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Characterization and Disruption of A Gene in the Maize Pathogen *Cochliobolus carbonum* Encoding a Cellulase Lacking a Cellulose Binding Domain and Hinge Region

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A gene, *CEL1*, in the maize pathogen *Cochliobolus carbonum* was identified using the *cbh1-3* gene of *Phanerochaete chrysosporium* as a heterologous probe. The predicted product of *CEL1*, Cel1, is 62% identical and 71% similar to the product of *cbh1-3* and 54 to 62% identical to five cellobiohydrolases from other filamentous fungi. The location of the polyadenylation site 221 bp downstream of the stop codon and the location of a single intron of 55 bp were identified by comparison of the sequences of genomic and cDNA copies of *CEL1*. The transcriptional start site was determined by rapid amplification of cDNA ends (RACE) to be 39 bp upstream of the putative translational start site. *CEL1* mRNA abundance is high when *C. carbonum* is grown on cellulose or maize cell walls but is undetectable when grown on 2% sucrose or cellulose plus sucrose. Cel1 has a predicted signal peptide of 18 amino acids and therefore a mature size of 46.4 kDa. Like the product of *cbh1-1* of *P. chrysosporium*, but unlike most other endoglucanases and cellobiohydrolases (including the predicted product of *cbh1-3*), Cel1 does not have a putative cellulose binding domain or associated hinge region. The codon bias of *CEL1* is stronger than the bias of *cbh1-1* and comparable to that of *cbh1-3* and that of the *C. carbonum* genes *PGN1* and *XYL1*, (encoding endopolygalacturonase and endo-xylanase, respectively). A strain of *C. carbonum* specifically mutated at *CEL1* was produced by transformation with a truncated copy of *CEL1*. Integration and disruption of *CEL1* in the mutant was confirmed by DNA and RNA blotting. Pathogenicity of the *CEL1* mutant was indistinguishable from the wild-type, indicating that *CEL1* by itself is not a critical disease determinant. Culture filtrates of *C. carbonum* grown on cellulose or maize cell walls had several cellobiohydrolase, endoglucanase, and β-glucosidase activities that were separable by chromatofocusing, hydrophobic interaction, or ion-exchange high-performance liquid chromatography. However, all of the activities that were found were present in both the wild type and the *CEL1* mutant and therefore are not Cel1.

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The plant cell wall is a major barrier to infection and spread by potentially pathogenic microorganisms. Extracellular enzymes that can degrade the complex polymers of the plant cell wall are produced by most if not all filamentous fungi, and for plant pathogens these cell wall-degrading enzymes have been postulated to play specific roles in the invasion of living plant tissue and in the release of nutrients to support growth. Alone or in combination, cell wall-degrading enzymes could be important determinants of pathogenicity or virulence in fungal-plant interactions (Walton 1994).

Cellulose is the single most abundant polymer in plant cell walls, and constitutes approximately 20 to 30% of the dry weight of maize cell walls (Kato and Nevins 1984). Typically, three classes of enzymes (cellobiohydrolase, endo-β1, 4-glucanase and β-glucosidase) are required for the complete depolymerization of native, crystalline cellulose. Because of the importance of these enzymes in the natural recycling of lignocellulose, they and their genes have been extensively studied in a number of saprophytic bacteria and fungi (Henrissat et al. 1989). The role of cellulolytic enzymes in plant pathogenesis, however, is less well understood. Cellulases have been demonstrated to contribute to virulence in some bacterial diseases. Extracellular endo-β1,4-glucanase contributes significantly to the virulence of *Pseudomonas solanacearum* on tomato (Roberts et al. 1988) and of *Erwinia carotovora* subsp. *carotovora* on potato tubers (Walker et al. 1994), but the major endoglucanase of *Xanthomonas campestris* pv. *campestris* has only a minor role in pathogenicity on turnip and radish (Gough et al. 1988). Studies on the role of cellulases in pathogenicity by fungi are more limited. *Colletotrichum lindemuthianum* and *Cochliobolus heterostrophus* (*Helminthosporium maydis*) produce β-glucosidase and carboxymethylcellulase (CMCase)(Anderson 1978). *Fusarium roseum* produces CMCase in culture and in planta (Mullen and Bateman 1975). An endo-β1,4-glucanase and two β-glucosidases were purified from *Phytophthora infestans* (Bodenmann et al. 1985). In a study of uncharacterized mutants of *Colletotrichum lagenarium*, cellulase was suggested to play a role in initial penetration (Katoh et al. 1988). Three monocot fungal pathogens, *Rhizoctonia cerealis*, *Fusarium culmorum*, and *Pseudocercosporella herpotrichoides*, produce low levels of cellulases that can degrade crystalline cellulose and CMC in culture. In plants infected with *R. cerealis*, cellulases with activity against CMC but not crystalline cellu-

lose or *p*-nitrophenyl-β-glucoside (pNPG) were detected (Cooper et al. 1988). The cellulases of *Fusarium oxysporum* have been examined from the point of view of biomass conversion (Christakopoulos et al. 1990). The apple scab pathogen, *Venturia inaequalis*, produces several cellulases both in culture and in planta (Kollar 1994). None of these cellulases have been purified nor their genes characterized.

Cochliobolus carbonum, a filamentous fungus that causes leaf spot of maize, produces extracellular cellulases, including enzymes with activity against insoluble cellulose, *p*-nitrophenyl-β-cellobioside (pNPCB), soluble CMC, and pNPG (P. Sposato and J. D. Walton, unpublished results). As a first step towards analyzing the role(s) of these enzymes in pathogenicity of *C. carbonum*, we report here the cloning and gene disruption of a putative cellobiohydrolase-encoding gene.

RESULTS

Isolation and analysis of *CEL1*.

The *CEL1* gene was cloned from a genomic DNA library of *C. carbonum* using the *cbh1-3* gene from *P. chrysosporium* as a heterologous probe. On DNA blots, the major hybridization signal with *cbh1-3* as probe at low stringency (Fig. 1A) corresponded in size to the signal obtained with *CEL1* itself at high stringency (Fig. 1B). At low stringency, *cbh1-3* hybridized to additional sequences of *C. carbonum* DNA (Fig. 1A), and at medium stringency *CEL1* itself hybridizes to other sequences in *C. carbonum* (Fig. 1C). These results indicate that *C. carbonum* has additional sequences, perhaps encoding other cellulases, related to *CEL1*.

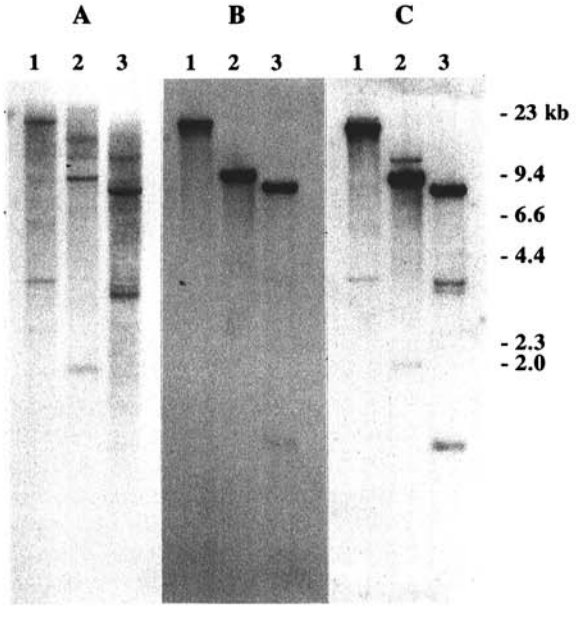

Fig. 1. A: DNA blot of *Cochliobolus carbonum* genomic DNA probed under low- stringency conditions (48°C) with the *cbh1-3* gene from *P. chrysosporium*. **B,** DNA blot of *C. carbonum* genomic DNA probed under high-stringency conditions (65°C) with *CEL1*. **C,** DNA blot of *C. carbonum* genomic DNA probed under medium stringency conditions (55°C) with *CEL1*. In all three blots, the DNA was cut with *Bam*HI, *Eco*RI, or *Hin*dIII (lanes 1 to 3, respectively).

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CGCTTCGTATTATTCCCCACCCATCCACTATCTGCTTAAGGCAAGGCACT     50
GGTTCCTGTGGCGTAGAGATCTCCACAGTACCCCGGAATACCCCACGGGC    100
AAGAAGCAGCCAAGGATGCTTCATCTACTCTTCATTAGTTTGGCACTTCT    150
AGCAACGTTCTACACTCCCGACGTCCCGCTTTACTTGTGGTATTGGTGAG    200
AACGCTCTGCAGGAGAGCCTTGGTATGCAGATCTCCTGCAGCGTGCAACG    250
CCCAAGGAGGTCACGTAGGTACTTTTTCCAAAGTACCACCGATAGTACTC    300
AAGAGCAGGAGATATTTTTTTCTGATATAAAAGGGGGAGTCCCCAACAAG    350
                +
CAGAAAAGTTCGTGGATCCAGCAGCAGACTCTTTACTCAAGCCAGTCAAC    400
 M  Y  R  T  L  A  F  A  S  L  S  L  Y  G  A  A  R     17
ATGTACAGGACACTTGCTTTCGCCTCTCTTTCGCTCTACGGAGCCGCCCG    450
  A  Q  Q  V  G  T  S  T  A  E  N  H  P  K  L  T      33
CGCTCAGCAGGTTGGCACCAGCACTGCTGAGAACCACCCCAAGCTGACCT    500
W  Q  T  C  T  G  T  G  G  T  N  C  S  N  K#  S  G     50
GGCAAACCTGCACGGGTACCGGTGGTACCAACTGCTCCAACAAGTCTGGT    550
 S  V  V  L  D  S  N  W  R  W  A  H  N  V  G  G  Y     67
                          ApaI
TCCGTTGTGCTCGACTCCAACTGGCGATGGGCCCACAATGTTGGCGGATA    600
  T  N  C  Y  T  G  N  S  W  S  T  Q  Y  C  P  D      83
CACCAACTGCTACACTGGCAACTCCTGGAGCACCCAGTACTGCCCCGATG    650
G  D  S  C  T  K  N  C  A  I  D  G  A  D  Y  S        93
GTGACTCTTGCACCAAGAACTGCGCTATCGACGGTGCTGACTACTCTGGT    700
AAGTTTGCTCGACTGAGTGGAAAAAGGAGGGAAGTTTGTTCTAACATATC    750
   G  T  Y  G  I  T  T  S  N  N  A  L  S  L  K  F     115
CAGGCACTTATGGTATCACTACCAGCAACAACGCTCTCTCCCTCAAGTTC    800
 V  T  K  G  S  F  S  S  N  I  G  S  R  T  Y  L  M    132
GTCACCAAGGGCTCCTTCTCCAGCAACATTGGTTCGCGTACCTACCTCAT    850
  E  T  D  T  K  Y  Q  M  F  N  L  I  N  K  E  F     148
GGAGACGGACACCAAGTACCAGATGTTCAACCTCATCAACAAGGAGTTCA    900
T  F  D  V  D  V  S  K  L  P  C  G  L  N  G  A  L     165
CCTTTGACGTCGATGTCTCCAAGCTTCCTTGCGGTCTGAACGGTGCCCTC    950
 Y  F  V  E  M  A  A  D  G  G  I  G  K  G  N  N  K    182
TACTTTGTCGAGATGGCCGCCGACGGTGGCATCGGCAAGGGCAACAACAA   1000
  A  G  A  K  Y  G  T  G  Y  C  D  S  Q  C  P  H     198
GGCTGGTGCCAAGTACGGAACTGGATACTGCGACTCCCAGTGCCCTCACG   1050
D  I  K  F  I  N  G  K  A  N  V  E  G  W  N  P  S     215
ACATCAAGTTCATCAACGGCAAGGCCAACGTCGAGGGCTGGAACCCCTCT   1100
 D  A  D  P  N  G  G  A  G  K  I  G  A  C  C  P  E    232
GATGCCGACCCCAACGGTGGCGCCGGCAAGATCGGTGCCTGCTGCCCTGA   1150
  M  D  I  W  E  A  N  S  I  S  T  A  Y  T  P  H     248
AATGGACATTTGGGAGGCCAACTCCATCTCGACTGCCTACACGCCTCACC   1200
P  C  R  G  V  G  L  Q  E  C  S  D  A  A  S  C  G     265
CTTGCCGCGGCGTAGGTCTCCAGGAGTGCTCCGACGCCGCGAGCTGCGGT   1250
 D  G  S  N  R  Y  D  G  Q  C  D  K  D  G  C  D  F    282
GACGGCTCCAACCGCTACGACGGCCAGTGCGACAAGGACGGATGCGACTT   1300
  N  S  Y  R  M  G  V  K  D  F  Y  G  P  G  A  T      298
CAACAGCTACCGCATGGGCGTCAAGGACTTCTACGGTCCCGGCGCCACCC   1350
L  D  T  T  K  K  M  T  V  I  T  Q  F  L  G  S  G     315
TCGACACCACCAAGAAGATGACGGTCATCACGCAGTTCCTCGGCTCCGGC   1400
 S  S  L  S  E  I  K  R  F  Y  V  Q  N  G  K  V  Y    332
                                        AccI
TCCAGCCTCTCCGAGATCAAGCGTTTCTACGTGCAGAACGGCAAGGTCTA   1450
  K  N  S  Q  S  A  V  A  G  V  T  G  N  S  I  T      348
CAAGAACTCGCAGTCGGCCGTTGCAGGCGTCACCGGCAACTCCATCACCG   1500
E  S  F  C  T  A  Q  K  K  A  F  G  D  T  S  S  F     365
AGAGCTTCTGTACCGCACAGAAGAAGGCCTTTGGCGACACCTCGTCGTTT   1550
 A  A  L  G  G  L  N  E  M  G  A  S  L  A  R  G  H    382
GCCGCTCTCGGCGGCCTCAACGAGATGGGTGCGTCGCTTGCTCGCGGCCA   1600
  V  L  I  M  S  L  W  G  D  H  A  V  N  M  L  W      398
CGTCCTCATCATGTCCCTCTGGGGCGACCACGCCGTCAACATGCTCTGGC   1650
L  D  S  T  Y  P  T  D  A  D  P  S  K  P  G  A  A     415
TCGACTCTACCTATCCCACCGACGCTGACCCATCCAAGCCCGGTGCTGCC   1700
 R  G  T  C  P  T  T  S  G  K  P  E  D  V  E  K  N    432
CGTGGTACCTGCCCTACCACCTCTGGTAAGCCAGAGGATGTCGAGAAGAA   1750
  S  P  D  A  T  V  V  F  S  N  I  K  F  G  P  I      448
CTCTCCCGATGCTACCGTTGTCTTCTCCAACATCAAGTTTGGCCCTATTG   1800
G  S  T  F  A  Q  P  A  *                             456
GCTCTACTTTTGCTCAGCCCGCATAAAGCGCTTTCTCTGAAGAGCGCGCC   1850
CCCCGTTATGGATGGACTTTGCACTCCTCTTGATCTTGATGGGAGACGTG   1900
TAGGTAGACCTTCTTCTTCTTCTTCTTGTTCGATACTTTCGCTTCGACAA   1950
TCTCGGAAATTGTACATAACTTTTTGTTGAGTGGTGGAAAGTGGGTGGGA   2000
                                                  $
CTACTAGAAATGTGTGCATTACAAAGTAGAACAAATGGAAATTTGCATTC   2050
AAAAAAAAGGGGGGACATTTTTGTTTAGTGAACTAGAAAATGTGATTTCG   2100
TGTGAAACATCTCTTATCTATGTTGGGCGTTGATGTTTTCGCGCCGATCT   2150
ACTCTTATAAACGCCGGGCCAACTATGTTAGTATGTGTACTCGCTAAGAA   2200
CGCTTTGACGAGGGAGAAGAGGGAGAGAAATATCTAAAAGAAAAGAAGAA   2250
AATAAAAATAAAAATAAAACGCCCATGCAGATCAACGGGTGCGAGTTCGC   2300
AGATATAAAAAAAAAAGAAAGAATACCCCGCCATTTAGACCAACGTCCTC   2350
GCACGTTGAGCAGTCATTCTAGCCATATCCTCAGCCGCACTCGTCACTG    2399
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Fig. 2. DNA sequence and predicted amino acid sequence of *CEL1* of *C. carbonum*. The single intron is indicated in bold type. The indicated *Apa*I and *Acc*I restriction sites are those used to construct the disruption vector pCEL1. The start of the 1.4-kb cDNA is indicated by #, the transcriptional start site, determined by RACE, is indicated by +, and the polyadenylation site is indicated by $ (all symbols and amino acid codes refer to the nucleotides immediately underneath).

A 3.7-kb *Xba*I fragment of genomic DNA containing *CEL1* was subcloned and entirely sequenced on both strands. Using an internal *PstI/Acc*I fragment from *CEL1* as probe, a 1,472-bp cDNA was isolated by screening a *C. carbonum* cDNA library and sequenced. The genomic and cDNA sequences were entirely colinear with the exception of a 55-bp intron occurring at nucleotides 699 to 753 (Fig. 2). The intron contains conserved 5′(GGTAAGTTTGCTCG; consensus GGTAAGTNNYCNYY, where Y stands for T or C) and 3′ (TCCAG; consensus WACAG, where W stands for A or T) splice junctions as well as a conserved internal sequence (TTCTAACA; consensus WRCTRACM, where R stands for A or G, and M stands for C or A) (Edelmann and Staben 1994). Based on the cDNA sequence, the polyadenylation site is 221 bp past the stop codon (Fig. 2). By comparison with the genomic sequence, the 1.4-kb cDNA was not full length, ending in the 5′ direction at nucleotide 544 (Fig. 2). The transcriptional start site of *CEL1* was determined using RACE (rapid amplification of cDNA ends) (Frohman et al. 1988). Four RACE products, all cloned from a single PCR reaction, were sequenced, and all indicated that the transcriptional start site was at nucleotide 362, 39 bp upstream of a methionine codon (Fig. 2). The sequences of the RACE products and of the genomic copy of *CEL1* were identical between nucleotide 362 and the start of the 1.4-kb cDNA (nucleotide 544), indicating that there are no introns in this region of *CEL1*. Between these two points there is a single ATG codon, at nucleotide 401 (Fig. 2), which is therefore assumed to be the translational start of *CEL1*. The nucleotide sequence immediately upstream of the putative translational start (CAACATGTAC) is in good agreement with the consensus translational start site of *Neurospora* (CAMMATGGCT) (Edelmann and Staben 1994). Other *C. carbonum* genes show a similar level of agreement with the *Neurospora* translational start consensus (e.g., *XYL1*-CAAAATGGTT;*PGN1*-CAAAATGGTC) (Apel et al. 1993; Scott-Craig et al. 1990).

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                    1
C. carbonum CEL1    MY----RTLA FASLSLYGAA ----RAQQVG TSTAENHPKL TWQTCTGTGGT
P. chrysosporium cbh1-1 MFRAAYRTLA FASLSLYGAA ----VAQQVG TYIPENHPLL ATQSCTASG--
P. chrysosporium cbh1-3 MVDIQAL-LA FTCLAMVSG- ------QQAG TNTAENHPQL QSQQCT-TSG-
T. reesei CBH1      MY----RKLA VISAFLATA- ----RAQSAC TLQSETHPPL TWQKCS-SGG-
P. janthinellum cbh1 MKGSI SYQI YKGALLLSAL LNSVSAQQVG TLTAETHPAL TWSKCT-AG--
T. viride           MY----QKLA LISAFLATA- ----RAQSAC TLQAETHPPL TWQKCS-SGG-
H. grisea cbh-1     M-----RTAK FATLAALVAS AA---AQQAC SLTTERHPSL SWNKCT-AGG-
                    52
C. carbonum CEL1    NCSNKSGSVV LDSNWRWAHN VGGYTNCYTG NSWSTQYCPD GDSCTKNCAI
P. chrysosporium cbh1-1 GCTTSSSKIV LDANRRWIHS TLGTTSCLTA NGWDPTLCPD GITCANYCAL
P. chrysosporium cbh1-3 GCKPLSTKVV LDSNWRWVHS TSGYTNCYTG NEWDTSLCPD GKTCAANCAL
T. reesei CBH1      TCTQQTGSVV IDANWRWTHA TNSSTNCYDG NTWSSTLCPD NETCAKNCCL
P. janthinellum cbh1 XCSQVSGSVV IDANWPXVHS TSGSTNCYTG NTWDATLCPD DVTCAANCAV
T. viride           TCTQQTGSVV IDANWRWTHA TNSSTNCYDG NTWSSTLCPD NETCAKNCCL
H. grisea cbh-1     QCQTVQASIT LDSNWRWTHQ VSGSTNCYTG NKWDTSICTD AKSCAQNCCV
                    402
C. carbonum CEL1    ARGHVLIMSL WGDHAVNMLW LDSTYPTDAD PSKPGAARGT CPTTSGKPED
P. chrysosporium cbh1-1 RTGMVLAFSI SDDPANHMLW LDSNFPPSAN PAVPGVARGM CSITSGNPAD
P. chrysosporium cbh1-3 GNGMVLALSI WDDHAANMLW LDSDYPTDKD PSAPGVARGT CATTSGVPSD
T. reesei CBH1      SGGMVLVMSL WDDYYANMLW LDSTYPTNET SSTPGAVRGS CSTSSGVPAQ
P. janthinellum cbh1 ADGMVLVMSL WDDHASDMLW LDSTYPTNAT STTPGAKRGT CDISR-RPNT
T. viride           SGGMVLVMSL WDDYYANMLW LDSTYPTDET SSTPGAVRGS SSTSSGVPAQ
H. grisea cbh-1     AGPMVLVMSI WDDHASNMLW LDSTFPVDA- AGKPGAERGA CPTTSGVPAE
                    452
C. carbonum CEL1    VEKNSPDATV VFSNIKFGPI GSTFAQPA*- ---------- ----------
P. chrysosporium cbh1-1 VGILNPSPYV SFLNIKFGSI GTTF-RPA*- ---------- ----------
P. chrysosporium cbh1-3 VESQVPNSQV VFSNIKFGDI GSTFS-GTSS PNPPGGSTTS SPVT------
T. reesei CBH1      VESQSPNAKV TFSNIKFGPI GS--TGNPSG GNPPGGNRGT TTTRRP----
P. janthinellum cbh1 VESTYPNAYV IYSNIKTGPL NSTFTGGTTS SSSTTTTTSK STSTSSSSKT
T. viride           LESNSPNAKV VYSNIKFGPI GS--TGNPSG GNPPGGNPPG TTTPRP----
H. grisea cbh-1     VEAEAPNSNV VFSNIRFGPI GSTVAG---- --LPGAGNGG NNGGNPPPPT
                    502
C. carbonum CEL1    ---------- ---------- ---------- ---------- ----------
P. chrysosporium cbh1-1 ---------- ---------- ---------- ---------- ----------
P. chrysosporium cbh1-3 IGYSGSTTCA SPYTCHVLNP -------TSP TPPPTGPTVP QWGQCGGIGY
T. reesei CBH1      IGYSGPTVCA SGTTCQVLNP -------ATT TGSSPGPTQS HYGQCGGIGY
P. janthinellum cbh1 NGWTGPTTCV SPYTCTKQND TTTVTT-TTT SSGSSGTGAR DWAQCGGNGW
T. viride           IGYIGPTVCA SGSTCQVLNP ------ATST GSSPGP-TQT HYGQCGGIGY
H. grisea cbh-1     IGFTGPTQCE EPYICTKLND TTTSSAPATT TTASAGPKAG RWQQCGGIGF
                    552
C. carbonum CEL1    ---------- ---------- ---------- ---------- ----------
P. chrysosporium cbh1-1 ---------- ---------- ---------- ---------- ----------
P. chrysosporium cbh1-3 SGSTTCASPY TCHVLNPCES ILSLQRSSNA DQYLQTTRSA TKRRLDTALQ
T. reesei CBH1      SGPTVCASGT TCQVLNPYYS QCL*------ ---------- ----------
P. janthinellum cbh1 TGPTTCVSPY TCTKQNDWYS QCL*------ ---------- ----------
T. viride           IGPTVCASGS TCQVLNPYYS QCL*------ ---------- ----------
H. grisea cbh-1     TGPTQCEEPY ICTKLNDWYS QCL*------ ---------- ----------
                    602
C. carbonum CEL1    ---
P. chrysosporium cbh1-1 ---
P. chrysosporium cbh1-3 PRK*
T. reesei CBH1      ---
P. janthinellum cbh1 ---
T. viride           ---
H. grisea cbh-1     ---
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	Similarity %	Identity %
P. chrysosporium cbh1-1	67	54
P. chrysosporium cbh1-3	71	62
T. reesei CBH1	70	58
P. janthinellum cbh1	72	62
T. viride	70	59
H. grisea cbh-1	73	61

Fig. 3. Alignment of portions of the predicted amino acid sequence of *CEL1* and six closely related cellobiohydrolase genes from other filamentous fungi. Sequence references—*Phanerochaete chrysosporium cbh1-1*, Covert et al. (1992); *P. chrysosporium cbh1-3*, Sims et al. (1988); *Trichoderma reesei CBH1*, Teeri et al. (1983); *Penicillium janthinellum cbh1*, Koch et al. (1993); *T. viride*, Cheng et al. 1990; *Humicola grisea cbh-1*, Azevedo et al. (1990). Alignment was done using PILEUP (Devereux et al. 1984). Bold type indicates residues highly conserved between *CEL1* and the other genes. Stop codons are indicated by *.

The DNA sequence of *CEL1* predicts a protein coding sequence of 456 amino acids. The PSORT program (Nakai and Kanehisa 1992) predicts that Cel1 has an 18-amino acid signal peptide, with a score of 7.36 by the method of von Heijne (1986). Therefore, the mature, secreted form of Cel1 is predicted to have a molecular mass of 46.4 kDa, assuming no further posttranslational modification.

The amino acid sequence of Cel1 has a high degree of similarity to that of several other known or putative cellobiohydrolase genes from filamentous fungi (Fig. 3). The identity at the amino acid level ranges from 55 to 62%, and the similarity ranges from 67 to 73%. The most striking feature of Cel1 is the absence of a cellulose binding domain and its associated serine- and threonine-rich linker region at either the N terminus or the C terminus, a common feature of endoglucanases and cellobiohydrolases (Knowles et al. 1987). Neither the 5′ nor the 3′ untranslated regions of *CEL1* show any evidence of a hinge region or cellulose-binding domain in any reading frame (Fig. 2).

A *CEL1* mRNA of 1.6 kb was detected when the fungus was grown on maize cell walls or cellulose but not when grown on sucrose or cellulose plus sucrose (Fig. 4). Substrate induction and glucose repression is common among cellulases and other extracellular enzymes in *C. carbonum* and other fungi (Knowles et al. 1987; Kubicek et al. 1993).

The degree of codon bias in a gene is related to its level of expression; more highly expressed genes tend to have higher bias. The Codon Bias Index developed by Bennetzen and Hall (1982) gives a measure of the degree of bias in a gene relative to the highly expressed, and highly biased, gene encoding glyceraldehyde-3-phosphate dehydrogenase. Covert et al. (1992) showed that *cbh1-3* is expressed at a level 1,000 times higher than *cbh1-1*, and, consistent with this, that *cbh1-1* has considerably lower codon bias than *cbh1-3* (Covert al. 1992; Table 1). We calculated a Codon Bias Index for *CEL1* and compared it to *cbh1-1* and *cbh1-3* of *P. chrysosporium* and to *XYL1*, *PGN1*, and *TOXA* of *C. carbonum*. *GPD1* encoding glyceraldehyde-3-phosphate dehydrogenase of *C. heterostrophus*, which shares greater than 95% nucleotide sequence identity to the same gene from *C. carbonum* (P. Sposato and J. D. Walton, unpublished data), was used as the representative highly expressed gene (Bennetzen and Hall 1982). Codon bias in *C. carbonum* is similar to that in *P. chrysosporium*, except for Pro and Ala, which were therefore not used in calculation of the Codon Bias Index. As expected, the highly expressed *C. carbonum* genes *PGN1* and *XYL1* have high bias

(Table 1). In contrast, *TOXA*, a part of the *TOX2* gene cluster which is expressed constitutively at low levels (Pitkin and J. D. Walton, unpublished results), has very low bias. The bias in the highly expressed gene *cbh1-3* is similar to that in *GPD1* and somewhat higher than in *XYL1* and *PGN1*. *CEL1* has a codon bias index considerably higher than *cbh1-1* and comparable to *XYL1* and *PGN1* (Table 1). Therefore, although *CEL1* resembles *cbh1-1* in lacking a hinge region and cellulose-binding domain, it more closely resembles *cbh1-3* in its codon bias and therefore predicted level of expression. Experimentally, the level of *CEL1* mRNA was moderately high relative to *GPD1* when *C. carbonum* was grown on cellulose or maize cell walls (Figs. 4, 6).

To determine the chromosomal location of *CEL1*, chromosomes of *C. carbonum* were separated by pulsed-field gel electrophoresis, blotted, and probed with the *CEL1* cDNA. *CEL1* is located on the second largest chromosome, which is 3.2 Mb in size, in strains SB111 (TOX2$^+$) and SB114 (TOX2$^-$) (data not shown).

Transformation-mediated gene disruption.

The linearized transformation plasmid pCEL1 was used to transform *C. carbonum* strain SB111 (TOX2$^+$) to hygromycin

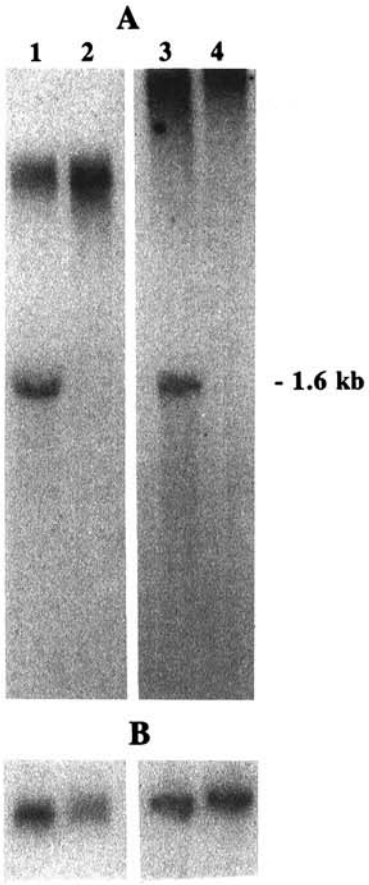

Fig. 4. RNA blot of total RNA (30 µg/lane) extracted from *Cochliobolus carbonum* grown for 4 days on 1% maize cell walls (lane 1), 2% sucrose (lane 2), 1% cellulose (lane 3), or 1% cellulose plus 2% sucrose (lane 4). **A,** Blot was probed with *CEL1* cDNA (2 days of exposure). **B,** Blot was stripped and reprobed with *GPD1* (6 h of exposure). High molecular weight hybridization is due to contaminating DNA.

Table 1. Codon usage in cellulase and other genes; shown are *CEL1* from *C. carbonum* and *cbh1-1* and *cbh1-3* from *P. chrysosporium*

Codon	*CEL1*	*cbh1-1*	*cbh1-3*	*PGN*	*XYL1*	*TOXA*	*GPD1*
TTT-Phe	6	6	0	1	2	20	1
TTC-Phe	12	16	18	6	4	10	8
TTA-Leu	0	0	0	0	0 8	0	
TTG-Leu	0	6	2	2	0	12	4
CTT-Leu	4	6	0	5	4	13	2
CTC-Leu	17	12	24	8	2	16	10
CTA-Leu	0	3	0	0	0	13	0
CTG-Leu	2	5	4	3	0	4	2
ATT-Ile	3	6	1	5	1	17	8
ATC-Ile	13	14	14	24	6	15	18
ATA-Ile	0	1	0	0	0	13 0	
ATG-Met	10	7	12	3	3	9	8
GTT-Val	5	6	3	6	6	19	8
GTC-Val	13	14	21	20	7	10	23
GTA-Val	1	2	0	0	1	12	1
GTG-Val	2	6	2	2	1	7	0
TCT-Ser	9	3	3	4	10	9	4
TCC-Ser	19	10	19	21	9	3	10
TCA-Ser	0	1	0	3	0	10	1
TCG-Ser	8	10	18	2	1	6	2
AGT-Ser	0	3	2	0	0	8	0
AGC-Ser	8	12	7	13	5	6	4
CCT-Pro	7	3	3	3	0	9	4
CCC-Pro	9	5	7	4	5	4	8
CCA-Pro	2	2	2	2	2	6	1
CCG-Pro	0	9	8	0	0	5	0
ACT-Thr	7	7	12	10	5	9	9
ACC-Thr	26	18	38	23	19	10	12
ACA-Thr	1	8	0	4	0	6	2
ACG-Thr	5	16	14	3	0	4	0
GCT-Ala	13	6	7	9	6	20	9
GCC-Ala	21	11	13	17	12	15	19
GCA-Ala	3	8	4	2	0	15	3
GCG-Ala	2	14	18	2	1	12	0
TAT-Tyr	2	3	1	1	1	7	1
TAC-Tyr	17	10	22	6	14	8	12
CAT-His	0	2	0	0	0	5	0
CAC-His	6	4	8	5	3	1	8
CAA-Gln	1	2	0	1	0	8	1
CAG-Gln	12	17	24	5	11	14	3
AAT-Asn	1	7	4	0	1	9	2
AAC-Asn	29	26	31	23	17	7	19
AAA-Lys	0	1	0	1	0	11	0
AAG-Lys	27	8	17	21	6	6	26
GAT-Asp	5	6	5	4	4	9	2
GAC-Asp	24	18	26	14	2	4	20
GAA-Gl	1	0	0	2	0	8	0
GAG-Glu	12	6	9	3	4	4	16
TGT-Cys	1	8	1	0	0	5	0
TGC-Cys	17	10	21	19	2	5	2
TGG-Trp	8	5	8	5	8	11	4
CGT-Arg	3	3	2	0	3	2	5
CGC-Arg	5	8	9	4	3	3	6
CGA-Arg	1	0	0	0	0	1	0
CGG-Arg	0	1	1	0	1	4	0
AGA-Arg	0	0	0	0	0	3	0
AGG-Arg	1	2	0	0	1	2 0	
GGT-Gly	21	6	11	12	11	15	15
GGC-Gly	29	26	37	32	12	15	12
GGA-Gly	5	9	1	9	5	19	2
GGG-Gly	0	5	2	0	0	7	0
Codon Bias Index[a]	0.76	0.42	0.82	0.72	0.73	0.03	0.83

Included for comparison are genes from *C. carbonum* encoding endo-polygalacturonase (*PGN1*), endo-xylanase (*XYL1*), and a putative HC-toxin efflux pump (*TOXA*), and the gene from *C. heterostrophus* encoding glyceraldehyde-3-phosphate dehydrogenase (*GPD1*).

[a] Codon Bias Index was calculated using the formula of Bennetzen and Hall (1982). Preferred codons are those used more than 75% of the time in *GPD1* from *C. heterostrophus*. Preferred codons are: TTC, TTG, and CTC, ATC, GTT, and GTC, ACT and ACC, TAC, CAC, CAG, AAC, AAG, GAC, GAG, TGC, CGT, and CGC, GGT and GGC. Codons for Met and Trp were not used because they show no redundancy; others were eliminated because they show insufficient codon bias in *GPD1* (Ser) or a bias that was different between *P. chrysosporium* and *C. carbonum* (Pro, Ala).

resistance. Ten transformants were isolated, purified by two rounds of single-sporing, and two of them were analyzed by DNA blotting. A restriction map of the genomic DNA region containing *CEL1* and the predicted map of the disruption mutants is presented in Figure 5A. The pattern of hybridization using the *CEL1* cDNA as probe was consistent with homologous integration of multiple copies of the transforming DNA (Fig. 5B). When grown on maize cell walls as the carbon source, the two disruption mutants lacked the 1.6-kb *CEL1* mRNA (Fig. 6A). Approximately equivalent loading of RNA in each lane was confirmed by stripping and probing the same blot with *GPD1* (Fig. 6B).

There was no significant difference in growth between the wild type and *CEL1*-disrupted strains on either maize cell walls or cellulose (data not shown). The pathogencity of the two *CEL1* disruptant mutants were compared with two wild-type strains of *C. carbonum*, SB111 (TOX2$^+$) and SB114 (TOX2$^-$). The infected plants were examined daily for 10 days. In three independent tests, no consistent differences in lesion size, lesion number, or rate of disease development were observed between SB111 and the two disruptants (Fig. 7). After reisolation from the infected plants, the mutant fungi were still resistant to hygromycin, excluding the possibility that the mutants had regained pathogenicity by excision of pCEL1. Therefore, we conclude that *CEL1* is not, by itself, a necessary pathogenicity determinant.

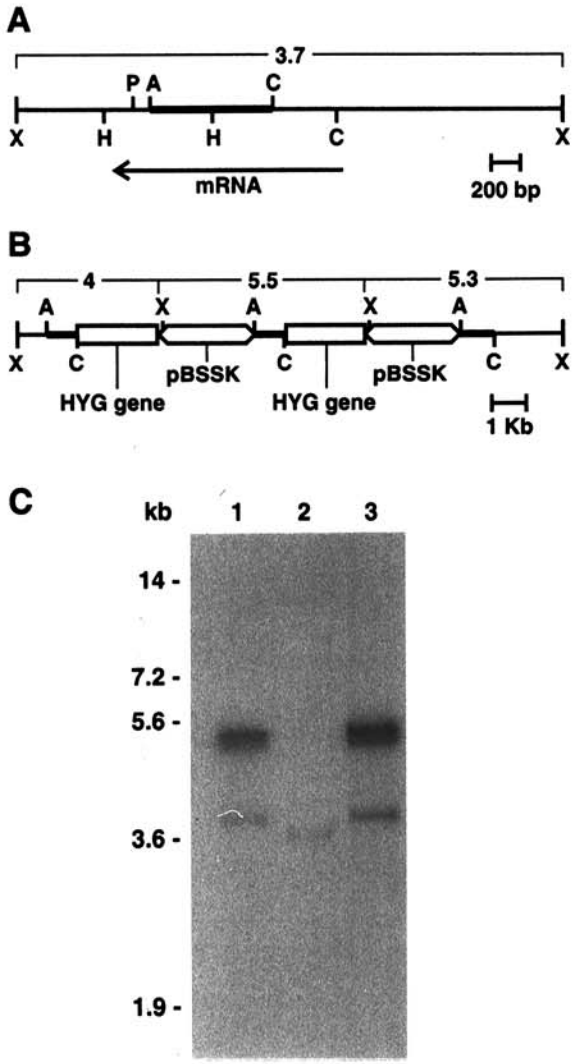

Fig. 5. A, Restriction map of wild-type copy of *CEL1*, showing important restriction enzyme sites and the location of the *CEL1* mRNA. X = *Xba*I; H = *Hin*dIII; P = *Pst*I; A = *Apa*I; C = *Acc*I. The *ApaI*/*Acc*I fragment used to construct pCEL1 is indicated by a heavy line. **B,** Predicted restriction map of *CEL1* disruption mutant. Predicted *Xba*I fragment sizes are indicated (in kb). **C,** DNA blot of transformant T414-1 (lane 1), wild type (lane 2), and transformant T414-2 (lane 3). Isolated DNA was cut with *Xba*I and the blot was probed with the *ApaI*/*Acc*I fragment used to construct pCEL1. The intensity of the 5.5-kb band is consistent with multiple tandem integrations of pCEL1.

Biochemical analysis of cellulases in *C. carbonum* wild type and *CEL1*-mutant strains.

The wild type and mutant strains were grown on insoluble cellulose, soluble CMC, or maize cell walls for 4, 6, or 8 days. Culture filtrates were assayed for cellulase activity using either pNPCB, CMC, or insoluble cellulose as substrates. β-Glucosidase activity was measured using pNPG. Both wild

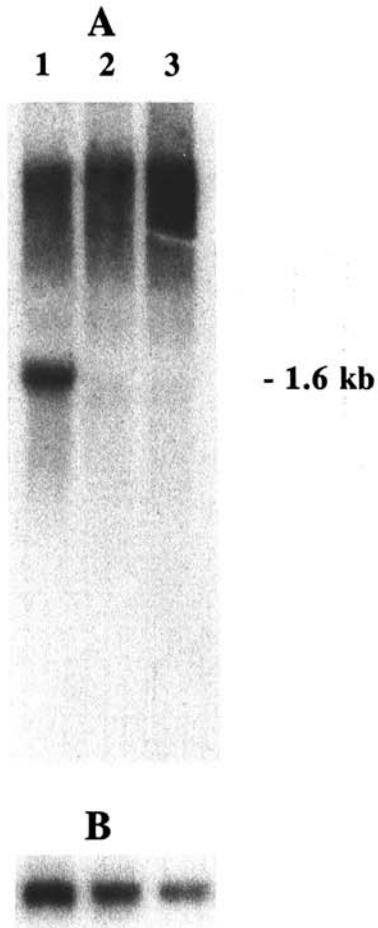

Fig. 6. RNA blot of total RNA (30 μg/lane) extracted from *Cochlibolus carbonum* wild type (lane 1) and two *CEL1* mutants (T414-1—lane 2; T414-2—lane 3) grown on 1% maize cell walls for 4 days. **A,** Blot was probed with *CEL1* cDNA (3 days of exposure). **B,** Blot was stripped and reprobed with *GPD1* (6 h of exposure). High molecular weight hybridization is due to contaminating DNA.

type and mutant culture filtrates had strong and comparable activity with pNPCB and pNPG, and weak but comparable activity against CMC or cellulose. Culture filtrates were concentrated by rotary evaporation and/or acetone precipitation, and desalted by gel filtration, ultrafiltration, and/or dialysis. The filtrates were fractionated by HPLC using chromatofocusing, hydrophobic interaction, and/or cation exchange. Ion-exchange chromatography resolved two major peaks of activity (one of which could be resolved into three peaks by subsequent hydrophobic interaction chromatography) and three minor peaks of activity with pNPCB as substrate. However, all the peaks of activity were identical in both wild type and *CEL1*-mutant strains. *C. carbonum* culture filtrates also had several separable peaks of activity against pNPG which likewise did not differ between wild type and the *CEL1* mutants (data not shown).

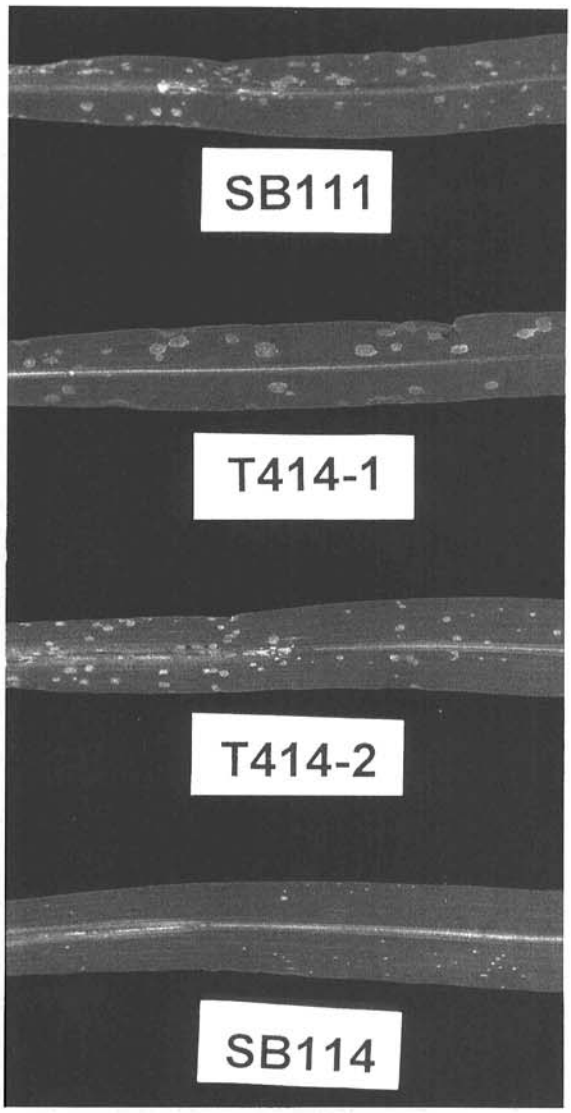

Fig. 7. Pathogenicity test of wild type and *CEL1* disruptant mutants. SB111: wildtype TOX2$^+$ isolate. T414-1 and T414-2: Two independent CEL1 mutants of SB111. SB114: wildtype TOX2$^-$ isolate. Photograph was taken 4 days after inoculation.

DISCUSSION

The fungal plant pathogen *Cochliobolus carbonum* has a gene, *CEL1*, that encodes a cellulase lacking a hinge region and cellulose binding domain. This gene is not required for pathogenicity on maize.

Many cellulases are composed of three structural domains: a core which contains the catalytic site, a hinge which protrudes from the core and tends to be glycosylated, and, separated from the catalytically active core by the hinge, a putative cellulose-binding domain (Knowles et al. 1987; Rouvinen et al. 1990). The hinge and cellulose-binding domains can be proteolytically removed with only small changes in substrate preference and catalytic activity (Gilkes et al. 1988; Tomme et al. 1988; Van Tilbeurgh et al. 1986). Among related cellulases, only *cbh1-1* of *P. chrysosporium* (Covert et al. 1992) also lacks both a hinge and cellulose-binding domain. The predicted coding regions of *CEL1* and *cbh1-1* end at the same place just before the hinge region (Fig. 3). In addition, *CEL1* and *cbh1-1* have very similar predicted signal sequences (Fig. 3).

CEL1 mRNA abundance is high when *C. carbonum* is grown on maize cell walls or cellulose (Fig. 4). Under identical growth conditions, *CEL1* mRNA is not present in mutant strains (Fig. 6). This indicates that *CEL1* was successfully disrupted, and allows the conclusion that *CEL1* is not required for pathogenicity (Fig. 7). However, attempts to find a cellobiohydrolase activity that was specifically lacking in culture filtrates of the *CEL1* mutant were unsuccessful. There are several possible explanations for this. First, Cel1 could be a minor component of the total cellulase activity secreted by *C. carbonum* and therefore be obscured by more abundant enzymes with the same or overlapping activity. *C. carbonum* culture filtrates contain several separable cellobiohydrolase, endoglucanase, and β-glucosidase activities (P. Sposato and J. D. Walton, unpublished data). Second, Cel1 might be unstable either in culture or during purification. Other *C. carbonum* extracellular glycosidases, however, are quite stable to standard protein purification protocols (e.g., Van Hoof et al. 1991). Third, Cel1 might not have activity against the cellulase substrates that were tried. There are a number of different assays for cellobiohydrolase, and none of them are completely specific nor suitable for all cellobiohydrolases (Sharrock 1988). *CEL1* was cloned using *cbh1-3* of *P. chrysosporium*, which itself was cloned using *CBH1* of *Trichoderma reesei* (Teeri et al. 1983). Since cellobiohydrolases and endoglucanases often have a high level of sequence similarity (Henrissat et al. 1989), Cel1 might be an endoglucanase; the name chosen for the *C. carbonum* gene, *CEL1*, reflects our uncertainty of the enzymatic activity of Cel1. Fifth, Cel1 might not be secreted. *O*-Glycosylation, which occurs, like *N*-glycosylation, mainly in the hinge region, may be important for secretion of cellulases (Kubicek et al. 1993). Although Cel1 appears to have a secretion signal, we have no experimental evidence that it is, in fact, secreted.

Previously, genes encoding cell wall-degrading enzymes of *C. carbonum* have been cloned in this laboratory starting with amino acid sequences derived from purified enzymes of known catalytic activity. As a result, the subsequent biochemical analyses of engineered mutants of those genes was straightforward (Scott-Craig et al. 1990; Apel et al. 1993;

Schaeffer et al. 1994). On the other hand, the case of *CEL1* demonstrates that although heterologous probes are effective for isolating related genes from other organisms, subsequent biochemical analyses of the gene products can be difficult. This is particularly true for enzymes, like cellulases, that occur in families with related and cooperative and/or overlapping activities.

Although our experiments exclude an important role for *CEL1* by itself in pathogenicity of *C. carbonum*, we cannot draw any conclusions about the importance of cellulose degradation in general in pathogenesis, due to the existence of multiple cellulases (including endoglucanase, cellobiohydrolase, and β-glucosidase) in *C. carbonum* culture filtrates. Polygalacturonase and xylanase activities of *C. carbonum* are also redundant (Scott-Craig et al. 1990; Apel et al. 1993).

MATERIALS AND METHODS

Fungal cultures and manipulations.

Conidia of *C. carbonum* SB111 (race 1) (ATCC #90305) were stored in glycerol at -80°C and used to inoculate V8 juice agar plates. The fungus was maintained and grown as described (Walton and Cervone 1990). Mycelia for protoplast preparation or DNA extraction were obtained from germinating conidia (Apel et al. 1993).

For enzyme production, *C. carbonum* was grown on mineral salts medium supplemented with trace elements and 0.2% yeast extract (van Hoof et al. 1991). As a carbon source, sucrose, maize cell walls, or powdered cellulose (Sigma C-6288) were used at a concentration of 2, 1, or 1%, respectively.

Maize cell walls were prepared from greenhouse-grown 5-week-old maize plants. Plants were chopped into approximately 2-cm^2 pieces, lyophilized, and ground in liquid nitrogen with a kitchen blender. The resulting powder was stirred at 21°C in 0.1 M KH_2PO_4, pH 7, for 1 h. The resulting slurry was filtered through eight layers of cheesecloth and twice resuspended in phosphate buffer and refiltered. The maize walls were then washed twice with water, twice with methanol, twice with chloroform:methanol (1:1), and then again with methanol until little or no additional pigment was extracted. The walls were then washed twice with acetone and dried in trays in an open fume hood.

Nucleic acid manipulations and sequencing.

RNA and DNA extractions and DNA and RNA blotting and hybridizations were done as described (Apel et al. 1993). The constitutively expressed gene encoding glyceraldehyde-3-phosphate dehydrogenase from *C. carbonum* was cloned using *GPD1* from *C. heterostrophus* (Van Wert and Yoder 1992; P. Sposato and J. D. Walton, unpublished data). Routine (high-stringency) DNA blotting and hybridizations were done with Zeta-Probe membranes (BioRad, Richmond, Calif.) and random primer-labeled DNA probes in 5× SPE, 7% SDS, 0.5% nonfat dry milk, and 0.1 mg/ml denatured salmon sperm DNA (Apel et al. 1989) at 65°C overnight. The blots were washed twice in 2× SSPE and 0.1% SDS at room temperature for 15 min and twice in 0.1× SSPE and 0.1% SDS at 65°C for 20 min. Low- or medium-stringency hybridizations were done in the same hybridization solution at 48°C or 55°C, respectively, and were then washed twice at room temperature with 2× SSPE and 0.1% SDS for 15 min and twice at 48°C or 55°C, respectively, with 0.5× SSPE and 0.1% SDS for 20 min.

For sequencing, nested deletions were made using the Erase-a-base kit (Promega, Madison, Wisc.). Automated fluorescent sequencing was done at the MSU-DOE-PRL Plant Biochemistry Facility using an Applied Biosystems (Foster City, Calif.) Catalyst 800 for Taq cycle sequencing and an Applied Biosystems 373A Sequencer for analysis of the products.

The transcriptional start site of *CEL1* was determined using the 5′ end of the longest cDNA clone and the Amplifinder RACE Kit, following the manufacturer's protocols (Clontech Laboratories, Palo Alto, Calif.) (Frohman et al. 1988). Reverse transcription was primed with an oligonucleotide of sequence CGCTCTCTCCCTCAAGTTCGTCACC (starting at nucleotide 781, Fig. 2) and the first-strand cDNA product amplifed by PCR using the supplied 5′ oligonucleotide "anchor" and a primer of sequence GGTGACTCTTGCACCAAGAACTGCGCTATCG (starting at nucleotide 649, Fig. 2). Sequence data were analyzed with the DNASIS and PROSIS programs (Hitachi Software Engineering Co., San Bruno, Calif.), and the University of Wisconsin GCG package (Devereux et al. 1984).

Clamped homogeneous electric field (CHEF) electrophoresis was performed on a Bio-Rad CHEF-II apparatus. The chromosomes were separated on an 0.8% agarose gel at 14°C and 45V using the following switching intervals: 40 to 50 min for 72 h, 15 to 30 min for 72 h, and finally 10 to 20 min for 72 h.

Isolation and disruption of *CEL1*.

The *C. carbonum* genomic library in EMBL3 has been described (Scott-Craig et al. 1990). The cDNA library was prepared from poly(A)$^+$-RNA extracted from *C. carbonum* grown on maize cell walls (J. Pitkin and J. D. Walton, manuscript in preparation). The *cbh1-3* gene of *P. chrysosporium* was obtained from Dan Cullen, Forest Products Laboratory, University of Wisconsin, Madison (Covert et al. 1992). This gene is probably an allele of the gene cloned by Sims et al. (1988). The *P. chrysosporium cbh1-3* gene had originally been isolated using the *CBH1* gene of *Trichoderma reesei* as a heterologous probe (Sims et al. 1988; Teeri et al. 1983).

The transformation disruption vector was created by subcloning the *SalI**Hin*dIII fragment of pUCH1, which contains a gene for hygromycin resistance driven by a promoter from *C. heterostrophus*, into pBlueScriptSK II to produce pHYG1. The single *Hin*dIII site in pHYG1 was removed by linearizing the plasmid with *Hin*dIII, filling in the ends with T4 polymerase and Klenow enzyme, and religating. An internal *ApaI*/*Acc*I fragment of *CEL1* (Fig. 2) was subcloned into pHYG1. The resulting plasmid, pCEL1, was linearized at the unique *Hin*dIII site in *CEL1* (Fig. 5A) and used to transform protoplasts of *C. carbonum* isolate SB111 to hygromycin resistance as described (Apel et al. 1993; Scott-Craig et al. 1990). Transformants were selected on V8 juice agar containing hygromycin (Calbiochem) and purified to nuclear homogeneity by two rounds of single spore isolation.

Pathogenicity tests were conducted by inoculation of 10-day old susceptible maize plants (hybrid Pr × K61, genotype *hm**hm*) with a conidial suspension (10^4 conidia per milliliter)

of *C. carbonum* SB111 and two independent transformants, T414-1 and T414-2. Disease progression was evaluated daily.

Enzyme assays.

Cellobiohydrolase and β-glucosidase activities were assayed colorimetrically with pNPCB and pNPG (Sigma), respectively. Hydrolysis of CMC and cellulose was assayed using the reducing sugar assay of Lever (1972).

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