

Cloning and Targeted Gene Disruption of *XYL1*, a β 1,4-Xylanase Gene from the Maize Pathogen *Cochliobolus carbonum*

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The gene, *XYL1*, encoding the major extracellular endo- β 1,4-xylanase from the maize pathogen *Cochliobolus carbonum* was cloned using a synthetic, degenerate oligonucleotide based on a tryptic fragment from the purified enzyme. The deduced product of *XYL1* has a M_r of 20,869 and a predicted pI of 9.1, in good agreement with the measured M_r and pI of the purified enzyme. The *XYL1* product has strong amino acid identity to seven endo- β 1,4-xylanases from six prokaryotes but no obvious similarity to 10 other prokaryotic endoxylanases or a yeast endoxylanase. An internal fragment of the gene was used to create a specific xylanase mutant by transformation-mediated gene disruption via homologous recombination. Total extracellular xylanase activity in the mutant was reduced by 85–94%. When analyzed by cation exchange HPLC, culture filtrates of the mutant and wild type had identical protein profiles, but the mutant lacked the major peak of UV absorption corresponding to the major xylanase activity. Xylanase II activity was also missing in the mutant, but xylanase III activity was still present. The *XYL1* mutant grew as well as the wild type on sucrose, on corn cell walls, and on xylan. The pathogenicity of the mutant was indistinguishable from the wild type, indicating that *XYL1* is not required for pathogenicity.

Additional keywords: cell wall degrading enzyme, homologous recombination, *Helminthosporium*.

The plant cell wall is a major element in the environment of plant pathogens. All land plants have xylan in their cell walls, and the monocot primary cell wall contains up to 40% β -1,4 xylans (Cooper 1984; Darvill *et al.* 1980; Wada and Ray 1978; Kato and Nevins 1984; Labavitch and Ray 1978). In maize, the host of the filamentous fungal pathogen *Cochliobolus carbonum* Nelson, arabinoxylan

is the major water-insoluble and noncellulosic component of the primary cell wall (Kato and Nevins 1984).

Most, if not all, cellular plant pathogens produce enzymes that degrade plant polymers, but the role of most of these enzymes in pathogenesis is not known. For example, endoglucanase contributes to virulence of *Pseudomonas solanacearum* on tomato (Roberts *et al.* 1988), but not of *Xanthomonas campestris* pv. *campestris* on radish and turnip (Glough *et al.* 1988). Endopolygalacturonase is not required for pathogenicity of *C. carbonum* on maize (Scott-Craig *et al.* 1990). A genetically engineered cutinase mutant of *Magnaporthe grisea* retained full pathogenicity on rice, barley, and weeping lovegrass (Sweigard *et al.* 1992a, 1992b). Likewise, the cutinase gene of *Nectria haematococca* appears not to be required for pathogenicity and virulence on pea (Stahl and Schäfer 1992).

Cell wall degrading enzymes are usually thought to contribute to penetration and invasion of plant tissues by pathogens. However, some cell wall degrading enzymes also induce putative plant defense responses. Endopolygalacturonase induces phytoalexin biosynthesis (Cervone *et al.* 1987; Lee and West 1981). Xylanases from several fungi elicit electrolyte leakage, necrosis, ethylene biosynthesis, and synthesis of pathogenesis-related proteins (Bailey *et al.* 1990; Dean and Anderson 1991; Dean *et al.* 1989; Fuchs *et al.* 1989).

Because of the abundance of xylan in the cell walls of monocotyledons, xylanase might be an important pathogenicity factor in diseases of this group of plants. We previously described three xylan-degrading enzymes that are secreted by *C. carbonum* when grown on xylan or corn cell walls (Holden and Walton 1992). We report here the cloning, sequencing, and transformation-mediated gene disruption of *XYL1*, which encodes xylanase I of *C. carbonum*. This enzyme is responsible for approximately 90% of the total xylanase activity of *C. carbonum* when grown on corn cell walls (Holden and Walton 1992).

RESULTS

XYL1 isolation and characterization.

Six tryptic peptides were isolated from xylanase I and sequenced (Table 1) (Holden and Walton 1992). A completely degenerate 17-mer oligonucleotide coding for the amino acid sequence HFDWA was used to screen an EMBL3 genomic library. The xylanase-encoding sequences were subsequently subcloned into pBluescript and pUC18.

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The nucleotide and amino acid sequences of *XYL1* are in GenBank, accession number L13596.

Table 1. Comparison of peptide sequences from tryptic digests of xylanase I and the corresponding peptide sequences deduced from the DNA sequence of *XYLI*

Tryptic peptide sequence ^a	Deduced amino sequence ^b
ATYTNAGAGSYSVSWGSGGNNV	ATYTNAGAGSYSVSWGSGGNIV
GINPGTAR	GwNPGTAR
LAV(Y)A	LAVYg
NPLVEYYVVENFGTYDP(P/S)SQWQNK	NPLVEYYVVENFGyYDPSSQsQNK
TFQYWSVR	TFQYWSVR
THFDAWA(S) (A/K)	THFDAWASK

^a Amino acids in parentheses indicate uncertainties in the peptide sequencing.

^b Lowercase letters denote mismatches between the protein sequence of the tryptic digests and the deduced amino acids from the DNA sequence.

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1  GATCTACACCCTAGCTAATATTACTCTAGCAGATGGAATACCGCTATCATGACGAGGATG
61  TTTGGCTTGGCTGTGCACAACATCCGGCTTGTTCCTCACTACATGCCATGCGAGGGGA
121  GTATAAAGCTATCCCTGAGTGGCTCCATGGCACACTTTCCTACCAACCATCACCAACAC
181  TACCACCTCCCTTCGCTTCATTCACTCAACAACACCATCAAATGGTTTCTTTCACC
      M V S F T
241  TCCATCATCACCGCTGCTGTGGCGGTACCGGCGCTCTTGCCGCCCCGCCCACTGATGTG
6   S I I T A A V A A T G A L A A P A T D V
      KpnI
301  TCTCTCGTTGCCGTCAGAACCCCCAACGGCGAGGGTACCCACAACGGTCTGCTCTGG
26  S L V A R N T P N Y N G E G T H N G C F W
      KpnI
361  TCTTGGTGTCTGATGGCGGTGCCCGCTACCTACACCAACGGTGGCGGTGGTAGCTAC
46  S W W S D G G A R A T Y T N G A G G S Y
421  AGCGTAAGCTGGGAAGCGGTGGCAACCTCGTGGTGAAGGGATGGAACCCAGGAAT
66  S V S W G S G G N L V G K G W N P G T
481  GCCCGTATGGACTGCTCACTCAACTCTGATAGACTACATACACTAACATTGATACAGT
86  A R
541  ACCATCACCTACTCTGGTACTTACAACACCAACCGCACTCCTACCTTGGCCGCTACGGC
88  T I T Y S G T Y P N Y N G N S Y L A A V Y G
      SacI
601  TGGACCCGCAACCCCTTGTGAGTACTACGTCGTTGAGAATTCGGCACCTACGACCCC
108  W T R N P L V E Y Y V V E N F G T Y D P
      KpnI
661  TCTTCCAGTCCCAGAACCAAGGTCACCTCAGCTGATGGATCTTCTACAAGATCGCT
128  S S Q S Q N K G T V T S D G S S Y K I A
      SalI
721  CAGTCGACCCGTCACAACCGCTCCATCGATGGCACGAGACCTTCAGCAGTATTGG
148  Q S T R T N Q P S I D G T R T F Q Q Y W
      oligo
781  TCTGTTGTCAGAACAGCGCTCTCCCGCTCCGTCATATGAAGACTCACTTTGAGGCC
168  S V R Q N K R S S G S V N M K T H F D A
      #1 Ball
841  TGGCCAGCAGCAAGGCATGAACCTTGGCCAGCACTACAGAGTTTGGCCACCGAGGGT
188  W A S K G M N L G Q H Y Y Q I V A T E G
901  TACTTCTCCACTGGTAAGCCGATACCGCTCAACTGCCATAAATTTCTTACCACGAA
208  Y F S T G N A Q I T V N C P *
961  GATTAACGAATGGCCCTGATGGCTCTCTGAAGACTGGAAGATGACAGGCGTGGTTTT
1021  GGTACGCTGCTGTGCTAGCTAGCTGGTGGATTCTTTCTGTATATACTACTTTTGTCAA
1081  CCCGATTTGCTATAGCTTAAACAATGTCAAATCTTGGCATCTACACAACGGGCTTTT
1141  CATTTTGGCTCTAAGTGAATTTGTGATGGTCTTGACGCATGATGACGCTACTACGAGCA
1201  TTCTCTCCGGTGTCCCTCTAATGATAAAAAATAACAACTCAATACTCGCAATATGAAA
1261  CTCGGCAGTACCAAAATTTAGATGTTGACTTCAACAATAGGCTGGCCTATCTACGCTGCC
1321  CATCTATTACATACACAGGATCGTACAGGAGTCACTCCTCAGTGGCTCCTCAATGATTC
1381  CCAATGCAACATGCAACTCCTCTCCATAACAGTAATCGGGTGTGCAATCTGAAAACAC
1441  CACCAAGCGAGCCATTTGTCGCAACTGCGCCCAAAAACCCATTTTCTCCATCTTTGCT
1501  ACACAGCAGCTCCGAAATCAGGAGTCCCAACCGTAAGAAGTGCAGCCTCAGTGTACT
1561  GGAGGACCCGCTAGTGGTGTCTTCCCAATATAGCTTAGTGCAATAGTAGTGGG
      SacI
1621  TAGCATCTCGCTGAGAGCTC

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Fig. 1. Nucleotide and deduced amino acid sequences of *XYLI*. The double underlined regions correspond to the sequences obtained from tryptic fragments from the purified protein. The overlined region indicates the location of the oligonucleotide used to clone the gene. The italicized sequence indicates the intron.

Figure 1 shows the genomic sequence of *XYLI* and the deduced amino acid sequence. The region identified by the probe is overlined. The sequenced peptides corresponding to those in Table 1 are double underlined. A 53-bp intron confirmed by sequencing a corresponding cDNA (data not shown) is indicated by italics in Figure 1. The intron contains two stop codons and a frameshift, as well as 5' G↓GTATGG (consensus G↓GTANGT) and 3' ACAG (consensus ACAG) intron splice sites and an internal CAC-TAAC (consensus TACTAAC) sequence (Ballance 1986).

A possible translational start site at nucleotide 226 (Fig. 1) has a proper consensus context of CAAAATGGT

(consensus CAMMATGNC, where M = A or C) (Ballance 1991). Since the N-terminus of the enzyme was blocked (Holden and Walton 1992), the exact start of the mature protein is not known. Eighteen of the first 30 amino acids, including the putative start methionine, are hydrophobic amino acid residues (Leu, Phe, Val, Ile, and Ala). The known signal peptide of the endopolygalacturonase from *C. carbonum* (Scott-Craig *et al.* 1990) ends in LDAR; *XYLI* contains LVAR starting at amino acid 27 (Fig. 1). Therefore, the mature xylanase protein probably begins at amino acid 31. Based on the cDNA sequence, the polyadenylation site is at nucleotide 1130, 184 bp past the stop codon (Fig. 1). There is no apparent polyadenylation consensus sequence (Ballance 1986). The predicted mature protein encoded by *XYLI* has 191 amino acids, a M_r of 20,869, and a pI of 9.1. These figures are close to the size (22,000 to 24,000) and pI (greater than 9.3) measured for the enzyme (Holden and Walton 1992).

XYLI has a high degree of similarity to seven prokaryotic xylanases (Fig. 2). Between amino acid 30 and 209 of the *C. carbonum* xylanase, the identity ranges from 38 to 60%. The amino acid sequence TFXQYWSVR obtained from the enzyme (Holden and Walton 1992) is one of the most highly conserved motifs in these xylanases (Fig. 2). *XYLI* has no apparent similarity to 11 other xylanases in GenBank version 72, of which 10 are prokaryotic and one is from the basidiomycetous yeast *Cryptococcus albidus*.

Transformation-mediated gene disruption.

The linearized transformation plasmid, pCC167, was used to transform *C. carbonum* to hygromycin resistance. Nineteen hygromycin-resistant transformants were obtained, and nine of these were purified by single-spore. Five of the single-spore isolates were analyzed at the molecular level by digestion of their DNA with *ApaI* and blotting; the *KpnI/KpnI* and *KpnI/SacI* (nucleotides 342–685 and 686–1634; Fig. 1) fragments of *XYLI* were used together as a hybridization probe. Based on the hybridization pattern, one transformant (T2-4) was a single insertional mutant, two transformants (one of which is T2-8) were multiple insertional mutants, and the remaining two transformants that were analyzed appeared to have ectopic integration. The pattern of hybridization of transformant T2-4 with band sizes of 4.8 and 8.1 kb, and no band at 6.9 kb, is consistent with homologous recombination of a single copy of pCC167 at *XYLI* (Fig. 3). The pattern obtained with DNA from transformant T2-8 is consistent with multiple tandem integrations at *XYLI*,

as evidenced by bands at 4.8 and 8.1 kb and a strong band at 5.7 kb (Fig. 3).

The *XYLI* mutant T2-8 lacks a 1.0-kb RNA that hybridizes to *XYLI* (Fig. 4). Equivalent loading of RNA in each lane was confirmed by stripping and probing the same blot with the glyceraldehyde-3-phosphate dehydrogenase gene of *C. heterostrophus* (Fig. 4B).

Growth and pathogenicity of a *XYLI* mutant.

Wild-type and mutant T2-8 strains were grown on a mineral salts medium supplemented with trace elements and yeast extract and containing either corn cell walls, oat spelt xylan, or sucrose as the sole carbon source. There was no significant difference in growth between the wild type and the disruption mutant T2-8 on any of the three substrates (Fig. 5A).

Culture filtrates from the growth studies in Figure 5A were assayed for xylanase activity. When grown on corn cell walls for 8–16 days, the mutant had a 85–94% reduction in extracellular xylanase activity compared to the wild type (Fig. 5B). Xylanase activity in mutant T2-4 was decreased by a similar amount (data not shown). Disruption of *XYLI* had minimal effect on the relatively low levels of xylanase made on oat spelt xylan (Fig. 5B).

Xylanase activities from mutant T2-8 and wild type were purified in parallel through cation exchange HPLC

(Holden and Walton 1992). The *XYLI* mutant specifically lacked the major peak of UV absorption, corresponding to the major xylanase, xylanase I, in fraction 7 (Fig. 6) (Holden and Walton 1992). The transformant also lacked the second peak of activity (xylanase II), in fraction 9, but maintained the third peak of activity (xylanase III), in fraction 12 (Holden and Walton 1992). Barring any epistatic effects of *XYLI* expression on the expression of xylanase II, we conclude that xylanases I and II are both encoded by *XYLI*, but that xylanase III is encoded by a different gene.

The rate of development, size, and morphology of the lesions produced by the wild-type and mutant T2-8 on susceptible maize appeared the same up to 7 days after inoculation (Fig. 7).

DISCUSSION

XYLI, the gene for the major xylan-degrading enzyme in *C. carbonum*, was cloned and sequenced. Transforma-

Cc	1	MVSEFT	SIITAAVAAT	GALAAPATDV	S.....	LVA
Bc	3	KFKKNFLVVG	..LSAALMSI	SLFSATASAA	S.....	TD.
Bp	4	RKRLRLFLVVC	IGLTLILTAV	PAHA
Bs	3	KFKKNFLVVG	..LSAALMSI	SLFSATASAA	S.....	TD.
Ca	1	MLRRKVIIF	VLAALVMSL	TIYDNTAFAA	TNLWTESTF	SKEVLSTQKT	YSAPNTQAAP
Rf	7	SKIKVLSGT	VSALMIASAA	PVV.....	A
Sb	20	RSAMA.....	..VALAR.SP	LMLPGTAQAD	T.....	VV
Sc	21	MSRRGFLGGA	GTLALATASG	LLELPGTAHAA	T.....	TI.
Cc	30	RONTPNHEGT	HNGCFWWSNS	DGGARATYTN	GAGGSYSVSW	GSGGNLVGGK	GWNPGTA.RT
BcYQNWNT	DGGGIYNAVN	GSGGNYSVNW	SNTGNFVVGK	GWTGSPFPRT
Bp	HTIEMEMGN	HSGYDVELMK	DYGNTSMTLN	NG.GAFSAGW	NNIGNALFRK	GKKFDS.TRT
BsYQNWNT	DGGGIYNAVN	GSGGNYSVNW	SNTGNFVVGK	GWTGSPFPRT
Ca	KTITSNEIGV	NGGYDYELMK	DYGNTSMTLN	NG.GAFSAGW	NNIGNALFRK	GKKFDS.TRT
Rf	SAADQQTRGN	VGGYDYEMNW	QNGQGSAMN	PGAGSFTCSW	SNIENFLARM	GKNVDSOKKN
SbTTNDEGT	NNGYYSFWT	DSQGTVMNMH	GSGGQYSTSW	RNTGNFVAGK	GNANGGR.RT
ScTTNDEGT	..DGMYSFWT	DGGGSVSMTL	NGGGSYSQW	TNCGNFVAGK	GNSTGD..GN
Cc	89IT	YS.GTHYVNG	NSYLAVYGMT	RNPLVEYYIV	ENFGTYDESS	.QSQNKGTVT
BcIN	YNAGVWAPNG	NCYLILYGMT	RSPLIEYYIV	DSWGTYRPT	..GTYKGTVK
Bp	HHQLGNISIN	YNAS.FNPSG	NSYLCVYGMT	QSPLEAYIIV	DSWGTYRPT	..AV.KGSPY
BsIN	YNAGVWAPNG	NCYLILYGMT	RSPLIEYYIV	DSWGTYRPT	..GTYKGTVK
Ca	YKQLGNISIN	YDCN.YQPYG	NSYLCVYGMT	SSPLIEYYIV	DSWGTYRPT	..GTYKGTVK
Rf	YKAFGNIVLT	YDVE.YTPRG	NSYLCVYGMT	RNPLVEYYIV	ENFGTYRPT	..GTYKGTVK
SbVQ	YS.GSFPNSG	NAVYALYGMT	SNPLVEYYIV	DNWGTYRPT	..GEYKGTVT
ScVR	YN.GYFNPVG	NGYGLYGMT	SNPLVEYYIV	DNWGTYRPT	..GTYKGTVK
Cc	139	SDGSSYKIAQ	STRTNQPSID	GTR.TFQQYW	SVRQKRR..SSGSV	NMKTHFDANA
Bc	SDGGTYDIYT	TTRYNAPSID	GDRTTFYQYW	SVRQSKRPTG	S.....NATI	TFTNHVNAWK
Bp	ADGGTYDIYE	TTRYNQPSII	GI.ATFFQYW	SVRQTKRT..SGTV	SVSAHRKWE
Bs	SDGGTYDIYT	TTRYNAPSID	GDRTTFYQYW	SVRQSKRPTG	S.....NATI	TFSAHVNAWK
Ca	VDDGIYDIYE	TTRYNQPSIQ	GN.TTFQYW	SVRTRKRT..SGTI	SVSKHFAANE
Rf	ANGTYDIRK	TTRYNAPSID	GT.ATFFQYW	SVRQTSGSAN	NOTNYMKGTI	DVTKHFDANS
Sb	SDGGTYDIYK	TTRYNAPSVE	GTR.TFQQYW	SVRQSKR.TGGTI	TTGNHFDANA
Sc	SDGGTYDIYQ	TTRYNAPSVE	GTR.TFQQYW	SVRQSKV.TSSGTI	TTGNHFDANA
Cc	190	SKGMNLGQH.	YQIVATEGY	FSTGNAQITV	NCHKFFTKI	NEWPDRLSR	LEDORLGF*
Bc	SHGMNLGSNW	AYQVMATEGY	QSSGSSNVTV	W*
Bp	SLGHPM.GKM	YETAFTVEGY	QSSGSANVTM	NQLFIGN*
Bs	SHGMNLGSNW	AYQVMATEGY	QSSGSSNVTV	W*
Ca	SKGHPM.GKM	HETAFNIEGY	QSSGKADVNS	MSINIGK*
Rf	AAGLMSGTL	YEVSLNIEGY	RBSGSAVNS	VSVTGGSSD	NGGQQQNDW	NQNNNQNN
Sb	RAGHPZGNS	YVIMMATEGY	QSSGSSEIV	GDTGGDSSG	GDNGGGGGC	TRRCPPGRSG
Sc	RAGHNMGGFR	YVIMMATEGY	QSSGSSNVTV	SG*

Fig. 2. Comparison of bacterial xylanases to *XYLI* from *Cochliobolus carbonum*. Shading denotes identity between *XYLI* and at least one other xylanase. Cc = *Cochliobolus carbonum*; Bc = *Bacillus circulans* (GenBank accession number X07723), Bp = *B. pumilus* (X00660), Bs = *B. subtilis* (M36648), Ca = *Clostridium acetobutylicum* (M31726), Rf = *Ruminococcus flavefaciens* (Z11127), Sb = *Streptomyces lividans* xylanase B (M64552), and Sc = *S. lividans* xylanase C (M64553). Numbering is from the known or putative translational start sites. The sequence for the *R. flavefaciens* xylanase gene in GenBank is partial. Alignment was done using BESTFIT (Devereux *et al.* 1984).

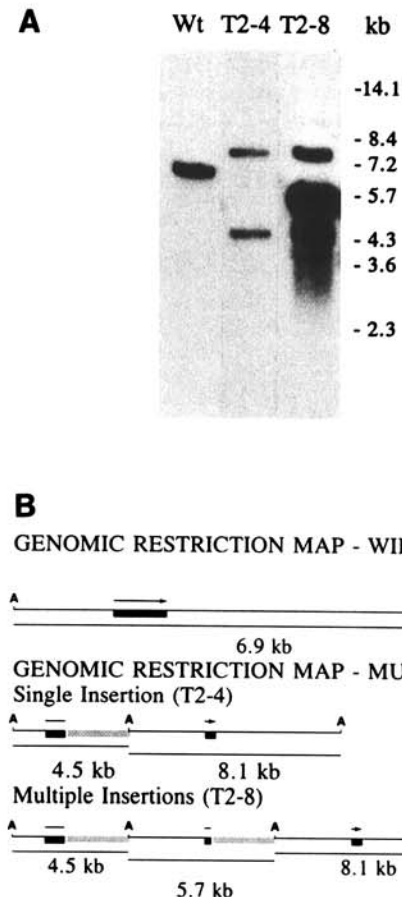


Fig. 3. DNA blot analysis of wild-type and disruption mutants. A, Genomic DNA from *Cochliobolus carbonum* wild type (Wt) and the putative *XYLI* disruption mutants T2-4 and T2-8 was digested with *ApaI*, fractionated, blotted, and hybridized with the *KpnI/KpnI* and *KpnI/SacI* fragments of *XYLI*. B, Predicted restriction map. The black shading indicates *XYLI* and the lightly shaded box denotes the gene for hygromycin phosphotransferase. The hybridization seen in part A is consistent with the predicted single and multiple integration patterns. The arrow indicates the direction of transcription of *XYLI*. A = *ApaI* endonuclease restriction sites.

tion-mediated gene disruption created a mutant specifically lacking a functional copy of this gene, as shown by DNA and RNA blotting and comparative biochemical characterization.

XYL1 appears to encode two forms of endoxylanase activity (xylanase I and II; Holden and Walton 1992), which together are responsible for 85–94% of the extracellular xylan-degrading activity of *C. carbonum* when it is grown on corn cell walls (Fig. 5B). However, growth of the mutant strain was indistinguishable from the wild type in media containing corn cell walls or xylan as the sole carbon source. The residual xylan-degrading activity in the mutant is apparently sufficient to support wild-type growth rates on xylan. The pathogenicity tests indicate that *XYL1* is dispensible for pathogenicity on maize. Whether xylan degradation is important or not in pathogenicity is still an open question, however, because of the small but significant amounts of xylanase remaining when *XYL1* is disrupted (Fig. 5).

The oat spelt xylan used in our growth studies and enzyme assays has been estimated to contain only 60% xylose, with the remainder being mainly arabinose and glucose (Weimer 1985). Therefore, metabolism of the non-xylan components of oat spelt “xylan” might account for

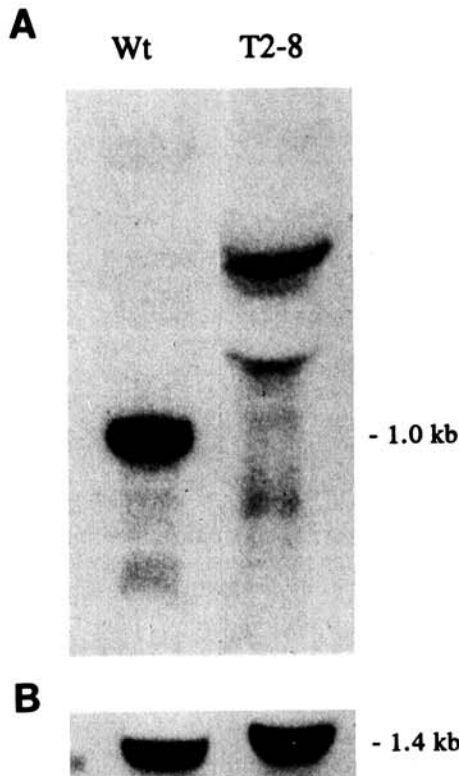


Fig. 4. RNA blot of wild-type and disruption mutant T2-8. **A**, Approximately 30 μ g of total RNA, isolated from fungal mats of the wild type and T2-8 grown for 6 days on corn cell walls, was fractionated on a 1.2% agarose gel containing formaldehyde (Selden 1987) and blotted to a nylon membrane. The blot was probed with the *KpnI/SacI* genomic DNA fragment (pCC157) corresponding to nucleotides 686–1639 of *XYL1* (Fig. 1). **B**, The blot was stripped and reprobed with the glyceraldehyde-3-phosphate dehydrogenase gene.

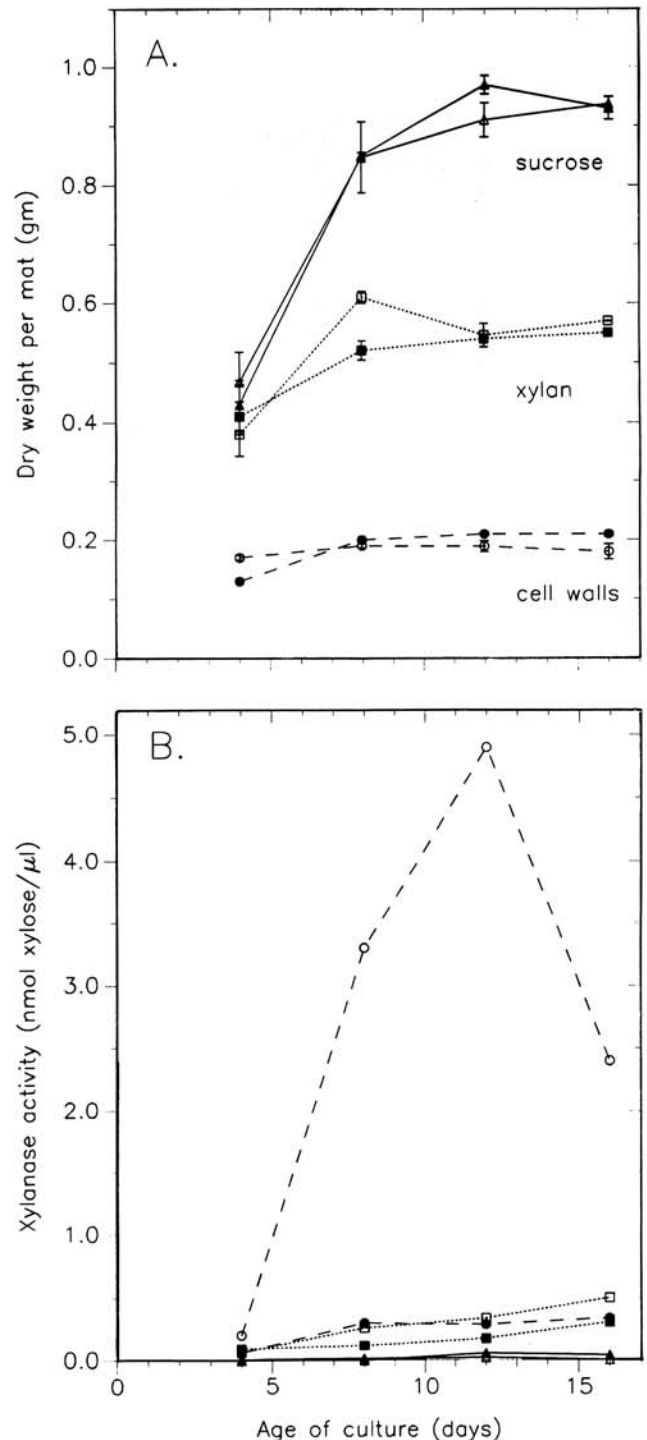


Fig. 5. Growth and xylan-degrading activity in wild-type and mutant T2-8 strains. **A**, Dry weights of fungal mats. **B**, Xylanase activity in the culture filtrates from the same experiment. Δ = Wild type grown on sucrose; \blacktriangle = Mutant grown on sucrose; \square = Wild type grown on oat spelt xylan; \blacksquare = Mutant grown on oat spelt xylan; \circ = Wild type grown on corn cell walls; \bullet = Mutant grown on corn cell walls. All treatments were done in duplicate. Error bars indicate the range.

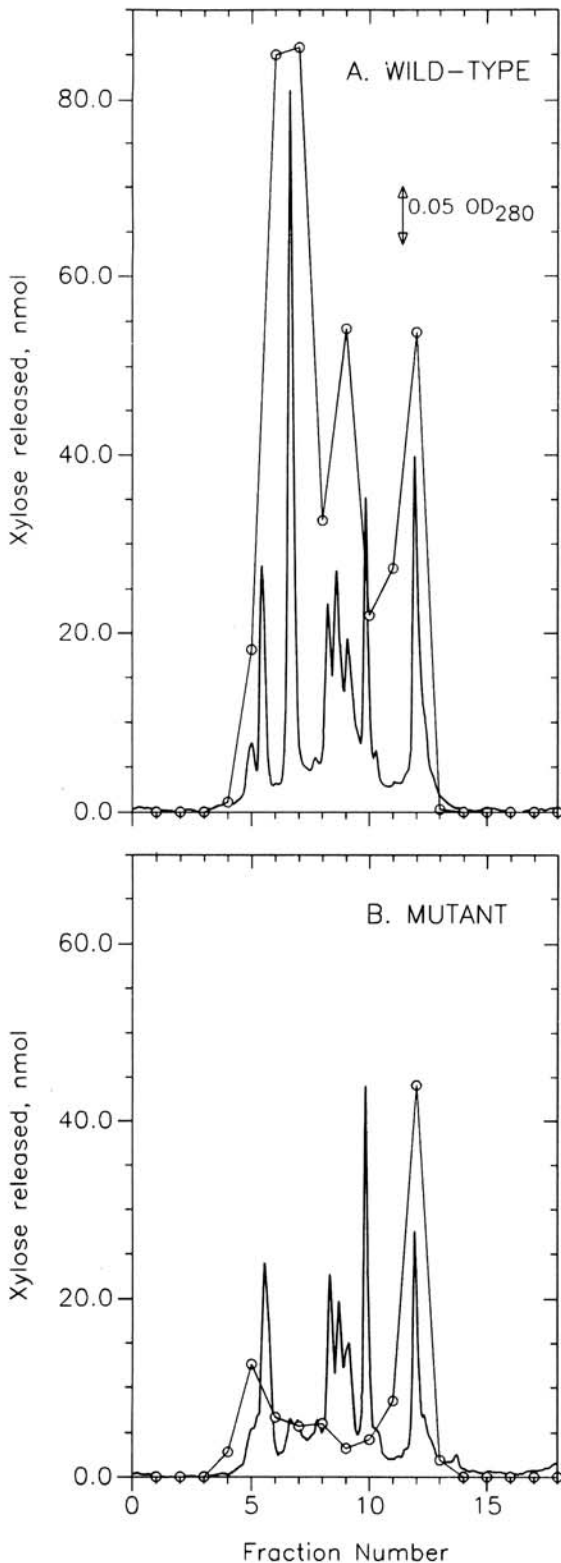


Fig. 6. Cation exchange high-performance liquid chromatography analysis of extracellular xylanase activity in the wild type and mutant. One milligram of total protein (Bradford 1976) from partially purified culture filtrate of each was loaded on the column. The solid line is the absorbance at 280 nm and circles are enzyme activity. The peaks of activity for xylanases I, II, and III appear in fractions 7, 9, and 12, respectively (Walton and Holden 1992). **A**, wild type; **B**, mutant T2-8.

both the growth of the *XYL1* mutant on xylan and for at least some of the apparent residual "xylanase" activity in culture filtrates of the mutant. Xylanase I is clearly a xylanase by its similarity to other fungal and bacterial xylanases, but xylanase III and any other xylanases made by *C. carbonum* might actually catalyze the depolymerization of substrates other than β 1,4-linked xylose. Some studies allude to the possible importance in pathogenicity of enzymes such as α -arabinosidase and arabinanase that degrade native plant xylans (Howell 1975; Cooper *et al.* 1988).

MATERIALS AND METHODS

C. carbonum was maintained and grown as previously described (Walton 1987). For enzyme production, the fungus was grown on mineral salts medium supplemented with trace elements (Van Hoof *et al.* 1991), 0.2% yeast extract, and 0.2% sucrose. When added, corn cell walls (English *et al.* 1971), oat spelt xylan (Fluka), or additional sucrose were at final concentrations of 0.8, 1.2, and 2.0%, respectively.

Mycelia for protoplast preparations or DNA extractions were obtained from germinating conidia. Conidia were collected from fungal cultures grown on V8 juice agar plates with 0.1% Tween 20. The conidia were inoculated into modified Fries medium (Walton and Cervone 1990) and grown for 14 hr at room temperature in a gyratory shaker at 125 rpm.

Nucleic acid manipulations.

A genomic library of DNA from *C. carbonum*, race 1 (isolate SB111) in lambda EMBL3 (Scott-Craig *et al.*

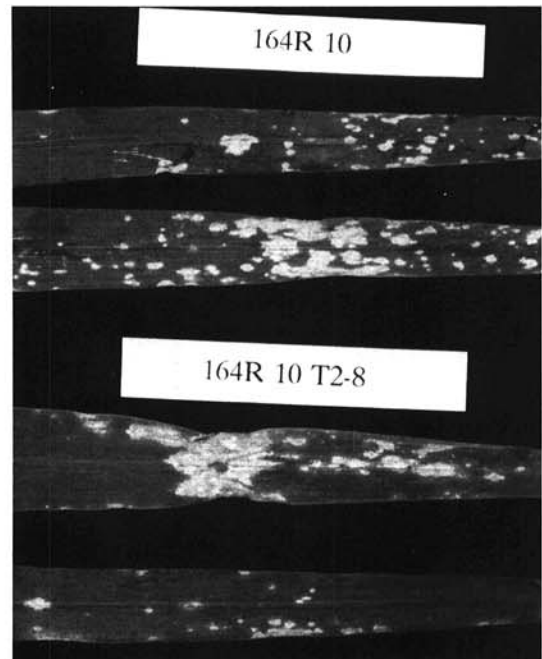


Fig. 7. Pathogenicity test of xylanase mutant T2-8. Corn leaves were inoculated with 1×10^4 spores per milliliter of the wild-type strain 164R10 and the disruption mutant T2-8. Photograph was taken 7 days after inoculation.

1990) was screened with a 32-fold degenerate 17-mer oligonucleotide with the sequence CAYTTYGAYGCNTGGC. This sequence was based on an amino acid sequence, HFDWA, from a tryptic fragment of the purified β 1,4-endoxylanase of *C. carbonum*, isolate SB111 (race 1) (Holden and Walton 1992). The oligonucleotide was labeled at the 5' end with T4 polynucleotide kinase (Sambrook *et al.* 1989). Hybridizations with the oligonucleotide were done overnight at 50° C in 5× SSPE (1× SSPE = 0.15 M NaCl, 50 mM sodium phosphate, 1 mM EDTA, pH 7.7), 5% SDS, 0.5% nonfat dry milk, and 0.1 mg of denatured salmon sperm DNA per milliliter (Sambrook *et al.* 1989). Nitrocellulose and Zeta-probe (Bio-Rad, Richmond, CA) membrane blots were washed three times in 2× SSPE and 0.1% SDS at 50° C for 20 min.

DNA was transferred to Zeta-probe nylon membrane with 0.4 M NaOH; RNA was transferred with 20× SSPE. Routine hybridizations with subcloned DNA fragments were done in 5× SSPE, 7% (w/v) SDS, 0.5% nonfat dry milk, and 0.1 mg of denatured salmon sperm DNA per milliliter at 65° C overnight. The blots were washed in 2× SSPE and 0.1% SDS with the final wash at 65° C for 1 hr.

DNA and RNA were isolated from *C. carbonum* mycelium by the method of Yoder (1988) except cresol and glass beads were omitted from the RNA isolation protocol. For the genomic DNA blot 4 μ g of DNA was loaded per lane and for the RNA blot 30 μ g of total RNA was loaded per lane. RNA electrophoresis was done in the presence of formaldehyde (Selden 1987). GIBCO-BRL RNA standards (0.24–9.5 kb) were used for transcript size estimation. The clone of the glyceraldehyde-3-phosphate dehydrogenase gene from *C. heterostrophus* was used as a reference for constitutive expression (Van Wert and Yoder 1992).

Sequence and analysis.

The complete nucleotide sequences of both strands of a genomic clone for *XYL1* were obtained by the dideoxynucleotide method. Sequencing reactions were performed with Sequenase (United States Biochemicals, Cleveland, OH) and double-stranded templates. When necessary, all junctions created by subcloning were sequenced using the appropriate template and sequence-specific primers. Sequence data were analyzed with DNASIS and PROSIS programs (Hitachi Software Engineering Co., San Bruno, CA), and the University of Wisconsin GCG software package (Devereux *et al.* 1984).

Transformation-mediated gene disruption.

The transformation vector was created by first subcloning an internal 241 bp *ScaI/BalI* fragment of the *XYL1* gene into the *SmaI* site of pBluescript II KS to create pCC166. This plasmid was then digested with *XhoI* and *HindIII* and ligated with the 2.5-kb *SalI/HindIII* fragment of pUCH1, which contains a gene conferring hygromycin resistance (Schäfer *et al.* 1989). The product of these manipulations (pCC167) was linearized at a unique *SalI* site contained within the *XYL1* sequence prior to transformation of *C. carbonum* isolate 164R10 (*TOX2*⁺, *MAT1-2*).

Protoplasts for transformation were prepared by the

method of Yoder (1988) except Driselase (10 mg/ml) (Sigma D-9515) and Novozym 234 (10 mg/ml) (Novo Laboratories, Wilton, CT) were the only enzymes used. Transformation of *C. carbonum* to hygromycin resistance was as described by Scott-Craig *et al.* (1990).

Disruption mutant analysis.

The transformants were single-spored twice to purify them to nuclear homogeneity. Xylanase was purified from culture filtrates of the wild-type and mutant grown on 0.8% corn cell walls for 8 days. DEAE-cellulose and CM-cellulose chromatography were followed by cation exchange HPLC using a linear gradient from 0–100% B in 30 min (Holden and Walton 1992). Buffer A was 25 mM sodium acetate, pH 5.0, and buffer B was buffer A plus 0.4 M KCl. Xylanase activity was assayed by a reducing sugar assay using 1% oat spelt xylan (Fluka) as substrate (Lever 1972). Assays were done in 50 mM sodium acetate, pH 5.0, at 37° C.

Pathogenicity was tested by inoculating leaves of maize inbred K61 (genotype *hm/hm*) with a suspension of conidia (10^4 /ml) in 0.1% Tween 20.

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