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Characterisation of the Polygalacturonase Gene of the Dutch Elm Disease Pathogen *Ophiostoma novo-ulmi*

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Abstract

The fungal pathogens *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, the causal agents of Dutch elm disease, are responsible for decimation of elms in the Northern Hemisphere during the twentieth and twenty-first centuries. Pectinolytic enzymes, including polygalacturonase, have been implicated as virulence factors in the life history of many fungi. To determine the role of polygalacturonase in Dutch elm disease, we cloned and characterised the polygalacturonase gene from the highly aggressive *O. novo-ulmi*. This gene was similar to polygalacturonase genes of other fungi, and was present as a single copy in the genome. Genetic disruption of the gene was not lethal to the pathogen but led to a reduction of pectinolytic activity *in vitro*. It appears, therefore, that polygalacturonase has a limited role in fungal virulence and parasitic fitness in the life history of *O. novo-ulmi.*

Keywords: phytopathogen, parasitic fitness factor, virulence, cell wall degrading enzymes, pectinase, polygalacturonase, dutch elm disease.

Introduction

The fungal pathogens *Ophiostoma ulmi* and *O. novo-ulmi*, the causal agents of Dutch elm disease, have been responsible for a catastrophic pandemic that has resulted in widespread death of elms (*Ulmus* species). The various populations of the pathogen have been separated on the basis of aggressiveness and phenotypic characteristics, and comprise two species: the highly aggressive *O. novo-ulmi* and the less aggressive *O. ulmi* (Brasier, 1991). *Ophiostoma novo-ulmi* is further differentiated by phenotype and geography to delineate the subspecies *novo-ulmi* and subsp. *americana*, respectively (Brasier & Kirk, 2001).

Cell wall degrading enzymes, including the pectinolytic enzyme polygalacturonase, can be important contributors to the virulence of phytopathogens. The contribution of polygalacturonase genes to virulence and parasitic fitness has been analysed in several fungi through targeted gene disruption and over-expression of polygalacturonase. Polygalacturonase genes have been implicated in virulence in *Botrytis cinerea* (ten Have et al., 1998) and *Alternaria citri* (Isshiki et al., 2001) and in parasitic fitness in *Aspergillus flavus* (Shieh et al., 1997; García-Maceira et al., 2001). In contrast, polygalacturonase genes did not appear to influence virulence in *Fusarium oxysporum* (Di Pietro

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& Roncero, 1998), *Cochliobolus carbonum* (Scott-Craig et al., 1990), *Alternaria alternata* (Isshiki et al., 2001) or *Cryphonectria parasitica* (Gao et al., 1996).

The question thus arises as to the main function of polygalacturonase in *O. novo-ulmi*. Data concerning the role of cell wall degrading enzymes in the virulence of *O. ulmi* and *O. novo-ulmi* have been contradictory. While Elgersma (1976) found no correlation between polygalacturonase production *in vitro* and virulence in the two pathogens *O. ulmi* and *O. novo-ulmi*, other studies have reported that the more aggressive *O. novo-ulmi* had an increased capacity to destroy the cell wall of host plants (Svaldi & Elgersma, 1982), seemingly due to secreted enzymes (Scheffer & Elgersma, 1982). It would, therefore, seem likely that polygalacturonase could have a role in virulence, in which the enzyme could assist host invasion, tissue destruction and like processes associated with plant disease. Alternatively, as a parasitic fitness factor, polygalacturonase could increase survival during the saprophytic phase of the pathogen's life cycle when the pathogen must surmount the considerable challenge of spreading to new hosts while undergoing intense competition not only from other genotypes of the pathogen, but also elm-associated fungi and bacteria, thus improving the available spores and viability of the pathogen in the saprophytic stage.

Our approach to assessing the role of polygalacturonase in parasitic fitness and virulence was to disrupt the polygalacturonase gene from *O. novo-ulmi* subsp. *americana* and measure any changes in survivability or aggressiveness of the pathogen. To this end, we modified the expression of the polygalacturonase gene of *O. novo-ulmi* subsp. *americana* by identifying and disrupting the gene sequence for polygalacturonase.

Materials and Methods

Isolates, culture conditions and DNA extraction

Cultures of three *O. novo-ulmi* subsp. *americana* strains (VA30 (isolated by L. Schreiber and A. Townsend, Virginia), MH75 (isolated by M. Hubbes, Toronto) and 416R14) and *O. ulmi* strain Q412 were maintained for long-term storage in glycerol (10% v/v) at -70 °C. During experiments, cultures were maintained on solid *Ophiostoma* complete medium (OCM) (Bernier & Hubbes, 1990) and kept at 4 °C. Cultures were grown on OCM or minimal pectin medium at 21 °C for 7 days. Minimal pectin medium was prepared using Bacto Agar (Difco, Lawrence, KS, USA; 1% w/v) supplemented with citrus pectin (Sigma-Aldrich; Oakville, ON, Canada; 1% w/v). Cultures for DNA extraction were grown in stationary liquid cultures for 7 days in OCM (5 mL) at 23 °C. Mycelium was harvested, freeze-dried and ground into a fine powder. Total genomic DNA was extracted as per Temple et al. (1997).

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) and NQDDCVAVNS (base pairs 641-668). 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 OUEPGF (0.4 μM) and OUEPGR (0.4 μM), 0.2 μL *Taq* polymerase (Pharmacia; Uppsala, Sweden; 2 units) and nucleotides (dATP, dCTP, dTTP, and dGTP; each at 0.1 μM) in a final volume of 50 μL containing *Taq* polymerase buffer (Pharmacia Uppsala, Sweden). All denaturing steps took place at 94 °C for 60 seconds; all primer extensions were denatured at 72 °C for 120 seconds. The first 5 cycles of primer annealing took place at 50 °C for 90 seconds followed by 25 additional cycles at 60 °C. The amplification products were separated by electrophoresis for one hour on an agarose gel (Sigma-Aldrich; Oakville, ON, Canada; 1.0%) made with Tris-acetate (0.04 M) and EDTA (1mM). Bands were visualised on a transilluminator, excised from the gel, purified using the Wizard polymerase chain reaction (PCR) preparation kit (Promega; Madison, WI, USA), ligated into pGEM®-T Vector using the TA cloning system (Promega) and cloned into *Escherichia coli* strain DH5α.

Genomic library screening and subcloning of the endopolygalacturonase (*epg1*) gene

Genomic DNA from *Ophiostoma novo-ulmi* subsp. *americana* strain MH75 was digested with the restriction enzyme *Mbo* I and ligated in bacteriophage lambda EMBL3. *Escherichia coli* strain VCS257 (Stratagene, La Jolla, CA, USA) was transfected with genomic library as described by Bowden et al. (1994). Hybridisation 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* gene fragment derived using oligonucleotide primers OUEPGF and OUEPGR. Plaques hybridising to the ^{32}P-dCTP *epg* gene probe DNA were re-screened a second time and phage DNA was isolated using polyethylene glycol (PEG) precipitation (Sambrook & Russell, 2001). The position of the polygalacturonase gene (designated *epg1*) in the recombinant phage was determined by restriction mapping of the isolated recombinant phage followed

by hybridisation analysis (Sambrook & Russell, 2001). A single 3.5 KB *Sal* I DNA fragment containing the *epg1* gene was subcloned into the pUC18 plasmid vector, and the entire gene sequence was determined by automated fluorescent sequencing using the LI-COR model 4200 (LI-COR; Lincoln, NE, USA).

Qualitative Polygalacturonic acid plate assay of polygalacturonase (EPG1ase) activity

To determine the level of secreted *epg1*-encoded polygalacturonase (designated as EPG1ase), *Ophiostoma novo-ulmi* subsp. *americana* wild-type strain VA30 and the modified VA30 strain with the *epg1*$^{-}$ gene mutant were inoculated into liquid OCM (5 mL) and grown in stationary culture at 23 °C for 7 days. Assay plates were prepared containing potassium acetate (50 mM), EDTA (5 mM), polygalacturonic acid (Sigma-Aldrich; Oakville, ON, Canada; 0.1% w/v) and agarose (Sigma-Aldrich; Oakville, ON, Canada; 1.0%), pH 4.5 (Bussink et al., 1992). Polygalacturonic acid was utilized as the substrate in these assays as it is predominately the enzymatic target of polygalacturonase, and thus limits background interference from other pectic enzymes. After solidification, a cork borer (5 mm diameter) was used to remove agarose plugs and create 3 wells per plate. Culture filtrate (1 mL) was lyophilised overnight. The resultant pellet was re-suspended in an aliquot (0.1 mL) of a solution of potassium acetate (50 mM) and, EDTA (5.0 mM) at pH 4.5 and applied to each well on the assay plates. After 24 hours of incubation at room temperature, the undigested polygalacturonic acid remaining in the plates was stained for 20 minutes using Ruthenium red (Sigma-Aldrich; Oakville, ON, Canada; 0.05% w/v in water) and washed with three changes of distilled water. The activity of EPG1ase was characterised by visually assessing the staining, or lack thereof, in the halo surrounding the wells.

Disruption of the *epg1* gene in *O. novo-ulmi* subsp. *americana*

The *epg1* gene locus of *O. novo-ulmi* subsp. *americana* strain VA30 was disrupted via recombination by the hygromycin phosphotransferase gene (*hph*) (Punt et al., 1987) flanked by endogenous *epg1* gene sequences. Protoplast formation and transformation of *O. novo-ulmi* subsp. *americana* was as previously detailed in Temple et al. (1997). This strategy took advantage of four unique restriction sites bounding the *hph* gene to introduce flanking DNA derived from, and homologous to, *O. novo-ulmi epg1*. The flanking *epg1* DNA was amplified with the following two primer sets: OUHPH F1 5' CATCACAAGATCTGGCATGGGCAGTTC 3' (tailed with *Bgl*II) and OUHPH R1 5' TTGAAGAGGTCACCCGACGCGC3'(tailed with *Bst*EII) 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 unique restriction sites corresponding to sites flanking the *hph* gene. The resulting construct (Figure 1) was used for homologous gene targeting of e*pg1*. Isolates carrying a putative disruption in the *epg1* gene locus 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'. The identity of the amplified 1131 bp diagnostic fragment was confirmed by sequence analysis. The isolate having a disrupted *epg1* gene was named *epg1*$^{-}$.

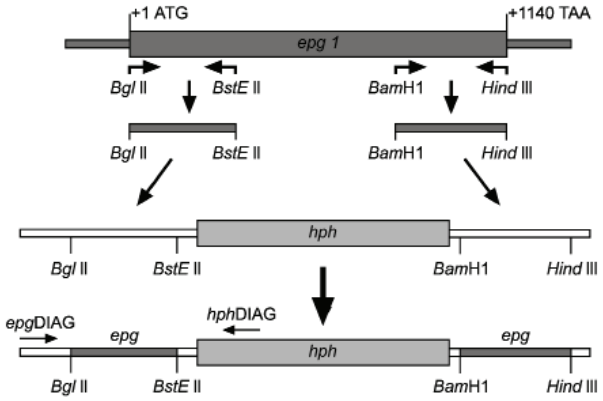

FIGURE 1: Diagramatic representation of the targeted disruption of the *epg1* gene in *O. novo-ulmi* subsp. *americana* strain VA30. Expression of the *epg1* gene was disrupted by the insertion of the selectable marker for hygromycin resistance (*hph*) at the *epg1* locus. The *hph* marker was flanked with DNA amplified from the endogenous *epg1* locus and inserted immediately up and downstream of the hph marker. Putative *epg1*$^{-}$ mutant isolates 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).

Genetic mapping of *epg1*

Ophiostoma novo-ulmi and *O. ulmi epg1* alleles were readily distinguished from each other by the presence of an *Ssp1* restriction site in the *epg1* allele of the latter. This allowed the localisation of the *epg1* gene locus on an existing *Ophiostoma* genetic map (Dusabenyagasani et al., 2000). The map, which includes over 200 loci, was derived from the analysis of 90 F_1 progeny from an interspecific cross between

O. novo-ulmi subsp. *americana* laboratory strain 416R14 and *O. ulmi* strain Q412. The coding region for the *epg1* gene was PCR-amplified, and digested with the restriction enzyme *Ssp1*. The resultant DNA fragments were separated by gel electrophoresis and the two polymorphic types were scored in the 416R14 x Q412 progeny. Mendelian segregation of the *epg1* alleles was verified by a χ^2 test of the two polymorphic types. Recombination between the *epg1* gene and the other loci was studied using MAPMAKER for the Macintosh™, version 2.0 software (Lander et al., 1987) with a LOD score value of 4.0 and a theta of 0.25.

Virulence trials on *Ulmus parvifolia* x *U. americana*

Yeast-like cells of *O. novo-ulmi* subsp. *americana* wild-type strain VA30, or from strain VA30 with the polygalacturonase mutant gene (*epg1*$^-$) were isolated from 3-day-old OCM shake cultures kept at room temperature and 110 revolutions per minute. 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 x 10^6 cells/mL. Two-year-old seedlings of clonally propagated *Ulmus parvifolia* x *U. americana* F1 hybrid clone 2245 (Smalley & Guries, 1993) grown in a perlite/peat moss (25% : 75%) substrate were used as hosts. Seedlings were inoculated by injecting spore suspension or water (for the controls) (100 μL) through a 1 cm long vertical slit (positioned 5 cm above the root crown) that was made with a sterile scalpel. The inoculation wound was then wrapped in parafilm and trees were distributed in eight randomly distributed blocks, with one replicate of each treatment 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-hour 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 days post inoculation. Re-isolation of the fungal strains from the infected elms was not performed because the experiment was conducted in an environmentally-controlled growth chamber where fortuitous infections by other fungi were unlikely. Also, the water and wild-type controls showed the expected range of responses to inoculation (with either water or *O. novo-ulmi*). Further, as the symptoms of Dutch elm disease are very characteristic, it was very easy to ensure that the symptoms seen in infected elms were, in fact, attributable to *O. novo-ulmi*. As the goal of the experiment was to determine the pathogenic effects of knocking out a major cell wall degrading enzyme, re-isolation of the strains from inoculated trees was considered unnecessary.

Results and Discussion

Structural analysis of the *epg1* gene

The full length *O. novo-ulmi* subsp. *americana* MH75 *epg1* gene was recovered from a genomic library (Genbank accession AF052061). DNA sequencing of ten separate clones identified that the amplification products corresponded to a single polygalacturonase gene. As all of the amplification products yielded the same sequence, it was unlikely that there were multiple copies of this gene present in the genome. This was further supported by hybridization analysis which supported the presence of a single copy of the *epg1* gene in *O. novo-ulmi* subsp. *americana* VA30. Mapping data indicated that the *O. ulmi* and *O. novo-ulmi epg1* alleles segregated 1:1 in the 416R14 x Q412 progeny. The *epg1* gene locus co-segregated with 40 genetic markers previously assigned to linkage group II-1. This linkage group corresponds to a 3.5 Mb chromosome (chDNA II), which harbours another putative pathogenicity gene locus, *pat1* (Et-Touil et al., 1999). Recombination between the *epg1* gene and Opk3-1050 (a Random Amplification of Polymorphic DNA (RAPD) marker tightly linked to the *pat1* gene) was 29%. The function of *pat1* remains unknown, but *epg1* and *pat1* are obviously different loci since close to 30% of the 416R14 x Q412 F_1 progeny were recombinants for the *epg1* gene and the marker Opk3-1050, which is tightly linked to *pat1* (Et-Touil et al., 1999).

The cloned DNA sequence of the *epg1* gene comprised the entire 1140 base pair coding region of the *epg1* gene locus plus 370 base pairs (bp) of upstream sequence (identified as the promoter), and 244 bp of downstream sequence. The *epg1* promoter sequence of *O. novo-ulmi* subsp. *americana* showed a putative TATA box at position -30. Areas of pyrimidine-rich sequence, characteristic of fungal promoters, were also found in the upstream region. There were no frame-shifts in the 1140 bp coding 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 start site for filamentous fungi proposed by Ballance (1986). The putative active site at amino acid positions 231 to 243 was in 100% agreement with consensus sequence reported by Reymond et al. (1994) to be characteristic of the active site of polygalacturonases in general. Multiple sequence alignment showed close segregation of the *epg1* gene with other fungal endopolygalacturonase proteins.

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MH75    MLGITTLLLTLAVAANASPVVAPANTPTPSPVLAERATSCTFSGANGASLAIQSQAACAT   60
H175    MLGITTLLLTLAVAANASPVVAPANTPTPSPVLAERATSCTFSGANGASLAIQSQAACAT   60
Q412    MLGITTLLLTLAVAANASPVVAPANTPTPSPVLAERATSCTFSGANGASLAIQSQAACAT   60

MH75    IVLNNVAVPSGTTLDLSKLADGATVIFEGETTWGYKEWAGPLLQIKGNGITVQGASGAVF  120
H175    IVLNNVAVPSGTTLDLSKLADGATVIFEGETTWGYKEWAGPLLQIKGNGITVQGASGAVF  120
Q412    IVLNNVAVPSGTTLDLSKLADGATVIFEGETTWGYKEWAGPLLQIKGNGITVQGALGAVL  120
                                                        ↑    ↑

MH75    NANGARWWDGKGSNSGKTKPKFFYAHGLTNSKILNLSIKNTPIQAVSINGCDGLTITDMT  180
H175    NANGARWWDGKGSNSGKTKPKFFYAHGLTNSKILNLSIKNTPIQAVSINGCDGLTITDMT  180
Q412    NANGARWWDGKGSNSGKTKPKFFYAHGLTNSKILNLSIRNTPIQAVSINGCDGLTITDMT  180
                                            ↑

MH75    IDSSAGDSAGGHNTDGFDIGTSKNIIIDGAKVYNQDDCVAVNSGTKITFQNGLCSGGHGL  240
H175    IDSSAGDSAGGHNTDGFDIGTSKNIIIDGAKVYNQDDCVAVNSGTKITFQNGLCSGGHGL  240
Q412    IDSSAGDSAGGHNTDGFDIGTSKNIIIDGAKVYNQDDCVAVNSGTKITFQNGLCSGGHGL  240

MH75    SIGSVGGRSDNTVDTVTFYNSQIKNSVNGIRVKGTVGTTGTIKGVTYNKITLSGITNYGV  300
H175    SIGSVGGRSDNTVDTVTFYNSQIKNSVNGIRVKGTVGTTGTIKGVTYNKITLSGITNYGV  300
Q412    SIGSVGGRSDNTVDTVTFYNSQIKNSVNGIRVKGTVGTTGTIKGVTYNKITLSGITNYGV  300

MH75    LIEQNYNGGDLKGTATSGVPITGLTIKDISGTKAIASSGYNVVITCGSGACSGWTWSGVS  360
H175    LIEQNYNGGDLKGTATSGVPITGLTIKDISGTKAIASSGYNVVITCGSGACSGWTWSGVS  360
Q412    LIEQNYNGGDLKGTATSGVPITGLTIKDISGTKAIASSGYNVVITCGSGACSGWTWSGVS  360

MH75    VTGGKSYDKCTNVPNGISC                                            379
H175    VTGGKSYDKCTNVPNGISC                                            379
Q412    VTGGKSYDKCTNVPNGISC                                            379
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FIGURE 2: Comparison of the predicted poly-galacturonase sequences from *O. novo-ulmi* strains MH75 and H175, and *O. ulmi* strain Q412. Both *O. novo-ulmi* isolates show 100% sequence homology, while *O. ulmi* shows over 99% homology with the two aggressive isolates. The three amino acid differences are marked with arrows. The underlined NLSI sequence represents the potential N-glycosylation site. The active site of polygalacturonase is shown in bold in a box.

Comparison of the predicted polygalacturonase protein sequences from *O. ulmi* and *O. novo-ulmi*

Following our cloning and Genbank disclosure of the *O. novo-ulmi* subsp. *americana* strain MH75 *epg1* gene sequence, the sequences of the *epg1* mRNAs from the closely related *O. novo ulmi* subsp. *americana* strain H175 and *O. ulmi* strain Q412 were reported on Genbank (DQ078293 and DQ078292, respectively, by Nasmith, C. G., Jeng, R. S. & Hubbes, M., 2005). Interestingly, strain Q412 is representative of the less aggressive and nearly extinct species *O. ulmi*, and the report of these sequences allowed us to compare polygalacturonase sequences between *O. ulmi* and two different isolates (H175 and MH75) of *O. novo-ulmi*. It should be noted that while our disruption experiments were carried out in strain VA30, our *epg1* gene sequence was derived from strain MH75. However, as both strain VA30 and strain MH75 are isolates of the highly clonal *O. novo-ulmi* subsp. *americana* (Temple et al., 2006) the two strains are likely identical.

Given the close evolutionary relationships between *O. ulmi* and *O. novo-ulmi*, it is expected that polygalacturonase sequences would show a high degree of similarity. Isolates from both *O. novo-ulmi* strains MH75 and H175 show 100% sequence homology between the predicted protein sequences their respective EPG1ases (Figure 2). Interestingly, the predicted protein sequence for the EPG1ase from *O. ulmi* Q412 showed over 99% homology with the two *O. novo-ulmi* isolates with only three amino acid changes (from serine (S) to leucine (L) at position 116, from phenylanaline (F) to leucine (L) at position 120, and from lysine (K) to arginine (R) at position 159) (Figure 2). One of the few differences between the *O. novo-ulmi* and *O. ulmi* EPG1ase sequences was found immediately downstream of the only potential N-glycosylation site in the protein (Figure 2). While this change does not directly alter the N-glycosylation site, it has the potential to alter glycosylation efficiency. It follows that *O. novo-ulmi* and *O. ulmi* may have differently glycosylated isoforms of polygalacturonase. At this point, it is unknown if the differences observed in EPG1ases between species *O. ulmi* and *O. novo-ulmi* are indicative of the small, but critical, differences that may influence the levels of pathogenicity when comparing *O. ulmi* and *O. novo-ulmi*. Alternatively,

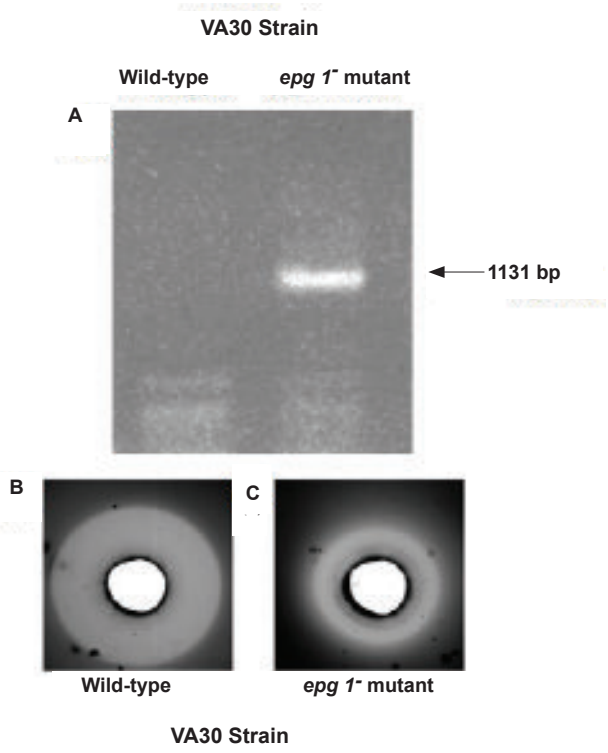

FIGURE 3: A - Gel electrophoretic image of an EPG1ase specific PCR-amplified diagnostic product in *O. novo-ulmi* subsp. *americana* wild-type strain VA30 and modified VA30 strain with the *epg1*$^{-}$ gene mutation. The diagnostic product of 1131 bp can only be amplified in the modified VA30 strain with the *epg1*$^{-}$ gene mutation.

B and C - Activity assays were used to confirm reduction of EPG1ase activity (shown by a smaller halo) in *O. novo-ulmi* subsp. *americana* modified VA30 strain with the *epg1*$^{-}$ gene mutant compared with the wild-type VA30 strain.

they may merely represent a diverging evolutionary lineage wherein the changes are functionally silent.

Targeted disruption of the *epg1* gene

Disruption of the *epg1* gene in *O. novo-ulmi* subsp. *americana* strain VA30 was accomplished by the homologous recombination of a selectable marker specifically targeted to the endogenous *epg1* gene locus. DNA recombination at the endogenous *epg1* genomic locus resulted in the insertion of 3588 bp of the hygromycin phosphotransferase gene *(hph*) driven by the *A. nidulans gpd* (glyceraldehydes-3-phosphate dehydrogenase) gene promoter derived from the vector pAN7-1 (Punt et al. 1987). Incorporation of the *hph* gene at the endogenous *epg1* locus simultaneously provided a dominant selectable marker and prohibited translation of the complete EPG1ase protein (Figure 1). Disruptions in the *epg1* gene were confirmed using a PCR based strategy such that a diagnostic 1131 bp PCR product resulted only in the presence of the correctly integrated disruption vector (Figure 3A). Neither the disruption vector nor the endogenous gene alone would produce an amplification product. The amplicon produced from the mutant *epg1*$^{-}$ gene was recovered, cloned and sequenced to verify accurate disruption, which showed disruption occurred at the expected locations within the mutant *epg1*$^{-}$ gene. It should be noted that targeted disruption by insertional mutagenesis of specific genes has proven to be an extremely difficult proposition in *O. novo-ulmi*, and this study represents only the second time this approach has been reported as being successful in this fungus, the first instance being the targeted disruption of cerato-ulmin (Bowden et al., 1996). In both the study by Bowden et al. (1996) and the current study, a large number of isolates needed to be screened in order to find an isolate with a single disruption in the desired gene locus. For example, in the current study, we screened over 50 putative isolates that carried the dominant selectable marker and only found two isolates where the transforming DNA had integrated at the *epg1* gene locus to produce *epg1*$^{-}$ mutants. Of these two *epg1*$^{-}$ mutants, we were able to recover only one mutant that carried a single integration at the *epg1* locus. The other mutant contained multiple copies of the transforming DNA at various sites throughout the genome due to random integration and was unsuitable for our studies.

Targeted disruption of the *epg1* gene led to substantial reduction of pectinase activity *in vitro* as shown by qualitative pectinase activity assays (Figures 3B and 3C, typical results shown) and prevented growth of the strain with the mutant *epg1*$^{-}$ gene on media containing citrus pectin as the sole carbon source. A number of other studies have reported that the disruption of genes with pectinolytic genes in other fungi has had varying impacts on growth and substrate utilisation. For example, disruption of the *pgn1* gene of *Cochliobolus carbonum* (Scott-Craig et al., 1990), the *Bcpg1* gene in *Botrytis cinerea* (ten Have et al., 1998), and the *Aapg1* gene of *Alternaria alternata* (Isshiki et al., 2001) did not halt growth of these fungi on media in which pectin was the only carbon source. This suggested the presence of other compensatory enzymes that are able to digest pectin. Only one case has been reported in which disruption of a polygalacturonase gene (the *Acpg1* gene in *A. citri*), led to a phenotype that grew very poorly on pectin-supplemented media (Isshiki et al., 2001).

Virulence trials on *Ulmus parvifolia* x *U. americana*

One of the goals of this study was to determine the contribution of a single cell wall degrading enzyme to the virulence of *O. novo-ulmi* subsp. *americana* strain VA30. Inoculation of two-year-old seedlings of

clonally propagated *Ulmus parvifolia* x *U. americana* F1 hybrid clone 2245 with the wild type *O. novo-ulmi* subsp. *americana* strain VA30 led to the rapid onset of symptoms and wilting by six days post inoculation (qualitative observation). The onset of wilt symptoms in the modified VA30 strain with the *epg1*$^{-}$ gene mutation was slower and less severe compared to the wild type VA30 strain. Trees were assessed for the extent of defoliation at 22 days post inoculation and the modified VA30 strain with the *epg1*$^{-}$ gene mutant was found to produce a lower mean percent defoliation (54.5 ± 38.5, reported as mean ± standard deviation in all instances) compared to that of the wild type strain VA30 (81.9 ± 34.5). Water controls demonstrated no wilt symptoms at six days post inoculation and showed a mean percent defoliation of 13.1 ± 11.3 at 22 days post inoculation. The non-parametric Wilcoxon-Mann-Whitney test was used to compare the mean percent defoliations statistically. This analysis found that the wild type VA30 strain showed significantly ($p < 0.05$) higher levels of mean defoliation relative to the water control. However, the modified VA30 strain with the *epg1*$^{-}$ gene mutant showed no significant differences between the water control and the wild type VA30 strain. This suggested that the mutant *epg1*$^{-}$ gene produced an intermediate phenotype on the elm clone tested (Figure 4). Disruption of the *epg1* gene in *O. novo-ulmi* strain VA30 permitted testing of the role of the enzyme *in planta* and suggested a role for this enzyme in establishment of disease on the elm hybrid *Ulmus parvifolia* x *U. americana* F1 hybrid clone 2245. However, based on the data presented here, we feel the contribution of the *epg1* gene to virulence is relatively minor, and other gene loci must certainly be involved. Our data favors the hypothesis that *epg1* could be more accurately classified as a parasitic fitness factor in *O. novo-ulmi*. 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 pathogenicity in a fungus such as *O. novo-ulmi* is difficult to extrapolate to other combinations of pathogen and host. Therefore, at present, our results indicate that pathogenicity in *O. novo-ulmi* is only partly dependent on polygalacturonase.

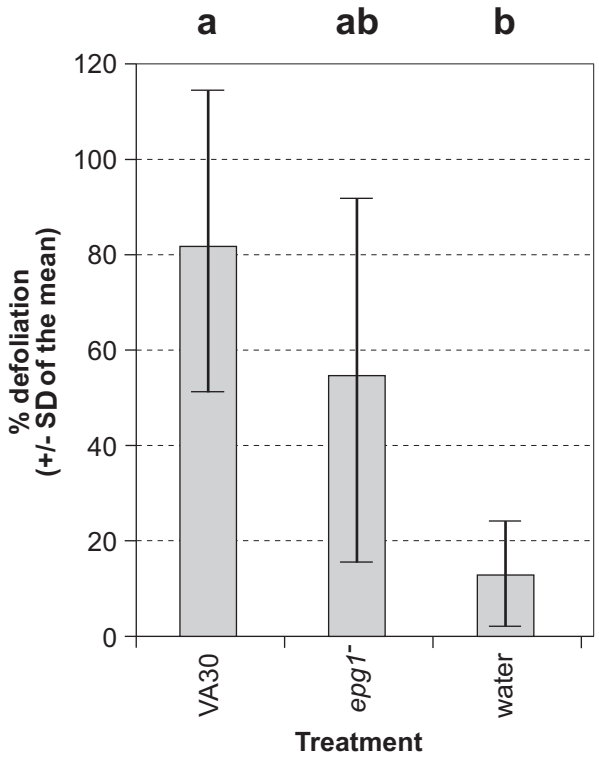

FIGURE 4: Percent defoliation in two-year-old seedlings of clonally propagated *Ulmus parvifolia x U. americana* F1 hybrid clone 2245, 22 days post inoculation with *O. novo-ulmi* subsp. *americana* wild-type strain VA30, modified VA30 strain with the *epg1*$^{-}$ gene mutant and water control. Statistically different treatments are designated by different letters at the top of the bars when differences are significant ($p < 0.05$), and by the same letter when no difference is detectable.

Conclusions

To clarify the specific role of polygalacturonase in the life history of *Ophiostoma novo-ulmi* and determine the contribution of a single gene towards the overall virulence of *O. novo-ulmi*, we cloned the polygalacturonase encoding *epg1* gene of *O. novo-ulmi* and determined the effects of disrupting the expression of this gene locus.

The polygalacturonase gene occurred as a single copy that was similar to the polygalacturonase genes of other fungi. Targeted disruption of this gene led to reduction of pectinolytic activity *in vitro* but did not result in a lethal phenotype. Instead, a phenotype of intermediate virulence rather than a lethal one was observed in pathogenicity trials.

It would seem that the cell wall degrading enzyme polygalacturonase is, at best, a minor virulence factor in *O. novo-ulmi*, as the disruption of the gene (*epg1*) responsible for polygalacturonase activity alone gave rise to a variable phenotype with virulence only slightly lower than the highly aggressive wild type isolate. Our results suggest that there was a single locus for the *epg1* gene (based on the lack of hybridisation to other gene variants and the lack of amplification of other forms of the gene using the degenerate *epg*-specific primers). However, there are certainly other pectinase enzymes present in *O. novo-ulmi* and these enzymes likely act in concert to contribute to the successful invasion of the host. The elimination of one enzyme could be compensated by the expression of any number of other enzymes during the infection process. Thus, our data suggest that polygalacturonse

is only one component in the pathogenic artillery of *O. novo-ulmi* and the loss of *epg1* activity is probably compensated for by other pectinases.

While there may be a small role for polygalacturonase in contributing to the disease symptoms in the host, it is more likely that polygalacturonase functions as a parasitic fitness factor. It has been previously demonstrated that *O. ulmi* has a lower parasitic fitness relative to *O. novo-ulmi* (Temple et al., 1997), and the more aggressive *O. novo-ulmi* is currently replacing *O. ulmi* in natural populations (Brasier, 2000; Gibbs et al., 1979). *Ophiostoma novo-ulmi* shows superior bark colonisation relative to *O. ulmi* (Webber & Hedger, 1986) and demonstrates higher survival rates in both resistant and susceptible elm cultivars (Scheffer & Elgersma, 1982). Further, as disruption of the *epg1* gene led to a delay in onset of wilt symptoms, our results suggest that the *epg1* gene is involved in establishing infection in elm. Indeed, it has been suggested that infection of *Ulmus* species by *O. ulmi* or *O. novo-ulmi* requires degradation of host cell wall polymers through secretion of several cell wall degrading enzymes (Svaldi & Elgersma, 1982). In this regard, differential production of cell wall degrading enzymes may influence parasitic fitness in *O. novo-ulmi*.

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