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Lack of Association between Cerato-ulmin Production and Virulence in *Ophiostoma novo-ulmi*

Christine G. Bowden[1], Eugene Smalley[2], R. P. Guries[3], Martin Hubbes[4], Bradley Temple[1], and Paul A. Horgen[1]

[1]Department of Botany, University of Toronto, Erindale Campus, Mississauga, Ontario, L5L 1C6, Canada; [2]Department of Plant Pathology, University of Wisconsin, Madison 53706 U.S.A.; [3]Department of Forestry, University of Wisconsin, Madison 53706 U.S.A.; and [4]Faculty of Forestry, University of Toronto, Toronto, Ontario, M5S 3B3, Canada
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Cerato-ulmin (CU), a hydrophobin produced by *Ophiostoma novo-ulmi*, has been implicated in the pathogenicity of this fungus on elm. We have generated a CU⁻ mutant by transformation-mediated gene disruption of a highly virulent (aggressive) strain of *O. novo-ulmi*. The inability of the mutant to synthesize CU was confirmed by transcript analysis as well as turbidity and immunological measurements. Bioassay of the CU⁻ strain in highly susceptible elm trees indicated no difference in the virulence parameters, percent vascular discoloration, and percent foliar wilting, when compared with the wild type. Our results indicate that the inability to produce CU had no measurable effect on the ability of *O. novo-ulmi* to produce symptoms of Dutch elm disease on inoculated elms.

Wilt pathogens are unique in that they do not invade healthy host tissue but are limited to the xylem of the tree. Since some microorganisms are capable of entering and colonizing the vascular system without inducing wilt symptoms, the virulence of a pathogen is measured by its ability to cause wilting symptoms. There is still a considerable amount of debate as to how the pathogen induces water stress. Both formation of embolisms and vascular plugging, particularly of the pit membranes, may affect extracellular water potential (Van Alfen 1989). Alternatively, plant membrane function may be affected directly. Although a variety of compounds have been suggested as probable agents involved in disease expression, at present no virulence genes have been isolated from wilt pathogens.

The protein cerato-ulmin (CU) produced by the fungal wilt pathogen *Ophiostoma novo-ulmi* Brasier (Brasier 1991) (= *Ceratocystis ulmi* Buisman) has been proposed as a pathogenicity factor in Dutch elm disease. The in vitro production of this small, 7.5-kDa, hydrophobic peptide appears in most cases to be correlated with the virulence of wild-type isolates on elm (Takai 1980; Brasier et al. 1990). Exceptions to this association have been shown in naturally occurring non-CU-producing isolates (Brasier et al. 1995) and progeny derived from crosses of highly and mildly virulent isolates (Takai 1989). CU is a member of a group of secreted fungal proteins known as hydrophobins. They are characterized as having low molecular weight, hydrophobic domains, and conserved cysteine residue patterns (Stringer and Timberlake 1993; Wessels 1994, in press). Hydrophobins have been isolated from a variety of fungi including *Aspergillus nidulans* (Stringer et al. 1991), *Schizophyllum commune* (Wessels 1992), *Metarhizium anisopliae* (St. Leger et al. 1992), *Neurospora crassa* (Bell-Pederson et al. 1992; Lauter et al. 1992), *Cryphonectria parasitica* (Zhang et al. 1994), *Magnaporthe grisea* (Talbot et al. 1993) and *O. novo-ulmi* (Bowden et al. 1994). Several hydrophobins have been identified as the products of genes expressed during the development of fungal aerial structures (Wessels et al. 1991). At present, the *mpg1* gene product of the rice pathogen *M. grisea* is the only hydrophobin shown to play an important role in pathogenicity (Talbot et al. 1993). In this study, we have constructed a vector for the targeted disruption of the CU gene of *O. novo-ulmi*. A mutant was recovered that lacked the ability to synthesize CU, based on turbidity measurements, immunological measurements, and transcript analysis. The mutant was utilized in greenhouse trials on several varieties of *Ulmus*. Based on the results from these studies, we conclude that the hydrophobin CU is not a major pathogenicity factor in Dutch elm disease.

Corresponding author: Paul A. Horgen
E-mail: phorgen@tuzo.erin.utoronto.ca

RESULTS

Transformation-mediated gene disruption.

The linearized transformation plasmid pHEK5 was used to transform *O. novo-ulmi* to hygromycin resistance (Fig. 1A, B). Initially, 2,500 hygromycin-resistant transformants were prescreened for CU production by immunoassay and/or turbidity analyses. These prescreening measurements resulted in the identification of 17 transformants that produced no visible color in the immunoassay and/or A_{400} turbidity readings of <0.074. These transformants were then evaluated for interruption of the *cu* gene. Hybridization of *Bgl*II-digested DNA with a ^{32}P-labeled *cu* fragment indicated that only one transformant (THEK5-8) possessed an insertion in the 1.9-kb *Bgl*II fragment containing the *cu* gene (Fig. 2A). All other transformants appeared to have ectopic integration, which did not disrupt the *cu* gene, but in some way affected the synthesis or secretion of the hydrophobin. THEK5-8 lacked the 1.9-kb *Bgl*II band but

contained a 30.5-kb band, indicating a tandem insertion of the vector into this site (Fig. 2B). Further evaluation of the multiple vector insertion in THEK5-8 was made with digestions of the DNA with *Hin*dIII, *Bst*XI, and *Eco*RI. *Hin*dIII was used to linearize the vector (single site was in multiple cloning site of plasmid); *Bst*XI cuts once just 3′ of *cu* coding region and therefore once in the vector; and *Eco*RI cuts twice in the hygromycin insert only. Although a 6.5-kb band of the intact vector was predicted after *Hin*dIII digestion and hybridization with pCU-1, which contains the *cu* gene (Fig. 2D), pAMP, which contains the ampicillin gene (Fig. 2E), and pAN7-1, which contains the hygromycin gene (Fig. 2F), only a high molecular weight band was seen. It appeared that any *Hin*dIII sites present upon formation of tandem vector copies had either been lost or modified. The other two enzymes did cut the high molecular weight band into smaller fragments. Digestion with *Eco*RI produced a prominent 4.2-kb band that hybridized to pCU-1 (Fig. 2D), pAMP (Fig. 2E), and pAN7-1 (Fig. 2F). Although this was the largest band present, it was smaller than could be explained by head-to-head (7.1-kb) or head-to-tail (5.1-kb) multiple copies. Likewise, hybridization of *Bst*XI digested THEK5-8 with pCU-1 and *hph* (hygromycin) produced

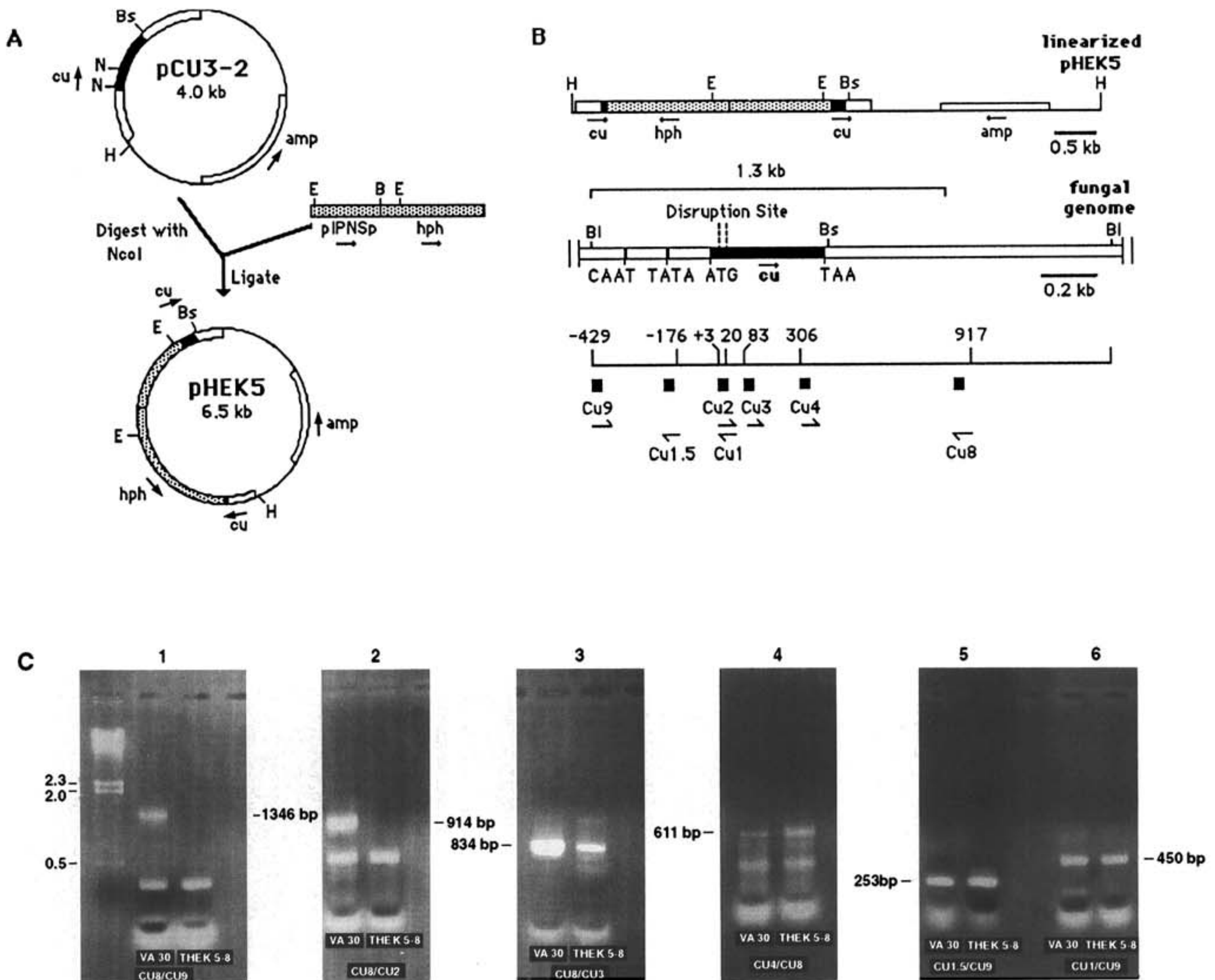

Fig. 1. Transformation-mediated gene disruption of the cerato-ulmin gene. **A,** Construction of the transformation vector pHEK5 utilized the plasmid pCU3-2, which contained the cerato-ulmin gene of strain MH75 located on a 1.3-kb fragment. A 121-bp *Nco*I fragment from the coding region of the *cu* gene was replaced by a 2.6-kb fragment containing the isopenicillin N synthetase (*ipns*) promoter from *P. chrysogenum* and the hygromycin B phosphotransferase (*hph*) gene from *Escherichia coli*. The *Hin*dIII site is in the plasmid. Solid black band represents the *cu* coding region. B, *Bam*HI; Bl, *Bgl*II; Bs, *Bst*XI; E, *Eco*RI; H, *Hin*dIII; and N, *Nco*I. **B,** Maps of *Hin*dIII-linearized vector and the wild-type *Ophiostoma novo-ulmi* cerato-ulmin gene with vector insertion site. Polymerase chain reaction (PCR) primers are shown below that were used for location of the insertion site in THEK5-8. **C,** Comparisons of wild-type VA30 and CU⁻ mutant THEK5-8 PCR products with pairwise primers. PCR utilized primers corresponding with positions +3 to +20 (CU2), +83 to +100 (CU3), +306 to +323 (CU4) in the coding sequence of the *cu* gene and −429 to −410 (CU9) located 5′ to the coding sequence, paired with +917 to +896 (CU8) located 3′ to the coding sequence. Primers corresponding with positions +21 to +3 (CU1) and −176 to −200 (CU1.5) were also paired with CU9. Base pair numbers on the side indicate the size of band predicted for each primer pair. Size markers are given in kilobases at far left. Product size in gel 4 is correct ; gel was not electrophoresed as long as others.

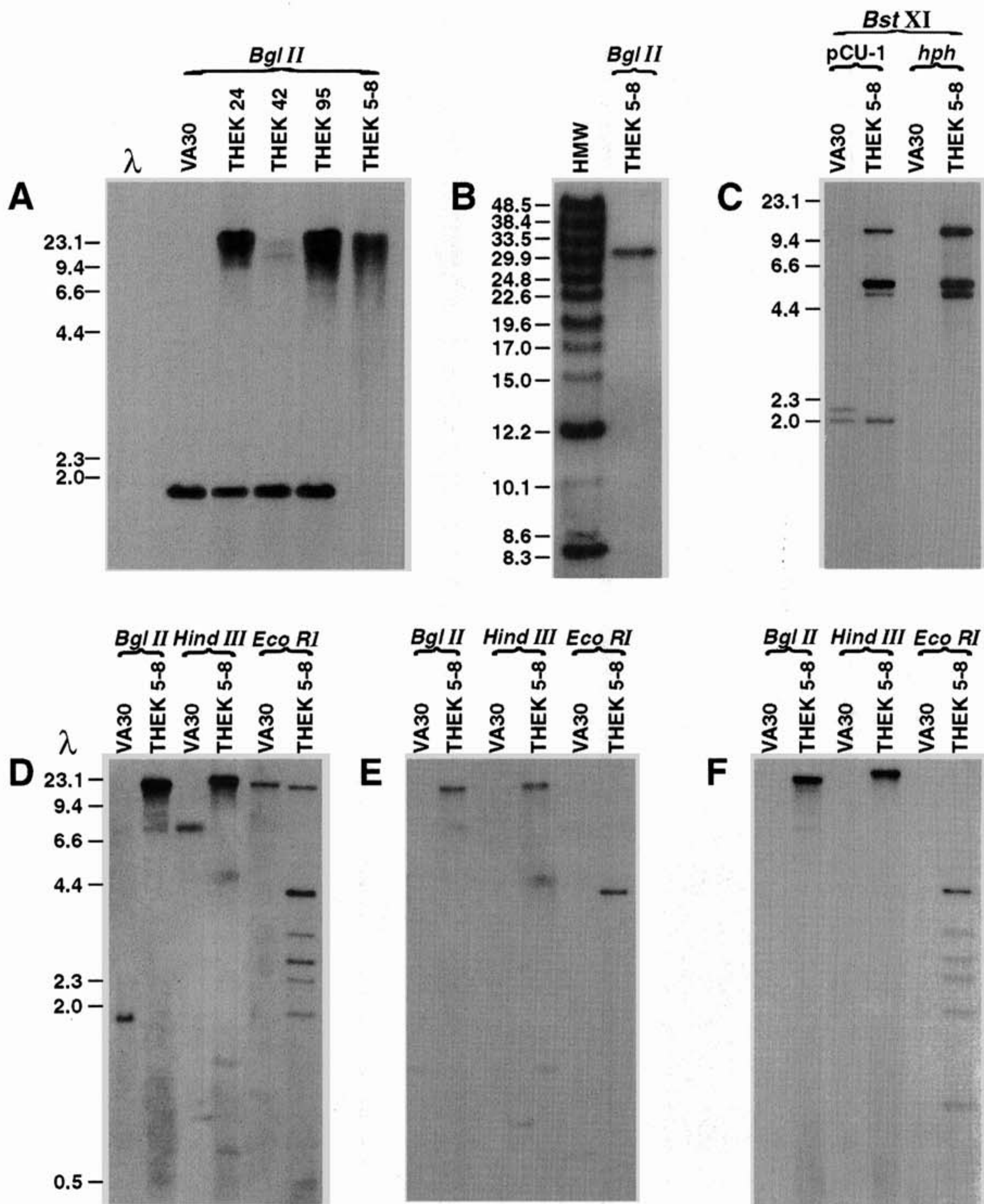

Fig. 2. Southern analysis of putative disrupted mutants. **A,** Digestion of the wild-type VA30 and four nonsecreting transformants with *Bgl*II. For this representative gel, hybridization was with the 1.9-kb *Bgl*II fragment containing the entire *cu* gene. All 17 putative CU⁻ mutants were analyzed by Southern hybridization; only THEK5-8 showed evidence for disruption of the *cu* gene by the absence of the 1.9-kb band. Size markers are given in kilobases. **B,** Electrophoresis of *Bgl*II-digested THEK5-8 DNA in a 0.4% gel and hybridization with pHEK5. VA30 was also run but is not shown here, since the 1.9-kb band is off the gel. Sizes (kilobases) of high molecular weight markers are shown at left. **C,** Digestion of VA30 and THEK5-8 with *Bst*XI and hybridization with pCU-1 and a 2.6-kb fragment containing the isopenicillin N synthetase promoter and the hygromycin B phosphotransferase gene (*hph*). **D, E, F,** Digestion of VA30 and THEK5-8 with *Bgl*II, *Hind*III, and *Eco*RI and hybridization with (**D**) pCU-1, (**E**) pAMP, which contains *amp,* and (**F**) pAN7-1, which contains *hph*.

a prominent 5.5-kb band rather than the 6.0- or 6.5-kb bands predicted with intact tandem copies in head-to-head or head-to-tail orientations (Fig. 2C). Some bands appeared that were of the predicted sizes. Two (2.0- and 2.2-kb) bands are present in VA30 digested with *Bst*XI and probed with pCU-1 (Fig. 2C). These bands occur due to the *Bst*XI restriction site that occurs just 3′ to the *cu* coding region (position +571 in fungal genome; Fig. 1B), a site that is present 1.3-kb upstream of the 5′ *Bgl*II site and a third site 1.0 kb downstream of the 3′ *Bgl*II site. The 2.0-kb band is present in THEK5-8 probed with pCU-1 (Fig. 2C), indicating that the DNA is intact from +571 to the 3′ *Bst*XI site. Absence of the 2.2-kb band in THEK5-8 indicates that an insertion has occurred between the 5′ *Bst*XI site and +571. A 1.2-kb band is unique to the *Eco*RI restriction probed with *hyg* (Fig. 2F) because *Eco*RI sites only occur in the *ipns* promoter-*hph* insert (Fig. 1A, B). Other bands that occur are not predicted by intact tandem copies of the vector. We hypothesize that tandem copies inserted, folded back on themselves, and then rearrangements and deletions occurred within the insertion. The presence of *cu*, *hyg*, and *amp* in the insertion confirm that the tandem plasmid copies initially integrated by a single crossover event, but the subsequent deletions make mapping of the insertion virtually impossible.

The approximate location of the vector insertion into the *cu* gene was determined by a comparison of polymerase chain reaction (PCR) products resulting from amplifications of DNA isolated from THEK5-8 and from VA30. Forward primers were constructed at –429, +3, +83, and +306 (Fig. 1B). Reverse primers were designed for –176, +21, and +917. With VA30, products of the predicted sizes were produced with each pairwise combination of primers. The following primer combinations produced the expected PCR products (Fig. 1C) in both THEK5-8 and VA30: CU3 and CU8 (834 bases), CU4 and CU8 (611 bases), CU9 and CU1.5 (252 bases), CU9 and CU1 (450 bases). The primer combination CU8 and CU9 produced the expected 1,346-base product for VA30 but no product was observed for THEK5-8 since the PCR product was too large. Likewise, the primer pair CU2 and CU8 produced a 914-base product that was only observed for VA30. These data establish that the interruption occurred between +21 and +83 in the 5′ end of the coding sequence.

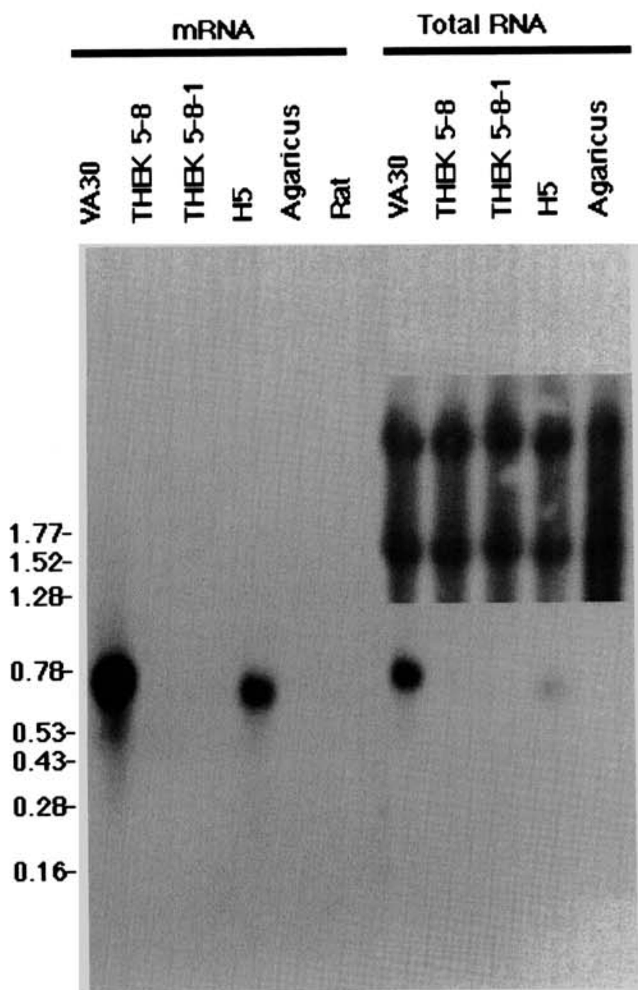

Fig. 3. Northern (RNA) analysis of cerato-ulmin expression. The Northern blot was hybridized with a 1.9-kb *Bgl*II fragment containing the *cu* gene. Blot was stripped and a *Schizophyllum commune* rDNA cassette was used as a constitutively expressed gene for hybridization to the total RNA. Data has been overlaid (top right) to illustrate even loading in all total RNA lanes. Samples include wild-type VA30 and the original *Ophiostoma novo-ulmi* transformant THEK5-8, as well as the single-spore isolate designated THEK5-8-1, *O. ulmi* H5, and two negative controls, *Agaricus bisporus* and rat poly(A)$^+$ RNA. RNA ladder in kilobases is shown at left.

Northern analysis.

Northern (blot) analysis with both total and poly(A)$^+$ RNA revealed that the *cu* mutant THEK5-8 did not possess the 0.72-kb mRNA band observed in the nontransformed isolates (Fig. 3). Although the previous Southern hybridizations used a single-spore isolate of THEK5-8, the Northern analysis evaluated both the initial transformant (THEK5-8) as well as a single-spore isolate (THEK5-8-1). The Northern blots were exposed for 1, 5, and 8 days (extremely overexposed) and we specifically looked for transcripts smaller than the 720-base intact *cu* transcript. Our results did not suggest that any truncated RNA fragments complementary to portions of the *cu* gene had accumulated. *Agaricus bisporus* and rat RNA were utilized as negative controls; rDNA was utilized as a constitutively transcribed gene to probe the total RNA lanes to ensure near-equal amounts of RNA were loaded into each well (Fig. 3).

Growth measurements of THEK5-8.

There was no statistically significant difference ($P < 0.01$) in radial colony growth extension on *Ophiostoma* complete medium (OCM) between wild-type VA30 (4.18 ± 0.14 mm/day) and the THEK5-8 transformant (4.14 ± 0.06 mm/day). There was also no statistically significant difference ($P < 0.01$) in dry mycelial weight between wild-type VA30 (311 ± 12 mg) and the THEK5-8 transformant (307 ± 15 mg).

Macroscopic and microscopic phenotype.

The macroscopic appearance of the THEK5-8 mutant was different from that of VA30 and of the nonaggressive isolate H5. The mutant colony produced sparse aerial mycelia compared with the wild type, which produced abundant aerial hyphae. The aerial hyphae produced by THEK5-8 gave the colony an appearance very different from that of the flat, waxy-appearing nonaggressive H5. After application of a drop of

water to the mycelial surface of the three colonies, only the CU⁻ mutant showed a water-soaked appearance. In comparison, the water drop stayed suspended on top of the aggressive VA30 mycelial surface or spread along but did not absorb into the waxy surface of the nonaggressive *O. ulmi* H5 isolate. Microscopic examination with a dissecting microscope at 10 to 50× revealed the same aerial hyphal differences as those seen macroscopically. Examination of hyphae with a compound microscope at 100 to 400× revealed no observable differences between the mutant and VA30.

Virulence of CU⁻ mutant on elm.

Analysis of percent vascular discoloration (including length and volume vascular discoloration) and percent foliar wilting (Proctor et al. 1994) in the extremely susceptible clonal *U. americana* L. cv. LA1-1 trees indicated that there were no significant differences ($P \leq 0.05$) in the virulence parameters of the CU⁻ mutant THEK5-8 (initial isolate), the single-spore isolate THEK5-8-1, or the VA30 aggressive isolate (Fig. 4). The corresponding percent volume vascular discoloration and percent foliar symptoms were significantly less for the nonaggressive *O. ulmi* H5 isolate. The analysis of differences in the *U. americana* cv. Amer seedlings was less clear-cut. Although there was no significant difference in the percent length of vascular discoloration between THEK5-8, THEK5-8-1, and VA30, there were slight differences in percent volume vascular discoloration. These differences are most likely the result of the genetic variability of the *U. americana* cv. Amer seedlings because the variability between seedling replicates was much higher in the *U. americana* cv. Amer seedlings than in the clonal *U. americana* cv. LA1-1 trees. The values of all three parameters were quite low for all the isolates inoculated in the highly resistant *U. carpinifolia* cv. 380-1 trees, and there were no significant differences among isolates in any of the parameters measured (data not shown).

The appropriate *O. ulmi* and *O. novo-ulmi* cultures were reisolated 50 cm above the inoculation point from previously inoculated test trees. Confirmation of the appropriate isolates was made using hybridization of *Bgl*II-digested DNA with a labeled *cu* fragment, and by confirmation of hygromycin resistance in the transformed cultures.

DISCUSSION

A transformant of *O. novo-ulmi* has been recovered in which the *cu* gene has been interrupted by a modified tandem vector insert. The disruption mutant produced no detectable CU mRNA and no detectable CU protein as measured by either immunoassay or a turbidity analysis. Our virulence evaluations of the CU⁻ mutant would indicate that, as a single primary factor, CU is not significantly involved in the pathogenicity of *O. novo-ulmi* on elm. This result, along with recent findings by Brasier et al. (1995) of naturally occurring non-CU-producing isolates with normal pathogenic abilities, is contrary to previous suggestions that reported that CU was a toxin directly, and primarily, involved in the wilt and vascular discoloration symptoms of Dutch elm disease (Richards and Takai 1984; Richards 1993). A considerable amount of effort has focused on the importance of CU in the development of Dutch elm disease, including examinations of effects on electrolyte loss, wilting, reduction of transpiration, and increases in leaf tissue respiration. Previous studies (Okamoto et al. 1986; Richards and Takai 1984; Landis and Hart 1972; Scheffer et al. 1987; Takai 1989) determined that either *O. novo-ulmi* or CU alone disrupted plasma membrane permeability with a subsequent electrolyte loss from the cells. The presence of *O. novo-ulmi* or purified CU alone was capable of inducing wilting in cuttings of *U. americanus* (Takai and Richards 1978; Stevenson et al. 1979). Since the hydrophobin CU is very surface-active and very insoluble in water, Russo et al. (1982) theorized that CU coated and stabilized air bubbles present in the xylem. These coated bubbles would then block intercellular openings or pits, resulting in wilt. Richards and Takai (1984) determined that a reduction in transpiration as well as an increase in respiration of leaf tissue occurred in the presence of either *O. novo-ulmi* or CU alone. Takai and Hirat-

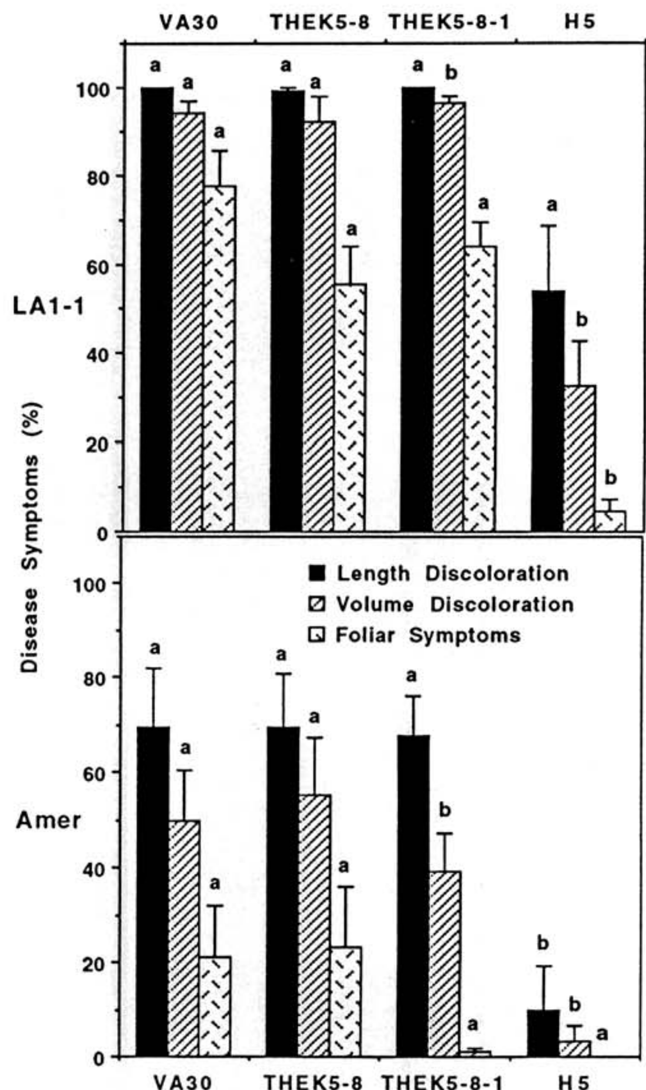

Fig. 4. Virulence assessment of *Ophiostoma novo-ulmi* and *O. ulmi* strains in elm (*U. americana*). Samples include the nonaggressive *O. ulmi* H5, aggressive *O. novo-ulmi* VA30, THEK5-8 (original *cu* transformant), and THEK5-8-1 (single-spore culture of THEK5-8). Columns labeled with the same letter indicate no significant differences ($P \leq 0.05$) for that virulence parameter. Bars at tops of columns represent standard error of the mean. **A,** Extremely susceptible clonal cv. LA1-1 rooted cuttings. **B,** Year-old cv. Amer seedlings.

suka (1984) observed identical, internal, vascular symptoms in white elm subjected to either *O. novo-ulmi* infection or CU alone.

In this study we wanted to determine whether a CU-deficient mutant of *O. novo-ulmi* would affect the virulence of this fungus on elm. Many previous studies have found a strong correlation between in vitro CU production and virulence in wild-type isolates (Brasier et al. 1990; Takai 1980, 1989). Our findings would suggest that CU is not a primary pathogenicity factor in the development of Dutch elm disease symptoms caused by an aggressive isolate of *O. novo-ulmi*. These results are consistent with the recent findings by Brasier et al. (1995), in which two naturally occurring CU-deficient isolates were found to be highly pathogenic. Our findings may also explain an earlier analysis of F_1 progeny from crosses between aggressive and nonaggressive isolates, which indicated that in some cases there was no correlation between CU production and virulence (Takai 1989). Although we have no evidence at this time, one possible explanation for the strong correlation found in the previously mentioned studies of Brasier et al. (1990) and Takai (1980) would be linkage of the *cu* locus to another gene or genes that code for primary pathogenicity factors.

There is considerable evidence in the literature that would suggest the nonaggressive isolates of *O. ulmi* produce lower quantities of the hydrophobin than the aggressive isolates (Takai 1974, 1980; Hubbes and Jeng 1981; Pusey and Wilson 1981; Richards and Takai 1984; Scheffer et al. 1987; Brasier et al. 1990). If near-equal quantities of total RNA are loaded onto a gel and hybridized with the *cu* gene, autoradiograms suggest a significantly higher level of the *cu* transcript in aggressive versus nonaggressive isolates (Jeng et al. 1996; Fig. 3). Further transformation studies, in which the *cu* gene from aggressive *O. novo-ulmi* is inserted into nonaggressive isolates of *O. ulmi*, would answer the question whether introduction into a mildly pathogenic background has any effect on the level of symptom development. These mutants exist, and are in pathogenicity trials beginning spring 1996 (P. Horgen, University of Toronto).

If CU is not necessary for disease symptom development in trees susceptible to *O. novo-ulmi*, then what are the possible roles that CU might play in this fungus? The protein CU is part of a family of secreted fungal proteins known as hydrophobins. These proteins have been characterized from a variety of fungi and share common characteristics of having low molecular weight, being strongly hydrophobic, and containing eight cysteine residues in a conserved pattern (Stringer and Timberlake 1993; Wessels 1994, 1996). A number of reviews have been written that discuss specific fungi and the nature of their hydrophobins (Wessels 1994, 1996). Most currently identified fungal hydrophobins appear to have developmental functions (Wessels 1994, 1996).

Three hydrophobins are of particular interest because they are also present in pathogenic fungi. The hydrophobin cryparin, isolated from the chestnut blight fungus *Cryphonectria parasitica* (Carpenter et al. 1992; Zhang et al. 1994), shares several physical characteristics with CU. These include accumulation on aerial fungal surfaces, assembly at a water-air interface, and solubility in aqueous alcohol. In addition, a comparison of the amino acid sequences of these two hydrophobins indicates a high degree of similarity (Bowden et al. 1994; Zhang et al. 1994). Cryparin does not appear to be phytotoxic and was down-regulated in hypo-virulent strains carrying viruslike dsRNAs (Carpenter et al. 1992). The entomopathogenic fungus *Metarhizium anisopliae* produces the hydrophobin SSGA during appressorial formation and conditions of starvation (St. Leger et al. 1992). St. Leger et al. (1992) have suggested that SSGA may be involved in attachment to hydrophobic insect surfaces and that the hydrophobin may be involved in pathogenesis, since there was a synchronous expression of the *ssgA* gene and the *Pr1* gene that codes for a cuticle-degrading protease. The hydrophobin MPG1 is important in the pathogenesis of *Magnaporthe grisea* on rice (Talbot et al. 1993). *Mpg1* mutants are impaired in their ability to form appressoria, which results in a reduction of disease symptoms. As with the CU$^-$ mutant, *Mpg1* mutants were also shown to possess a water-soaked phenotype. This observation would suggest that these two hydrophobins are involved in the surface hydrophobicity of aerial hyphae.

Wessels (1994) suggested that fungal hydrophobins can be classified into two categories. Class I hydrophobins have similar alignments of the eight cysteine residues, and all seem to be developmentally regulated. Class II hydrophobins, which include CU and cryparin, have distinct alignments of the eight cysteine residues compared with those of the Class I hydrophobins. Furthermore, the Class II hydrophobins appear to be constitutively produced (Wessels 1994). In a more recent review, Wessel (1996) suggests that both cryparin and CUs may coat the surface of aerial structures and conidiospores; this may be suggestive of developmental functions similar to those of the Class I hydrophobins.

Since many of the hydrophobins discussed appear to be involved in surface hydrophobicity of aerial hyphae and conidiospores, it would be reasonable to suggest that CU has a similarly important role for *O. novo-ulmi*. Since an insect vector is involved in transmission of the fungus from an infected elm to an uninfected elm, CU may be important in the adherence of the conidiospores to the surface of the elm bark beetle. The validity of our hypothesis may be tested by comparing the adherence of budding unicells of *O. ulmi* and *O. novo-ulmi* with adherence of the unicells from the CU$^-$ mutant to the elm bark beetle.

The results of our study suggest that the biological role of CU has yet to be determined. Whether it has some role in the spread of Dutch elm disease or has a solely developmental role in the life history of *O. novo-ulmi* awaits further experimentation.

MATERIALS AND METHODS

Organism and culture conditions.

O. novo-ulmi (= *O. ulmi* aggressive) strain MH75 was isolated by M. Hubbes in Toronto. *O. novo-ulmi* strain VA30 was originally isolated in Virginia by L. Schreiber and A. Townsend. *O. novo-ulmi* strains 70-99 and 70-25 were isolated by E. B. Smalley in Minnesota and Wisconsin, respectively. H5 is a nonaggressive *O. ulmi* (Buisman) Nannf. strain isolated by J. Gibbs north of Doulogne, France. *O. ulmi* nonaggressive strains 70-116 and 70-32 were isolated by E. Smalley in Maine and Kansas, respectively. MH75, VA30, and H5 cultures were maintained on OCM agar plates (Royer et al. 1991) and frozen at –70°C in 10% glycerol for long-term storage.

Cultures of wild-type field strains 70-99, 70-25, 70-116, and 70-32 were maintained in potato dextrose agar slants and stored frozen on twigs. Budding yeast-phase cells were cultured in a liquid minimal medium similar to OCM except for the deletion of yeast extract and malt extract, and the substitution of 1.1 g per liter of L-proline for the $(NH_4)_2SO_4$ (Bernier 1988). Liquid minimal medium was inoculated with mycelial plugs (3 mm) from OCM agar plates and incubated on a rotary shaker (160 rpm) (Controlled Environment Incubator Shaker, New Brunswick Scientific, New Brunswick, NJ) at 25°C for 6 days. All plasmid subcloning was performed in *Escherichia coli* strains DH5α (Gibco BRL, Gaithersburg, MD) according to standard procedures (Sambrook et al. 1989).

Construction of disruption vector pHEK5.

The plasmid pCU3-2 was an *Exo*III-derived deletion from the original plasmid pCU-1 (Bowden et al. 1994). pCU-1 consisted of a 1.9-kb *Bgl*II fragment containing the *cu* gene cloned into pUC18. pCU3-2 retained 1,263 bp from the 5′ end of the original 1.9-kb *Bgl*II fragment. An internal 121-bp *Nco*I fragment containing a portion of the *cu* coding sequence was deleted from pCU3-2. The *Nco*I sites were made blunt with Klenow polymerase and treated with calf intestinal alkaline phosphatase (CIAP). A 2.6-kb fragment containing the isopenicillin N synthetase promoter from *Penicillium chrysogenum* and the hygromycin B phosphotransferase gene from *E. coli* was inserted at the treated sites (Fig. 1A). The promoter and hygromycin B phosphotransferase gene were derived from plasmid pPS57, obtained from P. Skatrud (Eli Lilly Corp., Indianapolis, IN).

Transformation of *O. novo-ulmi*.

VA30 protoplasts were prepared by the second method of Royer et al. (1991). Each transformation reaction contained 2 × 10^7 protoplasts in 200 µl of 1 M sorbitol, 25 mM Tris-HCl, pH 7.5, 50 mM $CaCl_2$, mixed with 0.5 µg of *Hin*dIII linearized vector DNA, 1 µl of 2-mercaptoethanol, and 50 µl of polyethylene glycol 4000 (66%; BDH, Inc., Darmstadt, Germany) in 25 mM Tris-HCl, pH 7.5, 50 mM $CaCl_2$. The transformation procedure followed was that of Royer et al. (1991). The last step of the procedure involved incubation of the centrifuged protoplasts in 1 ml of OCM containing 0.6 M sucrose for 4 h. Aliquots of 125 µl were overlaid onto OCM containing 0.6 M sucrose plus 200 µg of hygromycin per ml with low melting temperature agarose (Sigma, St. Louis, MO) containing the same medium without selection. Hygromycin-resistant colonies appeared in 4 to 6 days. Transformant was single spored to homogeneity for use in subsequent analyses.

In situ immunoblotting.

Mycelial plugs from *O. novo-ulmi* transformants were transferred to a 90-mm petri dish containing solid OCM supplemented with 0.01% sodium deoxycholate (Bernier 1988). Each plate contained 52 samples. Cultures were incubated at room temperature for 6 days. Cellulose nitrate membranes (0.2 µm) were pre-wetted in 20 mM Tris-buffered saline (TBS), pH 7.5, for 15 min and air dried. Dried membrane was layered on top of the colonies and gently pressed to ensure contact between membrane and colonies. After 3 min, the membrane was transferred to a blocking solution composed of 5% skim milk powder in a solution of 0.05% Tween-20 in TBS (TTBS), incubated for 1 h, and washed twice in TTBS. The membrane was incubated for 3 h in a polyclonal antibody solution consisting of 1 ml of CU antiserum (Svircev et al. 1988) added to a 100 ml solution containing 5% skim milk powder in TTBS. This was followed by two washes in TTBS and one wash in TBS. The membrane was incubated 1 h in a solution consisting of protein A- horseradish peroxidase conjugate (Sigma), 0.5 mg/ml, diluted 1:10,000 in TBS containing 5% skim milk powder. This step was followed by two washes in TTBS and one wash in TBS. Membrane was immersed in a staining solution consisting of 60 mg of 4-chloro-1 naphtha in 20 ml of ice-cold methanol mixed immediately prior to use with 60 µl of 30% hydrogen peroxide and 100 ml of TBS. After a 45-min incubation, the reaction was stopped by a 10-min incubation in distilled water. Incubations and washes were done with gentle agitation at room temperature. Washes were for 5 min. This prescreening methodology for mutants gave either + or – results based on color change.

Turbidity analysis.

Cultures of yeastlike cells were grown in modified Salemink medium (Takai 1978) containing 2 g of yeast extract, 20 g of sucrose, 2 g of L-asparagine, 6 g of KH_2PO_4, 4 g of $MgSO_4 \cdot 7H_20$, 80 mg of $ZnSO_4$ and 64 mg of $FeCl_3$ per liter of distilled water. Thirteen-day-old cultures were centrifuged at 1,830 × *g* for 20 min, then filtered through a 0.45-µm cellulose-acetate membrane. The same filtrate was transferred into a 50-ml Falcon tube, hand shaken for 10 s, and then placed horizontally on a rotary shaker for 30 min. Filtrates of cultures from the first transformation experiment were evaluated spectrophotometrically at a wavelength of 400 nm. Filtrates that were clear to the unaided eye yielded absorbance values of ≤0.073. Subsequent transformant turbidities were evaluated visually for clear filtrates. Although this assay was subjective, it provided a rapid methodology for prescreening putative mutants.

DNA isolation.

Yeastlike cells were grown in liquid shake cultures of OMMP (Royer et al. 1991) or modified Salemink media. Cultures were centrifuged at 1,830 × *g* for 20 min, the supernatants were removed, and the pellets were transferred to 1.5-ml microtubes for drying in a Spin-Vac system (Savant Instruments, Inc., Farmingdale, NY). DNA was isolated from the dried material by a miniprep procedure (Zolan and Pukkila 1986). Bacterial plasmid DNA was isolated by alkaline lysis and $CsCl_2$ centrifugation according to Sambrook et al. (1989). DNA fragments from restriction endonuclease digested plasmids were isolated from preparative gels with Gelase (Cederlane, Mississauga, Ontario).

Northern analysis.

Total RNA was isolated from 6-day cultures of *O. novo-ulmi* (both the original *cu* transformant 5-8 as well as the single-spore isolate designated 5-8-1), *O. ulmi,* and a negative control, *Agaricus bisporus,* following the procedure of Raha et al. (1990). Poly(A)$^+$ RNA was purified from 20 µg of total RNA with a Quick Prep mRNA Purification kit (Pharmacia, Montreal, Quebec). All of the poly(A)$^+$ RNA recovered was separated in a 0.66 M formaldehyde/1.5% agarose gel, transferred to a GeneScreen Plus membrane, and hybridized with a

^{32}P-labeled 1.9-kb *BglII* fragment containing the entire *cu* gene. Hybridizations were performed at 42°C according to the manufacturer's recommendations (Dupont, Boston, MA). The rat poly(A)$^+$ RNA was a gift of T. Jin (Department of Medicine, University of Toronto). To establish that near-equal amounts of total RNA were loaded into each lane, the blot was also stripped and reprobed with the entire rDNA cassette from *Schizophyllum commune* (clone from R. Ullrich, University of Vermont).

Southern hybridization.

Chromosomal DNA was digested with *BglII*, *BstXI*, *HindIII*, and *EcoRI*, and electrophoresed in agarose gels with 1× Tris-borate-EDTA (TBE) (Sambrook et al. 1989). For evaluation of *cu* gene interruptions, the restricted DNA was electrophoresed in a 0.8% gel (Fig. 2A). Tandem insertion of the vector was evaluated on 0.4% gel (Fig. 2B) and restriction analysis on 0.8% gels (Fig. 2C,D,E,F). After electrophoresis, transfer was made to GeneScreen Plus according to the manufacturer's instructions (Dupont, Boston, MA). The hybridization probe for *cu* gene interruptions (Fig. 2A) was a ^{32}P-labeled 1.9-kb *BglII* fragment containing the entire *cu* gene. The pHEK5 plasmid was the hybridization probe used for evaluating tandem insertion of the vector (Fig. 2B). *BstXI* restricted DNA was hybridized with a 1.9-kb *BglII* fragment containing the entire *cu* gene in pUC 18 (pCU-1) or a 2.6-kb fragment containing the isopenicillin N synthetase promoter and the hygromycin B phosphotransferase gene (*hph*). The *HindIII*- and *EcoRI*-restricted DNA was probed with pCU-1, pAMP (Carolina Biological Supply Co., Burlington, NC), which contains the *amp* gene, or pAN7-1 (Punt et al. 1987), which contains the hygromycin B phosphotransferase gene (*hph*). Hybridization was performed at 65°C according to standard protocols (Sambrook et al. 1989).

PCR analysis.

PCR amplifications were carried out using primers CU1, 5′-GGTGGCAATGGAGAACTGC; CU1.5, 5′- CATAGGTCAA GCTGAAGACAAAACG; CU2, 5′-GCAGTTCTCCATTGC CAC; CU3, 5′-CCTACGACCCTTGCACTG; CU4, 5′-GCAG ATCGGCCCGGTGCT; and CU8, 5′-CACGTGAGGTTTA TACCAATGC; and CU9, 5′-TAACCTCTTGCCCTGCCATA. Primers CU2, CU3, and CU4 correspond to positions +3 to +20, +83 to +100, and +306 to +323 within the coding region of the *cu* gene (Fig. 1B; Bowden et al. 1994) and were paired with CU8 (+917 to +896), which is 3′ to the coding region as well as to the sequence used for vector construction. Primers CU1 (+21 to +3), and CU1.5 (–176 to –200) were paired with CU9, which corresponds to positions –429 to –410 and is 5′ to the coding region. Primers CU8 and CU9 bracket the *cu* gene.

PCR amplifications were carried out in 20-μl reaction mixtures containing 200 μM deoxynucleoside triphosphate, 0.5 μM primers, 0.5 units of *Taq* DNA polymerase, 1× DNA polymerase buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl), 2 mM $MgSO_4$, and 50 ng of DNA. The reaction was performed with 30 cycles of denaturation at 94°C for 90 s, annealing at 50°C for 60 s, and extension at 72°C for 90 s. PCR products were separated by electrophoresis in 1.0% agarose gels in TAE (40 mM Tris-acetate and 1 mM EDTA) buffer (Sambrook et al. 1989).

Growth measurements.

Radial colony growth extensions on 5 replicate OCM plates were measured on days 2 and 7 and subsequent growth rates were determined using the following equation (Brasier and Webber (1987): $D_2 - D_1 / 2(T_2 - T_1)$ = radial growth rate per day, where D_1 = colony diameter on day 2, D_2 = colony diameter on day 7, T_1 = day 2, and T_2 = day 7. Dry mycelial weights were taken on five replicate OCM flask cultures. After 2 weeks of stationary growth at 20°C, cultures were filtered and oven dried at 60°C prior to dry weight measurement.

Greenhouse inoculations and evaluations.

Preparation of plant material, inoculation, and evaluation of virulence were according to Proctor et al. (1994). Rooted cuttings LA1-1 (*Ulmus americana*) and W380-1 (*U. carpinifolia*) and year-old elm cv. Amer (*U. americana*) seedlings were transplanted into 5-inch clay pots and cut back to allow a single dominant bud to develop into an unbranched stem. The clonal material LA1-1 is extremely susceptible to Dutch elm disease while W380-1 is very insensitive to Dutch elm disease. The tree inoculations and evaluations were performed at the University of Wisconsin–Madison, Department of Plant Pathology. Strains supplied from the University of Toronto included *O. ulmi* H5, *O. novo-ulmi* VA30, THEK5-8 (original *cu* transformant), THEK5-8-1 (single-spore culture of THEK5-8), THEK5-24 (transformed to hygromycin resistance but no change in CU production), and a VA30 protoplast culture taken through the transformation process minus the vector. Control cultures supplied by the University of Wisconsin included *O. novo-ulmi* 70-99 and 70-25, and *O. ulmi* 70-116 and 70-32. Plants were inoculated about 2 inches above the ground in 1/32-inch holes drilled with a pin vise. Inoculum concentration used was 1×10^7 spores/ml. Symptom evaluations were made at 6 weeks and included percent length vascular discoloration, percent volume vascular discoloration, and percent foliar wilt (Proctor et al. 1994). The symptoms observed and measured were similar to those illustrated in Stipes and Campana (1981) and Proctor et al. (1994). *O. ulmi* and *O. novo-ulmi* were reisolated from at least 50 cm above the inoculation point from all previously inoculated trees. The recovered *O. ulmi* and *O. novo-ulmi* cultures were examined for sensitivity to hygromycin, turbidity analysis of CU production, and Southern analysis of vector insertion.

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