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# Cloning, Disruption, and Expression of Two Endo-β1,4-Xylanase Genes, *XYL2* and *XYL3*, from *Cochliobolus carbonum*

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**In culture, the filamentous fungus *Cochliobolus carbonum*, a pathogen of maize, makes three cationic xylanases. *XYL1*, which encodes the major endoxylanase (Xyl1), was earlier cloned and shown by gene disruption to encode the first and second peaks of xylanase activity (P. C. Apel, D. G. Panaccione, F. R. Holden, and J. D. Walton, Mol. Plant-Microbe Interact. 6:467–473, 1993). Two additional xylanase genes, *XYL2* and *XYL3*, have now been cloned from *C. carbonum*. *XYL2* and *XYL3* are predicted to encode 22-kDa family G xylanases similar to Xyl1. Xyl2 and Xyl3 are 60% and 42% identical, respectively, to Xyl1, and Xyl2 and Xyl3 are 39% identical. *XYL1* and *XYL2* but not *XYL3* mRNAs are present in *C. carbonum* grown in culture, and *XYL1* and *XYL3* but not *XYL2* mRNAs are present in infected plants. Transformation-mediated gene disruption was used to construct strains mutated in *XYL1*, *XYL2*, and *XYL3*. Xyl1 accounts for most of the total xylanase activity in culture, and disruption of *XYL2* or *XYL3* does not result in the further loss of any xylanase activity. In particular, the third peak of cationic xylanase activity is still present in a *xyl1 xyl2 xyl3* triple mutant, and therefore this xylanase must be encoded by yet a fourth xylanase gene. A minor protein of 22 kDa that can be detected immunologically in the *xyl1* mutant disappears in the *xyl2* mutant and is therefore proposed to be the product of *XYL2*. The single xylanase mutants were crossed with each other to obtain multiple xylanase disruptions within the same strain. Strains disrupted in combinations of two and in all three xylanases were obtained. The triple mutant grows at the same rate as the wild type on xylan and on maize cell walls. The triple mutant is still fully pathogenic on maize with regard to lesion size, morphology, and rate of lesion development.**

Although all bacterial and fungal plant-pathogenic organisms make a variety of enzymes that can degrade the polysaccharides of plant cell walls, the role of most enzymes in most host-pathogen interactions is unclear. Xylans are major hemicellulosic components of the cell walls of all land plants and are abundant in the walls of cereals. Therefore, xylanases might be important virulence factors for at least some pathogenic organisms. Xylanases are known to be made by a number of plant pathogens, and their genes have been cloned from at least two plant-pathogenic fungi, *Cochliobolus carbonum* and *Magnaporthe grisea* (2, 22). An additional role for xylanases in inducing plant defense responses is suggested by the finding that fungal xylanases are elicitors of necrosis, electrolyte leakage, and synthesis of pathogenesis-related proteins (3). Response to the elicitor activity of a fungal xylanase is controlled by a single dominant gene in tobacco (4).

When fractionated by cation-exchange chromatography, culture filtrates of *C. carbonum* contain three peaks of xylanase activity (9). Production of xylanase is suppressed by sucrose and induced by xylan and purified maize cell walls. *XYL1*, the gene encoding the major xylan-degrading activity, was cloned and disrupted (2). *xyl1* mutants have highly reduced xylanase activity in culture but still grow on xylan or maize cell walls and are still pathogenic (2). The presence of residual xylanase activity in culture filtrates of *xyl1* mutants indicates that *C. carbonum* has additional genes encoding enzymes with xylan-degrading activity. Therefore, it remains an open question whether xylanase activity is required for pathogenicity of this fungus.

A definitive approach to evaluating the role of xylan degradation in the interaction between *C. carbonum* and its host, maize, is to create specific mutations in the pathogen's xylanase genes. As a step in that direction, we report the discovery and analysis of two additional xylanase genes related to *XYL1*, called *XYL2* and *XYL3*.

## MATERIALS AND METHODS

**Fungal cultures, media, and growth conditions.** The wild-type strains of *C. carbonum* were 367-2A and 367-1a, both race 1 strains which were derived from strain SB111 (ATCC 90305). The fungus was maintained and grown as previously described (2). When added to cultures, maize cell walls (18) and oat spelt xylan (Fluka) were used at concentrations of 0.8% and 1.2%, respectively. For growth measurements, mycelial mats were washed briefly in distilled water, frozen, lyophilized, and weighed. Mycelia for protoplasts were obtained from germinating conidia (2). Fungal crosses were completed as described previously (12).

**Nucleic acid manipulations.** The *C. carbonum* genomic and cDNA libraries have been described previously (12, 16). DNA was extracted from lyophilized mycelial mats by vortexing the mats with glass beads (12), and RNA was extracted by the method of Yoder (24) except that cresol was omitted.

For the study of xylanase expression in infected leaves, mRNA was isolated from infected lesions 7 days postinoculation and mRNA was isolated by using the mini-oligo(dT) cellulose Spin Column Kit (5′-3′ Inc., Boulder, Colo.) according to the manufacturer's instructions.

DNA and RNA electrophoresis were performed in accordance with published protocols (14, 17). Typically, 4 μg of DNA or 10 μg of total or poly(A)$^+$ mRNA was loaded per gel lane. RNA standards (Gibco-BRL) were used for transcript size estimation. The *Cochliobolus heterostrophus GPD1* gene, encoding glyceraldehyde-3-phosphate dehydrogenase, was used as a reference (21).

DNA was transferred to Zeta-Probe nylon membranes with 0.4 M NaOH, and RNA was transferred with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (14). Nucleic acids were fixed to membranes by using 1,200 mJ of UV radiation (Stratalinker 1800; Stratagene) in accordance with the manufacturer's instructions. Routine high-stringency DNA hybridizations with subcloned DNA fragments labeled with [$^{32}$P]dCTP by random-primer labeling (14) were done in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaH_2PO_4$, and 1 mM EDTA [pH 7.4]) with 7% (wt/vol) sodium dodecyl sulfate (SDS), 0.5% nonfat

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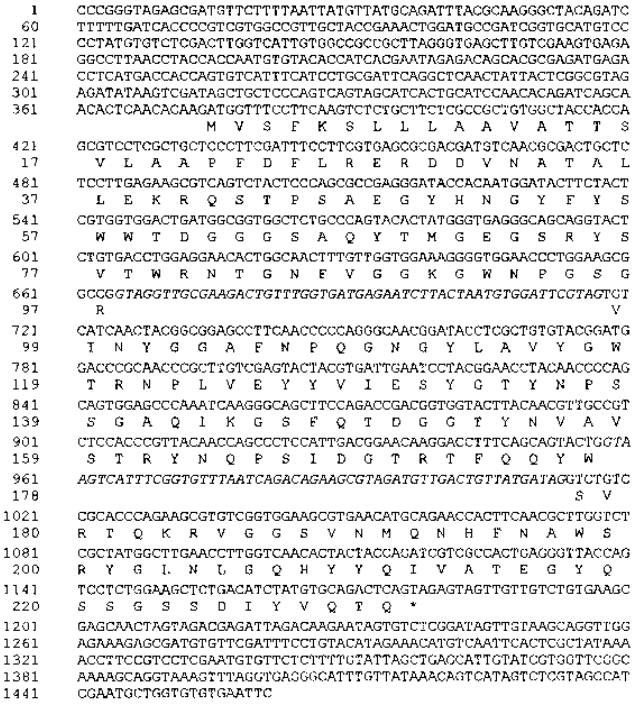

FIG. 1. Nucleotide and deduced amino acid sequences of *XYL2*. The two introns are indicated in italic type. Amino acids are shown under their corresponding codons.

dry milk, and 0.1 mg of denatured salmon sperm DNA per ml at 65°C overnight. RNA blots were hybridized at 42°C in a solution containing 50% (vol/vol) formamide, 5× SSPE, 2× Denhardt's solution (14), 4% (wt/vol) SDS, and 0.1 mg of denatured salmon sperm DNA per ml. Blots were washed in 0.1× SSPE with 0.1% SDS, with a final wash at 65°C for 1 h or three washes of 20 min each.

For isolation of *XYL3*, the genomic DNA library was screened with the same 32-fold-degenerate 17-mer oligonucleotide (sequence CAYTTYGAYGCNT GGGC, where Y is C or T and N is any nucleotide) that had been used to isolate *XYL1* (2). This sequence was based on an amino acid sequence, HFDAWA, from a tryptic fragment of Xyl1, the major endo-β1,4-xylanase (2, 9). The oligonucleotide was labeled and hybridized as described for the isolation of *XYL1* (2). For the isolation of a *XYL2* cDNA, the cDNA library was screened with a subcloned fragment of *XYL1* labeled with [$^{32}$P]dCTP by random-primer labeling (14). A *XYL2* genomic clone was obtained by screening the genomic library with the *XYL2* cDNA as a probe.

The sequences of both strands of the *XYL2* cDNA clone and of the *XYL2* and *XYL3* genomic clones were determined by automated fluorescence sequencing at the Department of Energy Plant Research Laboratory Plant Biochemistry facility at Michigan State University. Sequence data were analyzed with the DNASIS and PROSIS programs (Hitachi Software Engineering Co., San Bruno, Calif.).

The probes used to detect the xylanase genes were a *XYL1* cDNA, a *XYL2* cDNA, and the *Kpn*I-*Xba*I or *Sca*I-*Bst*EII fragments of the *XYL3* gene.

**Construction of disruption vectors.** The transformation vector for disrupting *XYL2* was created by subcloning a 532-bp *Hin*dII fragment of *XYL2* into the *Sma*I site of pHYG1, which is derived from pUCH1 and contains the *hph* gene, conferring hygromycin resistance, driven by a *C. heterostrophus* promoter (15, 18). The product of these manipulations (pXLB37-2) was linearized at a unique *Bam*HI site prior to transformation of *C. carbonum* 367-2A as described previously (2, 16, 18).

pHYG2 was created by subcloning the *Sal*I-*Hin*dIII fragment of pUCH1 (15), which contains the *hph* gene (conferring hygromycin resistance), into pSP72 (Promega). A 323-bp *Kpn*I-*Xba*I fragment of the *XYL3* gene was then cloned into pHYG2 cut with *Kpn*I and *Xba*I. The resulting plasmid (pXLC42-1) was linearized at the unique *Kpn*I site prior to transformation of *C. carbonum* 367-2A.

**Analysis of disruption mutants.** Transformants were purified to nuclear homogeneity by two rounds of single-spore isolation. Xylanase was purified from culture filtrates grown for 8 days on MS medium containing 0.2% sucrose and 0.8% maize cell walls (2, 9). Culture filtrates were filtered through Whatman #1 filter paper and, after twofold dilution with 25 mM sodium acetate (pH 5), passed through a column of DEAE-cellulose (to which the cationic xylanases do not bind). Following the DEAE-cellulose column chromatography, the filtrate was loaded onto a 33-ml column of CM-cellulose and the xylanase was eluted with 25 mM sodium acetate–0.4 M KCl, pH 5.0. The xylanase was dialyzed (Spectrapor; molecular weight cutoff, 6,000 to 8,000) overnight against 12.5 mM sodium acetate, pH 5.0, and subjected to cation-exchange high-performance liquid chromatography (HPLC) on a sulfoethylaspartamide column (200 by 4.6 mm; The Nest Group, Southboro, Mass.) with a linear gradient from 0 to 100% buffer B in 30 min (9). Buffer A was 25 mM sodium acetate, pH 5.0, and buffer B was buffer A plus 0.4 M KCl. The flow rate was 1 ml/min, and 1-ml fractions were collected.

**Xylanase assay.** Xylanase activity was assayed with a reducing sugar assay (11). Thirty microliters per HPLC cation-exchange fraction or 2 μg of protein from the total culture filtrate was assayed in a 300-μl reaction volume containing 1% oat spelt xylan (Fluka) and 50 mM sodium acetate, pH 5.0, at 37°C for 30 min (9). A 25- or 100-μl aliquot of the reaction mixture was mixed with 1.5 ml of a working solution of *p*-hydroxybenzoic acid hydrazide, the mixture was heated at 100°C for 10 min, and the $A_{410}$ was read (11).

**Pathogenicity test.** Leaves of maize inbred K61 (genotype *hm*/*hm*) at the three- to four-leaf stage were inoculated with a suspension of conidia ($10^4$/ml) in 0.1% Tween 20. Plants were covered in plastic bags overnight and then grown in a greenhouse. Plants were observed twice daily for 2 weeks.

**Immunoblot analysis.** Twenty milliliters of *C. carbonum* culture filtrate was passed through a column of DEAE-cellulose and then concentrated by precipitation with 1/10 volume of 100% (wt/vol) trichloroacetic acid. Eighty micrograms of protein was loaded into each lane of a 15% (wt/vol) acrylamide SDS-polyacrylamide gel. After electrophoresis, the gel was blotted to nitrocellulose (0.2-μm pore size) and treated with a 1:1,000 dilution of a rabbit antiserum raised against the 22-kDa xylanase of *Trichoderma viride* (8). The antixylanase antiserum was obtained from James Anderson (U.S. Department of Agriculture, Beltsville, Md.). The bound antibody was visualized with goat anti-rabbit antibody conjugated to alkaline phosphatase (Cooper Biomedical, Malvern, Pa.). Molecular weights were calculated by using prestained markers (Gibco-BRL).

**Nucleotide sequence accession numbers.** The GenBank accession numbers for *XYL1*, *XYL2*, and *XYL3* are L13596, U58915, and U58916, respectively.

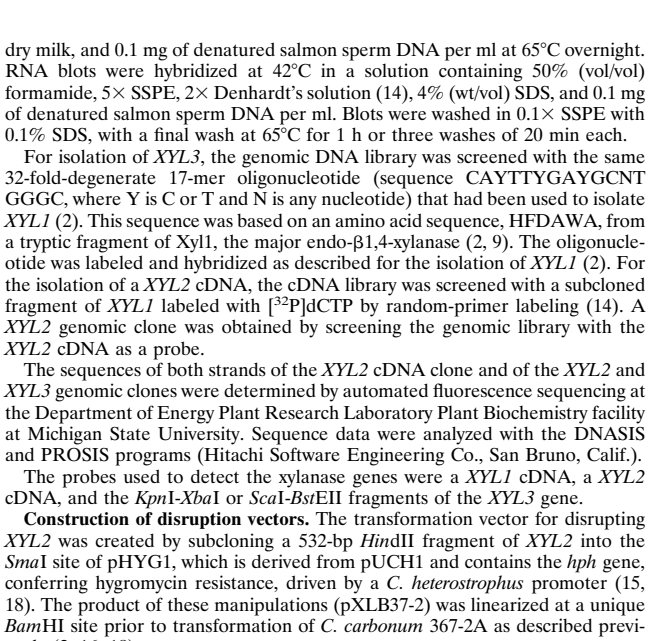

FIG. 2. Nucleotide and deduced amino acid sequences of *XYL3*. The location of the oligonucleotide (oligo #1) used to clone *XYL1* and *XYL3* is indicated. The putative intron is indicated in italic type. Amino acids are shown under their corresponding codons.

```
XYL1    MVSFTSIITA AVAATGALAA PAT......D VS........ .LVARQNTPN
        ::::.:.. : :::.:. ::: :        : :           :  :: ::
XYL2    MVSFKSLLLA AVATTSVLAA PFDFLRERDD VNATAL.... .LEKRQSTPS
        ::.:::.. .   : ..: :: : .       : :.          : :::.  .
XYL3    MVAFTSVLLG LSAIGSAFAA PVA......D VPDFEEFSGPK HLAARQDYNQ

XYL1    GEGTHNGCFW SWWSDGGARA TYTNGAGGSY SVSWGSGGNL VGGKGWNPGT
        .:: ::: :. :::.:::. :  :: : :. : ::.:  .:: :::::::::.
XYL2    AEGYHNGYFY SWWTDGGGSA QYTMGEGSRY SVTWRNTGNF VGGKGWNPGS
                .  ::    :: :... .: ::. ... .  : :::: :: 
XYL3    .......... .NYKTGG.NI QY.NPTSNGY SVTFSGAQDF VLGKGWKQGT

XYL1    ARTITYSGTY NYNGNSYLAV .YGWTR.NPL VEYYVVENFG TYDPSSQSQN
        .: : :.:.. : .::.:::: :::: ::: ::::.: .: ::.::: .:
XYL2    GRVINYGGAF NPQGNGYLAV .YGWTR.NPL VEYYVIESYG TYNPSSGAQI
        .::. :.:.  . .. . :    :::::   : :::: .  . .  .:.:.:
XYL3    TRTVKYTGST QAQAGTVLVA LYGWTRGSKL VEYYIQDFTS GGSGSAQGQK

XYL1    KGTVTSDGSS YKIAQSTRTN QPSIDGTRTF QQYWSVRQNK RS.SGSVNMK
        ::   .::.. : .: ::: : :::::::::: :::::: .: :  .:::::
XYL2    KGSFQTDGGT YNVAVSTRYN QPSIDGTRTF QQYWSVRTQK RV.GGSVNMQ
         : :: ::: : : : :  : :::: :: ::  :: : :  : :: .:..  :
XYL3    MGQVTCDGSV YDIWQHTQVN QPSIVGTTTF VQYISNRVSK RSTGGTIITK
          Oligo1
XYL1    THFDAWASKG MNLG.QHYYQ IVATEGYFST .GNAQITVNC HKFETTKINE
         ::.::.  : .::: ::::: :::::: :.  : ..: :.
XYL2    NHFNAWSRYG LNLG.QHYYQ IVATEGYQSS .GSSDIYVQT Q*
         :::::  : :::: :  ::  ..:::.  .  : .: ::
XYL3    CHFDAWAKLG MNLGNQWDYQ TISTEGWGNA AGKSQYTVSA A*

XYL1    WPDRLSERLE DDRLGF*
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FIG. 3. Alignment of the deduced amino acid sequences of *XYL1*, *XYL2*, and *XYL3*. Colons indicate identity between Xyl1 and Xyl2 or Xyl3, and periods indicate similarity. The location of the oligonucleotide (Oligo1) used to clone *XYL1* and *XYL3* is indicated.

TABLE 1. *C. carbonum* strains used in this study

| Strain[a] | Transformed strain | Cross parents | *XYL* genotype[b] |
|---|---|---|---|
| 367-2A | | | *XYL1 XYL2 XYL3* |
| T216-4a | 164R10 T2-4 | | *xyl1 XYL2 XYL3* |
| 312-6A | | T216-4a × 164R17A | *xyl1 XYL2 XYL3* |
| T446-17A | 367-2A | | *XYL1 xyl2 XYL3* |
| 447-8a | | 312-6A × 367-1a | *xyl1 XYL2 XYL3* |
| 477-3a | | T446-17A × 447-8a | *xyl1 xyl2 XYL3* |
| 477-7a | | T446-17A × 447-8a | *xyl1 xyl2 XYL3* |
| T448-4A | 367-2A | | *XYL1 XYL2 xyl3* |
| 556-11 | | T448-4A × 477-3a | *xyl1 XYL2 XYL3* |
| 556-8 | | T448-4A × 477-3a | *XYL1 xyl2 XYL3* |
| 556-1 | | T448-4A × 477-3a | *XYL1 XYL2 xyl3* |
| 556-16 | | T448-4A × 477-3a | *xyl1 xyl2 XYL3* |
| 556-4 | | T448-4A × 477-3a | *xyl1 XYL2 xyl3* |
| 556-12 | | T448-4A × 477-3a | *XYL1 xyl2 xyl3* |
| 556-2 | | T448-4A × 477-3a | *XYL1 XYL2 XYL3* |
| 556-13 | | T448-4A × 477-3a | *xyl1 xyl2 xyl3* |

[a] Except for 367-2A and 556-2, all strains carry at least one copy of the *hph* gene, which confers hygromycin resistance. When known, the mating type is indicated with an A or a after the strain name.

[b] Uppercase italic type is used to indicate the wild-type copy of a gene; lowercase italic type is used to indicate a mutated copy.

## RESULTS

**Isolation of *XYL2* and *XYL3*.** When a cDNA library was screened with a *XYL1* genomic clone (2), two classes of cDNAs were obtained, one corresponding to *XYL1* and the other corresponding to a related sequence called *XYL2*. The 17-mer oligonucleotide that was used to clone *XYL1* (2) hybridized to a third related sequence, *XYL3*, in a genomic-DNA library.

cDNA and genomic clones of *XYL2* were sequenced. *XYL2* has two introns, one of 53 bp and the other of 56 bp (Fig. 1). The first intron of *XYL2* produces a shift in the reading frame and has 5′ G↓GTAGGT (consensus, G↓GTANGT) and 3′ GTAG (consensus, ACAG) intron splice sites (5). The second intron also produces a frame shift and has 5′ G↓GTAAGT and 3′ ATAG intron splice sites. A possible translational start site (Fig. 1) has a context of CAAGATGGT (consensus, CAMMATGNC, where M is A or C) (6). Nineteen of the first 40 amino acids following this start site are hydrophobic. Based on similarity to the predicted sequence of *XYL1* (2), the known signal peptide of *PGN1* of *C. carbonum* (16), and the sequence of the *M. grisea* 22-kDa xylanase (22), the mature xylanase protein probably begins at amino acid 41 (Fig. 1). The product of *XYL2*, Xyl2, has one potential N-glycosylation site at amino acid 32 (Fig. 1). The polyadenylation site of *XYL2* is at nucleotide 1315.

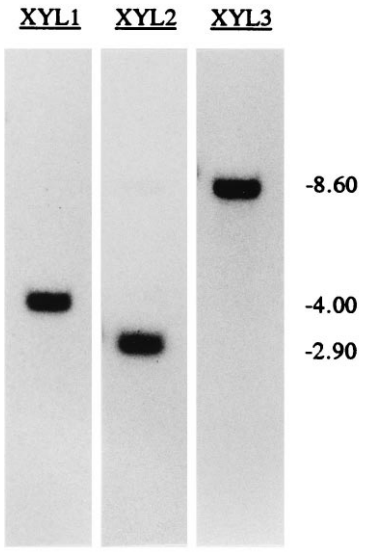


FIG. 4. DNA blot of *XYL1*, *XYL2*, and *XYL3*. Genomic DNA from wild-type *C. carbonum* was digested with *Hin*dIII and, after blotting, hybridized sequentially at high stringency with probes representing *XYL1*, *XYL2*, and *XYL3*. Size markers are shown on the right (in kilobases).

A genomic clone of *XYL3* was sequenced. No *XYL3* cDNA could be found in the cDNA library, which is consistent with the fact that *XYL3* mRNA is not detectable by RNA blotting when the fungus is grown in culture on maize cell walls (see below). By comparison to the sequences of *XYL1* and *XYL2*, *XYL3* is predicted to have an intron of 60 bp in the same position as the single intron of *XYL1* and the first intron of *XYL2*. The putative intron of *XYL3* contains three stop codons as well as 5′ G↓GTATGC and 3′ ACCG intron splice sites. A possible translational start site (Fig. 2) has a context of CAA AATGGT. Nineteen of the first 39 amino acids of the predicted product of *XYL3*, Xyl3, are hydrophobic. The mature Xyl3 protein probably begins at amino acid 40 (Fig. 2). Xyl3 has no consensus N-glycosylation sites.

The sequence of Xyl1 is 75% similar and 60% identical to Xyl2 and is 60% similar and 42% identical to Xyl3; Xyl2 is 59% similar and 39% identical to Xyl3 (Fig. 3). Despite their high degree of similarity, the three genes can be distinguished from each other on DNA blots under high-stringency hybridization conditions (Fig. 4).

**Disruption of *XYL2* and *XYL3*.** Transformation-mediated gene disruption was used to create specific *XYL2* and *XYL3* mutants. The plasmid pXLB37-2 was used to disrupt *XYL2*. Two of five hygromycin-resistant transformants had single copies of the vector integrated at *XYL2*, and three had undergone ectopic integration. Transformant T446-17A, with a single

TABLE 2. Crosses to obtain triple xylanase mutants in *C. carbonum*[a]

| Cross no. | Progeny genotype[b] | Number obtained |
|---|---|---|
| 1: T448-4A (*XYL1 XYL2 xyl3*) × 477-3a (*xyl1 xyl2 XYL3*) | *xyl1 xyl2 XYL3* | 5 |
| | *XYL1 XYL2 xyl3* | 4 |
| | *xyl1 XYL2 XYL3* | 3 |
| | *XYL1 xyl2 XYL3* | 6 |
| | *xyl1 XYL2 xyl3* | 5 |
| | *XYL1 xyl2 xyl3* | 1 |
| | *XYL1 XYL2 XYL3* | 2 |
| | *xyl1 xyl2 xyl3* | 1 |
| 2: T448-4A (*XYL1 XYL2 xyl3*) × 477-7a (*xyl1 xyl2 XYL3*) | *xyl1 xyl2 XYL3* | 0 |
| | *XYL1 XYL2 xyl3* | 3 |
| | *xyl1 XYL2 XYL3* | 0 |
| | *XYL1 xyl2 XYL3* | 7 |
| | *xyl1 XYL2 xyl3* | 3 |
| | *XYL1 xyl2 xyl3* | 1 |
| | *XYL1 XYL2 XYL3* | 5 |
| | *xyl1 xyl2 xyl3* | 2 |

[a] Progeny genotypes were determined by DNA blotting. Strains numbered 556 in Table 1 are progeny from cross 1.

[b] Uppercase italic type is used to indicate the wild-type copy of a gene; lowercase italic type is used to indicate a mutated copy.

copy of pXLB37-2 integrated at *XYL2*, was used for further studies.

The plasmid pXLC42-1 was used to disrupt *XYL3*. Although homologous integration of the plasmids used to disrupt *XYL1* (2) and *XYL2* occurred at a high frequency (greater than 40%), over 50 independent transformants, which were obtained by using several different vectors, were analyzed before a single transformant, T448-4A, was found in which the vector had integrated homologously at *XYL3*. The reason for the atypical difficulty in disrupting *XYL3* is not known. Based on the hybridization pattern, transformant T448-4A has undergone homologous integration of a single copy of pXLC42-1.

**Creation and characterization of double and triple xylanase mutants.** The *xyl1* mutant 312-6A used in the current experiments was derived from the original *xyl1* mutant 164R10 T2-4 (Table 1) (2). Strain 312-6A was crossed with 367-1a (wild type) to create 447-8a, a *xyl1* strain of mating type a. Strain 447-8a was then crossed with the *xyl2* mutant T446-17A to create the strains 477-3a and 477-7a (both *xyl1 xyl2*) (Table 1).

A triple xylanase mutant was obtained by crossing strains T448-4A (*XYL1 XYL2 xyl3*) and 477-3a (*xyl1 xyl2 XYL3*). Because no selection could be used to analyze the progeny, they were analyzed by DNA blotting. Progeny in all eight possible classes were obtained (Table 2). Selected progeny from this cross are indicated in Table 1 with the designation 556, and DNA blots of these progeny are shown in Fig. 5. Strain T448-4A was also crossed with 477-7a (Table 2).

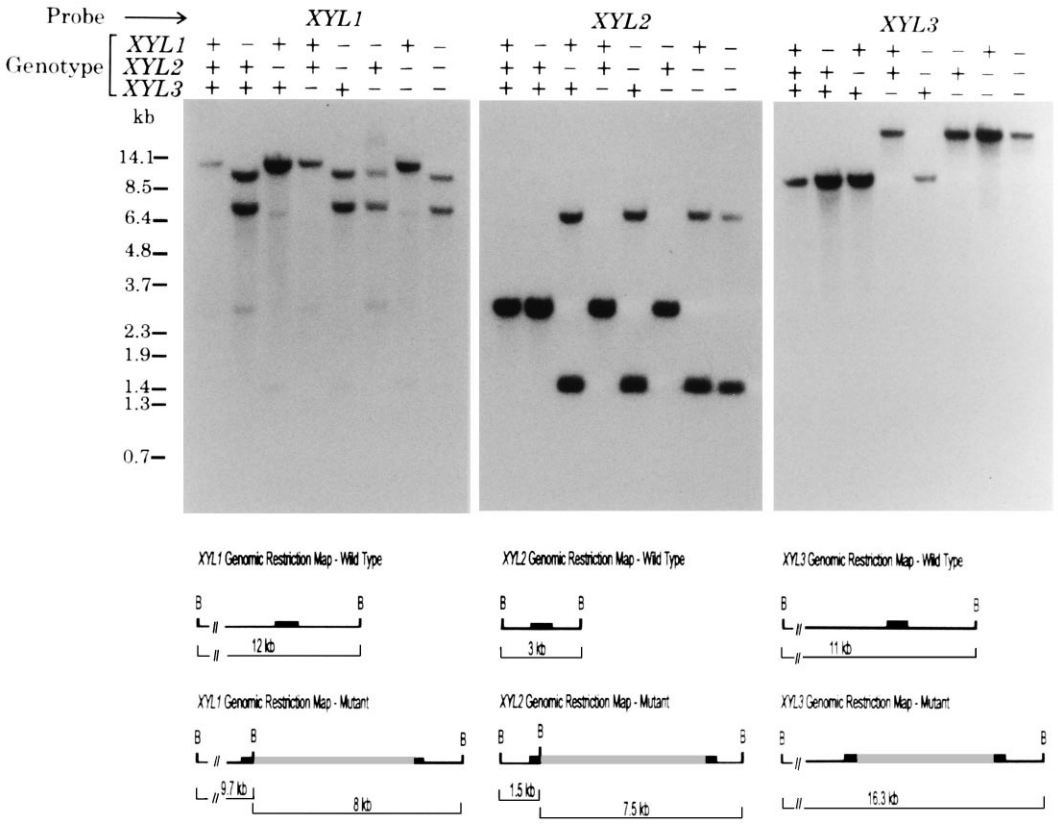


FIG. 5. DNA blot analysis of wild-type and xylanase disruption mutants. From left to right, the strains are 556-2, 556-11, 556-8, 556-1, 556-16, 556-4, 556-12, and 556-13 (see Table 1). DNA was digested with *Bam*HI (sites indicated by B) and electrophoresed on three separate gels. One blot was hybridized with *XYL1*, one was hybridized with *XYL2*, and one was hybridized with *XYL3*. The predicted wild-type and disruption restriction maps for single, homologous integration events are shown below the DNA blots. Black shading indicates the xylanase gene, and light shading indicates the vector. The faint hybridizing bands of approximately 3 kb in the *XYL1* panel are due to cross-hybridization of the *XYL1* probe with *XYL2*. Genotypes are indicated by + (wild type) or − (mutant).

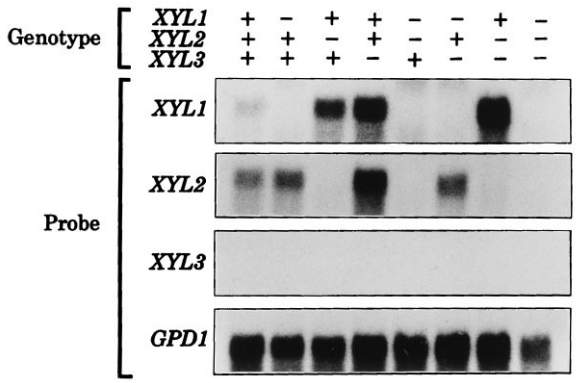


FIG. 6. Expression of xylanase genes in culture. The same strains (in the same order as in the legend to Fig. 5) were grown on maize cell walls for 6 days, and 10 μg of total RNA from each was fractionated and blotted. The blot was hybridized sequentially with probes representing *XYL1*, *XYL2*, *XYL3*, and *GPD1*. Genotypes are indicated by + (wild type) or − (mutant).

The chromosomes of *C. carbonum* were separated by pulsed-field gel electrophoresis (1), and the blotted gel was probed sequentially with *XYL1*, *XYL2*, and *XYL3*. *XYL1* and *XYL2* hybridized to bands of very different sizes and are therefore on different chromosomes. *XYL1* and *XYL3*, on the other hand, hybridized to the same band, which, based on its ethidium bromide staining intensity, contained two or more chromosomes. Therefore, *XYL1* and *XYL3* might be on the same chromosome. The cross data, however, indicate that *XYL1* and *XYL3* are not tightly linked (Table 2).

**Characterization of xylanase expression in the wild type and mutants.** *C. carbonum* culture filtrates have three peaks of cationic xylanase activity (9). *xyl1* mutants lack the first two of these peaks, which together account for 80 to 90% of the total xylanase activity, and therefore it was concluded that these two peaks are both encoded by *XYL1* (2). In cultures grown on maize cell walls, *XYL2* mRNA is present at a level equal to or higher than that of *XYL1*, but *XYL3* mRNA is not detectable (Fig. 6). *XYL3* mRNA has also not been detected in wild-type *C. carbonum* grown on sucrose or maize cell walls from day 1 through day 12 (7).

As expected, *XYL1*, *XYL2*, and *XYL3* mRNAs are not detectable in the respective mutants (Fig. 6). It appears that disruption of *XYL2* or *XYL3* results in an increase in the level of *XYL1* mRNA (Fig. 6). However, determination of the extent to which *XYL1*, *XYL2*, and *XYL3* influence each other's expression will require further study.

As expected, the first two peaks of cationic xylanase activity (9) are missing in all of the mutants (Table 1) that are disrupted in *XYL1* (data not shown). Because *xyl1* mutants still have a prominent peak of xylanase activity (2), and because *XYL2* is expressed in culture, it originally seemed plausible that *XYL2* encoded the third peak of xylanase. However, *xyl2* and *xyl3* mutants have cation-exchange xylanase activity profiles identical to that of the wild type (data not shown). In particular, the third peak of xylanase is still present in *xyl2* and *xyl3* mutants. Therefore, *C. carbonum* has at least one more xylanase gene besides *XYL1*, *XYL2*, and *XYL3*. The protein that accounts for the third peak of xylanase activity (9) has been purified, and the cloning of its gene, *XYL4*, is in progress.

Culture filtrates of the *xyl1 xyl2* double mutant and the *xyl1 xyl2 xyl3* triple mutant have the same level of total xylanase activity as the culture filtrate of the *xyl1* single mutant (data not shown). Since no enzyme activity disappears in the *xyl2* mutant,

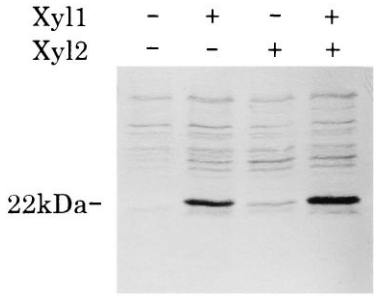


FIG. 7. Immunoblot analysis of culture filtrates of a wild-type strain (367-2A), a *xyl1* mutant (477-8a), a *xyl2* mutant (T446-17A), and a *xyl1 xyl2* double mutant (477-7a). Each lane of an SDS-polyacrylamide gel was loaded with 80 μg of partially purified protein from a culture filtrate. The blot was treated with an antiserum raised against the 22-kDa xylanase of *T. viride* (8) and visualized with goat anti-rabbit antibody coupled to alkaline phosphatase.

culture filtrates were examined immunologically by using an antibody raised against the 22-kDa cationic xylanase of *T. viride* (8). This antibody recognizes multiple proteins in *C. carbonum* culture filtrates, but the strongest band in the wild-type fungus has a molecular mass of 22 kDa (Fig. 7). This band is also prominent in a *xyl2* mutant, is greatly reduced in a *xyl1* mutant, and is undetectable in a *xyl1 xyl2* double mutant (Fig. 7). This protein is a candidate for Xyl2. It is not known why Xyl2 protein should be scarce and Xyl2 xylanase activity is undetectable despite the fact that *XYL2* mRNA is abundant (Fig. 6). It is possible that *XYL2* mRNA is less efficiently translated than *XYL1* mRNA or that Xyl2 is less efficiently secreted or less stable.

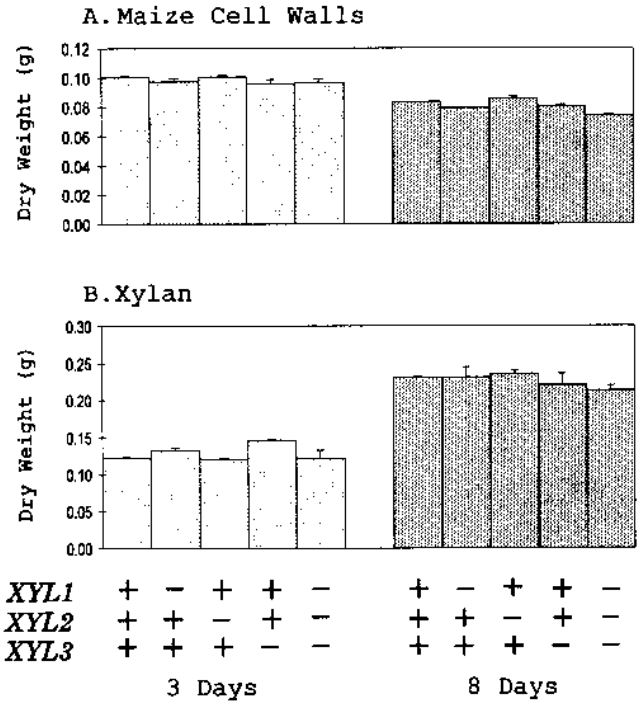


FIG. 8. Dry weights of mycelial mats of wild-type strain 367-2A and mutant strains 556-11 (*xyl1*), 556-8 (*xyl2*), 556-1 (*xyl3*), and 556-13 (*xyl1 xyl2 xyl3*) after growth for 3 or 8 days on (A) maize cell walls or (B) oat spelt xylan. Error bars indicate the ranges of duplicate samples. Genotypes are indicated by + (wild type) or − (mutant).

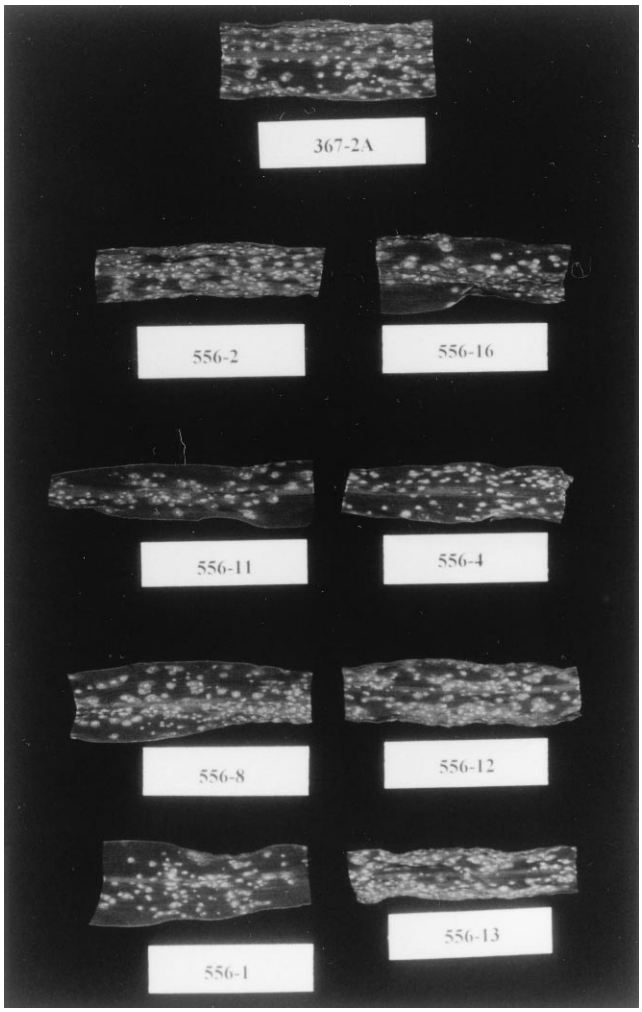


FIG. 9. Pathogenicity of the wild type and xylanase mutants. Leaves were harvested 5 days after inoculation. The genotype of each strain is given in Table 1.

**Growth and pathogenicity of xylanase mutants.** In culture, all of the mutants grow as well as the wild type on maize cell walls or xylan (Fig. 8). On plants, no detectable differences are seen between any of the xylanase mutants and the wild type in the rate of lesion development, in lesion size, or in lesion morphology (Fig. 9).

**Xylanase mRNA expression in infected leaves.** *XYL1* and *XYL3* mRNAs, but not *XYL2* mRNA, are detectable by RNA blotting in infected leaves 7 days postinoculation (Fig. 10). *XYL2* mRNA is also not detectable in plants from 12 h to 14 days after inoculation (7).

## DISCUSSION

*C. carbonum* has three xylanase genes that encode, or are predicted to encode, extracellular 22-kDa family G enzymes. Although the sequences of the three genes are highly similar, their patterns of expression are distinct. *XYL1* encodes the most abundant xylanase made in culture and is also expressed in plants. Although *XYL2* is strongly expressed at the mRNA level in culture, no Xyl2 enzyme activity is detectable. *XYL2* expression is not detectable in plants. *XYL3* expression is detectable in plants but not in culture.

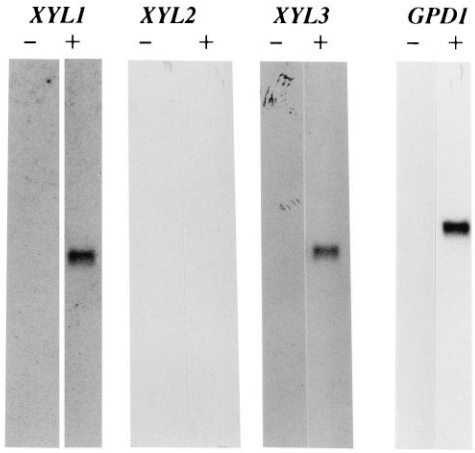


FIG. 10. Expression of *XYL1*, *XYL2*, and *XYL3* in plants. Poly(A)$^+$ mRNA was isolated from infected maize leaves 7 days after inoculation with wild-type isolate 367-2A, and 10 μg was electrophoresed and blotted. The blot was hybridized sequentially with probes representing *XYL1*, *XYL2*, *XYL3*, and *GPD1*. + and − indicate mRNA from infected and uninfected leaves, respectively.

Because *XYL2* and *XYL3* do not contribute to the extracellular xylanase activity in culture, the total xylanase activity levels and the xylanase HPLC activity profiles of the *xyl2* and *xyl3* mutants are identical to that of the wild type (2). The *xyl1 xyl2 xyl3* triple mutant retains significant extracellular xylanase activity because of the third of the three xylanase activities originally characterized (9), which we now call Xyl4.

*XYL1*, *XYL2*, and *XYL3*, either alone or in combination, are not required for growth on xylan or on maize cell walls. Growth of the triple mutant might be supported by the enzymatic activities of Xyl4, β-xylosidase (9, 13), and perhaps other xylanases or other enzymes that can degrade the minor sugars present in commercial oat spelt xylan. It is not surprising that *XYL2* is not required for pathogenicity since it is not expressed in plants. However, *XYL3*, which is expressed only in plants, is also not required either by itself or in combination with *XYL1*.

Redundancy is emerging as a common theme among the cell wall-degrading enzymes of plant-pathogenic bacteria and fungi (see, e.g., references 10, 19, 20, and 23), and the xylanases of *C. carbonum* are no exception. Biologically, the significance of redundancy might lie in the fact that the redundant enzymes are not identical in their pattern of expression and/or activity. Because Xyl2 and Xyl3 have not been purified, it is not known if their enzymatic activities differ from that of Xyl1; however, as shown here, the three encoding genes have distinct patterns of expression. From a study of the cutinases and esterases of *Alternaria brassicicola*, Yao and Köller (23) have drawn a distinction between enzymes typical of saprophytic growth and those associated with pathogenic growth. Because of their different yet overlapping patterns of expression in culture and in plants, the xylanase genes of *C. carbonum* are not readily classifiable as either saprophytic or pathogenic.

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## REFERENCES

1. **Ahn, J.-H., and J. D. Walton.** 1996. Chromosomal organization of *TOX2*, a complex locus controlling host-selective toxin biosynthesis in *Cochliobolus*

*carbonum*. Plant Cell **8:**887–897.
2. **Apel, P. C., D. G. Panaccione, F. R. Holden, and J. D. Walton.** 1993. Cloning and targeted gene disruption of *XYL1*, a β1,4-xylanase gene from the maize pathogen *Cochliobolus carbonum*. Mol. Plant-Microbe Interact. **6:**467–473.
3. **Bailey, B. A., J. F. D. Dean, and J. D. Anderson.** 1990. An ethylene biosynthesis-inducing endoxylanase elicits electrolyte leakage and necrosis in *Nicotiana tabacum* cv Xanthi leaves. Plant Physiol. **94:**1849–1854.
4. **Bailey, B. A., R. F. Korcak, and J. D. Anderson.** 1993. Sensitivity to an ethylene biosynthesis-inducing endoxylanase in *Nicotiana tabacum* L. cv Xanthi is controlled by a single dominant gene. Plant Physiol. (Rockville) **101:**1081–1088.
5. **Ballance, D. J.** 1986. Sequences important for gene expression in filamentous fungi. Yeast **2:**229–236.
6. **Ballance, D. J.** 1991. Transformation systems for filamentous fungi and an overview of fungal gene structure, p. 1–29. *In* S. A. Leong and R. M. Berka (ed.), Molecular industrial mycology; systems and applications for filamentous fungi. Marcel Dekker, New York.
7. **Cheng, Y., and J. D. Walton.** Unpublished data.
8. **Dean, J. F. D., H. R. Gamble, and J. D. Anderson.** 1989. The ethylene biosynthesis-inducing xylanase: its induction in *Trichoderma viride* and certain plant pathogens. Phytopathology **79:**1071–1078.
9. **Holden, F., and J. D. Walton.** 1992. Xylanases from the fungal maize pathogen *Cochliobolus carbonum*. Physiol. Mol. Plant Pathol. **40:**39–47.
10. **Kelemu, S., and A. Collmer.** 1993. *Erwinia chrysanthemi* EC16 produces a second set of plant-inducible pectate lyase isozymes. Appl. Environ. Microbiol. **59:**1756–1761.
11. **Lever, M.** 1972. A new reaction for colorimetric determination of carbohydrates. Anal. Biochem. **47:**273–279.
12. **Pitkin, J. P., D. G. Panaccione, and J. D. Walton.** 1996. A putative cyclic peptide efflux pump encoded by the *TOXA* gene of the plant pathogenic fungus *Cochliobolus carbonum*. Microbiology **142:**1557–1565.
13. **Ransom, R., and J. D. Walton.** Unpublished data.
14. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Schäfer, W., D. Straney, L. Cuiffetti, H. D. Van Etten, and O. C. Yoder.** 1989. One enzyme makes a fungal pathogen, but not a saprophyte, virulent on a new host plant. Science **246:**247–249.
16. **Scott-Craig, J. S., D. G. Panaccione, F. Cervone, and J. D. Walton.** 1990. Endopolygalacturonase is not required for pathogenicity of *Cochliobolus carbonum* on maize. Plant Cell **2:**1191–1200.
17. **Selden, R. F.** 1987. Analysis of RNA by northern hybridization. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, New York.
18. **Sposato, P., J.-H. Ahn, and J. D. Walton.** 1995. Characterization and disruption of a gene in the maize pathogen *Cochliobolus carbonum* encoding a cellulase lacking a cellulose binding domain and hinge region. Mol. Plant-Microbe Interact. **8:**602–609.
19. **Sweigard, J. A., F. G. Chumley, and B. Valent.** 1992. Disruption of a *Magnaporthe grisea* cutinase gene. Mol. Gen. Genet. **232:**183–190.
20. **Templeton, M. D., K. R. Sharrock, J. K. Bowen, R. N. Crowhurst, and E. H. Rikkerink.** 1994. The pectin lyase-encoding gene (*pnl*) family from *Glomerella cingulata*: characterization of *pnlA* and its expression in yeast. Gene **142:**141–146.
21. **Van Wert, S. L., and O. C. Yoder.** 1992. Structure of the *Cochliobolus heterostrophus* glyceraldehyde-3-phosphate dehydrogenase gene. Curr. Genet. **22:**29–35.
22. **Wu, S.-C., S. Kaufman, A. G. Darvill, and P. Albersheim.** 1994. Purification, cloning, and characterization of two xylanases from *Magnaporthe grisea*, the rice blast fungus. Mol. Plant-Microbe Interact. **8:**506–514.
23. **Yao, C., and W. Köller.** 1995. Diversity of cutinases from plant pathogenic fungi: different cutinases are expressed during saprophytic and pathogenic stages of *Alternaria brassicicola*. Mol. Plant-Microbe Interact. **8:**122–130.
24. **Yoder, O. C.** 1988. *Cochliobolus heterostrophus*, cause of Southern corn leaf blight. Adv. Plant Pathol. **6:**93–112.