249 of the predicted protein sequence. The putative consensus start sequence is underlined beginning at –4 and a putative CAAT sequence is underlined at –88.

```
-1236 ACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGTAAATATGCTATTATTTTAACTCCCCTGTCACCCCTA
GCCAGTGCTATCACCCTCTCCAAGCAACCTATCCTTTTCGTGTTCTGTTCCACTTAATACGAATGTGGCCGTCGTCGT
CGACATCCCATTTACCCTCACTCACGTCTTCAATCTGCTTCACGCCCCCTCTAAGCCTCAACTTTTCCTTGACGTCGT
TTCTCTCTCGATTTGTTTGCTGCTGCTTCTCTTCTCCCCTCTCCTTTATCCCCATTTCTTCATACAGTTTGAGACTCG
TTTGTCTGGGACTCCCGCACCGGACGGGTTGACACGCTGGTCCCTTTGTTTTCTCTTGCTTTTAGCTCCCCTACTCGT
TGAGCATACCCGCTACTCGTCGTTGGCTGCTATTTGGCTTGTCACTCCTATCTTTTTGGGCCACGACCCGCCTACTTC
CCGCGGTCTTCTCTTCAAATCGACCTCTACTGACCTCGACGAATCGACGAATCGACGACTACCTCGACGACTACCTTG
ACGATCACCTCGACCGCCGTCTAACCCCCCTAATTGATTCACCTGACATAGCGGTCAAGAGGGTGTCTCACCAAACTA
GACAGATCGAGACTCGGGACGACAAGACCACACAGACTGGCCTACGACTGGGGAGTAAGCTGTTGAGCATCGATTGCG
CACACTCGACGTCGTGCGTATCAACACCATCCCTACCTCCTATCTACACCTCCCTTTTCTCACCCACCCATCGCATGC
CATGTTGCCCTCGTACGCTGCGCCGGGGTCCAAGGTGACCCAAGACACAATGCCAACTCGGCGTCCTACTCCAATGGC
TACCCGCGAGGCTCCAACACACTAGAGTTCTCACCGCATCGGTATGTACTTTGTCGGCATCGTCCACCCGTCCACCTA
GAAATATAGGCTAACCTCCCAGCTTCCAACCTCGCAACTTTCCTCCCTTCGTGGCCCGGGCGGCGGAAGCTTTCACGCG
TCTCGGAATCGTCGTCGTCATCATCCTCTTTGCCGCCTTCGTCTGGCCCGCCGCCCCCGTGGCCTCGCTCATCTCCCT
TAGCTTCCTGACTGGCGGTGACCGCATCCAGCTCGAGACTGTGCGCTACTACGACTTGTCCAATGTCCAGGGTACTGC
GCGCGGTTGGGAGCGCGAGGAGCGCATTCTTCTGTGCGTGCCTCTTCGTGACGCCGAGACGCATCTGCAC - 1

 M  F  F  S  H  L  R  N  F  T  Y  P  H  H  L  I  D  L  A  F
ATGTTTTTCTCGCATCTGCGCAACTTTACCTACCCGCACCACCTCATCGACCTGGCCTTT   60

 L  V  S  D  S  K  D  H  T  L  Q  V  L  I  D  N  L  E  S  I
TTGGTTTCCGACTCCAAGGACCACACCCTTCAGGTTCTCATCGATAACCTCGAGTCCATT  120

 Q  G  D  V  D  A  A  E  H  F  G  E  I  S  I  I  E  K  D  F
CAGGGCGACGTCGACGCGGCCGAGCACTTTGGCGAGATCTCCATCATTGAGAAGGACTTT  180

 G  Q  K  V  N  Q  D  V  E  S  R  H  G  F  A  A  Q  A  S  R
GGCCAAAAGGTCAACCAGGACGTCGAGTCTCGCCACGGTTTCGCCGCTCAGGCCAGCCGT  240

 R  K  L  M  A  Q  A  R  N  W  L  L  S  A  S  L  R  P  Y  H
CGTAAGCTCATGGCTCAGGCACGCAACTGGCTTCTCAGTGCCTCACTGCGGCCCTACCAC  300

 S  W  V  Y  W  R  D  V  D  V  E  T  A  P  F  T  I  L  E  D
TCGTGGGTGTACTGGCGTGATGTCGATGTCGAGACTGCGCCCTTTACCATTCTGGAGGAT  360

 L  M  R  H  N  K  D  V  I  V  P  D
CTGATGCGCCATAACAAGGATGTCATTGTGCCGAGTAAGTTTTTCTCTCCTTGATGTTTT  420

                N  K  E  D  V  W  R  P  L  P  D  W  L  G  G
TTAAACACATGTACTAACAAAGAAGACGTCTGGCGTCCTCTTCCCGACTGGCTTGGCGGC  480

 E  Q  P  Y  D  L  N  S  W  Q  E  S  E  T  A  L  A  L  A  D
GAGCAGCCGTATGATCTAAACTCGTGGCAGGAGTCTGAGACCGCCCTTGCCCTTGCCGAT  540

 T  L  D  E  D  A  V  I  V  E  G  Y  A  E  Y  A  T  W  R  P
ACCCTGGACGAAGACGCCGTCATTGTTGAGGGTTACGCCGAGTACGCCACGTGGCGGCCC  600

 H  L  A  Y  L  R  D  P  Y  G  D  P  D  M  E  M  E  I  D  G
CATCTTGCCTACCTGCGCGACCCCTACGGCGACCCGGACATGGAGATGGAGATTGACGGT  660

 V  G  G  V  S  I  L  A  K  A  L  V  F  R  H  G  V  H  F  P
GTTGGTGGTGTCAGTATTTTGGCCAAGGCTCTTGTCTTCCGTCACGGTGTCCACTTCCCT  720

 A  F  S  F  E  K  H  A  E  T  E  G  F  G  K  V  S  P  P  K
GCTTTCAGTTTCGAGAAGCACGCCGAGACTGAGGGTTTCGGAAAGGTCAGTCCTCCGAAA  780

 #
TAA                                                            783

784 TAGGCTACATTGGCAACATACTAACTGATATACTAGATGGCAAAGCGCATGGGTTTCTCTGTTGTCGGCCTC
CTCACTACACCATCTGGCATTTGTACGAGCCTAGTCTCGACGATATCCGCCACATGGAGGTAAGCTTCTTAACGTCC
CTGTAAACTATGCAATCTATGCCACGAAACAAATACTGACTACCTAGGAAATGGAAGCTGAACGTATCGCTCGTGAG
GAAGAGGAGAAGGAAAAGGCTGAAAAGGCCGAGAAGATGAAGAAGACCTTTGGTGACGCTGGTGGCCAGTGGGAGAA
GGACAAGGCTGCCCTGCAGGACAACGCCAGAGAAGACAACCCGACAGAGAACGAAGCCCCACACGCCGCCGCCGCCC
CTGCTGCTGCCGCCGCTCCTGCTGTTGCCGCTGCTCCTGTTGCCGCTGCTGCTCCTGCTGTTGGTGCCGATGCTGCT
GCTGCCGCCGCCGCCGCCGCCCCGGCTGCCCCGGCTGCCCCTGTGGTTGCTGCCGCTCCTGTGCCGCTCCCCCCGTT
GCTGCTGGAGGTGCTGATGTTTTTCTCG  1351
```

Figure 4. The predicted protein sequence of *O. novo-ulmi* MNT1 in alignment with other fungal mannosyltransferase protein sequences. The *O. novo-ulmi* MNT1 segregated most closely with the MNN9 of *Candida albicans*, suggesting a similar activity. Genebank accession numbers are shown in brackets.


Ophiostoma novo-ulmi putative glycosyltransferase
Candida albicans Mnn9p [AAB05924.1]
Neurospora crassa probable ANP1 [CAE85509.1]
Yarrowia lipolytica α 1-6 mannosyltransferase [AAL32468.1]
Ajellomyces capsulatus N-glycosyl-transferase [AAF90184.1]
Pichia angusta Mnn9p [AAK40024.1]
Yarrowia lipolytica Mnn9p [CAD91564.1]
Saccharomyces cerevisiae ANP1p [AAB28440.1]
Schizosaccharomyces pombe glycosyltransferase [CAA21290.1]]
Saccharomyces cerevisiae vanadate resistance Van1p [S53957]
Saccharomyces cerevisiae MNN9p [S43746]

CHAPTER FOUR

A nine-year genetic survey of the causal agent of Dutch elm disease, *Ophiostoma novo-ulmi* in Winnipeg, Canada

Chapter Summary

The causal agent of Dutch elm disease, *Ophiostoma ulmi sensu lato,* has been spreading across North America since the 1920's. The population of the pathogen in Winnipeg, Manitoba, Canada was surveyed in 1993 and 2002 using a combination of RAPD marker analysis, vegetative compatibility tests and surveys for viral double stranded RNA (dsRNA). The data presented here show that the population, based on the vegetative compatibility and RAPD analysis, was highly genetically uniform, and has remained so for the nine-year duration of the study. The pathogen population was also monitored for the presence and spread of dsRNA molecules that, depending on identity, can be associated with a diseased phenotype and reduction of virulence in the pathogen. The current study found a very low incidence of dsRNA, and did not find any evidence for spread of these molecules through the population, even though there appeared to be no barriers to the transfer of the dsRNA between Winnipeg isolates. Despite the observation that isolates infected with the Winnipeg dsRNA showed no obvious phenotypic differences, the low incidence of dsRNA in general suggests that infected isolates do not compete as successfully as the uninfected isolates. The highly clonal nature of the pathogen population may be exploited in a control strategy.

Introduction

Dutch elm disease is caused by *Ophiostoma ulmi sensu lato* (*s.l.*), with two epidemics having been recorded in North America. The first epidemic, starting in the 1920's, was caused by *O. ulmi* (Buism.) Nannf. The source of the initial epidemic in North America was speculated to have been by introduction of the bark beetle vector, *Scolytus multistriatus*, over-wintering on pathogen infected elm logs shipped to North America for furniture production. The second epidemic, starting in the 1940's and continuing to this day, was caused by the more aggressive *O. novo-ulmi* Brasier (Brasier 1991). The source of the more aggressive *O. novo-ulmi* is currently unknown (Brasier 1996). Population genetic studies utilizing analysis of molecular genetic data suggested that aggressive *O. novo-ulmi* North America (NAN) races were derived from the Eurasian aggressive races (EAN) (Bates, Buck & Brasier 1993a; Bates, Buck & Brasier 1993b). The current epidemic in North America is likely the result of an introduction from Europe or Asia. In general, the populations of *O. novo-ulmi* NAN isolates in North America have been shown to be more genetically uniform than *O. novo-ulmi* EAN isolates in Europe. This is supported by the greater diversity of vegetative compatibility (vc) types in Europe, as compared to North America (Brasier 1996). In Europe, where a similar epidemic of Dutch elm disease has decimated elms, there was a high initial uniformity in vc types at the disease fronts; however, after the disease had become entrenched, there was a diversification of vc types (Brasier 1988).

The genetic diversity of the population of *O. novo-ulmi* NAN in North America has been previously assessed using restriction fragment length polymorphisms (Hintz et al. 1993) and by vc types (Brasier 1996); however, the variation in genetic diversity generated by a highly clonal disease front of *O. novo-ulmi* NAN has not been measured over a significant time span in a localized area. Use of RAPD markers has been useful for analysis of the population structures of *O. ulmi* and both the EAN and NAN races of *O. novo-ulmi*, and has proven effective in finding variation within closely related groups of the pathogens (Pipe, Buck & Brasier 1995). Assessment of vegetative compatibility has also proven to be a suitable measure of variation within *O. novo-ulmi* populations (Brasier 1996). The current study of *O. novo-ulmi*, was started in 1993 in Winnipeg, Manitoba, Canada, with Dutch elm disease firmly established in the city, and concluded with a second collection in 2002. Both vc and RAPD marker analysis was used to analyze the nature of Dutch elm disease population structure and genetic diversity in North America.

The lack of genetic diversity among the *O. novo-ulmi* isolates in North America implies that this disease may be more easily controlled here than in Europe where genetically diverse and interbreeding populations are common. It has been speculated that control of the fungus may be achieved by infecting the pathogen with double stranded RNA (dsRNA), also known as d factors (Brasier, 1983). These d factors have been demonstrated to be cytoplasmically transmitted (Rogers, Buck & Brasier 1986; Charter, Buck & Brasier 1993) and severely

reduced growth, reproductive vigor and parasitic fitness of infected isolates of *O. novo-ulmi* (Brasier 1983; Webber 1987; Sutherland and Brasier 1995); the avoidance of these deleterious effects has been suggested to be a driving force behind generation of genetic diversity in the pathogen (Brasier, 1983). Genetic variability and infection by dsRNA of *O. novo-ulmi* was surveyed in the city of Winnipeg, Manitoba, Canada in 1993 and 2002 in order to elucidate both the dynamics of the population in North America and to determine if infection of the population with dsRNA correlated with the generation of genetic diversity in the pathogen.

Materials and methods

Isolates and culture conditions

Working cultures of *O. novo-ulmi* were grown on solid *Ophiostoma* complete media (OCM) (Bernier and Hubbes 1990) at 22 °C and maintained at 4 °C for short-term storage. Stock cultures were kept at –196 °C or colder in liquid nitrogen, following infusion with either 15 % glycerol or 10 % DMSO. Culture collections were constructed by recovery of living *O. novo-ulmi* from twigs pruned from diseased American elm (*Ulmus americana*) trees growing in the city of Winnipeg, Manitoba, Canada by the City of Winnipeg, Forestry Branch in the summers of 1993 and 2002. To recover isolates of *O. novo-ulmi*, twigs were surface sterilized by a 70 % ethanol dip, the bark was peeled back and small sections of xylem were excised and placed on potato dextrose agar (PDA) without antibiotic supplementation. If contamination by other fungi and bacteria out-competed *O. novo-ulmi*, sections of xylem from twigs were sterilized as above and placed on OCM supplemented with chloramphenicol (30 mg/L), streptomycin sulfate (100 mg/L) and cycloheximide (100 mg/L). Cultures were tentatively identified as *O. novo-ulmi* based on microscopic features and culture morphologies, followed by several transfers on OCM agar to derive isogenic cultures. To further identify the isolates as *O. novo-ulmi*, the cultures were assayed for the production of cerato-ulmin, a feature common to all *O. novo-ulmi* isolates except two NAN isolates discovered in Europe (Takai 1974; Brasier, Kirk & Tegli 1995). Cultures were grown in 5 ml of liquid OCM at 22 °C for 7 d. One ml of culture filtrate was then sedimented by microcentrifuge for 3 min to remove

cells and cell debris. The clear filtrate was then shaken in 15 ml test tubes at 200 rpm for 30 min and assessed for turbidity characteristic of cerato-ulmin production (Bowden et al. 1996).

Transfer of dsRNA from 93-1224 to VA30

Cultures of *O. novo-ulmi* 93-1224 (dsRNA infected isolate from Winnipeg, 1993) and *O. novo-ulmi* VA30 (isolated by L. Schreiber and A. Townsend, Virginia) were inoculated onto opposing sides of an OCM agar petri dish and incubated until contact was made between the leading edges of the two growing colonies. Plates were then transferred to 4 °C for 14 d. To determine whether the dsRNA was transferred from 93-1224 to VA30, plugs of mycelium were recovered from the VA30 side of the plate at a distance of 1 to 5 mm from the zone of contact. These culture explants were grown in liquid OCM shake cultures for 2 d, placed in a microcentrifuge for 3 min to sediment the yeast-like cells followed by washing with sterile water. Single cell isolates were obtained by dilution plating. The recovered isolates were verified to be VA30 using the RAPD primer OPC-05 (Operon) (5'-GATGACCGCC-3') and then screened for the presence or absence of dsRNA.

Nucleic acid extraction and RAPD PCR

Cultures for DNA extraction were grown in stationary liquid cultures for 7 d in 5 ml OCM at 23 °C. Mycelia were harvested, freeze-dried and ground into a fine powder. Mycelia (200 mg) were re-suspended in 0.5 ml of 50 mM EDTA and 0.2

% SDS, mixed and incubated for 20 min at 65 °C. Samples were centrifuged to remove cell debris and protein was precipitated on ice with a 0.17 volume 3.0 M potassium acetate and 5.0 M acetic acid. After removal of the supernatant to a fresh tube, an equal volume of isopropanol was used to precipitate the DNA. The DNA was then re-suspended in 500 µl of 10 mM Tris-HCl (pH 7.5), 5.0 mM EDTA (TE), treated for 30 min with ribonuclease (100 µg per sample), extracted once with an equal volume TE-saturated phenol and extracted twice with equal volumes of chloroform: isoamyl alcohol (24:1). The DNA was then precipitated from the aqueous phase with 0.10 volume of 3 M sodium acetate (pH 5.3) and 2 volumes of 95 % ethanol and washed once with 70 % ethanol. The DNA pellet was then air-dried and re-suspended in 100 µl sterile water. The target genomic DNA (10-50 ng) was amplified using 200 pmol of either RAPD primer OPA-02, 04 or 13, two units of Taq DNA polymerase (Pharmacia) and 0.1 µM each of dATP, dCTP, dTTP, and dGTP in a 20 µl final volume containing the Pharmacia Taq polymerase buffer at 1X concentration. A total of 35 cycles of PCR were used for amplification. All denaturing steps took place at 94 °C for 60 s, annealing was at 35 °C for 120 s and primer extensions were for 120 s at 72 °C. The amplification products were separated on a 1.0% agarose gel in 1 X TAE (0.04 M Tris-acetate; 1 mM EDTA).

dsRNA extraction

Cultures for dsRNA extraction were grown in stationary liquid cultures for 7 d in 5 ml OCM at 23 °C. The dsRNA was extracted following a procedure modified

from Romaine et al. (1993). Mycelia were harvested by centrifugation at 2000 g for 10 min, followed by homogenization in 0.5 ml 2X STE (50 mM Tris-HCl; 0.1 M NaCl; 1 mM EDTA; pH 7.0) containing 1 % SDS, 0.5 ml aqueous phenol and 0.25 ml chloroform: isoamyl alcohol (24:1). The resulting emulsion was separated for 5 min in a microcentrifuge and the aqueous phase was transferred to a new microcentrifuge tube and adjusted to 15 % ethanol by the addition of an appropriate amount of 100 % ethanol. Following the addition of the ethanol, 100 mg cellulose powder was added, the tube was mixed briefly by vortex and the cellulose was precipitated in the microcentrifuge for five min. The cellulose pellet was washed with 2X STE containing 15 % ethanol and the dsRNA was eluted using 1.0 ml STE. The dsRNA was precipitated using two volumes of 95% ethanol and separated on a 1.0 % agarose gel in 1 X TAE (0.04 M Tris-acetate; 1 mM EDTA).

Vegetative compatibility tests

Vegetative compatibility was determined by pairing isolates of *O. novo-ulmi* 1.0 cm apart on malt extract agar plates (1.5 % weight per volume malt extract; 15 g/l agar). Liquid cultures used for inoculating the vc test plates were grown for 7 d at room temperature in stationary cultures using liquid OCM. Plates were inoculated with 1.0 µl of liquid culture and incubated for 7 d in complete darkness, followed by 7 d in natural light conditions. All cultures were grown at 22 °C. Cultures were then assessed for reactions characteristic of vegetative compatibility or incompatibility by observing the reaction of the mycelium in the

zone of contact. Incompatibility has previously been detected by the presence of wide, narrow, line or line gap reactions, showing characteristic mycelial barrages, synnemata formation and bilateral mycelial penetration (Brasier 1996).

Results

The genetic diversity of the *O. novo-ulmi* populations recovered from the disease front in Winnipeg, Manitoba was assessed by the analysis of random amplified polymorphic DNA (RAPD) markers. When used in conjunction with the polymerase chain reaction (PCR), RAPD is a fast and effective means of determining genetic variation in a population (Welsh and McClelland 1990; Williams et al. 1990). Historically, Western Canada was first subjected to invasion by *O. ulmi*, the less aggressive form of the Dutch elm pathogen (Brasier 1991). This less destructive epidemic was superceded by the much more aggressive *O. novo-ulmi* beginning in the 1940's after introduction of *O. novo-ulmi* to North America, presumably from Europe (Brasier 1991). An earlier survey of the population in 1990 did not detect the presence of the less aggressive *O. ulmi*, suggesting that either the more aggressive *O. novo-ulmi* had replaced *O. ulmi*, or was re-infecting territory that had completely recovered from the previous epidemic of *O. ulmi* (Hintz et al. 1991; Hintz et al. 1993). Analysis of our culture collections from both 1993 and 2002 confirmed that *O. ulmi* was not found in this part of the continent. In particular, it should be noted that cultures were isolated from the xylem of diseased trees, increasing the likelihood that the pathogenic phase of *O. novo-ulmi* had been isolated. The very high level of genetic uniformity found in both sampling periods verified that the sample sets were comprised of *O. novo-ulmi* exclusively, and not a mix of *O. ulmi* and *O. novo-ulmi*.

Several RAPD 10mer primers were screened for their suitability in discriminating between isolates. Because of the high uniformity of the isolates, only one primer, OPA-13, proved able to detect differences among isolates. Accordingly, OPA-13, along with OPA-02 and OPA-04 were used for classification of the entire culture collection which consisted of 50 isolates from 1993 and 44 isolates from 2002. OPA-02 (5'-TGCCGAGCTG-3') and OPA-04 (5'-AATCGGGCTG-3') gave reliable amplification patterns (Fig. 1) however neither primer showed any variation between isolates of either sample set. Only OPA-13 (5'-CAGCACCCAC-3') showed an ability to discriminate between isolates. The population of *O. novo-ulmi* in Winnipeg from 1993 surveyed with OPA-13 showed the population to be largely comprised of a single clone (74%) showing OPA-13 pattern 1 (Fig. 2) with a second closely related genotype, pattern 2, present in much lower numbers (26%). The 2002 data indicate that the population has actually become less diverse, with the OPA-13 pattern 1 genotype first identified in 1993 still predominant (80%). The second genotype, pattern 2, exhibited the same RAPD pattern as in 1993, represented an even smaller proportion of the population in 2002 (20%) and had a reduced range when compared to the area covered in 1993.

Variation in vegetative compatibility (vc) type has been used previously to assess the genetic diversity of the pathogen population (Brasier 1983; Brasier 1996). Vegetative compatibility is proposed to arise rapidly after the initial disease front of the pathogen passes through an area, and represents one metric by which the

genetic diversity of the population may be measured (Brasier 1996). Our measure of vc diversity showed the population to be completely homogenous in 1993 and 2002, with isolates from 1993 fully compatible with isolates from 2002, regardless of OPA-13 RAPD pattern. It has been noted that vegetative incompatibilities arise much more quickly in the pathogen than observable genetic mutations (Brasier 1996), so by combining RAPD analysis with vc assessment, an accurate assessment of changes in the population structure could be made. The possibility does exist that our RAPD analysis was not sensitive enough to detect variation that has arisen since the survey in 1993, but testing both sample sets with three different RAPD primers, in conjunction with vc assessment, allowed a greater probability of detecting any changes that might have occurred.

Due to the possibility that dsRNAs can be used as a control of *O. novo-ulmi* and may also exert selection pressure on the vc groups of the pathogen, the current study surveyed the pathogen population in Winnipeg for the presence of dsRNA viruses. In both the 1993 and 2002 sample set, only one isolate in each group infected with dsRNA was found. Both isolates were found in close proximity, and the dsRNA was identical in size and banding pattern, which suggests the spread of this Winnipeg dsRNA has been limited. To preclude the possibility that the NAN race is resistant to dsRNA spread, and demonstrate that the Winnipeg dsRNA can spread by cytoplasmic transmission, *O. novo-ulmi* NAN VA30 was infected experimentally with the Winnipeg dsRNA by transfer from 93-1224 (Fig. 3). Transfer of the dsRNA from the Winnipeg 93-1224 to VA30 (isolated in

Virginia, U.S.A.) was accomplished by hyphal anastomosis between the two isolates, which showed no vegetative incompatibilities. Samples of VA30 were taken from behind the zone of contact and single spore isolates were obtained from these samples and verified to be dsRNA infected VA30. This dsRNA virus appeared to have no phenotypic effects on *O. novo-ulmi* NAN as no ill effects were observed in VA30 subsequent to the dsRNA infection. As there appeared to be no barriers to inhibit the spread of the Winnipeg dsRNA, other factors must have influenced the lack of dsRNA in the population. Additionally, the lack of dsRNA in the population may also have been a factor in the maintenance of genetic uniformity of the Winnipeg population of *O. novo-ulmi* NAN, as the deleterious effects of dsRNA have been proposed to drive diversification of genotypes in other locales (Brasier 1988).

Discussion

As a follow-up to initial surveys of the population structure of *O. novo-ulmi* in Western Canada, this study assessed a localized culture collection of the pathogen from Winnipeg, Manitoba, Canada sampled at a 9-year interval to determine if and how differences in the genetic structure of the pathogen population arise over time in the same area. A library of pathogen genotypes like the one presented here has been previously unavailable for the pathogen in North America. In 1993, the pathogen was largely clonal on the disease front, thus if the dynamics of the NAN population in North America were similar to the EAN population in Europe, an increase in genetic diversity in the same area 9 years later would be expected as the disease front passed to new areas. This increase in diversity was not observed and the population of the pathogen surveyed in 2002 remained almost clonal.

Several factors may contribute to the disparity between the expected diversity and the largely clonal population that still remained in 2002. The first question that must be addressed is how genetic diversity appears in the population of *O. novo-ulmi*. The epidemic of Dutch elm disease in North America may have started due to the introduction of a limited number of genotypes of the pathogen to the continent and consequently may have undergone a genetic bottleneck, thus limiting the generation of new genotypes by sexual and parasexual recombination. In order to assess a highly clonal population, sensitive markers such as RAPDs, must be used and be capable of detecting small changes in a largely uniform

background; processes that have previously been shown to be hypervariable in generation of diversity, such as generation of vc types in the pathogen, are also valuable in measurement of diversity in uniform populations. It is likely that the lack of resistance in the American elm (*Ulmus americana* L) has contributed to the preferentially clonal propagation of the *O. novo-ulmi* population, as it has been established that rapidly expanding pathogen populations tend to have low genetic diversity (Frank 1992).

The question arises as to how fast genetic diversity is generated in the pathogen. It has been suggested that the pathogen very quickly establishes a variety of vc types behind the disease front (Brasier 1988), while another hypothesis suggested that genetic variation might arise by introduction of new genotypes of the pathogen to the area (Hintz et al. 1993). The data presented here indicated that genetic diversity did not arise rapidly from a clonal population and would appear to support the idea that rapidly increasing genetic diversity may be the result of multiple introductions, assuming the absence of other factors that may act to generate diversity. Thus, while the vc groups of the pathogen appeared to diversify rapidly behind the disease front in Europe, presumably through mutation or pseudo-selfing (Brasier 1988), our findings would appear to be at odds with that hypothesis.

The deleterious effect of d factors has been proposed to drive genetic diversification in *O. novo-ulmi* in Europe, with sexual recombination one way of

escaping the negative effects of a d factor, or possibly leading to the production of genotypes that are resistant to the negative effects of d factors (Brasier 1983; Brasier 1988; Brasier 1996). The appearance of dsRNA resistant genotypes has been observed in an isolate of *Cryphonectria parasitica* derived from a sector of dsRNA-infected culture; attempts to re-infect this isolate with a dsRNA virus proved unsuccessful (Polashock et al. 1994). To eliminate the possibility that the NAN race was resistant to dsRNA, *O. novo-ulmi* VA30 was infected experimentally with the Winnipeg dsRNA by transfer from the infected isolate 93-1224 (Fig. 4). There were no phenotypic effects in the infected VA30 typical of other dsRNA infections. This suggested that the NAN population is not resistant to the spread of dsRNA and that the dsRNA spreads by cytoplasmic transmission. Because of their conspicuous absence, d factors did not appear to have a major role in contributing to the diversity of *O. novo-ulmi* NAN in Winnipeg. In both the 1993 and 2002 sample set, only one isolate in each group (Fig. 3) was infected with dsRNA. Both isolates were found in close proximity to each other, suggesting that the dsRNAs are geographically limited and that spread of the dsRNA is not common. Alternately, as the two infected clones were collected approximately 1 km apart, a single infected clone may have been spreading on a local scale, a hypothesis supported by the finding that both infected isolates were of the same RAPD OPA-13 pattern 1 type.

In assessing the effects of d factors, wide variation has been observed in their efficacy in lowering fitness (Webber 1993). Certainly, the lack of diversity of vc

types in the Winnipeg population would not present a barrier to the spread of any dsRNA so another explanation must account for the lack of dsRNA in the population. The dsRNA discovered in Winnipeg did not appear to have any apparent effects on the phenotype of isolates from either 1993 or 2002, or in VA30. The original source of the dsRNA, isolate 93-1224, was recovered from the xylem of a diseased tree, as was the diseased isolate 02-0833 in the 2002 population, suggesting that isolates infected with the Winnipeg dsRNA were still virulent. However, the low frequency of infected isolates in the population suggested either the d factor lowered parasitic fitness in a manner that could not be detected, or that the rapid expansion of *O. novo-ulmi* NAN on the highly susceptible *Ulmus americana* in North America has contributed in some way to the lack of dsRNA and its spread in the population. Currently, it is difficult to predict what is occurring with respect to the dynamics of dsRNA within the Winnipeg population. Further monitoring of dsRNA occurrence would perhaps clarify if dsRNA infections were on the advance or decline in this population.

It does not appear that the presence of dsRNA in the Winnipeg population had an impact on the genetic diversity of the pathogen in this population. Why more dsRNA infected isolates were not found in the population is unknown, as is how these dsRNAs arise in the first place. The original source of the dsRNA found in the Winnipeg population is unknown. Presumably the dsRNA was generated *de novo* by the existing population, or was introduced by hyphal anastomosis from an infected isolate of a different genotype that later disappeared from the region,

or was present in such low numbers that the current survey did not detect its presence. Some clues as to the origin of dsRNAs suggest that these molecules may generate *de novo* from the mitochondrial genome (Charter et al. 1993; Abu-Amero et al. 1995), but if this is the case, this does not appear to be a frequent event for *O. novo-ulmi* NAN. Given that the dsRNA found in this population had no apparent negative effects on the pathogen, it is an unlikely candidate for a dsRNA based biocontrol strategy; however, this does not preclude the use of another, more effective d factor derived from other populations of the pathogen, in particular d factors from the EAN population. The d^2 factor has been extensively characterized and isolates of *O. novo-ulmi* and *O. ulmi* infected with the d^2 factor have shown a decrease in cytochrome aa_3 concentration (Brasier et al. 1993); the loss of cytochrome aa_3 has been correlated with disease symptoms in fungal isolates containing the d^2 factor (Rogers, Buck & Brasier 1987). Further data indicated that a diseased isolate of *O. novo-ulmi* contained mitochondrial DNA plasmids that appeared to have homology to the fungal mitochondria and therefore may have been derived from recombination events in the mitochondrial genome (Charter et al. 1993; Abu-Amero et al. 1995). These plasmids were suggested to code for an RNA maturase-like protein or a DNA endonuclease, giving rise to the speculation that some of the effects of dsRNAs may result indirectly from the actions of these proteins (Abu-Amero et al. 1995).

The possibility that d factors may be used to control Dutch elm disease is borne out by the effective use of dsRNA infected isolates of *Cryphonectria parasitica* to

control Chestnut blight (Tartaglia and Nuss 1988). In the case of *O. novo-ulmi*, it has been suggested that the best time for control attempts would be during the saprophytic phase of the pathogen's life cycle, which would target isolates growing in the vector breeding galleries. During the saprophytic phase, the pathogen exists as an intermingling mosaic of genotypes that would contribute to the spread of the d factor (Webber et al. 1987; Webber 1993). In particular, the d^2 factor appears to impair the pathogen's ability to colonize feeding grooves and initiate xylem infection (Webber 1987). Presence of the d^2 factor in pathogen populations raised the number of spores required for host infection from 1000 to 50000 on *U. procera* (Webber 1993). Since the beetle vectors, particularly the small *Scolytus multistriatus*, often carry loads of less than 1000 spores, the d^2 factor appeared to have some potential as a method to control of spread of this disease. The continued genetic uniformity of *O. novo-ulmi* in North America and the high transmissibility of dsRNA in genetically uniform populations represent an opportunity for control of this pathogen using d factors.

Figure 1. RAPD marker pattern for the 3 RAPD primers used to survey *O. novo-ulmi* in Winnipeg, MA, Canada. Genomic DNA was screened with one of RAPD primers OPA-02, 04 or 13 (Operon). The amplification products were separated on a 1.0% agarose gel in 1 X TAE (0.04 M Tris-acetate; 1mM EDTA) using lambda DNA digested with *Hind* III as size markers (shown on left in kb).

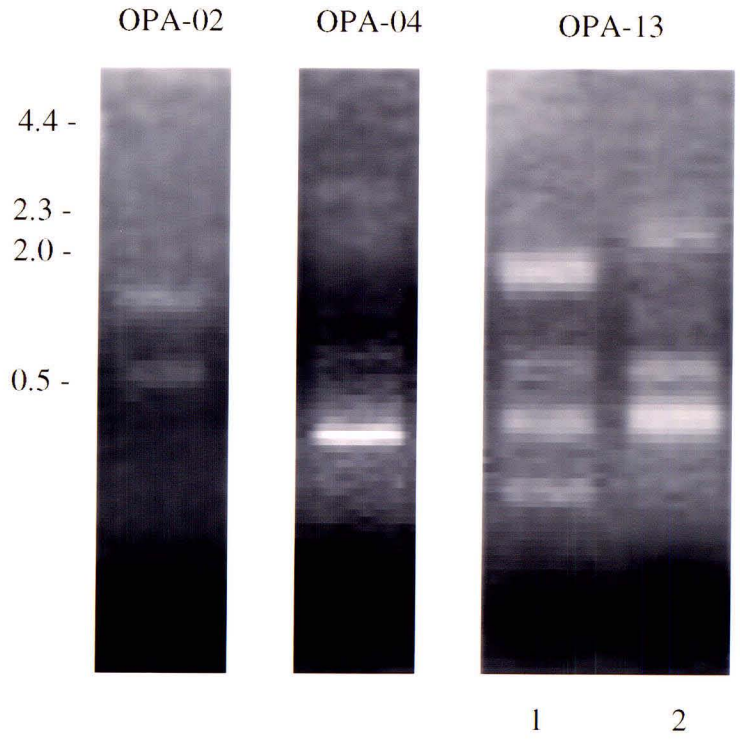

OPA-02
OPA-04
OPA-13
4.4 -
2.3 -
2.0 -
0.5 -
1
2

Figure 2. The distribution of OPA-13 RAPD marker types 1 and 2 for the years 1993 and 2002 in Winnipeg, Manitoba, Canada.

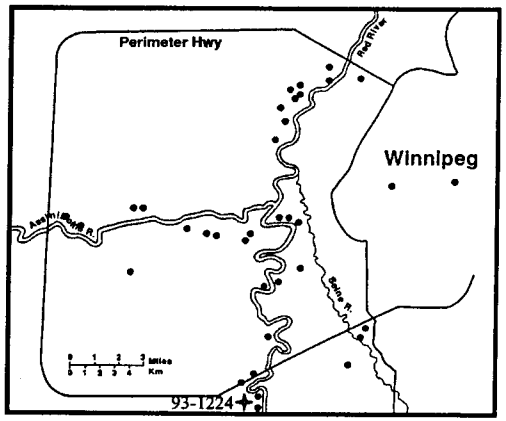
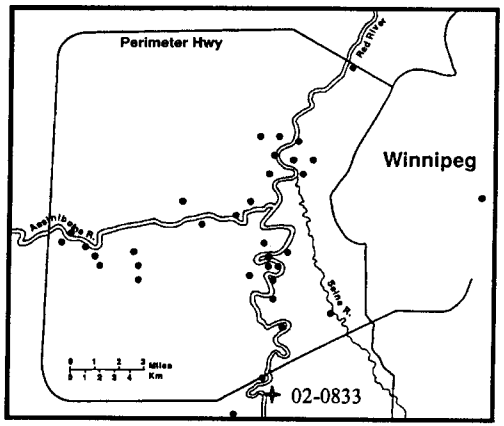
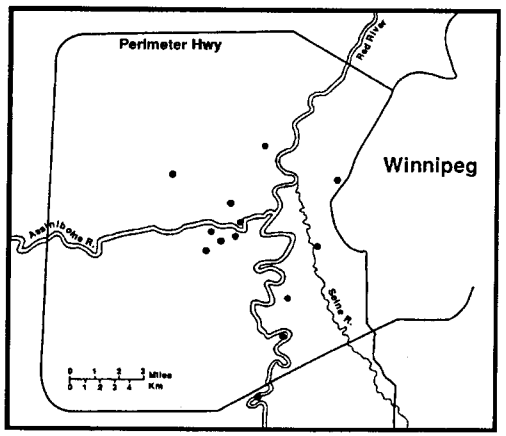
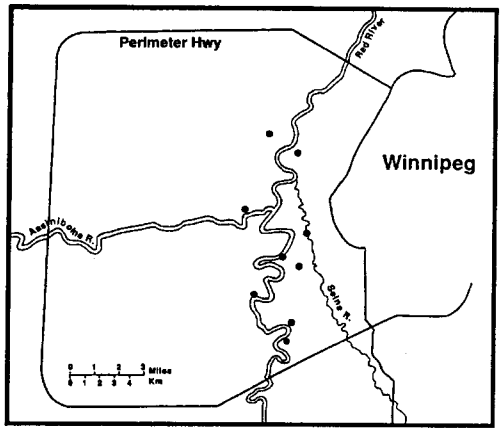
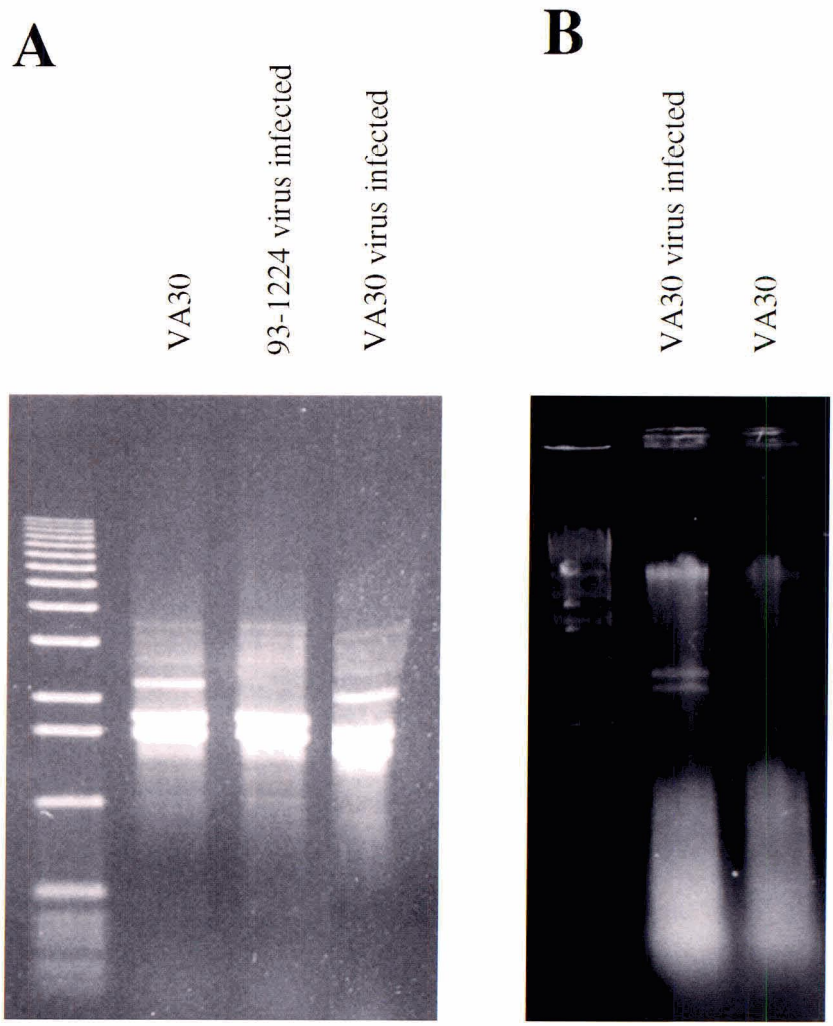
	1993	2002
OPA-13 Type 1		
OPA-13 Type 2		

Figure 3. RAPD OPC-05 analysis of VA30 and 93-1224 in conjunction with gel analysis of infection by the dsRNA from the 93-1224 source. Cultures of VA30 were dsRNA infected by co-culturing with 93-1224 for two weeks on OCM Single spore isolates were obtained and verified to be VA30 by OPC-05 RAPD analysis screened for infection by dsRNA using the dsRNA miniprep procedure published by Romaine et al. (1993). The dsRNA and OPC-05 amplification products were separated on a 1.0% agarose gel in 1 X TAE (0.04 M Tris-acetate; 1mM EDTA) using either 100 bp ladder (Pharmacia) for RAPD analysis (A) or lambda DNA digested with *Hind* III as size markers for dsRNA visualization (B).


A
VA30
93-1224 virus infected
VA30 virus infected
B
VA30 virus infected
VA30

CHAPTER FIVE

Summary and General Discussion

Dutch elm disease has been one of the most economically destructive plant diseases of the last 100 years. The American elm (*Ulmus americana)* has great value in the urban landscape, as it is attractive, hardy and resistant to pollution. Unfortunately, the elm has been decimated by *Ophiostoma ulmi sensu lato* across North America since the early 1920's with mortality in some areas as high as 95%. The highly aggressive elm pathogen *O. novo-ulmi* is fortunately amenable to *in vitro* culture and manipulation, which serves to make it an ideal model system for this and other destructive *Ophiostoma* species, as well as for other fungal plant pathogens. Deciphering the genetic basis of virulence in a fungus is an important step in determining the best methods of controlling destructive plant diseases caused by fungi. Evidence to date has increasingly suggested that single genes appear to be vitally important in the success or failure of infection and establishment of disease. Conceptually, these processes, described predominantly in agricultural systems and unified as the gene-for-gene hypothesis (Heath 1981) relate to tree diseases as well. This thesis has detailed the cloning, characterization and disruption of the gene encoding the cell wall-degrading enzyme, polygalacturonase, the characterization of glycosylation mutants of *O. novo-ulmi* and a survey of the population structure, vegetative compatibility and the occurrence of dsRNA virus infection in the pathogen population in Winnipeg, Manitoba, Canada.

Cell wall-degrading enzymes (CWDEs) have important roles in determining virulence of some plant pathogens (Collmer and Keen 1986). Polygalacturonase appears to be one of the most important in this class of enzymes due to its early secretion in infection processes and its ability, in purified form, to do substantial damage to cells. Pectin polymers are a major component of the cell wall and represent an early barrier that a pathogen must circumvent in order to establish an infection of the host. Secretion of CWDEs may enhance invasion of the host, enable the spread of the pathogen within the host tissue and cause damage to the host in their own right. The activity of CWDEs may affect host-pathogen recognition by uncovering elicitor receptors in the host cell wall, or by releasing biologically active cell wall fragments or other degradation products as a consequence of cell wall penetration (West 1981). However, the role of CWDEs in virulence depends heavily on the disease system under study, as both saprophytic and pathogenic fungi alike produce CWDEs (Collmer and Keen 1986).

Precisely how CWDEs in general, and polygalacturonase in particular, act in the host-pathogen interactions of the Dutch elm disease system was largely unknown until recently. *Ophiostoma novo-ulmi* was shown to have better cell wall-degrading abilities, when compared *O. ulmi* (Binz and Canevascini 1996a; Scheffer and Elgersma 1982). This correlated with more effective bark colonization by *O. novo-ulmi* (Webber and Hedger 1986) and suggested that CWDEs, polygalacturonase included, may contribute to virulence and parasitic

fitness of the pathogen on elm. Disruption of a single major polygalacturonase gene in *O. novo-ulmi* gave the pathogen a phenotype of intermediate virulence relative to the wild type strain VA30. This observed reduction in virulence has been observed previously in other systems, as the targeted disruption of polygalacturonase genes has resulted in a reduction in virulence for other fungi. For the *epg1* strain of *O. novo-ulmi* tested, the onset of symptoms was delayed in host trees infected by the strain carrying a disrupted polygalacturonase gene, suggesting that not only did expression of this enzyme affect symptom expression, but also influenced the ability of the fungus to invade the host. It thus appears that, in addition to contributing to the overall virulence of the *O. novo-ulmi*, polygalacturonase likely contributes to the parasitic fitness of the fungus as well.

Interactions between a pathogen and host may depend on the role of glycosylation enzymes, and how the activity of these enzymes affects recognition and signaling. Both the effect of these enzymes on the surface structure of the pathogen cell wall, and their overall requirement for the health of the organism, makes them likely candidates as targets for modifying host-pathogen interactions. Thus, while a gene may be considered to be necessary for the completion of the life cycle of the pathogen, it cannot be strictly defined as a virulence factor. Despite this, housekeeping genes such as those involved in coding for glycosyltransferases would still make obvious targets for control strategies, given the multitude of deleterious effects that may arise from the disruption of glycosylation pathways;

these negative effects can include the possible disruptions of secretion and function of secreted enzymes, including CWDEs. Genes encoding glycosyltransferases may also be involved in host-pathogen interactions in more direct ways. Given the known role these enzymes have in modifying the cell wall and secreted proteins, it is possible that the sugar chains created by these enzymes function to create or hide elicitors (Keen 1982), or even possibly function to suppress the plant defense response (Basse et al. 1992).

It is likely that some responses between *O. novo-ulmi* and elm may be predicted using a tissue culture based system and extrapolating from that to how *O. novo-ulmi* may interact with whole trees, (Corchete et al. 1993; Valle et al. 1997). Callus tissue of clonal *Ulmus americana* was used to determine if fungal isolates treated with random insertional mutagenesis and selected for mutations in glycosylation would show variation in their interaction with callus tissue. Our results suggested that glycosylation mutants appear to interact differently with callus tissue when compared to the wild type. These mutants produced a profusion of synnemata, while the wild type isolate did not produce any synnemata at all. Exactly how the mutations affected host-pathogen interactions is unknown. Mutants showed a lack of polygalacturonase activity, but showed no decrease in cerato-ulmin production, suggesting that glycosylation is required for the secretion or activity of PG (which has one site for glycosylation) and not for cerato-ulmin (which has no sites for glycosylation). Given that defects in some part of the glycosylation pathway affected the secretion of at least some of the

regular complement of CWDEs suggested one way that this class of mutations could have altered host-pathogen interactions. Another possible interpretation of our observations would be that the large alterations in the cell wall of the pathogen, due to the disruption of these genes, led to variation in recognition between host and pathogen. Why mutants were triggered to produce a profusion of synnemata is unknown, but this effect could be related to differences in biological signals generated and received by the mutants as compared to the wild type control. Synnemata production has been correlated to virulence in some isolates, but this was in a comparison between aggressive and less aggressive isolates of *O. ulmi sensu lato* (Brasier 1991). Thus, it cannot be conclusively said that altering glycosylation enzymes makes an isolate more or less virulent, but on callus tissue there appeared to have been a correlation between altered glycosylation and unusual interaction of the pathogen with callus tissue of American elm.

Complementary to searching for virulence factors in the pathogen, a survey of the pathogen population in Winnipeg, Manitoba, Canada was undertaken to determine how the population dynamics compare to data from Europe. Additionally, as the North American population of the pathogen has not been previously tested for dsRNA, survey of dsRNA virus infection of the population was completed. This survey allowed evaluation of the feasibility of developing a biocontrol strategy using mycoviruses for control of the pathogen. Analysis of randomly amplified polymorphic DNA (RAPD) markers and vegetative

compatibility comparisons showed that the pathogen population was highly uniform, and had remained so for the nine-year duration of the survey. Of particular interest was the lack of dsRNA viruses, or d factors, in the population of the pathogen. In general, d factors have a negative effect on the parasitic fitness of the pathogen and thus may be possible biological control agents (Sutherland and Brasier 1995). In using dsRNA to control Dutch elm disease, certain factors must be taken into account, including determining the most efficacious time and location to introduce the dsRNA into the population. The results of the survey in Winnipeg, which showed almost no dsRNA present in the population, suggested at least one reason for the continued uniformity of the pathogen population. Additionally, the lack of vegetative incompatibility in this population presents opportunity for control using deleterious dsRNA as no barriers to spread of mycoviruses existed in the population.

By developing an understanding of the host-pathogen interactions between elm and *O. novo-ulmi*, potential control strategies for the Dutch elm disease pathogen may be elucidated. The financial cost associated with Dutch elm disease and the elimination of elm trees from the North American landscape has been estimated in the hundreds of billions of dollars for landscaping values alone, making Dutch elm disease one of the most economically destructive plant diseases in North America. The development of control methods that would permit American elms to survive or become immune to the Dutch elm pathogen would have considerable economic benefit and horticultural value. A valuable step in determining the best

approach in the development of viable control strategies for Dutch elm disease is to fully understand the host-pathogen interactions, from the population dynamics of *O. novo-ulmi*, to the molecular basis of disease in the host. Research to date has determined that it is much more likely that interactions between host and pathogen are controlled, or at least broadly affected by one or a few genes, similar to gene-for-gene interactions described in agricultural model systems. The basis of pathogenicity in *O. novo-ulmi* appears to be a function of numerous genes, such as CWDEs, which can act in an additive manner; however, recognition processes may be ultimately controlling virulence, and recognition may be under the control of a single gene, or very few genes. Genes encoding glycosyltransferases in *O. novo-ulmi* represent one possible family of genes that may have a significant effect on recognition in particular and therefore on host-pathogen interactions in general. From an understanding of population structure, pathogenicity and virulence in the fungus, strategies may eventually be developed for the control of Dutch elm disease.

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