Mutational Analysis of β -Glucanase Genes from the Plant-Pathogenic Fungus *Cochliobolus carbonum*

Hoon Kim, Joong-Hoon Ahn, Jenifer M. Görlach, Claudio Caprari, John S. Scott-Craig, and Jonathan D. Walton

Department of Energy Plant Research Laboratory, Michigan State University, East Lansing 48824 U.S.A.

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Two new β -glucanase-encoding genes, *EXG2* and *MLG2*, were isolated from the plant-pathogenic fungus *Cochliobolus carbonum* using polymerase chain reaction based on amino acid sequences from the purified proteins. *EXG2* encodes a 46.6-kDa exo- β 1,3-glucanase and is located on the same 3.5-Mb chromosome that contains the genes of HC-toxin biosynthesis. *MLG2* encodes a 26.8-kDa mixed-linked (β 1,3- β 1,4) glucanase with low activity against β 1,4-glucan and no activity against β 1,3-glucan. Specific mutants of *EXG2* and *MLG2* were constructed by targeted gene replacement. Strains with multiple mutations (genotypes *exg1/mlg1*, *exg2/mlg1*, *mlg1/mlg2*, and *exg1/exg2/mlg1/mlg2*) were also constructed by sequential disruption and by crossing. Total mixed-linked glucanase activity in culture filtrates of *mlg1/mlg2* and *exg1/exg2/mlg1/mlg2* mutants was reduced by approximately 73%. Total β 1,3-glucanase activity was reduced by 10, 54, and 96% in *exg2*, *mlg1*, and *exg1/exg2/mlg1/mlg2* mutants, respectively. The quadruple mutant showed only a modest decrease in growth on β 1,3-glucan or mixed-linked glucan. None of the mutants showed any decrease in virulence.

Additional keyword: maize.

Filamentous fungi produce a battery of extracellular enzymes capable of degrading the polymers of plant cell walls. Many ultrastructural, biochemical, and genetic studies have implicated these enzymes in the process of plant pathogenesis as agents of penetration and ramification and as elicitors of plant defense responses. Recently, several pectin-degrading enzymes have been shown to contribute to virulence in several diseases of dicotyledonous plants, which contain much more pectin than do cereals (Isshiki et al. 2001). However, many

targeted mutational studies have found that individual enzyme genes play only a small, if any, role in pathogenesis (Annis and Goodwin 1997; Scott-Craig et al. 1998a; Walton 1994).

The major classes of polymers in plant cell walls are cellulose, hemicellulose, pectin, and proteins. The hemicelluloses of cereals (Poaceae family) include noncellulosic β -glucans such as callose (β 1,3-linked glucan) and mixed-linked glucan (β 1,3- β 1,4-glucan, also known as cereal β -glucan). Callose is present in phloem sieve plates, at sites of wounding, and in papillae (specialized structures formed in response to attempted pathogen penetration, especially in members of Poaceae) (Sherwood and Vance 1980). Mixed-linked glucan is relatively abundant in elongating cells of members of Poaceae and has been implicated in the regulation of wall expansion (Carpita 1996).

Cochliobolus carbonum (anamorph *Bipolaris zeicola* or *Helminthosporium carbonum*) is a foliar and ear pathogen of maize (*Zea mays* L.). Like other filamentous fungi, it produces a variety of extracellular polysaccharide depolymerases with redundant or overlapping activities (Annis and Goodwin 1997; Walton 1994). By the construction and biochemical analysis of strains that are mutated in specific depolymerase structural genes, we have demonstrated that the enzyme redundancy of *C. carbonum* is due, in some cases, to multiple genes encoding enzymes with overlapping enzymatic activities and, in other cases, to differential posttranslational processing (differential glycosylation) of the products of single genes (Apel-Birkhold and Walton 1996; Görlach et al. 1998; Murphy and Walton 1996; Scott-Craig et al. 1998a).

C. carbonum makes several extracellular β -glucanases. Insofar as β 1,3-glucan and mixed-linked glucans are important structural components of cereal cell walls, including the defense-related structures known as papillae, the ability to degrade these polymers might be important during the process of pathogenesis. Two previously characterized mixed-linked glucanases (MLGase), Mlg1a and Mlg1b, act in an endo manner on both mixed-linked (β 1,4- β 1,3) glucan and β 1,3-glucan and were shown to be glycosylation isoforms of the product of *MLG1* (Görlach et al. 1998). A third MLGase, Mlg2, is active against mixed-linked glucan and, to a lesser extent, β 1,4-glucan, but has no activity against pure β 1,3-glucan. *C. carbonum* also makes multiple β 1,3-glucanases. *Exg1* is an exo- β 1,3-glucanase encoded by *EXG1*. Mutants of *EXG1* still have significant residual β 1,3-glucanase activity (Nikolskaya et al. 1998; Schaeffer et al. 1994).

Corresponding author: Jonathan D. Walton; Telephone: +1-517-353-4885; Fax: +1-517-353-9168; E-mail: walton@msu.edu

Current address of H. Kim: Department of Agricultural Chemistry, Sunchon National University, Sunchon 540-742 Korea.

Current address of J.-H. Ahn: Department of Forest Resources, Kon-Kuk University, Seoul 143-701 Korea.

Current address of J. M. Görlach: Division of Infectious Diseases, Duke University Medical Center, Durham, NC 27710 U.S.A.

Current address of C. Caprari: Dipartimento di Biologia Vegetale, Università degli Studi di Roma "La Sapienza," 00185 Rome, Italy.

Here, we report the isolation of the genes encoding Mlg2 and a new α -1,3-glucanase, Exg2. Mutants lacking the individual activities and also all four known noncellulosic β -glucanase genes (*MLG1*, *MLG2*, *EXG1*, and *EXG2*) were constructed and tested for enzyme activity, growth, and pathogenicity.

RESULTS

Isolation of *EXG2*.

C. carbonum was grown on maize cell walls, and the culture filtrates were fractionated by anion-exchange and cation-exchange chromatography. Proteins from the void volume of the cation-exchange step were further separated by hydrophobic-interaction high-performance liquid chromatography (HPLC) (Görlach et al. 1998). Four fractions that had prominent peaks of absorbance at 280 nm were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and four of the most prominent protein bands (detected by staining with Coomassie blue) were excised and subjected to N-terminal amino acid sequencing. One protein gave no sequence, one had no similarity to anything in the nonredundant databases, one was Mlg1 (Görlach et al. 1998), and the other gave the sequence AVGFNWGSEKIRGVNIGGXLVLEPXTPSI, in which X indicates unknown. This sequence was similar to those of several yeast α -1,3-glucanases, including the products of *EXG1* from *Yarrowia lipolytica* (GenBank accession no. Z46872), *EXG1* from *Kluyveromyces lactis* (GenBank accession no. Z46869) (Esteban et al. 1999), and *SSG* (= *SPR1*) from *Saccharomyces cerevisiae* (GenBank accession no. X59259) (San Segundo et al. 1993). Therefore, it seemed possible that this protein was an α -1,3-glucanase distinct from Exg1 of *C. carbonum* (Schaeffer et al. 1994).

All of the known β 1,3-glucanases whose N termini are similar to this protein contain an internal motif, QNGFDN. Polymerase chain reaction (PCR) primers based on this sequence and on VGFNWG from the N-terminal peptide of Exg2 were used to amplify a portion of the gene, called *EXG2*. The 450-bp PCR product was used to isolate cDNA and genomic DNA copies of *EXG2*, which were sequenced on both strands (Fig. 1). *EXG2* has five introns of 70, 55, 47, 52, and 56 bp. The predicted protein has a molecular mass of 46.6 kDa and a pI of 6.4. It has no predicted N-glycosylation sites. Based on the experimentally determined N terminus and the most probable translational start methionine, 25 amino acids of Exg2 are removed during processing and secretion. The program SignalP (Nielsen et al. 1997) predicts that Exg2 has a signal peptide of 18 amino acids (cleavage site: VLA-APA), which suggests that a second processing event during secretion removes an additional seven amino acids.

Overall, Exg2 shows low but significant amino acid identity (33.2 to 35.9%) to the same yeast α -1,3-glucanases originally identified by the N-terminal peptide. It also shares 35.1% identity with a putative α -1,3-glucanase from the basidiomycete *Agaricus bisporus* (GenBank accession no. X92961). Exg2 also shows a clear relatedness (16.8% identity) to an endo- β 1,6-glucanase from the ascomycete *Trichoderma harzianum* (GenBank accession no. X79197) (Lora et al. 1995; van de Rhee et al. 1996). The conserved active site motif of β 1,3-glucanases, including the presumptive catalytic glutamic acid residue (A/V)GEW(S/T)XAXTDC(A/T)XXLNG, is represented in Exg2 by VGEWTSAMTDCARYLNG start-

ing at amino acid 311 (Chen et al. 1993; Mackenzie et al. 1997) (Fig. 1).

EXG2 was hybridized to intact chromosomes of *C. carbonum* separated by pulsed-field gel electrophoresis. *EXG2* is located on the 3.5-Mb "TOX2" chromosome of strain 367-2 (J.-H. Ahn and J. D. Walton, unpublished data), which contains the *TOX2* genes required for HC-toxin biosynthesis (Ahn and Walton 1996). This is the first active gene to be found on this chromosome other than the genes of *TOX2* and transposable elements.

Isolation of *MLG2*.

Of several substrates tested (maize cell walls, β 1,3-glucan, and other substrates given by Van Hoof and colleagues [1991]), maize bran was found to be the best inducer of Mlg2

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1  GGTAAAGTTGGCATTCTTTGTCTGCTCAGCGCTCGGATTTCAGTATAAAAGTACCTCCATG
61  ACCGGTCTTGAACCTCTCCACTCCGCAACTCATCGTGACAAACACAATGTGAGAGCAACA
121 TCATCTTTGCAACATGATTCTCACCAGAACTCGTTTCAACATCTGCTGCTATGGCTGCTGT
      M I L T K L V S T L S L C A A V
181 CTGGGCTGCTCTCGTgaagtgtatgcttccagcgcacatcactgcaaaacaaacatcacat
17  L A A P A
241 tgtgtctctgtgacttttctctagCCAGCAGAGCGTGACGTGGCTTCAACTGGGGAT
22      Q Q K R A V G F N W L G
301 CTGAGAAGATTGCGGGTGAACATTTGGTGGATGGTTGGTCTCTGGAGCGtaagtgcacat
33  S E K I R G V N I G G W L V L E P
361 gggttgatgatgctttgagacatctgctaactccttccacagCTGGATCACCCCATCA
50      W I T P S
421 ATCTTCGACAAACGCAACCGAGGCGCTCCACAAATGACTTTGTTGACGgtaagctgaat
55  I F D N A N R G R P Q N D F V D
481 gactttcatattgagactgatgctaataacgcgcagAGTACACATTTGGGTGAAAAGCTTG
71      E Y T L G E K L
541 CGAGCCAAATGCCCCCTAAACATCCTTCGTAAACATTGGGATCTTTCTGCTACCTGGCAAG
79  G S Q N A L N I L R N H W D T F V T W Q
601 ACTTCAACAAATCAAGCAGTCCGGTTTCAACGTTGTCCGTATCCCGTTGGCTACTGGG
99  D F N K I K Q S G F N V R I P V G Y W
661 CATACGATACCTTTGGCTCGCCCTACGTCAGTGGAGCAGCTGTCTACATTTGATGTGCCA
119 A Y D T F G S P Y V S G A A V Y I D A A
721 TCAGTCTGGCTCGTAGCCTGGGCTTGAAGATTATCATTTGACCTTACCGGTGACCTGGAT
139 I D W A R S L G L K I I I D L H G A P G
781 CCCGAACGGGTTTGACAACCTCTGGTCAACGCTGATCGCCCCAGTGGCAGCAAGGCG
159 S Q N G F D N S G Q R M D R P T W Q Q G
841 ACACGCTCGGAAGGACCTTCAAGTTTTCGCGACAATCTCTCAGAAATACGCACAACGA
179 D T V R R T L Q V L T I S Q K Y A Q T
901 GCTACCGAGGATGTCATCGTGGTATTCACTGCTCAACGAGCCCGCACTTTTACAACGGCC
199 S Y Q D V I V G I Q L L N E P A L Y N G
961 TCAGTCTGATGTTCTTGACAGTTCTATCGCGATGGCTATGGCCAGTGGCGAGGTTT
219 L S R D V L A Q F Y R D G Y G Q V R E V
1021 CCGACACGCGGTCATCATTTCTGACGGCTTCACTGCACCAAACTCCTGGAACGGCTTCC
239 S D T P V I I S D G F T A P N S W N G F
1081 TCACACCTCAGATGCCAAGCCGAGATGTAGCCATTGACAAACCAATACCAAGTTT
259 L T P S D A N A Q N V A I D N H Q Y Q V
1141 TCAGCTCTAATCTGCTCAAACTGTACCCGCTGGACACGCCCCAACAGGCTGCAGGAACA
279 F D S N L L K L S P A G H A Q Q A C R N
1201 CTGGCGCATATGGCGTGCAGACAAGTGACCTTTGtattgttcaagaagtgcgataca
299 T G A Y G G A D K W T F
1261 ataagcccttactgacttatatgttgagTTGGAGAGTGGACTTCCGCCATGACgtgagc
311      V G E W T S A M T
1321 atgactctttcacacataacacaaaagcacataaactgactgcccgcagGGACTCGCA
320      D C A
1381 CGTTACTTGAACGGCTACGGCCGTGGTGGCCGCTATGATGTTACCTACTTGGGCAACCCC
323 R Y L N G Y G R G A R Y D G T Y L G N P
1441 AAGTTGGCGGATGCGGCTGGCGAAACGACCTAGCGCAGTGGCCGCTCTTATAAGGAC
343 K L G E C G W R N D L A Q W P A S Y K D
1501 GACTCCAGGCGCTACATCGAGGCCAGATCCGCGCTTTTGAAGTCAGACCCCAAGGCTGG
363 D S R R Y I E A Q I R A F E S T T Q G W
1561 TTCTGGTGAACCTTCAAGACTGAGGCTGGGCTGAGTGGGATGCTTTTCAAGCTCATCGAC
383 F W W N F K T E G A A E W D A F R L I D
1621 GCTGGTCTTCTCCCGCCATCAGGAACGAGTAGAGTACAAGTTTGGCGCTGCCTGC
403 A G V F P A I R N G Q V E Y K F G A A C
1681 TAGAAACATGGCTTGACACCTGGCTTAAATTTACTTTTGTGACCTCTAGAAGGGGCTA
423 *
1741 GACGGCTGAACGCAATCGCAAAGAAAGCAAAGCAGCATGTGTGATGTCATAGAGGA
1801 GGCTTGACTTTTCATTGCTCAGTTCGATTAAACAATTATGATGATTTGGAATAACGAAGT
      +
1861 ACACGATAGCAATGAGATTTTTCATACACTTTTGTGCAAGCACAAGATAAGTAC

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Fig. 1. Nucleotide sequence and deduced amino acid sequence of *EXG2*. The experimentally determined N terminus of the mature protein is double-underlined, and the amino acid sequence that is conserved among other β 1,3-glucanases and used to design the reverse polymerase chain reaction primer for amplification of *EXG2* is single-underlined. Introns are indicated in lowercase letters. The polyadenylation site is indicated by + over the appropriate nucleotide.

(data not shown). Mlg2 was purified from culture filtrates by anion and cation exchange and hydrophobic-interaction HPLC and, as the final step, SDS-PAGE (Görlach et al. 1998). Because the N terminus was blocked, internal amino acid sequences were obtained from three peptides generated by digestion with trypsin (Görlach et al. 1998). PCR using primers based on these tryptic peptides amplified a 190-bp fragment that was subcloned and used to isolate genomic DNA and cDNA copies of *MLG2* (Fig. 2).

MLG2 has two introns of 70 and 59 bp. Based on analysis by random amplification of cDNA ends (RACE), the *MLG2* transcript has a 59-bp 5' untranslated region, similar to *MLG1* (64 bp) (Görlach et al. 1998). It is predicted to encode a protein with a molecular mass of 26.8 kDa and a pI of 8.5. All of the experimentally determined peptides from Mlg2 were present in the predicted translation product, with only a few individual amino acid discrepancies (Görlach et al. 1998). SignalP predicted a signal peptide of 17 amino acids. Mlg2 has no predicted *N*-glycosylation sites. There is little overall sequence similarity between Mlg2 and any other known MLGase, including Mlg1 of *C. carbonum*. The best matches are to several fungal proteins annotated as endo- β 1,4-glucanases belonging to family 12 of the glycosyl hydrolases. The protein with the

highest overall similarity (54% identity) is an endo- β 1,4-glucanase from *Aspergillus aculeatus* (GenBank accession no. P226690) (Ooi et al. 1990). Mlg2 has only weak cellulase activity (Görlach et al. 1998), and apparently none of the related cellulases were tested against mixed-linked glucan. Therefore, perhaps this family of enzymes might more properly be designated as mixed-linked glucanases.

Expression of *EXG2* and *MLG2*.

EXG2 RNA was expressed when the fungus was grown on sucrose, maize bran, oat bran, and maize cell walls (data not shown). In being expressed during growth on sucrose as well as on its substrate, *EXG2* is similar to *EXG1* but different from the other characterized extracellular depolymerases of *C. carbonum*. *MLG2* was expressed on mixed-linked glucan, maize bran, and maize cell walls but not on sucrose (data not shown). mRNAs for all four glucanase genes (*EXG1*, *EXG2*, *MLG1*, and *MLG2*) could be detected in planta at 4 days postinoculation with relative abundances of *EXG2* = *MLG2* > *EXG1* > *MLG1* (Fig. 3).

Gene disruption.

EXG2 was disrupted by replacing a portion of the 5' end with the *hph* gene. Three independent *exg2* mutant transformants were purified by single-spore isolation and analyzed. The DNA blot pattern and enzyme activity pattern of all three transformants were the same, and further experiments were done with transformant T682. In the wild type, a probe consisting of the *EXG2* cDNA hybridized to *Hind*III fragments of 1.7 and 4.0 kb (Figs. 4 and 5). As predicted, this probe hybridized to a 6.4-kb fragment in T682 (Figs. 4 and 5) and other transformants (data not shown). A probe con-

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1  TGAGGCATTCAGACCCCGCCCGTAGTCGGAAGAACACGCGGCAGATATCCAGGATAT
61  CACCTGTTGTTTCCAGGCGCGTGTTCGAGCGGAGACTAATTCGCCGATGCAGTAATAC
121  AAGTGGGTAGCCACGCGACATGATTTGGCGATTATTAATTTCCGCCCGAGGGCC
181  TGCTCAGTGTAGTGTGTTCCCGCATGAGACTGGGATACAACTTGTTCGAGCGGTG
241  CTTCGGAGACGCGTGGGAATCTAGGGAAGCATCGGACTGTGGAGGACTGAATTATAT
301  GAGGACCCACTGAAGCTTTCCCGCCGCTCTAAAGTCATGATCTAGACCATGTGGAGTCG
#
361  TGAAGGCAACTATGGGAAGTACAAAGGTTCGAGGATACGCACAGGAAAAACAGCACGCA
421  TCACAACAGCCAATCTTTCCAAAGCCAATCAACCGTCAACCATAAACCGCCCATGTGAAG
M K
481  GTTGTATCATTTCTCAGTGTCTAGCTCCCTCAGCGCCGCTCAGCAAGCCACCTC
V V S F I L S A L A S L T A A Q Q A T L
541  TGCAAGGACTTTGAGTACTACTCCAGCAACGGCTACGAGCTCAACAACAACATCTGGGC
C K D F E Y Y S S N G Y E L N N N I W G
601  CGCAGCTCAGCCACCTCTGGATCTCAATGCACCTACGTCAACAGCGTAAGCCAGACTGGT
R S S A T S G S Q C T Y V N S V S Q T G
661  GCCAAGTGGACCTCAACTGGCAGTGGCAGGTGGCGTGACAATGTCAAGAGCTACGTG
A K W T S N W Q W Q G G R D N V K S Y V
XhoI
721  TACTCTGGCGCTCAAAACACCAAGAGCCGTCAGCCAGTACAGAACCTCGAGACCGAG
Y S G R Q I T K K P V S Q Y S N L E T E
781  GCCTACTGGGCTACGACACCTCCACATCCGCTGCAATGTTGCCTACGATCTCTTACC
A Y W V Y D T S N I R C N V A Y D L F T
841  TCTGCCAAGCTCAACCAACCCAGCAGCGGTGACTACGAGCTCATGGTCTGGTgtagctt
S A N V N H P T S S G D Y E L M V W
901  ttacaccatcatccaaccttctagctcttctactatcacatataactaacgtttgaggattta
#
961  tagGCTCGGCAGGTACGATGTGTACCCCATTTGGATCCTCGCAAGGGATGGTCAACGTGCG
L G R Y D V Y P I G S S Q G M V N V A
1021  TGGCCGCCAATGGGAATCTTCTACGGCCTCAACGGCAACATGAAGGTCTACAGCTTCGT
G R Q W E L F Y G L N G N M K V Y S F V
ApaI
1081  TACTCCTTCGGGCCCCATCTACAACCTCAAGGCCAGCATGAAGACTTCTTCCAGTACCT
T P S G P I Y N F K A S M K D F F Q Y L
1141  CGCCAAACAAGGGTTTCCCATCAACAGCCAGAACCTCATTAgtaagtttttttaaaa
A N N K G F P I N S O N L I
1201  gtggatgatgatgatgcagaagatgctaacagccatgatagCCTACGATTCGGTACC
T Y Q F G T
1261  GAGCCCTTCACTGGTGGCCCCGCAAGTTCACCGTCAACCAAGTGGTCCGCAACGCTTAT
E A F T G G P A K F T V N Q W S A N A Y
1321  TAAATCAGAAACGAACAACCAACCAAGGCTCTTTTATTTCCTCCGACGAAATTCACCC
*
1381  CCTTTTGGTTAAATTTCTTCTGATCGAAGAGACACAAACAAAACAACTAAAAACA
1441  AGTTTATACAGCGGTAATGATGGAACCGGTGATATATTCGAGATGCTTTCGCGATTTT
1501  TTCTTCTTCTTCTTCAAACTTATTGAGCTGATATATCTAGCCTTACCTTCTCGAAACG
+
1561  CTCAAATTAACAAGTCAACGACTGCGGCAAGTTTCTCGCTTGTACTATCCCTACTA
1621  CCATGCAAGATATTGACCAACCAACCCACCGTTGACCCCGCTCCCGCTTCCAG

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Fig. 2. Nucleotide sequence and deduced amino acid sequence of *MLG2*. The experimentally determined peptide sequences are indicated by double underlining. The two introns are shown in lowercase letters. The DNA between the underlined *Xho*I and *Apa*I restriction sites was removed in the gene replacement mutant. The transcriptional start site and the polyadenylation site are indicated by # and +, respectively, over the appropriate nucleotides.

Fig. 3. mRNA expression in planta. RNA was purified from uninoculated (U) maize seedlings or 4 days after inoculation (I) with wild-type *Cochliobolus carbonum*. Five micrograms of polyA⁺-RNA was loaded in each lane. *SBP1* is a constitutive maize gene serving as loading control. Sizes of the RNAs are *EXG1*, 2.7 kb; *MLG1*, 1.5 kb; *EXG2*, 1.2 kb; and *MLG2*, 1.7 kb.

sisting of the replaced *XhoI/BamHI* fragment of *EXG2* did not hybridize to DNA from T682, and the *hph* gene hybridized to DNA from T682 but not the wild type (data not shown).

To create multiple mutants by crossing, the genomic locations of *EXG1* and *MLG1* were first determined on chromosomes separated by pulsed-field gel electrophoresis. These two genes hybridized to the same band, which was a doublet based on the intensity of its staining with ethidium bromide (data not shown). Therefore, *EXG1* and *MLG1* might be physically linked. A genetic cross was made between *exg1* mutant T1 (Schaeffer et al. 1994) and *mlg1* mutant T503-1A (Görlach et al. 1998), both of which are resistant to hygromycin. Eleven hygromycin-resistant progeny were selected and analyzed by DNA blotting. Four were genotype *exg1*, three were genotype *mlg1*, and four were genotype *exg1/mlg1* (data not shown). This result indicates that *exg1* and *mlg1* are not genetically linked. Strain T669 (*exg1/mlg1*) was used for biochemical analysis and further experiments.

A triple *exg1/exg2/mlg1* mutant was generated by crossing T682 (*exg2*) and T669. Twenty hygromycin-resistant progeny were analyzed by DNA blotting, and eight of these were genotype *exg1/exg2/mlg1*. Triple mutant T685-12 was used for further biochemical analysis.

To create *mlg2* mutants, T503-1A (*mlg1*) and T685-12 (*exg1/exg2/mlg1*) were transformed with the gene for phleomycin resistance flanked by *mlg2* sequences from plasmid pKA8. Phleomycin-resistant transformants were characterized by DNA gel blot analysis. Successful homologous integration by a double-crossover event was predicted to result in the disappearance of DNA hybridizing to the internal *XhoI/ApaI* fragment of *MLG2* that had been replaced with the phleomycin gene (Fig. 4). Five transformants from each experiment were analyzed, and four putative double mutants (*mlg1/mlg2*) and three putative quadruple mutants (*exg1/exg2/mlg1/mlg2*) were verified by DNA blotting (Fig. 5). Further experiments were done with KA8-4 (genotype *mlg1/mlg2*) and QKA8-2 (*exg1/exg2/mlg1/mlg2*).

Fig. 4. Maps of the *EXG2* and *MLG2* loci in the wild type and mutants. **A**, *EXG2* wild type; **B**, *exg2* knockout transformant; **C**, *MLG2* wild type; and **D**, *mlg2* knockout transformant. H, *HindIII*; P, *PstI*; X, *XhoI*; B, *BamHI*; S, *SalI*; and A, *ApaI*.

Analysis of mutants.

MLGase and β 1,3-glucanase activities were assayed in culture filtrates of wild-type and mutant strains grown on 1% corn bran as carbon source (Fig. 6). Under these conditions, *MLG1* contributed approximately 46% and *EXG1* and *EXG2* together contributed approximately 53% of the total β 1,3-glucanase activity. *MLG1* contributed approximately 46% and *MLG2* contributed approximately 26% of the total MLGase activity (Fig. 6). From other experiments with the *exg1* single mutant, it was estimated that *EXG2* contributes approximately 10% of the total β 1,3-glucanase activity, and therefore, *EXG1* contributes approximately 43% (Schaeffer et al. 1994; and data not shown).

Fig. 5. DNA blot of the wild type and glucanase knockout transformants. Total genomic DNA was digested with *HindIII*. The DNA blot was probed with fragments of the genes indicated across the top. The *Sh-ble* probe was a 400-bp *BamHI/ApaI* fragment from pUT720 containing only the *Sh-ble* gene (Calmels et al. 1991). In each panel, the first lane is the wild type, the second lane is the *exg1/exg2/mlg1/mlg2* mutant QKA8-2, and the third lane is the *mlg1/mlg2* mutant KA8-4. The *exg1* and *mlg2* mutants were made by single-crossover gene disruption (Görlach et al. 1998; Schaeffer et al. 1994), whereas the *exg2* and *mlg2* mutants were made by double-crossover gene replacement.

Fig. 6. Mixed-linked glucanase and β 1,3-glucanase activities in culture filtrates of wild type and mutants. Wild-type, strain 367-2A; *mlg1*, strain T503-1A; *mlg1/mlg2*, strain KA8-4; *mlg1/exg1/exg2*, strain 368-12; and *mlg1/mlg2/exg1/exg2*, strain QKA8-2. Error bars indicate one standard error of the average of four replicates.

Despite the large decrease in extracellular enzyme activities in the mutants, growth was only slightly affected. Growth of the quadruple mutant was the same as the wild type on sucrose or 0.5% β -glucan, but was reduced by 12 and 17% on 0.5% laminarin or 0.2% β -glucan, respectively (Table 1).

Although its primary amino acid sequence is similar to other known or putative exo- β 1,3-glucanases, it had not been previously established that Exg2 actually belonged to this class of enzyme. Reduction of total β 1,3-glucanase activity in the *exg2* mutant by 10% was not strong evidence that *EXG2* encodes an enzyme with this activity. Therefore, the mutant was analyzed further by anion-exchange chromatography (van Hoof et al. 1991). The mutant lacked a peak, eluting at 19 min, that was of the appropriate relative magnitude (Fig. 7A). Based on its sequence similarity to other β 1,3-glucanases, the small but reproducible decrease in β 1,3-glucanase activity in crude filtrates of the *exg2* mutant, and the disappearance of a chromatographic peak of activity in the mutant, we conclude that *EXG2* does encode an enzymatically active β 1,3-glucanase.

EXG1 and *EXG2* did not contribute to the MLGase activity of *C. carbonum* culture filtrates, because the quadruple mutant had the same amount of MLGase activity as the *mlg1/mlg2* mutant (Fig. 6). However, the *mlg1/mlg2* mutant still retained residual MLGase activity. This activity could be resolved as a peak eluting just before Mlg2 on a cation-exchange column (retention time approximately 22 min) (Fig. 7B). When further analyzed by hydrophobic-interaction HPLC, this small peak (eluting at 38 min) could be resolved from the major peak eluting at 34 min (Fig. 7C). This residual peak of MLGase activity therefore represents a third MLGase (Mlg3) that is encoded by a gene different from *MLG1* or *MLG2*.

Pathogenicity of mutants.

Leaves of 14-day-old maize seedlings were spray-inoculated with the wild-type strain 367-2A, double mutant KA8-4 (*mlg1/mlg2*), or quadruple mutant QKA8-2 at different spore concentrations. The pathogenicity of the tested isolates was indistinguishable with regard to lesion size, color, and rate of lesion formation at any of three conidial suspensions. Thus, we conclude that none of these four genes make a significant contribution to the virulence of *C. carbonum*, either alone or in combination.

Table 1. Growth of a quadruple β -glucanase mutant (*exg1exg2mlg1mlg2*) on sucrose, laminarin, and β -glucan^a

Medium	Growth (g per mat)		Mutant as % of wild type
	Wild type	Quadruple mutant	
Experiment 1			
2% sucrose	0.092 ± 0.010	0.090 ± 0.009	98
0.5% laminarin	0.032 ± 0.002	0.028 ± 0.002	88
0.5% β-glucan	0.023 ± 0.001	0.023 ± 0.002	100
Experiment 2			
0.2% sucrose	0.022 ± 0.002	0.023 ± 0	105
0.2% β-glucan	0.023 ± 0	0.019 ± 0.003	83

^a In both experiments, *Cochliobolus carbonum* was grown in 125-ml Erlenmeyer flasks containing 15 ml of medium plus the indicated compound as the sole carbon source. Growth was for 12 days in still culture at 21°C. Each datum represents the average $\pm$ 1 standard error of the mean of the dry weights of mycelial mats, four per treatment.

DISCUSSION

Mutational analysis of two β -glucanase genes of *C. carbonum*, separately and in combination with two previously characterized β -glucanase genes, allowed the definition of their biochemical function and role in growth on the appropriate polysaccharide substrates. The results indicate that these four

Fig. 7. Chromatographic analysis of mixed-linked glucanase (MLGase) and β 1,3-glucanase activities in wild type and mutants. **A**, Anion-exchange high-performance liquid chromatography (HPLC) analysis of β 1,3-glucanase activity in culture filtrates of wild type and *exg2* mutant. **B**, Cation-exchange HPLC analysis of MLGase activity in culture filtrates of wild type and mutants of genotype *mlg1/exg1/exg2* and *mlg1/mlg2/exg1/exg2*. **C**, Hydrophobic-interaction HPLC analysis of MLGase activity of mutants of genotypes *mlg1/exg1/exg2* and *mlg1/mlg2/exg1/exg2* from the second peak (20 to 27 min) of the cation-exchange HPLC fractionation shown in **B**.

gene products contribute >98% of the total extracellular β 1,3-glucanase activity and approximately 70% of the total MLGase activity. Nonetheless, growth of the quadruple mutant on mixed-linked glucan was identical to the wild type and was only slight reduced on β 1,3-glucan (laminarin).

The phenomenon of a small decrease in growth on the appropriate substrate despite a large decrease in total extracellular activity was earlier observed for polygalacturonase gene mutants of *C. carbonum*. A double *pgn1/pgx1* mutant had <1% of wild-type extracellular polygalacturonase levels, but its growth on pectin was reduced by only approximately 20% (Scott-Craig et al. 1998a). A quadruple xylanase mutant had <25% of wild-type xylanase levels but no reduction in growth on xylan (J. S. Scott-Craig and J. D. Walton, *unpublished data*).

There are several possible explanations for these results. Either *C. carbonum* makes a huge excess of these enzymes, such that the minute amounts of activity remaining in the mutants is sufficient to support growth, or *C. carbonum* has other mechanisms to obtain energy from extracellular polymers. In regard to alternate mechanisms, one possibility is that *C. carbonum* has extracellular enzymes whose activity has not been detected for technical reasons. However, this seems unlikely because we have made efforts to keep the enzyme assay conditions (pH, salt concentration, substrate, etc.) identical or nearly identical to those under which the fungus was able to grow. Another possibility is that *C. carbonum* has depolymerases that remain within the fungal wall and are not secreted into the medium. Wall-bound enzymes could be a way for the fungus to obtain metabolizable sugars insofar as the substrate polymers were able to diffuse into the wall, and they would have the advantage of allowing more efficient capture of the released sugars. Wall-bound enzymes have been reported from filamentous fungi (Valsangiacomo and Gessler 1992), and the walls of *C. carbonum* contain detectable polygalacturonase and the majority of its invertase activities (K. Pedley and J. D. Walton, *unpublished data*). The walls of *C. carbonum* have not been tested for β -glucanase or xylanase activity. A third possibility is that fungi might have a mechanism to take up polymers by endocytosis and thus degrade them in the cytoplasm. *Neurospora crassa* has been reported to have an intracellular pectinase (Crotti et al. 1998). However, although endocytosis has been documented in filamentous fungi, its role in nutrient acquisition is unknown (Fischer-Parton et al. 2000).

The results in this paper indicate that none of the studied β -glucanase genes are necessary for pathogenesis, although small effects could not be excluded by the nature of the qualitative assay. Possible explanations are, first, that degradation of neither β 1,3-glucan nor mixed-linked glucan is important for penetration or growth of *C. carbonum* in maize leaves. Second, the small amounts of activity remaining may be sufficient for full virulence. Third, additional enzymes with β -glucanase activity, encoded by other genes that are expressed only in planta, might compensate for the loss of *EXG1*, *EXG2*, *MLG1*, and *MLG2*.

To date, more than 15 extracellular depolymerase genes, alone and in various combinations, have been disrupted in *C. carbonum*. None of the mutants have had a noticeable disease phenotype. However, disruption of a regulatory protein kinase, *ccSNF1*, which is essential for expression of glucose-repressed

genes, resulted in a strain with reduced depolymerase gene expression, reduced growth on alternate carbon sources (e.g., arabinose, pectin, and maize cell walls), and strongly reduced virulence (Tonukari et al. 2000). Although these results are consistent with extracellular depolymerases having a role in virulence, it cannot be excluded that the disease phenotype of the *ccsnf1* mutant is due to some other cause. Further studies will be necessary to identify the critical virulence determinants regulated by *ccSNF1*.

MATERIALS AND METHODS

Fungal cultures.

C. carbonum strain 367-2A (HC-toxin producing), which is a progeny of SB111 (ATCC 90305), was grown on V8 juice agar plates. The *mlg1* mutant T503-1A and *exg1* mutant T1 have been previously described (Görlach et al. 1998; Schaeffer et al. 1994). Sucrose, maize cell walls, maize bran (Country Life Natural Foods, Pullman, MI, U.S.A.), and oat bran (Quaker Oats Co., Chicago) were tested as carbon sources for optimization of Exg2 production. For routine production of Mlg2 and Exg2, the fungus was grown in a medium containing salts, yeast extract, 0.2% sucrose, and 1% maize bran in still culture for 8 days (Exg2) or 11 days (Mlg2) (van Hoof et al. 1991). Pathogenicity was assayed on maize seedlings of genotype *hm1/hm1* as described (Görlach et al. 1998). Plants were inoculated by spraying conidial suspensions at 10^4 , 10^5 , or 10^6 per ml in 0.1% Tween-20 and grown in a greenhouse. Plants were observed every day for 2 weeks or until dead.

Enzyme assays.

Glucanase activities were determined with a reducing sugar assay using 0.2% barley β -glucan (Sigma Chemical Co., St. Louis) as the substrate for MLGase and 0.2% laminarin (Sigma Chemical Co.) as the substrate for β 1,3-glucanase (Görlach et al. 1998; Schaeffer et al. 1994; van Hoof et al. 1991). One unit of activity was defined as the amount of the enzyme that liberated 1 μ mol of reducing sugar per min. Protein concentration was determined by UV absorption (Whitaker and Granun 1980).

Enzyme purification.

Exg2 was purified from *C. carbonum* wild-type strain 367-2A by anion-exchange HPLC, hydrophobic-interaction HPLC, and SDS-PAGE (van Hoof et al. 1991). Mlg2 was purified from the *mlg1* mutant strain T503-1A (Görlach et al. 1998). Mlg2 was purified on the basis of its enzymatic activity by cation exchange and hydrophobic-interaction HPLC, except that the 25 mM sodium acetate buffer used for cation exchange was adjusted to pH 5.0 instead of 4.0 (Görlach et al. 1998).

Nucleic acid manipulations.

The methods used for DNA and RNA isolation and analysis, genomic DNA and cDNA library screening, probe labeling, DNA blotting, and hybridization have been described previously (Murphy and Walton 1996; Pitkin et al. 1996; Scott-Craig et al. 1990, 1998a). DNA sequencing with gene-specific primers was performed by automated fluorescent sequencing at the Plant Research Laboratory Biochemistry Facility, Michigan State University, East Lansing.

EXG2 was isolated using PCR primers based on the amino acid sequences VGFNWG, which was experimentally deter-

mined, and QNGFDN, which is an internal sequence highly conserved among related fungal β 1,3-glucanases. The PCR primers were GTNGGNTTYAAYTGGG and TTRTCRAAN-CCRTTYTG (R = A or G, N = any nucleotide, and Y = C or T). A fragment of approximately 450 bp was amplified, subcloned, and used to screen genomic DNA and cDNA libraries.

MLG2 was isolated using PCR primers based on amino acid sequences obtained from tryptic peptides of purified Mlg2 (Görlach et al. 1998). The primers that were successfully used were CARGGGNATGGTNAAY, based on the amino acid sequence QGMVNV, and CCRAAYTGRTANGTRAT (reverse complement), based on ITYQFG (Görlach et al. 1996). A 190-bp fragment amplified by PCR using a phage lysate of a cDNA library as template (Pitkin et al. 1996) was subcloned and used to screen cDNA and genomic DNA libraries.

The transcription start site of *MLG2* was determined by RACE (version 2.0; Gibco-BRL, Bethesda, MD). First-strand cDNA synthesis was primed with the oligonucleotide CCGT-TGAGGCCGTAGAAG (starting at nucleotide 1,056). PCR primer CTGGCTTACGCTGTTGAC (starting at nucleotide 654) and the anchor primer provided with the RACE kit were used to amplify the 5' end of the *MLG2* transcript.

Targeted gene disruption.

A 2.4-kb *Pst*I fragment containing the 5' portion of the coding region of *EXG2* was subcloned into pGEM5 (Promega Corp., Madison, WI, U.S.A.). The resulting plasmid was cut with *Bam*HI and *Xho*I to remove 874 bp of the promoter and 5' end of the coding region, blunted, and ligated with the *Hpa*I fragment containing the *hph* gene encoding hygromycin phosphotransferase driven by the *Aspergillus nidulans trpC* promoter from pCB1004 (Carroll et al. 1994) to create pAJ56. This plasmid was cut with *Pst*I and the resulting 2.8-kb *Pst*I fragment used for transformation by gene replacement (double-crossover integration). Transformation of *C. carbonum* protoplasts was done as described (Scott-Craig et al. 1990, 1998a). Transformants were purified by three rounds of single-spore isolation.

For *MLG2*, the transformation vector was made by digesting a 6.4-kb *Sma*I/*Sma*I *MLG2* genomic clone, pAJ60, with *Xho*I and *Apa*I to liberate a 325-bp fragment internal to the coding sequence of *MLG2*. This was ligated to a 2.5-kb *Xho*I/*Apa*I fragment containing the phleomycin resistance marker from pUT737 (Calmels et al. 1991) (Cayla, Toulouse, France) to produce pKA8 (8.8 kb). This plasmid was then cut with *Bam*HI and *Pst*I and the resulting 5.8-kb fragment used to transform *C. carbonum* strain T503-1A (genotype *mlg1*; Görlach et al. 1998) and triple mutant strain 685-12 (genotype *exg1/exg2/mlg1*; this paper), both of which are resistant to hygromycin. Transformants were selected on 50 μ g of phleomycin per ml (Cayla).

In planta expression.

Three-week-old maize seedlings of genotype *hm1/hm1* were spray-inoculated with 10^5 conidia of *C. carbonum* wild-type 367-2 per ml. Infected leaf segments were collected 4 days later and frozen in liquid nitrogen. Total RNA was extracted as described (Scott-Craig et al. 1998a) and the polyA⁺ fraction-purified from 500 μ g of total RNA using a mini-oligo(dT) cellulose spin column (5 Prime - 3 Prime, Boulder, CO, U.S.A.). PolyA⁺-RNA (5 μ g) was fractionated on a 1.5% formaldehyde-

agarose gel and blotted onto Nytran SuperCharge membrane (Schleicher & Schuell, Keene, NH, U.S.A.). cDNA clones of *EXG1*, *EXG2*, *MLG1*, and *MLG2* and the maize gene *SBP1* were labeled by random priming. *SBP1* encodes a constitutively expressed herbicide safener-binding protein (Scott-Craig et al. 1998b). Following each round of hybridization, the filters were stripped with 0.1 $\times$ SSPE (1 $\times$ is 0.18 M NaCl, 10 mM NaPO₄, and 1 mM EDTA [pH 7.7]) and 0.1% SDS for 5 min at 100°C.

Nucleotide sequence accession numbers.

The nucleotide sequence data of *EXG2* and *MLG2* are available in the GenBank database under the accession numbers AF229446 and AF229447, respectively.

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