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Research Notes

Expression of a Modified Dutch Elm Disease Toxin in *Escherichia coli*

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The fungal toxin associated with Dutch elm disease, cerato-ulmin, has been produced in the bacterium *Escherichia coli* by the assembly of oligonucleotides according to the unpublished amino acid sequence of the toxin. This toxin was produced at approximately 80 μg/L of cell culture as a fusion to glutathione S-transferase. We synthesized the toxin as a fusion protein to improve purification and stability. Recombinant cerato-ulmin was analyzed by immunoblot analysis and then separated from its fusion partner by thrombin. We incorporated this molecule into an appropriate medium to test the activity of the toxin on the growth of American elm callus cultures.

Additional keywords: *Ophiostoma ulmi*, plant tissue culture, recombinant DNA, *Ulmus americana*.

The most devastating vascular wilt disease of the twentieth century is Dutch elm disease, which attacks susceptible elms, particularly the American elm (*Ulmus americana* L.) (for a recent review, see Sticklen *et al.* 1991). The disease is caused by the fungal pathogen *Ophiostoma ulmi* (Buisman) Nannf. (Nannfeldt 1932), which produces several toxins (Claydon *et al.* 1980). The hydrophobic 7.6-kDa polypeptide cerato-ulmin (CU) is one such toxin (Takai 1974). This toxin has been shown to cause the same symptoms as the intact fungus (Richards and Takai 1984). However, very little is known about the mode of action of this or any other wilt toxin (Van Alfen 1989). To study CU, one could produce the toxin by using recombinant DNA technology. Expression of CU in *Escherichia coli* (Migula) Castellani and Chalmers would establish a system for site-directed mutagenesis for further characterization of the amino acids involved in toxicity and specificity. To achieve this goal, we produced a recombinant CU molecule as a translational fusion with glutathione S-transferase (GST; Smith and Johnson 1988) in *E. coli*, then we tested the recombinant toxin on the growth of American elm callus.

A number of approaches have been taken in the study of Dutch elm disease with the goal of reducing the spread of the disease (for review see Mazzone and Peacock 1985). The possible applications of molecular techniques in studying host-vector-pathogen interactions include the isolation of disease-resistant genes from elms that are resistant to Dutch elm disease, the introduction into elms of DNA encoding proteins that prevent the spread of the fungus or colonization of the elm by the elm bark beetle (Jassim *et al.* 1990), or the isolation of genes from the fungus encoding pathogenesis-related products. We have studied one of the major fungal toxins by synthesizing and assembling synthetic DNA according to the unpublished amino acid sequence of the toxin. The use of synthetic DNA has also allowed us to optimize the codon usage for expression of proteins in *E. coli*.

Oligonucleotide sequences (Fig. 1A) were based on the amino acid sequence of CU (Yaguchi *et al.*, in press) and were optimized through the use of an *E. coli* codon usage table (Sharp and Li 1986). The synthetic oligonucleotides, varying in length from 28 to 41 nucleotides, were assembled as two sets of four pairs (each with overhanging ends complementary only to the pair of oligonucleotides to which it was to be ligated) and were cloned between the *Bam*HI and *Sal*I (5′ clones) or *Bam*HI and *Hin*dIII (3′ clones) sites of pUC19 (Fig. 1B). Positive 5′ clones (pCU1) were identified by digestion with *Eco*RV, because a unique restriction site for this enzyme was encoded in the oligonucleotides. The positive 3′ clones (pCU2) were identified by screening for the loss of a *Pst*I site (unique to pUC19), which was removed during the insertion of the oligonucleotides. All clones were sequenced to make sure no spurious mutations had occurred. Purified DNA encoding the two halves of CU was ligated in the proper orientation to form pCU3, which contained the complete 246-nucleotide insert, which was sequenced again.

The predicted small size (7.6 kDa), hydrophobicity, and toxicity of the protein suggested that recombinant CU might be highly unstable in *E. coli*. For these reasons, CU was produced as a fusion protein with GST. The GST fusion partner served not only to simplify purification, but also provided a means for stabilizing the toxin. The DNA encoding CU was inserted in-frame between the unique *Eco*RI and *Bam*HI sites in the expression vector pGEX-2T (Pharmacia, Piscataway, NJ). Positive clones (pCU4)

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were identified by *Eco*RI-*Bam*HI digestion to release the DNA encoding CU. Assembly of oligonucleotides on the basis of known amino acid sequences may prove to be a useful approach in producing and analyzing other proteins involved in plant-microbe interactions. The size of the protein that can be produced with this technique is limited only by the availability of the amino acid sequence, as well as the patience and resources of the investigator.

A fusion protein of the predicted size of 34 kDa (GST, 26 kDa; CU, 8 kDa), produced after induction with IPTG (isopropyl-β-D-thiogalactopyranoside), was detected by western blotting using an antiserum to CU. This protein was produced at approximately 80 μg/L of cell culture, as estimated by a comparison with a known concentration of native CU after immunoblot analysis. The CU-GST fusion protein was then purified by use of a glutathione-agarose affinity column, and an immunoblot assay (Fig. 2) was used to monitor the purification. The antiserum used for this procedure has been tested extensively against native CU using a double-diffusion assay (Krywienczyk *et al.* 1979; Richards and Takai 1984). Following this purification procedure, the fusion partners were separated using human thrombin, because a thrombin cleavage site is present between glutathione S-transferase and CU. The digestion was carried out while the fusion was still attached to the matrix, as described (Abath and Simpson 1991). The digestion supernatant was then removed, dried, resuspended in 70% ethanol, and approximately 250 ng of protein was subjected to reverse-phase high-performance liquid chromatography (HPLC) to purify the recombinant CU for amino terminal amino acid sequencing and for further analysis.

G S T T M S D S Y D P C T G L L Q K S
GATCCACAACAATGTCTGACTCTTACGATCCGTGC ACCGGTCTGCTGCAGAAATCT
GTGTTGTTAC AGACTGAGAATGCTAGGCACGTGGCCAGACGACGTCTTTAGA

P Q C C N T D I L G V A N L D C H G
CCGCAGTGCTGCAACACCGATATCCTCGGTGTTGCTAACCTGGACTGCCACGGT
GGCGTCACGACGTTGTGGCTATAGGAGCCACAACGATTGGACCTGACGGTGCCA

P P S V P T S P S Q F Q A S C V A D
CCGCCGTCT GTTCCGACTAGT/CCGTCT CAGTTCCAGGCTAGCTGCGTT GCTGAC
GGCGGCAGACAAGGCTGATCA/GGCAGAGTCAAGGTCCGATCGACGCAACGACTG

G G R S A R C C T L S L L G L A L V
GGCGGCCGTTCT GCTCGTT GCTGCACCCTGTCT CTGCT GGGTCTG GCTCTGGTG
CCGCCGGCAAGACGAGCAACGACGTGGGACAGAGACGACCCAGACCGAGACCAC

C T D P V G I stop
TGCACCGACCCGGTTGGTATCT GAGAGCTCGAATTCA
ACGTGGCTGGGCCAACCATAGACTCTCGAGCTTAAGTTCGA

Fig. 1A. Sequence of oligonucleotides encoding recombinant cerato-ulmin (CU). The single letter code for each amino acid of the recombinant CU protein is given in bold print above the appropriate codon. The first codon of the native CU sequence is underlined, whereas additional amino terminal amino acids are in italics. The oligonucleotides were assembled in two halves; each set was generated and had the capacity to be expressed independently. The bold slashes represent the dividing point between the two sets of oligonucleotides. The first glycine of the sequence is the first amino acid to follow the thrombin cleavage site. Sixteen oligonucleotides, varying in length from 28 to 41 nucleotides, were synthesized by the Macromolecular Structure Facility at Michigan State University, and then purified by high-performance liquid chromatography with a C18 reverse-phase column. These oligonucleotides were assembled (Snouwaert *et al.* 1987) to produce double-stranded DNA encoding CU according to the amino acid sequence of the polypeptide (Yaguchi *et al.*, in press). Briefly, 1 μg of one oligonucleotide from each set of four complementary pairs encoding the 5′ half of CU was phosphorylated with T_4 kinase (Promega Biotech, Madison, WI), heated to 95° C, and cooled to 50° C. At this point, the complementary oligonucleotide was added to allow annealing to proceed. The pairs were then cooled slowly to room temperature, and the four pairs were mixed and ligated with T_4 DNA ligase (New England Biolabs, Beverly, MA). This procedure was repeated with the second set of oligonucleotides encoding the 3′ half of the toxin. Assembled oligonucleotides were purified by polyacrylamide gel electrophoresis. DNA was then removed from the gel by electroelution followed by ethanol precipitation.

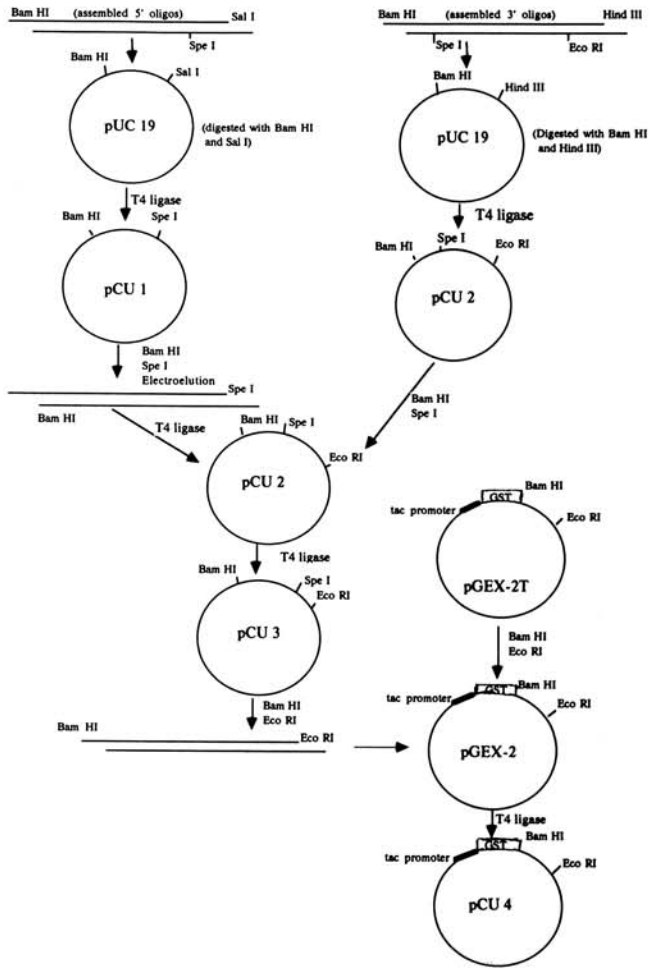

Fig. 1B. Cloning scheme for the production of pCU4, the cerato-ulmin (CU) expression vector. Purified DNA encoding each half of CU was cloned into pUC19, which had been previously digested with *Bam*HI and *Sal*I (5′ clones) or *Bam*HI and *Hind*III (3′ clones) (Promega Biotech). Ligated DNA was transformed into competent JM101 by the method of Hanahan (1983). Positive clones were screened by rapid plasmid isolation (Sambrook *et al.* 1989), followed by digestion of the purified plasmid DNA with *Eco*RV (BRL, Gaithersburg, MD). Universal primers (New England Biolabs) were then used for dideoxy sequencing (Biggin *et al.* 1982; Sanger *et al.* 1977) of the assembled oligonucleotides in both directions in pUC19. After the confirmation of the appropriate sequences, DNA encoding the two halves of CU and purified by cesium chloride was joined by inserting the 5′ oligonucleotides from pCU1 between *Bam*HI and *Spe*I sites of pCU2, yielding pCU3. This 246-nucleotide double-stranded DNA fragment was then resequenced to ensure correctness. The DNA encoding CU was inserted into the expression vector pGEX-2T (Pharmacia, Piscataway, NJ) to produce pCU4. Unless otherwise stated, cloning procedures were performed as described (Sambrook *et al.* 1989).

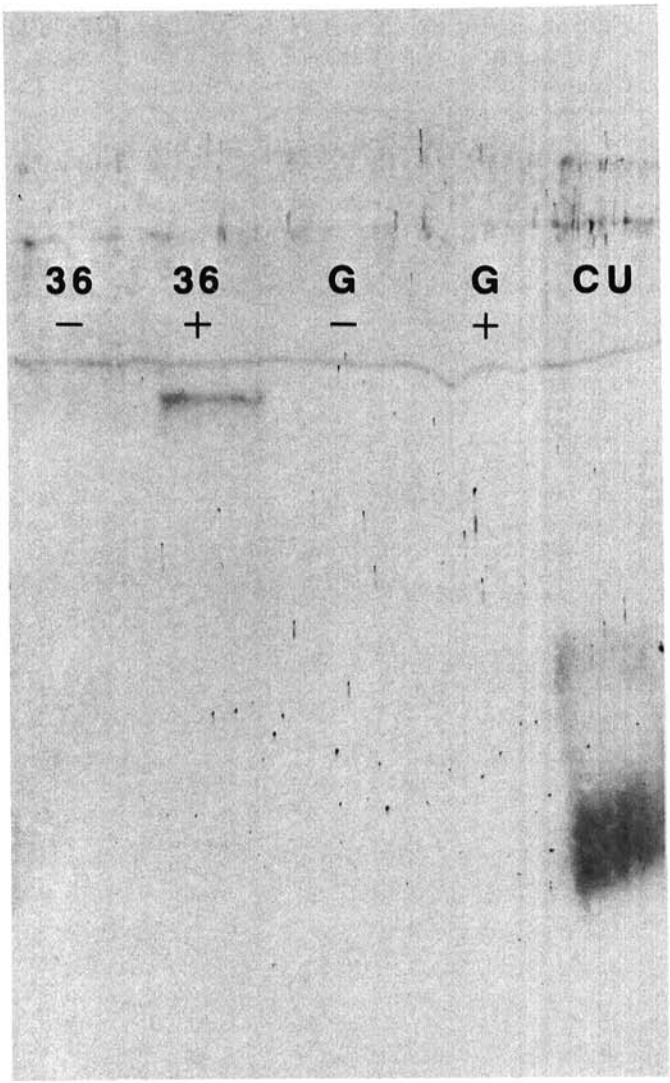

Fig. 2. Western analysis of IPTG (isopropyl-β-D-thiogalactopyranoside) induced (+) and uninduced (−) affinity-purified fusion proteins from cells containing pCU4 (lane 36) or pGEX-2T (lane G), with 800 ng of purified cerato-ulmin (CU), as detected by an antiserum against CU. Cultures of JM101 containing pCU4 were grown in fresh Luria-Bertani media containing ampicillin; protein production was induced by the addition of IPTG to 1 mM. Incubation continued for 45 min, then cells were pelleted and resuspended in 5× sodium dodecyl sulfate (SDS) gel sample buffer for SDS-polyacrylamide agarose gel electrophoresis (PAGE) analysis or MTPBS (150 mM NaCl, 4 mM sodium phosphate monobasic, 16 mM sodium phosphate dibasic, pH 7.3) for affinity purification (Smith and Johnson 1988). Cell pellets from a 5-ml culture, obtained as described above, were resuspended in 250 μl of MTPBS containing 1% Triton X-100, sonicated twice briefly, then centrifuged 5′ in a microcentrifuge. Supernatants were then incubated with an equal volume of 50% glutathione-agarose beads (prepared in MTPBS; sulfur linkage; Sigma, St. Louis, MO) for 5′ at room temperature. Beads to which the fusion proteins were bound were pelleted by a brief centrifugation at 500 *g*, followed by washing three times with 10 ml of MTPBS. Protein preparations were analyzed by immunoblot analysis with a rabbit polyclonal antibody to CU (provided by Wayne Richards; Richards and Takai 1984). Proteins were separated with 12 or 15% running, 6% stacking SDS-polyacrylamide gels and electroblotted onto 0.45 μm of nitrocellulose (Molecular Separations, Inc., Westborough, MA). Membranes were blocked with 0.25% gelatin, incubated with the primary antibody and with goat anti-rabbit alkaline phosphatase conjugated antisera (Promega, Madison, WI), and, finally, bound secondary antibody was visualized after the addition of the substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

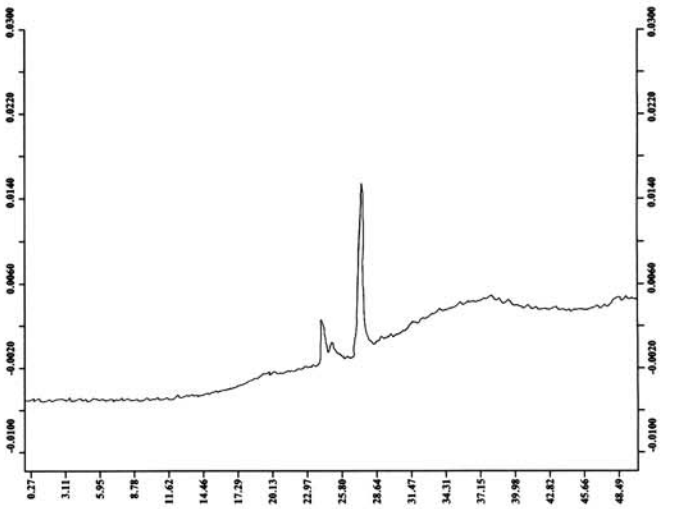

Fig. 3A. Reverse-phase high-performance liquid chromatography (HPLC) of affinity-purified cerato-ulmin (CU) similar to that analyzed in Figure 2. Approximately 250 ng of protein was injected during this run. The fraction containing the largest peak contained material that cross-reacted with the antiserum against CU. Recombinant CU was separated from its fusion partner with the serine protease thrombin, whereas the GST remained bound to the affinity matrix (Abath and Simpson 1991). Digestions were carried out overnight at ambient temperature in 150 mM NaCl, 2.5 mM $CaCl_2$, and 10 ng of human thrombin (Sigma, St. Louis, MO). After thrombin digestion, recombinant CU was purified by reverse-phase HPLC with a C8 RP300 column (1 × 260 mm; Applied Biosystems, Inc., Foster City, CA), with a trifluoroacetic acid (TFA) (1%), water, acetonitrile (0–90%) linear gradient. Detection was carried out at 214 nm; the detector was set at 0.05 AUFS with attenuation. Positive peaks were identified by immunoblot analysis as described above.

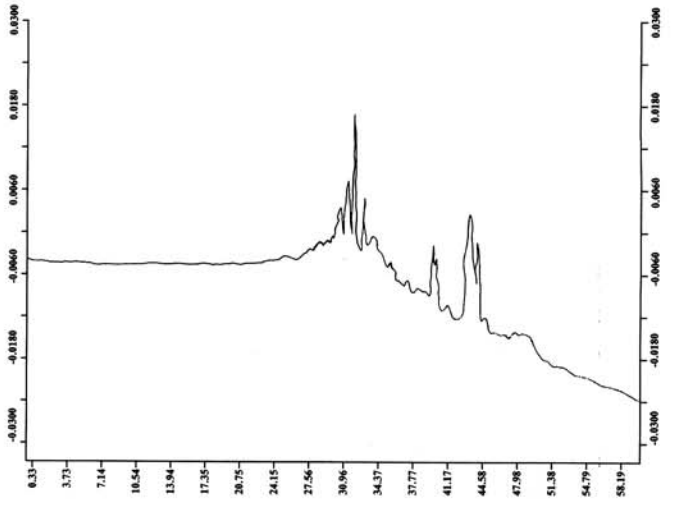

Fig. 3B. Reverse-phase HPLC of the material contained in the larger peak in Figure 3A (approximately 190 ng), after treatment with cyanogen bromide in 70% TFA, indicating that the peptide had been substantially degraded. Recombinant CU molecules were also digested with cyanogen bromide (CNBr; 25 mg/ml) in 70% TFA under nitrogen for 24 hr at 25° C in the dark (Shen 1984). Amino terminal amino acid sequence analysis was performed by automated Edman degradation (Hewick *et al.* 1981) with an Applied Biosystems (477A) peptide sequencer followed by reverse-phase HPLC of derivatized residues (Hunkapillar and Hood 1983).

Results of this purification demonstrated the presence of a single strong peak (Fig. 3A), which cross-reacted with the anti-CU antibody and was the correct size. However, the CU cleavage product produced by thrombin digestion contained five additional amino terminal amino acids (G-S-T-T-M), although these residues do not disrupt the overall hydrophobic nature of the peptide. Results of amino terminal amino acid sequencing also support the conclusion that recombinant CU, containing the five additional amino terminal residues, was being produced (not shown). Because the methionine at the junction between the sequence of native CU and the additional five amino acids was the only methionine in the peptide, cyanogen bromide (CNBr) digestion was undertaken on the material in the larger peak (190 ng) to remove those amino acids. Figure 3B shows the HPLC tracing of the resulting CNBr digest, which indicates that the strongly acidic environment that CNBr requires caused the cleavage of the toxin at a number of positions and rendered it unusable for further analysis. Therefore, the remaining studies were carried out with CU containing the five additional amino terminal residues. However, experiments to delete the DNA encoding these five amino acids by oligonucleotide-directed mutagenesis are underway.

To test the activity of the recombinant toxin on American elm callus cultures, as has been done previously with native CU (Pijut *et al.* 1990), we cultured American elm callus on an appropriate medium containing native or recombinant CU. Native CU was added to a final concentration of 1 μM, because it was previously shown that this concentration was effective in inhibiting the growth of American elm callus cultures (J. Cheng and M. B. Sticklen, unpublished). The OD_{280} of the recombinant CU solution after affinity chromatography was 0.622 (compared to 0.368 for 100 μM native CU). This higher initial concentration (relative to the concentration of the native CU solution) was used for recombinant CU because the preparation was not absolutely pure (as evident in Fig. 3A). Table 1 shows the composition of each medium on which callus pieces were cultured, the number of callus pieces per treatment, and the changes in the fresh weight of the callus relative to its initial weight.

Table 1. Effect of recombinant cerato-ulmin (CU) on the growth of American elm callus in culture[a]

Media supplement	Number of samples	Fresh weight (mg) mean ± S.D.
Control	7	223 ± 161
Escherichia coli protein + human thrombin	7	105 ± 135[b]
1 μM Native CU[c]	8	86 ± 41
1:1,000 Diluted recombinant CU	7	352 ± 180[d]
1:100 Diluted recombinant CU	7	137 ± 32
1:10 Diluted recombinant CU	7	62 ± 34
Undiluted recombinant CU[e]	4	−41 ± 3[d]

[a] The GST-Cu fusion was purified by affinity chromatography and cleaved with human thrombin to release CU. After the affinity resin containing bound GST was pelleted, the supernatant was dried and dissolved in 70% ethanol. The recombinant toxin was then incorporated at several concentrations into a modified elm callus medium (Sticklen *et al.* 1986), consisting of half-strength Murashige and Skoog (1962) basal medium (Sigma, St. Louis, MO), 1× B5 vitamins, 40 g/L of maltose, 100 mg/L of casein hydrolysate, 100 mg/L of thiamine, 2.5 μM 2,4-dichlorophenoxyacetic acid, 1 μM kinetin, 100 ml of coconut water (added after autoclaving; Sigma), pH 5.7 and solidified by 6.5 g/L of phytagar (BRL Life Technologies, Gaithersburg, MD). Media were supplemented with 0-, 10-, 100-, and 1,000-fold dilutions of recombinant CU, which were chosen after comparison to OD_{280} readings of a 100-μM solution of purified native CU (provided by Wayne Richards). The extinction coefficient for CU, on the basis of these readings in 70% ethanol, is approximately $\epsilon_{280} = 3.56$ (the OD_{280} of a 100-μM solution of purified CU was 0.368). One of the control media contained human thrombin (10 ng final) and *E. coli* extract at a concentration approximating that of the contaminating proteins in the undiluted solution of recombinant CU, based on high-performance liquid chromatography data. Media were distributed in 1-ml aliquots into wells of a 24-well plate, and one American elm callus piece, weighing between 50 and 60 mg, was cultured in each well. Final callus weights were taken 8 wk after the initial culture.

[b] Two of the seven samples failed to grow on this medium. $OD_{280} = 0.1$.

[c] 100 μM native CU $OD_{280} = 0.368$.

[d] Media were dessicated in some samples before the experiment was completed.

[e] $OD_{280} = 0.622$.

The amount of growth observed in the callus cultures varied greatly depending on the treatment used. Only callus grown on the medium containing undiluted recombinant CU showed a reduction in the weight of the callus over the course of the experiment. Part of this decrease in weight was due to the dessication of the medium before the final weight was measured. However, other pieces of callus cultured on media not containing CU also dried out before the final weight was taken, and these showed increases in fresh weight as well as obvious increases in size (not shown). Therefore, although we cannot be certain that the recombinant protein incorporated into this medium caused an absolute decrease in callus fresh weight, it is clear that the medium containing undiluted recombinant CU had the most detrimental effect on the growth of the callus.

Native CU caused a two- to threefold smaller increase in callus growth compared to callus grown on an unsupplemented medium. This change is consistent with that observed in previous experiments (J. Cheng and M. Sticklen, unpublished) in which callus growth was reduced 1.5- to threefold on medium containing 1 μM CU as compared to the control. The effect of the ethanol was held constant in the recombinant CU samples, as well as in the native CU sample, each containing 0.7% ethanol.

The presence of nonspecific *E. coli* proteins, as well as human thrombin, reduced the amount of callus growth slightly relative to callus grown on unsupplemented media. As indicated in the table, these samples also showed a great deal of variation, caused in part by the failure of two of the explants to grow on this medium (without these two data points, the data becomes mean = 155 ± 129). The amount of protein in this treatment was approximately equivalent to the amount of contaminating protein in the undiluted recombinant CU sample, as estimated from Figure 3A. Comparison of the means of the fresh weights after these treatments indicates that the contaminating proteins had little (if any) effect (155 ± 129 vs. −41 ± 3) on decreasing the rate of callus growth.

Finally, increases in callus size were positively correlated with decreases in the amount of recombinant CU in the media used in these experiments. When the results of the various control experiments are taken into account, the recombinant CU appears to have an overall negative effect on American elm callus growth. It appears that the recombinant toxin is within one order of magnitude in

terms of activity on American elm callus when compared to native CU. However, these preliminary experiments need to be standardized and studied in depth before any conclusions can be drawn about the quantitative effects of this recombinant toxin on callus cultures.

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