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THE CLONING, COMPARISON AND EXPRESSION OF THREE FAMILY G ENDO B-1,4-XYLANASE GENES OF THE MAIZE FUNGAL PATHOGEN COCHLIOBOLUS CARBONUM AND ANALYSIS OF THEIR IMPORTANCE FOR PATHOGENICITY ON MAIZE

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ABSTRACT

THE CLONING, COMPARISON, AND EXPRESSION OF THREE FAMILY G ENDO β-1,4-XYLANASE GENES OF THE MAIZE FUNGAL PATHOGEN *COCHLIOBOLUS CARBONUM* AND ANALYSIS OF THEIR IMPORTANCE FOR PATHOGENICITY ON MAIZE

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The filamentous fungus *Cochliobolus carbonum*, a pathogen of maize, makes three xylanases when grown in culture. A degenerate oligonucleotide based on the sequence of a tryptic peptide of the major xylanase, Xyl1, was used to clone the corresponding gene, *XYL1*. The oligonucleotide also hybridized to another xylanase gene, *XYL3*. A third xylanase gene, *XYL2*, was cloned by using *XYL1* as a heterologous probe. All three xylanase genes encode family G endo-β1,4-xylanases with basic pI's and predicted Mr's of approximately 22,000. At the amino acid level Xyl2 and Xyl3 are 60% and 42% identical to Xyl1. Xyl2 and Xyl3 are 39% identical. *XYL1* and *XYL2* but not *XYL3* are expressed at the mRNA level in fungus grown in culture. *XYL1* and *XYL3* but not *XYL2* are expressed in infected plants. Transformation-mediated gene disruption was

used to create strains mutated in all three xylanase genes. In the *XYL1* mutant, total xylanase activity decreased by 85% to 94% and two of the three previously characterized xylanase enzymes were gone. The *XYL2* mutant lacked *XYL2* mRNA but no enzyme activity or major protein disappeared. By immunoblotting using an antibody raised against a 22-kDa xylanase from *Trichoderma viride*, a minor protein of 22 kDa could be observed to disappear in the *XYL2* mutant. Since in all three xylanase mutants the third peak of xylanase activity remained in culture filtrates, this third xylanase must be encoded by yet another xylanase gene. The single xylanase mutants were crossed to 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 grew at the same rate as the wild type on xylan and maize cell walls. Additionally, the triple mutant was still fully pathogenic on maize as determined by lesion size, morphology, and rate of lesion development.

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LIST OF SYMBOLS

bp	Base pair
C	Celsius
cDNA	Complementary deoxyribonucleic acids
kb	Kilobase
kDa	Kilodalton
MS	Murashige and Skoog
XYL1	Gene for protein encoding Xyl1
Xyl1	Protein encoded by *XYL1* gene
XYL1$^-$	Disrupted copy of *XYL1* gene
XYL2	Gene for protein encoding Xyl2
Xyl2	Protein encoded by *XYL2* gene
XYL2$^-$	Disrupted copy of *XYL2* gene
XYL3	Gene for protein encoding Xyl3
Xyl3	Protein encoded by *XYL3* gene
XYL3$^-$	Disrupted copy of *XYL3* gene

CHAPTER 1

INTRODUCTION

For years, plant pathologists have been trying to determine what makes an organism a plant pathogen and what distinguishes saprophytic organisms from pathogenic organisms. During the process of infection, a plant pathogen must enter the plant. Pathogens may enter via stomata, wounds, directly by mechanical pressure from melanized appresoria, or enzymatically by cell wall degrading enzymes (Misaghi, 1982). Once inside, pathogens must have mechanisms to overcome plant defenses and to spread within the plant. Pathogens must also obtain nutrients once inside the plant (Misaghi, 1982).

For both penetration and ramification, pathogens must deal with the plant cell wall. The intact cell wall can be penetrated either by mechanical pressure or by enzymatic degradation (Walton, 1994). Mechanical pressure via melanized appresoria is required for penetration by two pathogens, *Magnaporthe* and *Colletotrichum* (Valent and Chumley, 1990; Kubo *et al.*, 1991). Other pathogens are thought to penetrate using cell wall degrading enzymes, but proof of this is lacking (Cooper, 1984). The importance

of enzymatic penetration has been suggested by indirect evidence such as the presence of cell wall degrading enzymes in infected tissue, by loss of host cell wall polysaccharides during infection, and by ultrastructural studies (Cooper, 1984). A *Colletotrichum lagenarium* mutant that has melanized appressoria is unable to form penetration hyphae (Katoh *et al.*, 1988). The authors propose that this may be due to the inability of the pathogen to secrete some cell wall degrading enzymes (Katoh *et al.*, 1988).

Cell wall structure

The plant cell wall is composed of many complex components which are interlinked (Roberts, 1989; Darvill *et al.*, 1980a). A major component of the walls of all land plants is xylan. Xylan is a polymer of ß-1,4-xylose with substitutions of short chains of arabinose and glucuronic acid (Wada and Ray, 1978; Kato and Nevins, 1984; Darvill *et al.*, 1980a; Labavitch and Ray, 1978). In the angiosperm's secondary cell wall the most abundant hemicelluloses are xylans (Aspinal, 1980; Burke *et al.*, 1974; Wada and Ray, 1979). A small percentage of the primary cell wall of dicots is xylan, whereas it is the major constituent of the secondary cell wall (Darvill *et al.*, 1980b). The monocot primary cell wall is at least 40% ß-1,4 xylan (Burke *et al.*, 1974; Cooper, 1984; Darvill *et al.*, 1980a). In maize, the host of the fungus *Cochliobolus carbonum*, arabinoxylan is the major non-cellulosic component of the primary cell wall (Kato and Nevins, 1984).

The role of cell wall degrading enzymes in pathogenesis

While the walls of plants contain many components such as xylan, cellulose, and pectin, plant pathogens produce a large array of enzymes that can degrade these components (Cooper, 1984). Some of the major groups of cell wall degrading enzymes made by plant pathogens are xylanases, cutinases, pectinases, cellulases and glucanases (Cooper, 1987). These enzymes may be important for a variety of reasons. Enzymatic degradation could be important for the plant pathogen to penetrate into the plant. It could also be important for a pathogen to be able to ramify through the cell walls as it moves through the tissues of the plant. The metabolism of the degraded cell wall polymers could provide nutrients to support the growth and development of the pathogen inside the plant (Cooper, 1984). Additionally, certain cell wall degrading enzymes may not be necessary for growth of the pathogen inside the plant, but may be necessary for nutrition during over-wintering or saprophytic growth when the host plant is not present.

Besides their possible role in pathogenesis, cell wall degrading enzymes might also have a role in triggering host defenses. Some cell wall degrading enzymes indirectly elicit host defense responses (Cervone *et al.*, 1987). For example, short oligogalacturonides produced by endo-polygalacturonase (EC 3.2.1.15) cause phytoalexin accumulation in soybean (Davis *et al.*, 1984). In addition, superoxide radical formation, a putative

defense response, is stimulated by pectin lyase in rice protoplasts (Ishii, 1988).

Previous studies on the role of cell wall degrading enzymes in pathogenesis

Some cell wall degrading enzymes may be involved in pathogenesis, but others may not be necessary (Walton, 1994). Only by creating mutants that specifically lack particular enzymes can the role of enzymes be unequivocally tested (Cooper, 1987; Walton, 1994). Mutants have been used to determine the relationship between specific cell wall degrading enzymes and pathogenicity in both bacterial and fungal host-pathogen interactions (Cooper, 1984; Payne *et al.*, 1987; Reid and Collmer, 1988; Roberts *et al.*, 1988; Gough *et al.*, 1988; Scott-Craig *et al.*, 1990). After transposon mutagenesis of the pectin methylesterase gene of *Erwinia chrysanthemi* 3937, the bacterium became non-invasive on Saintpaulia plants (Boccara and Chatain, 1989). While some cell wall degrading enzymes have been shown to be important for pathogenesis, others have been shown to be unimportant. When all four pectate lyase genes in *Erwinia chrysanthemi* are deleted, the bacterium's ability to degrade pectin is drastically reduced. However, the bacterium can still grow on pectin as a carbon source and the mutant still causes significant maceration of carrot, pepper, and potato tissue (Payne *et al.*, 1987; Reid and Collmer, 1988).

Pseudomonas solanacearum and *Xanthomonas campestris* are both root pathogens and cause wilting by blocking water flow in the plant. Both of these bacteria produce large amounts of endo-glucanase, which is thought to contribute to maceration of the plant tissue during infection (Roberts *et al.*, 1988; Gough *et al.*, 1988). Mutation of the endo-glucanase gene of *P. solanacearum* reduces virulence on tomato (Roberts *et al.*, 1988), but disruption of the same enzyme *in X. campestris* does not reduce virulence on radish and turnip (Gough *et al.*, 1988).

While there are some examples of bacterial cell wall degrading enzymes that are important in pathogenesis, there are no examples of fungal cell wall degrading enzymes that are definitely important for pathogenesis. The endo-polygalacturonase of *Cochliobolus carbonum* was demonstrated by targeted gene disruption to be non-essential for the infection of maize (Scott-Craig *et al.*, 1990). Additionally, an alkaline protease, a cellobiohydrolase and an exo-β1,3-glucanase have been shown not to be required for pathogenicity or contribute to virulence of *C. carbonum* on maize (Murphy and Walton, 1996; Sposato *et al.*, 1995; Schaeffer *et al.*, 1994).

In *Nectria haematococca* (*Fusarium solani* f. sp. *pisi*), a cutinase has been found to either contribute (Rogers *et al.*, 1994) or not contribute (Stahl *et al.*, 1994) to virulence on pea. Disruption of a cutinase gene in *Magnaporthe grisea* and in *Alternaria brassicicola* had no effect on pathogenicity (Sweigard *et al.*, 1992; Yao and Koller, 1995). However,

cutinase activity still remained in both cases (Sweigard *et al.*, 1992; Yao and Koller, 1995). A pectin lyase gene was disrupted in *Glomerella cingulata* but the mutant retained pathogenicity on apple and *Capsicum* (Bowen *et al.*, 1995).

Xylanases

Many micro-organisms produce xylanases. Xylanases and cellulases have been divided into families based on hydrophobic cluster analysis and analysis of the catalytic sites (Henrissat *et al.*, 1989). Xylanases are currently divided into two categories, Family G and Family F (Henrissat *et al.*, 1989; Gilkes *et al.*, 1991). Family G includes primarily low-molecular-mass alkaline xylanases such as the 22-kDa *Trichoderma viride* xylanase (Dean *et al.*, 1989; Gilkes *et al.*, 1991). Family F primarily includes high-molecular-mass xylanases that can be either basic or acidic.

Xylanases from plant pathogens

Because such a large proportion of the monocotyledonous cell wall is xylan, it is possible that enzymes that can degrade it play a major role in pathogenesis of monocots. Many fungal pathogens make xylanases (ß-1,4-endo-xylanase, EC 3.2.1.8) including *Cochliobolus heterostrophus* (*Helminthosporium maydis*), a species closely related to *Cochliobolus carbonum* (Anderson, 1978; Cooper *et al.*, 1988; Strobel, 1963; Bateman *et al.*, 1973, 1969; Reilly, 1981; Van Etten and Bateman, 1969; Mullen and Bateman, 1975). Xylanases have been found in infected plant tissue (Mullen

and Bateman, 1975; Bateman *et al.*, 1969; Cooper *et al.*, 1988). When monocot pathogens are grown on plant cell walls, xylanase is produced earlier than other cell wall degrading enzymes (Mullen and Bateman, 1975). In lesions on wheat caused by *Rhizoctonia cerealis*, xylanase is the first enzyme produced (Cooper *et al.*, 1988).

While xylanases may be important for penetration of the plant cell wall and ramification through the tissues, they may also play a role in the elicitation of plant defense responses. The xylanase from *Trichoderma viride* can elicit electrolyte leakage, ethylene biosynthesis, necrosis, and pathogenesis-related proteins in tobacco (Dean and Anderson, 1981; Fuchs *et al.*, 1989; Bailey *et al.*, 1990; Lotan and Fluhr, 1990; Raz and Fluhr, 1993). The plant recognizes the xylanase and not the enzymatic products (Fuchs *et al.*, 1989). A single gene controls sensitivity of tobacco to the xylanase (Bailey *et al.*, 1993).

The fungal pathogen *Cochliobolus carbonum*

Cochliobolus carbonum causes Northern leaf spot of maize (Jennings and Ullstrup, 1957). There are four races of the fungus. Race 1 produces a host selective toxin, HC-toxin, a primary virulence factor, and causes large oval lesions on lines of maize that are homozygous recessive at the *Hm* locus (Walton, 1987). Resistant maize has a dominant allele which encodes an enzyme that detoxifies the toxin (Meeley *et al.*, 1992). Race 2 does not produce a host selective toxin but causes small lesions on most inbred maize

lines (Jones and Dunkle, 1993). Both race 1 and race 2 cause small lesions on maize resistant to the toxin. The remaining races of *C. carbonum* vary in their morphological, pathogenic, and cultural characteristics (Jones and Dunkle, 1993). Histological studies have shown that *C. carbonum* spores germinate and penetrate resistant and susceptible leaves equally well. Approximately 90% of the penetrations are direct while approximately 10% of the infections are through the stomata (Jennings and Ullstrup, 1957).

Two lines of evidence suggest that cell wall degrading enzymes are important in pathogenicity of *C. carbonum*. First, *Cochliobolus* appresoria are only weakly melanized and melanin is not required for penetration (Walton *et al.*, 1995; Valent and Chumley, 1990; Kubo *et al.*, 1991). Melanin is not required for *C. carbonum* penetration into the host, suggesting that cell wall degrading enzymes rather than mechanical pressure are involved in penetration. Secondly, the mycelia of *C. carbonum* ramify both intercellularly and intracellularly as the fungus infects the chlorenchyma. The hyphae advance from cell to cell in advance of the necrotic lesions (Jennings and Ullstrup, 1957). Cell wall degrading enzymes may be important for growth through the walls of cells as the hyphae advance. Because of the large amount of xylan in maize cell walls, xylanases may be particularly important cell wall degrading enzymes during pathogenesis by *C. carbonum*.

Previous work characterized three major endo-xylanases from culture filtrates of *C. carbonum* grown on media containing xylan or isolated maize cell walls (Holden and Walton, 1992). In order to determine if these xylanases have any role, either as virulence factors or as triggers of plant defense responses, xylanase-deficient mutants were made by cloning the genes encoding xylanases and using transformation-mediated gene disruption to create mutants. The mutants were then inoculated onto the host and the lesion phenotype was observed. This was done for three xylanase genes. The mutants were individually tested for pathogenicity on maize. Additionally, the mutants were crossed to each other to obtain double and triple mutants and their pathogenicity was tested on maize. The expression of the three endo-xylanases was also examined during leaf infection.

CHAPTER 2

MATERIALS AND METHODS

Culture media and growth conditions

Cochliobolus carbonum was maintained and grown as previously described (Walton, 1987). Flask cultures were inoculated with two mycelia plugs each approximately 1 cm^2. For enzyme production, the fungus was grown in still culture on mineral salts medium supplemented with trace elements (Van Hoof *et al.*, 1991), 0.2% yeast extract, and 0.2% sucrose. When added, maize 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. For growth measurements, mycelial mats were harvested, washed briefly in distilled water, frozen, lyophilized to dryness, and weighed.

Mycelia for protoplast preparations or DNA extractions were obtained from germinating conidia. Conidia were collected with 0.1% Tween 20 from fungal cultures grown on V8 juice agar plates. 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. Protoplasts were prepared from the resultant mycelia as described by Panaccione *et al.* (1989).

Nucleic acid manipulations

A genomic library of DNA from *C. carbonum* race 1, isolate SB111, (ATCC 90305) in lambda EMBL3 vector with an average insert size of 15-20 kb (Scott-Craig *et al.*, 1990) was screened with a 32-fold degenerate 17mer oligonucleotide of sequence CAYTTYGAYGCNTGGGC. This sequence was based on an amino acid sequence, HFDAWA, from a tryptic fragment of the purified endo-β1,4-xylanase of *C. carbonum* (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 5X SSPE (1X SSPE = 0.15M 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 ml (Sambrook *et al.*, 1989). Nitrocellulose and Zeta-probe (BioRad, Richmond, CA) membrane blots were washed three times in 2X SSPE and 0.1% SDS at 50°C for 20 min.

A cDNA library constructed from poly A^+ mRNA from *C. carbonum* grown on corn cell walls for 8 days was made in the phagemid vector Uni-Zap XR with the Zap cDNA Synthesis Kit from Stratagene (Pitkin *et al.*, 1996). This library was screened with a sub-cloned fragment of *XYL1* DNA labeled with ^{32}P-dCTP by random prime labeling (Sambrook *et al.*, 1989). The *XYL1* and *XYL2* cDNAs were isolated from this library.

DNA was transferred to Zeta-probe nylon membranes with 0.4 M NaOH; RNA was transferred with 20X SSPE. Nucleic acids were fixed to the

membrane using a UV Stratalinker 1800 (Stratagene) at 1200 mjoule according to the manufacturer's instructions. Routine hybridizations with sub-cloned DNA fragments labeled with ^{32}P-dCTP by random prime labeling (Sambrook *et al.*, 1989) were done in 5X SSPE, 7% (w/v) SDS, 0.5% non-fat dry milk, and 0.1 mg of denatured salmon sperm DNA per ml at 65°C overnight. The blots were washed in 0.1X 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 xylanase expression studies in infected leaves, mRNA was isolated from infected lesions seven days post-inoculation. mRNA was isolated using the mini-oligo (dT) cellulose Spin Column Kit (5'-3' Inc., Boulder, CO) according to the manufacturer's instructions. For the genomic DNA blots, 4 µg of DNA were loaded per lane of a 0.7% agarose gel run for 17 hours at 17 V prior to blotting. For the RNA blots, 10 µg of total RNA or 4 µg of poly A+ mRNA was loaded per lane of a 1.2 % denaturing agarose gel (Sambrook *et al.*, 1989). RNA electrophoresis was done in the presence of formaldehyde at 70 V for 4 hours (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 of *XYL1* were obtained by the dideoxynucleotide method using ^{32}S-dATP (Sanger *et al.*, 1979). Sequencing reactions were performed with a Sequenase Kit (United States Biochemicals, Cleveland, OH) and double-stranded templates according to the manufacturer's instructions. Sequencing reactions were electrophoresed on 8% and 6% denaturing polyacrylamide gels, soaked in 12% acetic acid and 12% methanol, dried, and exposed to X-ray film according to Sambrook *et al.* (1989). When necessary, all restriction site junctions created by sub-cloning 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).

The sequences of both strands of all of the cDNA clones and the *XYL2* and *XYL3* genomic clones were determined by automated fluorescence sequencing by the MSU-DOE-PRL Plant Biochemistry facility using an ABI Catalyst 800 (Foster City, CA) for Taq cycle sequencing and an ABI 373A Sequencer (Foster City, CA) for analysis of the products. Sequence data were analyzed with DNASIS and PROSIS programs (Hitachi Software Engineering Co., San Bruno, CA).

Construction of the *XYL1* disruption vector

The transformation vector was created by first sub-cloning 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.

Construction of the *XYL2* disruption vector

pHYG1 was created by sub-cloning the SalI/HindIII fragment of pUCH1, which contains a gene conferring hygromycin resistance, into pBlueScript SK II. The transformation vector was created by sub-cloning a 532-bp HindII fragment of the *XYL2* gene into the SmaI site of pHYG1, which contains a gene conferring hygromycin resistance. The product of these manipulations (pXLB37-2) was linearized at a unique BamHI site prior to transformation of *C. carbonum* isolate 367-2A.

Construction of the *XYL3* disruption vector

pHYG2 was created by subcloning the SalI/HindIII fragment of pUCH1, which contains a gene conferring hygromycin resistance, into pSP72. The transformation vector was created by sub-cloning a 323-bp KpnI/XbaI fragment of the *XYL3* gene into the KpnI/XbaI fragment of pHYG2. The

product of these manipulations (pXLC42-1) was linearized at the unique KpnI site prior to transformation of *C. carbonum* isolate 367-2A.

Transformation-mediated gene disruption

Protoplasts for transformation were prepared by the method of Yoder (1988) except Driselase (10 mg/ml) (Sigma D-9515) and Novozyme 234 (10 mg/ml) (Novo Laboratories, Wilton, CT) were the only enzymes used. The transformation protocol was derived from those developed for *C. heterostrophus* (Turgeon *et al.*, 1987) and *Aspergillus nidulans* (Oakley *et al.*, 1987). Transformation of *C. carbonum* to hygromycin resistance was as described by Scott-Craig *et al.* (1990).

Analysis of disruption mutants

The transformants were single-spored twice to purify them to nuclear homogeneity. Xylanase was purified from 200 ml of culture filtrate of the wild-type and mutants grown on HMT supplemented with 0.8% maize cell walls for 8 days. The culture filtrate was filtered through cheesecloth and Whatman #1 filter paper and then diluted two-fold with 25 mM sodium acetate buffer, pH 5.0, and loaded onto a column of DEAE-cellulose (D-3764, Sigma) with a bed volume of 33 ml. Following the DEAE-cellulose was a column of CM-cellulose (C-4146, Sigma) with a bed volume of 33 ml. The CM-cellulose was eluted with a 75-ml linear gradient from 25 mM sodium acetate, pH 5.0, to 25 mM sodium acetate plus 0.4 M KCl, pH 5.0. The eluant from the CM-cellulose was dialyzed (Spectrapor 6,000 to 8,000 MW cutoff, 32

mm diameter) overnight in 12.5 mM sodium acetate, pH 5.0. DEAE-cellulose and CM-cellulose chromatography were followed by cation exchange HPLC. Ion exchange HPLC was done on a 200 X 4.6 mm polysulfoethyl aspartamide cation exchange column (The Nest Group, Inc., Southboro, Massachusetts) 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. One ml fractions were collected.

Xylanase activity assay

Xylanase activity was assayed by a reducing sugar assay (Lever, 1972). Thirty µl per HPLC cation exchange fraction or 2 µg of protein from total culture filtrate (protein was measured by the method of Bradford (1976)) was assayed in a 300 µl reaction volume containing 1% oat spelt xylan (Fluka), 50 mM sodium acetate, pH 5.0, at 37°C for 30 min. Twenty five µl aliquots from the reactions were taken at 0 min and 30 min and added to 1.5 ml of p-hydroxybenzoic acid hydrazide working solution which was heated to 100 °C for 10 min prior to measuring the absorption at 410 nm. The p-hydroxybenzoic acid hydrazide working solution was prepared from a 5% (w/v) stock solution in 0.5 N HCl by diluting 1:4 with 0.5 N NaOH. The 0 min sample absorbance was subtracted from the 30 min absorbance and xylose was used as a standard.

Pathogenicity tests

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 harvested from fungal cultures grown on V8 juice agar plates as previously described (Walton, 1987). All plants were inoculated at the 3 to 4 leaf stage and the development of symptoms was visually recorded.

Immunoblot analysis

Twenty ml of culture filtrates of *C. carbonum* grown for 8 days on maize cell walls was passed through a column of DEAE-cellulose chromatography and then concentrated by precipitation with one-tenth volume 100% (w/v) trichloroacetic acid. Eighty μg of protein of each sample was fractionated on a 15% acrylamide SDS-PAGE gel. The gel was blotted to nitrocellulose (0.2μ pore size) and hybridized with anti-serum raised in rabbit against the 22-kDa xylanase of *Trichoderma viride* (Dean *et al.*, 1989) at a 1:1000 dilution. The antibody was a gift from Dr. James Anderson (USDA Beltsville, MD). The protein was visualized by cross reacting with a goat anti-rabbit antibody conjugated with alkaline phosphatase (Cooper Biomedical, Inc., Malvern, PA). Markers correspond to the prestained low molecular weight standards from Gibco-BRL.

Fungal crosses

Fungal crosses were done as previously described (Yoder, 1988; Pitkin *et al.*, 1996).

CHAPTER 3*

CLONING AND TARGETED GENE DISRUPTION OF *XYL1* , A β1,4-XYLANASE GENE FROM THE MAIZE PATHOGEN *COCHLIOBOLUS CARBONUM*

INTRODUCTION

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. Holden and Walton (1992) identified three xylanases, xylanase I, xylanase II, and xylanase III, from the culture filtrate of *C. carbonum*. The protein, xylanase I (Xyl1), associated with the major xylan degrading activity, was purified to homogeneity (Holden and Walton, 1992). This xylanase was shown to be a basic, 22 kDa protein (Holden and Walton, 1992). The protein was digested with trypsin and several peptide fragments were sequenced (Holden and Walton, 1992).

*The majority of these results were originally published in Molecular Plant-Microbe Interactions

Apel, P.C., Panaccione, D.G., Holden, F.R., and Walton, F.D., (1993) Cloning and targeted gene disruption of *XYL1*, a β1,4-Xylanase gene from the maize pathogen *Cochliobolus carbonum*. Mol. Plant-Microbe Interactions 6(4):467-473.

RESULTS

XYL1 isolation and characterization.

Six tryptic peptides were isolated from xylanase I (Xyl1) and sequenced (Table 1) (Holden and Walton, 1992). A completely degenerate 17-mer oligonucleotide coding for the amino acid sequence HFDAWA was used to screen a lambda EMBL3 genomic library from *C. carbonum* (Scott-Craig *et al.*, 1990). The xylanase-encoding sequences were subcloned into pBluescript and pUC18. Figure 1 shows the genomic sequence of *XYL1* 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, identified by sequencing a corresponding cDNA, is indicated by italics in Figure 1. The intron contains two stop codons and a frame shift, as well as 5' G↓GTATGG (consensus G↓GTANGT) and 3' ACAG (consensus ACAG) intron splice sites and an internal CACTAAC (consensus TACTAAC) sequence (Ballance, 1986).

A possible translational start site at nucleotide 226 (Figure 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. Seventeen 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 endo-polygalacturonase from *C.*

Table 1. Comparison of peptide sequences from tryptic digests of xylanase I and the corresponding peptide sequences deduced from the DNA sequence of *XYL1*.

Tryptic Peptide Sequence[a]	Deduced Amino Acid Sequence[b]
ATYTNGAGGSYSVSWGSGGNNV	ATYTNGAGGSYSVSWGSGGNlV
GINPGTAR	GwNPGTAR
LAV(Y)A	LAVYg
NPLVEYYVVENFGTYDP(P/S)SQWQNK	NPLVEYYVVENFGyYDPSSQsQNK
TFQQYWSVR	TFQQYWSVR
THFDAWA(S)(A/K)	THFDAWASK

[a]Amino acids in parentheses indicate uncertainties in the peptide sequencing (Holden and Walton, 1992).
[b]Lower case letters denote mismatches between the protein sequence of the tryptic digests and the deduced amino acids from the DNA sequence (Apel *et al.*, 1993).

carbonum (Scott-Craig *et al.*, 1990) ends in LDAR; *XYL1* contains LVAR starting at amino acid 27 (Figure 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 (Figure 1). There is no apparent polyadenylation consensus sequence (Ballance, 1986). The predicted mature protein encoded by *XYL1* 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).

XYL1 has a high degree of similarity to several prokaryotic and eukaryotic xylanases (Figure 2). Between amino acid 30 and 209 of the *C. carbonum* xylanase, the identity ranges from 38% to 68%. The amino acid

sequence TFXQYWSVR obtained from the enzyme (Holden and Walton, 1992) is one of the most highly conserved motifs in these xylanases (Figure 2).

Transformation-mediated gene disruption.

In order to disrupt *XYL1*, 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-sporing. Five of the single-spored 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; Figure 1) fragments of *XYL1* 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 hybridizing band sizes of 4.8 kb and 8.1 kb, and no band at 6.9 kb, is consistent with homologous integration of a single copy of pCC167 at *XYL1* (Figure 3). The pattern obtained with DNA from transformant T2-8 is consistent with multiple tandem integrations at *XYL1*, as evidenced by bands at 4.8 kb and 8.1 kb and a strong band at 5.7 kb (Figure 3). The *XYL1* mutant T2-8 lacks a 1.0-kb *XYL1* RNA that hybridizes to *XYL1* (Figure 4).

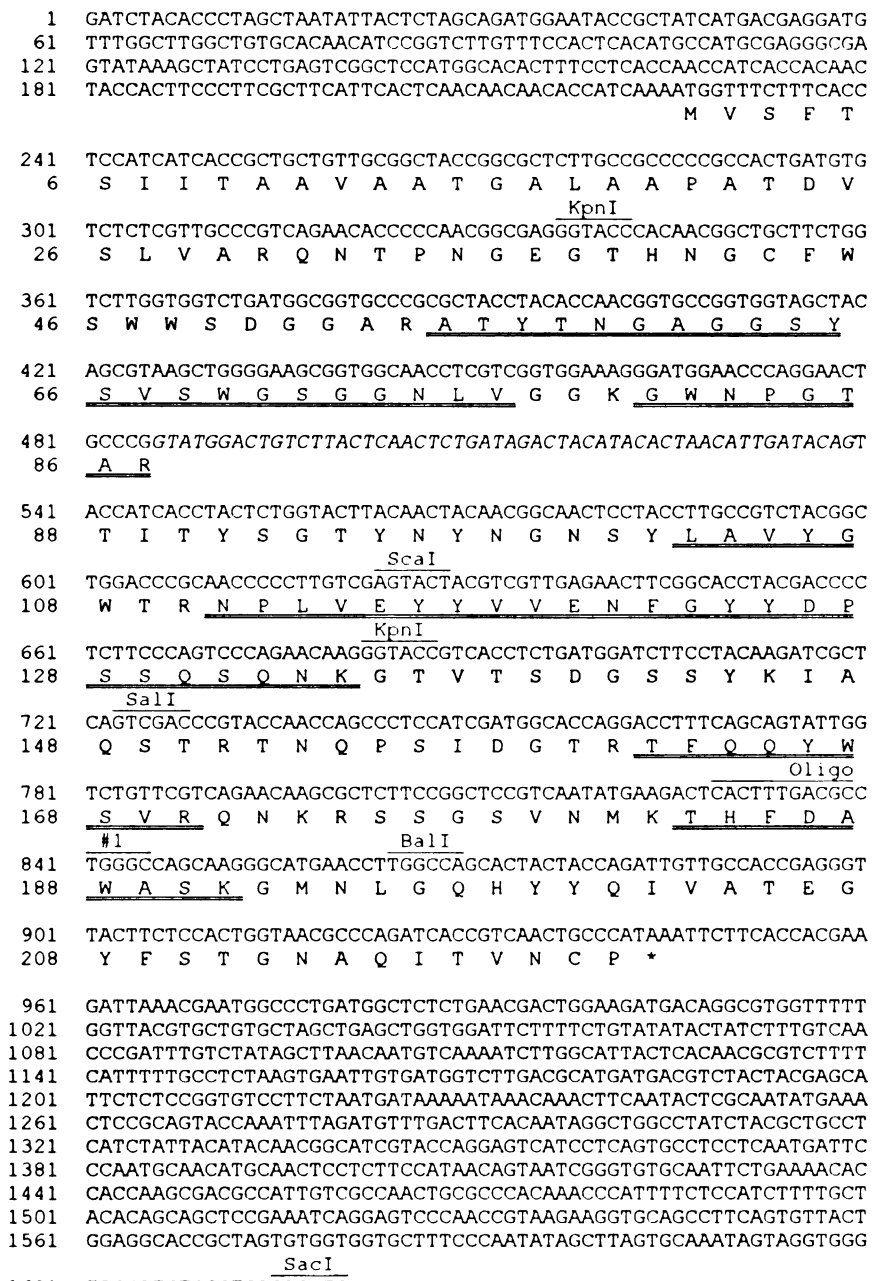

Figure 1. Nucleotide and deduced amino acid sequences of *XYL1*. The double underlined regions correspond to the sequences of tryptic fragments obtained from the purified protein (Table 1). The overlined region indicates the location of the oligonucleotide used to clone the gene. The italicized sequence indicates the intron. The endonuclease restriction sites KpnI, ScaI, SalI, BalI, and SacI are indicated.

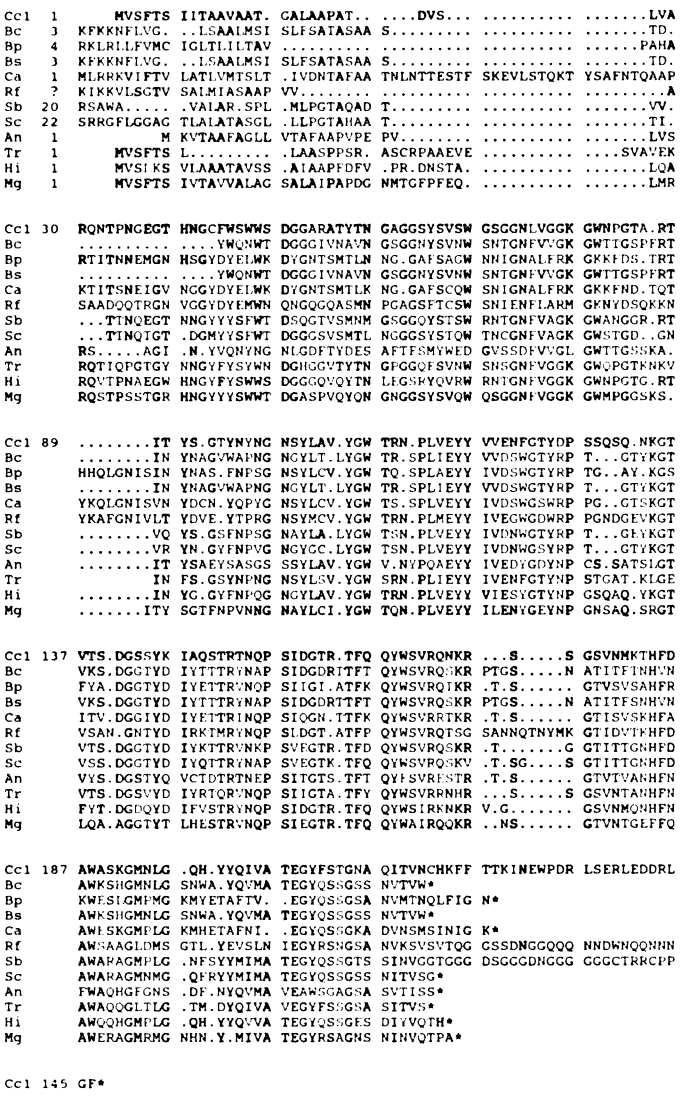

Figure 2. Comparison of bacterial and fungal xylanases to *XYL1* from *C. carbonum*. Shading denotes identity between *XYL1* and at least one other xylanase. Cc=*Cochliobolus carbonum*; Bc=*Bacillus circulans* (GenBank accession number X07723), Bp=*B. pumilus* (X00660), Bs=*B. subtilis* (M36648), Ca=*Clostridium acetobutylicum* (M31726), Rf=*Ruminococcus flavefaciens* (Z11127), Sb=*Streptomyces lividans* xylanase B (M64552), Sc=*S. lividans* xylanase C (M64553), An=*Aspergillus niger* (A19535), Tr=*Trichoderma reesei* xylanase I(S51973), Hi=*Humicola insolens* (X76047), and Mg=*Magnaporthe grisea* (L37529).

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.

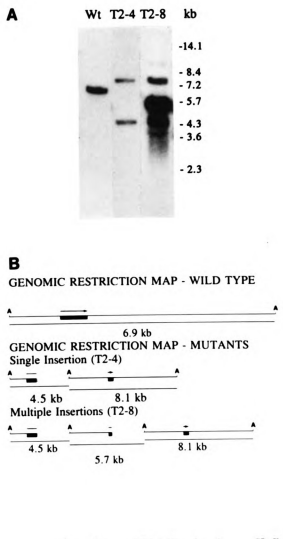

Figure 3. DNA blot analysis of wild type and disruption mutants.

A. Genomic DNA from *C. carbonum* wild type (Wt) and the putative *XYL1* disruption mutants T2-4 and T2-8 was digested with ApaI, fractionated, blotted, and hybridized with the KpnI/KpnI and KpnI/SacI fragments of *XYL1*.

B. Predicted restriction map. The black shading indicates *XYL1* 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 *XYL1*. A=ApaI endonuclease restriction sites.

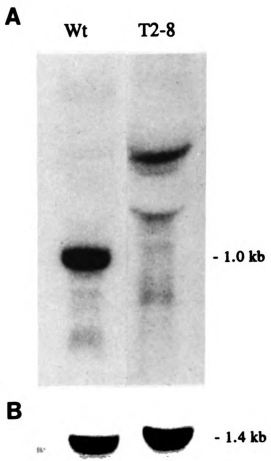

Figure 4. RNA blot of wild type and disruption mutant T2-8.

A. Approximately 30μg total RNA, isolated from fungal mats of the wild type and T2-8 grown for 6 days on maize cell walls, was fractionated on a 1.2% agarose gel containing formaldehyde 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* (Figure 1).

B. The blot was stripped and rehybridized with the glyceraldehyde-3-phosphate dehydrogenase gene.

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* (Figure 4B).

Growth and pathogenicity of a *XYL1* 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 maize cell walls, oat spelt xylan, or sucrose as the sole carbon source. There was no significant difference in growth of the wild type and the disruption mutant T2-8 when grown on the same substrate (Figure 5A).

Culture filtrates from the growth studies in Figure 5A were assayed for xylanase activity. When grown on maize cell walls for 8-16 days, the mutant had a 85%-94% reduction in extracellular xylanase activity compared to the wild type (Figure 5B). Xylanase activity in mutant T2-4 was decreased by a similar amount (data not shown).

Xylanase activities from mutant T2-8 and wild type were purified in parallel through cation exchange HPLC (Holden and Walton, 1992). The *XYL1* mutant specifically lacked the major peak of UV absorption, corresponding to the major xylanase, xylanase I, in fraction 7 (Figure 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 *XYL1* expression on the expression of xylanase II, we

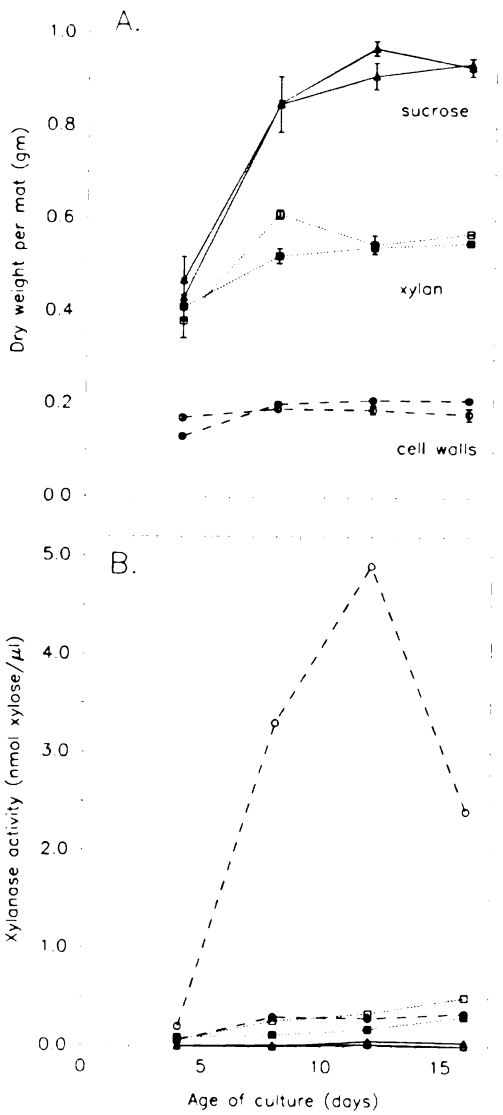

Figure 5. Growth and xylan-degrading activity in wild-type and mutant T2-8 strains.

A. Dry weights of fungal mats.

B. Xylanase activity in the culture filtrates from the same experiment. Δ = Wild type grown on sucrose; ▲ = Mutant grown on sucrose; □ = Wild type grown on oat spelt xylan; ■ = Mutant grown on oat spelt xylan; o = Wild type grown on maize cell walls; ● = Mutant grown on maize cell walls. All treatments were done in duplicate and the range was less than 10% of the average.

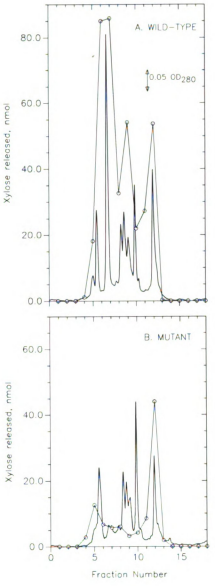

Figure 6. Cation exchange HPLC analysis of extracellular xylanase activity in the wild type and mutant. One mg of total protein (Bradford, 1976) from partially purified culture filtrate of each was loaded on the column. The solid line is the absorbance at 280nm and circles are enzyme activity. The peaks 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.

conclude that xylanases I and II are both encoded by *XYL1*, 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 seven days after inoculation (Figure 7).

DISCUSSION

XYL1, the gene for the major xylan-degrading enzyme in *C. carbonum*, was cloned and sequenced. Transformation-mediated gene disruption created a mutant specifically lacking a functional copy of this gene, as shown by DNA and RNA blotting and biochemical characterization.

XYL1 appears to encode two forms of endo-xylanase 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 maize cell walls (Figure 5B). However, growth of the mutant strain was indistinguishable from the wild type in media containing maize 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 dispensable for pathogenicity on maize. Whether xylan degradation is important or not in pathogenicity is still an open question, however, due to the low but significant amount of xylanase remaining when *XYL1* is disrupted (Figure 5).

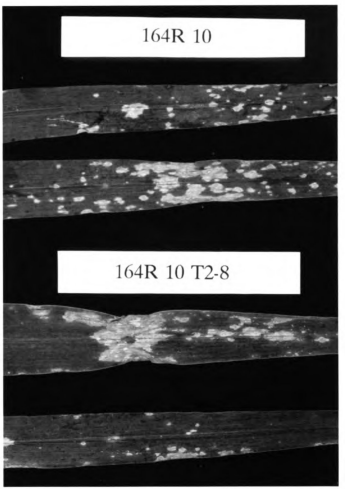

Figure 7. Pathogenicity test of xylanase mutant T2-8. Maize leaves were inoculated with 10^4 spores per ml of the wild-type strain 164R10 and the disruption mutant T2-8. Photograph was taken seven days after inoculation.

Some studies allude to the possible importance in pathogenicity of enzymes such as α-arabinosidase and arabinanase that can at least partially degrade native plant xylans (Howell, 1975; Cooper *et al.*, 1988). The oat spelt xylan used in our growth studies and enzyme assays to contains 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 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, Xyl1, 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 β1,4-linked xylose.

CHAPTER 4

CLONING AND TARGETED GENE DISRUPTION OF *XYL2* AND *XYL3*

INTRODUCTION

Cochliobolus carbonum makes three extracellular xylanases in culture (Holden and Walton, 1992; Apel *et al.*, 1993). *XYL1*, the gene encoding the major xylan degrading activity, was cloned and disrupted in order to test its involvement in pathogenicity (Apel *et al.*, 1993). *XYL1* is not required for pathogenicity nor does it contribute to virulence of *Cochliobolus carbonum* on maize. However, xylanase may still be important for *C. carbonum* infection of maize because the *XYL1* mutant still has xylan degrading activity (Apel *et al.*, 1993). The purpose of this work was to clone and disrupt two additional xylanase genes that are related to *XYL1*.

RESULTS

XYL2 and *XYL3* isolation

XYL2 was cloned using the *XYL1* genomic clone to screen a cDNA library constructed from mRNA from *C. carbonum* race 1 (isolate SB111) grown on maize cell walls for 8 days. A genomic clone for *XYL2* was obtained by screening a lambda EMBL3 genomic DNA library with the *XYL2* cDNA.

A *XYL3* genomic clone was obtained by screening the EMBL3 genomic library with the same 17-mer oligonucleotide that was used to clone the genomic *XYL1* clone.

Figure 8 shows the nucleotide sequence and the deduced amino acid sequence of *XYL2*. The region identified by the oligonucleotide is overlined. There are two introns, of 53-bp and 56-bp, based on a comparison of the genomic DNA and cDNA sequences of *XYL2*. The first intron contains five stop codons and a frame shift as well as a 5' G↓GTAGGT (consensus G↓GTANGT), 3' GTAG (consensus ACAG) intron splice sites and an internal TACTAAT (consensus TACTAAC) sequence (Ballance, 1986). The second intron has six stop codons and a frame shift as well as 5' G↓GTAAGT (consensus G↓GTANGT), 3' ATAG consensus ACAG) intron splice sites. A possible translational start site (Figure 8) has a consensus context of CAAGATGGT (consensus CAMMATGNC, where M = A or C)(Ballance, 1991). Twenty two of the first 40 amino acids including the putative start methionine are hydrophobic amino acid residues (Met, Leu, Phe, Val, Ile, and Ala). Based on similarity to *XYL1* (Apel *et al.*, 1993) and the known signal peptide of endo-polygalacturonase from *C. carbonum* (Scott-Craig *et al.*, 1990) the mature xylanase protein probably begins at amino acid 41 (Figure 8). There is one potential glycosylation site at amino acid 32 (Figure 8).

The DNA and deduced amino acid sequences of *XYL3* are shown in Figure 9. *XYL3* has a putative intron of 60 bp that occurs in the same

```
   1    CCCGGGTAGAGCGATGTTCTTTTAATTATGTTATGCAGATTTACGCAAGGGCTACAGATC
  60    TTTTTGATCACCCCGTCGTGGCCGTTGCTACCGAAACTGGATGCCGATCGGTGCATGTCC
 121    CCTATGTGTCTCGACTTGGTCATTGTGGCCGCCGCTTAGGGTGAGCTTGTCGAAGTGAGA
 181    GGCCTTAACCTACCACCAATGTGTACACCATCACGAATAGAGACAGCACGCGAGATGAGA
 241    CCTCATGACCACCAGTGTCATTTCATCCTGCGATTCAGGCTCAACTATTACTCGGCGTAG
 301    AGATATAAGTCGATAGCTGCTCCCAGTCAGTAGCATCACTGCATCCAACACAGATCAGCA
 361    ACACTCAACACAAGATGGTTTCCTTCAAGTCTCTGCTTCTCGCCGCTGTGGCTACCACCA
                    M  V  S  F  K  S  L  L  L  A  A  V  A  T  T  S

 421    GCGTCCTCGCTGCTCCCTTCGATTTCCTTCGTGAGCGCGACGATGTCAACGCGACTGCTC
  17      V  L  A  A  P  F  D  F  L  R  E  R  D  D  V  N  A  T  A  L

 481    TCCTTGAGAAGCGTCAGTCTACTCCCAGCGCCGAGGGATACCACAATGGATACTTCTACT
  37      L  E  K  R  Q  S  T  P  S  A  E  G  Y  H  N  G  Y  F  Y  S

 541    CGTGGTGGACTGATGGCGGTGGCTCTGCCCAGTACACTATGGGTGAGGGCAGCAGGTACT
  57      W  W  T  D  G  G  G  S  A  Q  Y  T  M  G  E  G  S  R  Y  S

 601    CTGTGACCTGGAGGAACACTGGCAACTTTGTTGGTGGAAAGGGGTGGAACCCTGGAAGCG
  77      V  T  W  R  N  T  G  N  F  V  G  G  K  G  W  N  P  G  S  G

 661    GCCG*GTAGGTTGCGAAGACTGTTTGGTGATGAGAATCTTACTAATGTGGATTCGTAG*TGT
  97      R                                                       V

 721    CATCAACTACGGCGGAGCCTTCAACCCCCAGGGCAACGGATACCTCGCTGTGTACGGATG
  99      I  N  Y  G  G  A  F  N  P  Q  G  N  G  Y  L  A  V  Y  G  W

 781    GACCCGCAACCCGCTTGTCGAGTACTACGTGATTGAATCCTACGGAACCTACAACCCCAG
 119      T  R  N  P  L  V  E  Y  Y  V  I  E  S  Y  G  T  Y  N  P  S

 841    CAGTGGAGCCCAAATCAAGGGCAGCTTCCAGACCGACGGTGGTACTTACAACGTTGCCGT
 139      S  G  A  Q  I  K  G  S  F  Q  T  D  G  G  T  Y  N  V  A  V

 901    CTCCACCCGTTACAACCAGCCCTCCATTGACGGAACAAGGACCTTTCAGCAGTACTG*GTA*
 159      S  T  R  Y  N  Q  P  S  I  D  G  T  R  T  F  Q  Q  Y  W

 961    *AGTCATTTCGGTGTTTAATCAGACAGAAGCGTAGATGTTGACTGTTATGATAG*GTCTGTC
 178                                                          S  V

1021    CGCACCCAGAAGCGTGTCGGTGGAAGCGTGAACATGCAGAACCACTTCAACGCTTGGTCT
 180    R  T  Q  K  R  V  G  G  S  V  N  M  Q  N  H  F  N  A  W  S

1081    CGCTATGGCTTGAACCTTGGTCAACACTACTACCAGATCGTCGCCACTGAGGGTTACCAG
 200    R  Y  G  L  N  L  G  Q  H  Y  Y  Q  I  V  A  T  E  G  Y  Q

1141    TCCTCTGGAAGCTCTGACATCTATGTGCAGACTCAGTAGAGTAGTTGTTGTCTGTGAAGC
 220    S  S  G  S  S  D  I  Y  V  Q  T  Q  *

1201    GAGCAACTAGTAGACGAGATTAGACAAGAATAGTGTCTCGGATAGTTGTAAGCAGGTTGG
1261    AGAAAGAGCGATGTGTTCGATTTCCTGTACATAGAAACATGTCAATTCACTCGCTATAAA
1321    ACCTTCCGTCCTCGAATGTGTTCTCTTTTGTATTAGCTGAGCATTGTATCGTGGTTCGGC
1381    AAAAGCAGGTAAAGTTTAGGTGAGGGCATTTGTTATAAACAGTCATAGTCTCGTAGCCAT
1441    CGAATGCTGGTGTGTGAATTC
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Figure 8. Nucleotide and deduced amino acid sequences of *XYL2*. The italicized sequences indicate the introns.

position as the intron of *XYL1* and the first intron of *XYL2*. The *XYL3* putative intron contains three termination codons as well as 5' G↓GTATGC (consensus G↓GTANGT), 3' ACCG (consensus ACAG) intron splice sites and an internal TACTAAC (consensus TACTAAC) sequence (Ballance, 1986). A possible translational start site (Figure 9) has a consensus context of CAAAATGGT (consensus CAMMATGNC, where M = A or C)(Ballance, 1991). Twenty two of the first 39 amino acids including the putative start methionine are hydrophobic amino acid residues (Met, Leu, Phe, Val, Ile, and Ala). Based on similarity to *XYL1* (Apel *et al.*, 1993) and the known signal peptide of endo-polygalacturonase from *C. carbonum* (Scott-Craig *et al.*, 1990) the mature Xyl3 protein probably begins at amino acid 40 (Figure 9).

Comparison of the three xylanases

In order to be certain that the three xylanase genes could be identified individually, a Southern blot of genomic DNA digested with HindIII was sequentially hybridized with *XYL1*, *XYL2*, and *XYL3*. Figure 10 shows that the three genes can be identified from each other on genomic Southern blots despite their high degree of similarity to each other (Figure 11). Xyl1 is 75% similar and 60% identical to Xyl2. Xyl1 is 60% similar and 42% identical to Xyl3. Xyl2 is 59% similar and 39% identical to Xyl3.

Transformation-mediated gene disruption of *XYL2* and *XYL3*

Transformation-mediated gene disruption was used to create *XYL2* and *XYL3* mutants. The linearized transformation plasmid, pXLB37-2, was

```
   1  TGCATGACTGGACCACTCAGCGCGCGGCTTCCCCACAAAAGTCTATATGAAGTATCCACC
  60  GCCGTGATGGAGCTTGATTAACTGTCGGGATGGCGACTTGGCACGCGCCTTGAAATGCAG
 121  GATAGCATCACGCCTAGGGGCAGGGCCGATTGCGCTCATTGGATACGAATACATCAAGAC
 181  ACTAGTGTAGAACGATTCAGACGATTCATCCATGGGCAACGAGGGGCGAGAGGTGGCCCC
 241  ATGACACAGGGAGGAAGACAGCGGGTGAAACAACCGCAATATGTTTCCTGTGGTGTGCAC
 301  CCACGCTCTTTAACCGTCTCTAAGTATATAGGTCAAGACTTCCAAGACCGAATAAGCTCA
 361  CCACCAAGCCTTTCATCTTCAACACTCAAGCAGTCTTTACTTCACTCACCCTCTTTTCAA
 421  TTCACTCCTAGCGAGAACCAGCTTCTTTTCACACAAACACAAACAAATCTTCAATCTATC
 481  AAAATGGTTGCCTTCACCTCCGTCCTCCTCGGCCTCTCCGCCATTGGCTCTGCCTTCGCC
        M  V  A  F  T  S  V  L  L  G  L  S  A  I  G  S  A  F  A

 541  GCCCCCGTTGCCGATGTCCCTGACTTCGAGTTCTCCGGCCCCAAGCACTTGGCTGCCCGC
  20  A  P  V  A  D  V  P  D  F  E  F  S  G  P  K  H  L  A  A  R

 601  CAGGACTACAACCAGAACTACAAGACTGGTGGTAACATCCAGTACAACCCCACCAGCAAC
  40  Q  D  Y  N  Q  N  Y  K  T  G  G  N  I  Q  Y  N  P  T  S  N

 661  GGTTACTCCGTCACCTTCTCTGGCGCCCAGGACTTCGTCCTTGGCAAGGGCTGGAAGCAA
  60  G  Y  S  V  T  F  S  G  A  Q  D  F  V  L  G  K  G  W  K  Q

 721  GGTACTACCAGG*TAAGCCACACACAGTGTTCTAGACCCAAATATCAATCAATACTAACTC*
  80  G  T  T  R

 781  *CTTTCCTTCAGG*ACCGTCAAGTACACTGGTTCCACCCAGGCCCAGGCCGGTACTGTCCTC
  84              T  V  K  Y  T  G  S  T  Q  A  Q  A  G  T  V  L

 841  GTCGCTCTCTACGGCTGGACCAGGGGCAGCAAGCTCGTCGAGTACTACATCCAGGACTTC
 100  V  A  L  Y  G  W  T  R  G  S  K  L  V  E  Y  Y  I  Q  D  F

 901  ACCTCTGGCGGCTCTGGCTCCGCCCAGGGCCAGAAGATGGGCCAAGTCACCTGCGACGGC
 120  T  S  G  G  S  G  S  A  Q  G  Q  K  M  G  Q  V  T  C  D  G

 961  TCCGTCTACGACATCTGGCAGCACACCCAGGTGAACCAGCCTTCCATCGTCGGCACGACC
 140  S  V  Y  D  I  W  Q  H  T  Q  V  N  Q  P  S  I  V  G  T  T

1021  ACCTTCGTCCAGTACATCAGCAACCGCGTCAGCAAGCGCTCCACCGGCGGTACCATCACC
 160  T  F  V  Q  Y  I  S  N  R  V  S  K  R  S  T  G  G  T  I  T
                 Oligo #1
1081  ACCAAGTGCCACTTCGACGCCTGGGCCAAGCTCGGCATGAACCTTGGTAACCAGTGGGAC
 180  T  K  C  H  F  D  A  W  A  K  L  G  M  N  L  G  N  Q  W  D

1141  TACCAGACCATCTCCACTGAGGGTTGGGGCAACGCTGCTGGAAAGTCCCAGTACACCGTC
 200  Y  Q  T  I  S  T  E  G  W  G  N  A  A  G  K  S  Q  Y  T  V

1201  TCCGCTGCTTAAATTGTGGGCGCGGTCCGTTCTTTGGCTCAAGGGGAATGAACCAGGGGT
 220  S  A  A  *

1261  TCGAGAGATTTGTGAAACATTGGCTTGCGCAATCATCAATTCCTCTCCAGAGGAGAGGAA
1321  ATATGATAGCTTCAAGGGCTTTGTAGGGGTGTTGATTTAGGGGATGCTGCCGCTGCTGTT
1381  GCTTGATTCATTGGTGTTTATCTTTCTTTCTTCTTGATTTGTTCAGGGCCTTGACACACC
1441  CTGGCTTGTACATACTTTTTTTACTCGTTTCTTGTAGAAACGGGTAAATCCTGAATATAC
1501  ACGTTGCCTTGTTTCTCCCTCGTTTGGATGACAATAGCAATTGCCCGGTTATGAACCGAA
1561  AAAAAAACTCCTCATCTTCTTCTGTGTTTCTTATCATTGTGACAAGTGACTTTTTTTTCT
1621  TTTCTTACTTAAATTTATTTTTCTAGGTTTTCACAAAAGTATATATATTCTTAGCCATGC
1681  TGGCTGCCCCATCTTACTTAGTAAACAGGTGAAACTTCCAATTATCGCTGCCAAACGCAG
1741  GCTCGGATGTCGGCGGCGGCGCGTGGATGGAGATACATAACATTGTGTTGCTTAAAACAA
1801  GGCTAGGTTGGCTGGGCTGGGGCTGAACAGCCGGTGGTAGGATGATTTATCGGGCAAATA
1861  TCAAGACACACACACATCTTCTTAAAACGCCCTTTTGGTGGCGAAGGTTCTTAACCAAGT
1921  GGGTACCCGGGGGATCCACTAGTTCTAGA
```

Figure 9. Nucleotide and deduced amino acid sequences of *XYL3*. The overlined region indicates the location of the oligonucleotide used to clone *XYL1* and *XYL3*. The italicized sequence indicates the putative intron.

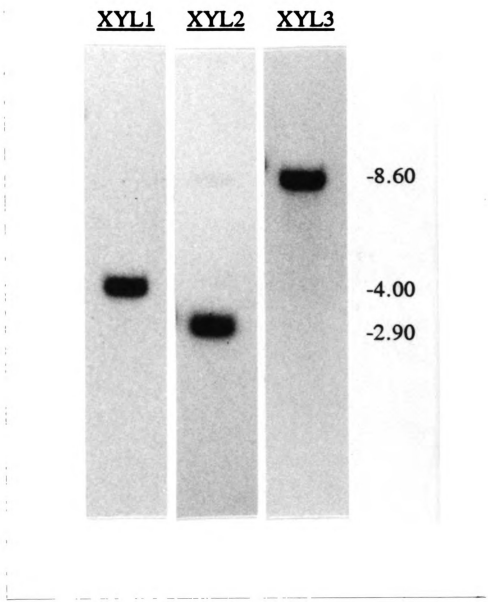

Figure 10. Genomic Southern of *XYL1*, *XYL2* and *XYL3*.
Genomic DNA from *C. carbonum* wild type was digested with HindIII, fractionated, blotted, and hybridized with the cDNA of *XYL1*, stripped and hybridized with the cDNA of *XYL2*, and then stripped and hybridized with the KpnI/XbaI fragment of *XYL3*.

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

        Oligo1
XYL1    THFDAWASKG MNLG.QHYYQ IVATEGYFST .GNAQITVNC HKFFTTKINE
         ::.::.  : .::: ::::: ::::::: :.  : ..: :.
XYL2    NHFNAWSRYG LNLG.QHYYQ IVATEGYQSS .GSSDIYVQT Q*
         ::::::  : :::: :  ::  ..:::.  .  : .: ::
XYL3    CHFDAWAKLG MNLGNQWDYQ TISTEGWGNA AGKSQYTVSA A*

XYL1    WPDRLSERLE DDRLGF*
```

Figure 11. Comparison of the deduced amino acid sequences of three *C. carbonum* xylanases. Double point marks indicate identity of *XYL2* or *XYL3* to *XYL1*. Single point marks indicate similarity.

used to disrupt *XYL2*. The isolates were analyzed by digestion of their DNA with BamHI and blotting. Two out of five transformants were single insertion mutants. The remaining three transformants had ectopic integration of the vector. Based on the hybridization pattern with a *XYL2* cDNA, transformant T446-17A is a single insertion mutant as seen in Figure 12.

The linearized transformation plasmid, pXLC42-1, was used to disrupt *XYL3*. The isolates were analyzed by digestion of their DNA with BamHI and blotting. The pattern of hybridization in T448-4A with the XbaI/KpnI fragment of *XYL3* to a band of 16.3 kb and no band at 11 kb is consistent with homologous recombination of a single copy of pXLC42-1 at *XYL3* (Figure 13).

Creation of a double *XYL1/XYL2* mutant

Strain 312-6A (*XYL1*$^-$/*XYL2*$^+$) was crossed with 367-1a (*XYL1*$^+$/*XYL2*$^+$) to obtain a strain, 447-8a (*XYL1*$^-$/*XYL2*$^+$), with the *XYL1* mutation in a strain of mating type "a". Strain 447-8a was then crossed with the *XYL2* mutant to obtain the double mutants 477-3a and 477-7a (*XYL1*$^-$/*XYL2*$^-$) (Table 2).

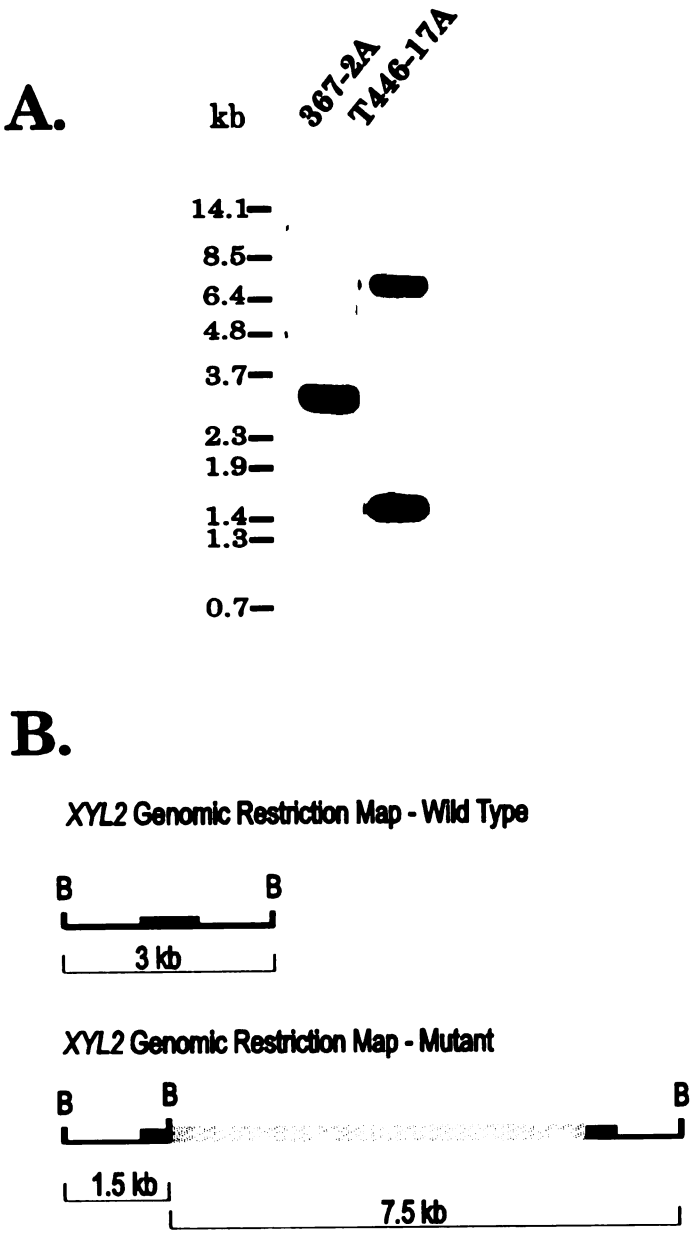

Figure 12. DNA blot analysis of wild-type and *XYL2* disruption mutant.

A. Genomic DNA from *Cochliobolus carbonum* wild type (367-2A) and the *XYL2* mutant (T446-17A) was digested with BamHI, fractionated, blotted, and hybridized with the *XYL2* cDNA.

B. Predicted restriction map. The black shading indicates *XYL2* and the lightly shaded box denote the disruption vector. The hybridization seen in part A is consistent with the predicted single integration pattern. B=BamHI endonuclease restriction sites.

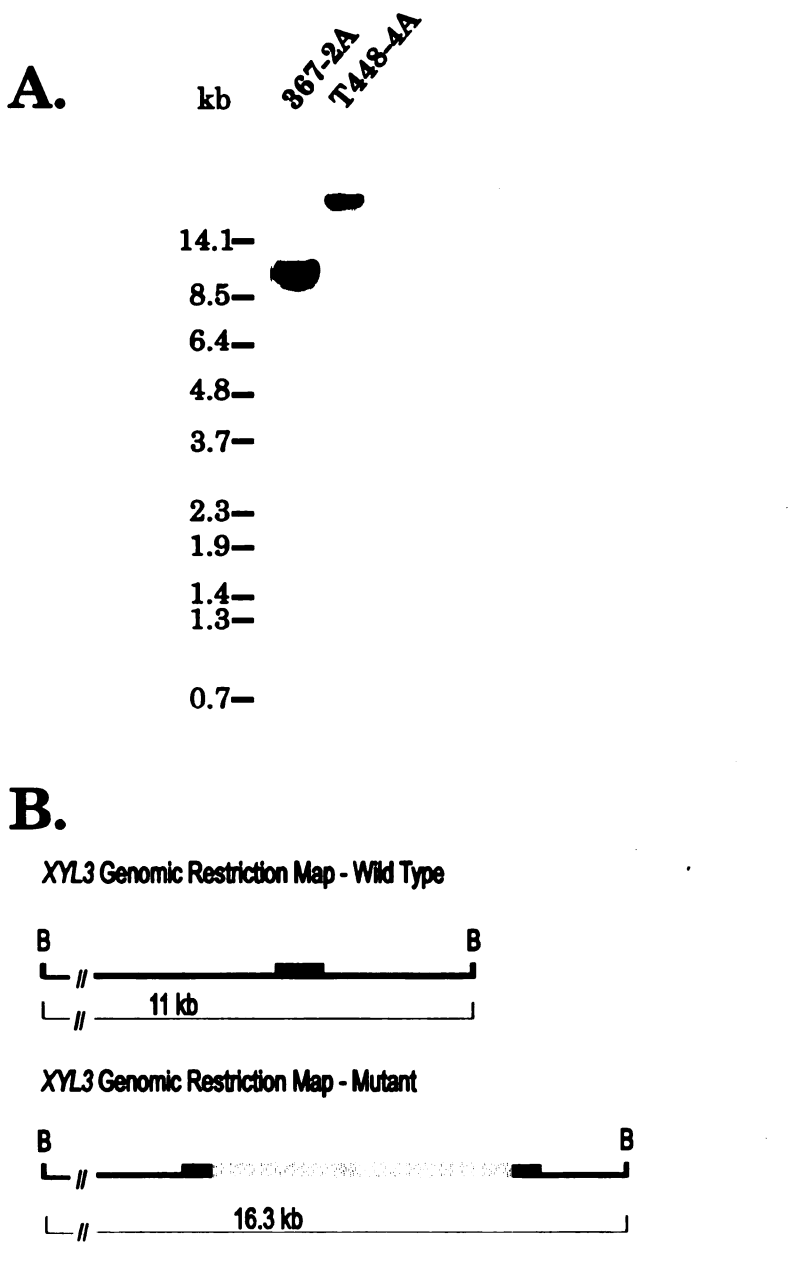

Figure 13. DNA blot analysis of wild-type and *XYL3* disruption mutant.

A. Genomic DNA from *Cochliobolus carbonum* wild type (367-2A) and the *XYL3* mutant (T448-4A) was digested with BamHI, fractionated, blotted, and hybridized with the *XYL3* KpnI/XbaI fragment.

B. Predicted restriction map. The black shading indicates *XYL3* and the lightly shaded box denote the disruption vector. The hybridization seen in part A is consistent with the predicted single integration pattern. B=BamHI endonuclease restriction sites.

Table 2. *C. carbonum* strains.

Strain #	Transformed Strain	Cross Parents	*XYL* Genotype	Antibiotic Resistance
367-2A			*XYL1*$^+$, *XYL2*$^+$, *XYL3*$^+$	
164R10			*XYL1*$^+$, *XYL2*$^+$, *XYL3*$^+$	
164R10 T2-4	164R10		*XYL1*$^-$, *XYL2*$^+$, *XYL3*$^+$	HYG
164R10 T2-8	164R10		*XYL1*$^-$, *XYL2*$^+$, *XYL3*$^+$	HYG
T216-4a	164R10 T2-4		*XYL1*$^-$, *XYL2*$^+$, *XYL3*$^+$	HYG
312-6A		T216-4a X 164R17A	*XYL1*$^-$, *XYL2*$^+$, *XYL3*$^+$	HYG
T446-17A	367-2A		*XYL1*$^+$, *XYL2*$^-$, *XYL3*$^+$	HYG
447-8a		312-6A X 367-1a	*XYL1*$^-$, *XYL2*$^+$, *XYL3*$^+$	HYG
477-3a		T446-17A X 447-8a	*XYL1*$^-$, *XYL2*$^-$, *XYL3*$^+$	HYG
477-7a		T446-17A X 447-8a	*XYL1*$^-$, *XYL2*$^-$, *XYL3*$^+$	HYG
T448-4A	367-2A		*XYL1*$^+$, *XYL2*$^+$, *XYL3*$^-$	HYG
556-11		T448-4A X 477-3a	*XYL1*$^-$, *XYL2*$^+$, *XYL3*$^+$	HYG
556-8		T448-4A X 477-3a	*XYL1*$^+$, *XYL2*$^-$, *XYL3*$^+$	HYG
556-1		T448-4A X 477-3a	*XYL1*$^+$, *XYL2*$^+$, *XYL3*$^-$	HYG
556-16		T448-4A X 477-3a	*XYL1*$^-$, *XYL2*$^-$, *XYL3*$^+$	HYG
556-4		T448-4A X 477-3a	*XYL1*$^-$, *XYL2*$^+$, *XYL3*$^-$	HYG
556-12		T448-4A X 477-3a	*XYL1*$^+$, *XYL2*$^-$, *XYL3*$^-$	HYG
556-2		T448-4A X 477-3a	*XYL1*$^+$, *XYL2*$^+$, *XYL3*$^+$	
556-13		T448-4A X 477-3a	*XYL1*$^-$, *XYL2*$^-$, *XYL3*$^-$	HYG

Growth and pathogenicity of the *XYL2* mutant, *XYL3* mutant, and the *XYL1/XYL2* double mutant

The wild-type and *XYL2* mutant were grown on mineral salts medium supplemented with trace elements and yeast extract and either maize cell walls or oat spelt xylan as the sole carbon source. There was no significant difference in growth between the wild type and the disruption mutant. Culture filtrates from the growth studies were assayed for xylanase activity, and no difference was seen between the wild type and the *XYL2* disruption mutant.

The *XYL3* mutant also did not show any visible difference in growth on xylan or maize cell walls when compared to the wild type. When compared to the wild type, the *XYL3* mutant did not have any loss of xylan degrading activity. This was expected since *XYL3* mRNA is not present when *C. carbonum* is grown in culture.

Xylanase activities from the wild type, the *XYL1* mutant, the *XYL2* mutant, and the *XYL1/XYL2* double mutant were purified in parallel through cation exchange HPLC (Apel *et al.*, 1993). There were no reproducible differences in the protein profiles of 367-2A and T447-17A (Figure 14). Xylanase activity was assayed in each fraction from the cation exchange HPLC. As shown in Figure 15, the wild-type gives the characteristic two peaks of xylan degrading activity in fractions 21 and 27. The second peak corresponding to xylanase II, which is missing in this sample, is encoded by *XYL1* and is unstable (Apel *et al.*, 1993). The *XYL1* mutant lacks the two peaks of activity in fraction 21 and 23 which correspond to xylanase I and xylanase II, as found previously (Apel *et al.*, 1993). The *XYL2* mutant has all three peaks of xylanase activity and does not appear to be missing any peaks of xylan degrading activity. The *XYL1/XYL2* double mutant is missing two peaks of activity in fractions 21 and 23, like the *XYL1* mutant. Because the third peak, called xylanase III (Holden and Walton, 1992), in fraction 27 does not disappear in the *XYL1*, *XYL2*, or *XYL3* mutants, it is probably encoded by a different gene.

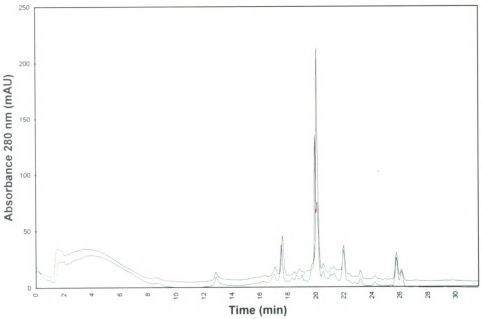

Figure 14. HPLC protein profiles of the wild type and *XYL2* mutant. Four milligrams of total protein (Bradford, 1976) from partially purified culture filtrate were loaded onto a cation exchange HPLC column. The solid lines are absorbance profiles at 280 nm. The bottom line corresponds to the wild type (367-2A) and the top line corresponds to the *XYL2* mutant (T447-17A).

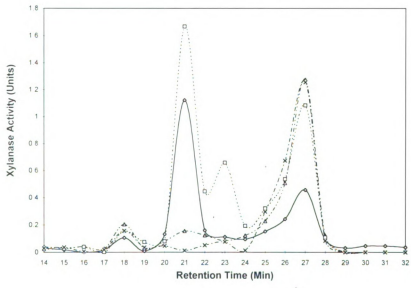

Figure 15. Xylanase activity from cation exchange HPLC of the *XYL2* mutant. The peaks of xylanase I, II, and III appear in fractions 21, 23, and 27 respectively. Xylanase I and II are encoded by *XYL1*.

Because the cation exchange HPLC protein profile for the *XYL2* mutant did not appear to lack a protein peak when compared to the wild type, an immunoblot of culture filtrate was done to see if any Xyl2 protein was produced. When the immunoblot was reacted with anti-serum to a 22-kDa xylanase from *Trichoderma viride* (gift of Dr. James Anderson, USDA, Beltsville, MD) many bands are seen but a 22-kDa band is the most prominent (Figure 16). This 22-kDa band is missing in the *XYL1* mutant and therefore we conclude that it is Xyl1. A faint 22-kDa band is present in the *XYL1* mutant. This faint band is seen in the $XYL1^{-}/XYL2^{+}$ mutant but not the $XYL1^{-}/XYL2^{-}$ double mutant (Figure 16). Therefore, it appears that *XYL2* is expressed at the protein level but that Xyl2 does not accumulate to levels as high as Xyl1. The protein encoded by *XYL2* may not be active or its activity may not be detected by the reducing sugar assay.

The rate of development, size, and morphology of the lesions produced by the wild-type, *XYL1* mutant, *XYL2* mutant, and *XYL1/XYL2* double mutant appear to be the same (Figure 17). Pathogenicity of the *XYL3* mutant was also tested on susceptible maize and no differences were seen in lesion size, rate of development, or morphology.

mRNA expression in culture and in infected leaves

The mRNA expression of *XYL1*, *XYL2*, and *XYL3* was studied in culture and in infected plant leaves. *XYL2* RNA is present in the wild type as well as in the *XYL1* mutant strain (Figure 18). The same RNA blot was

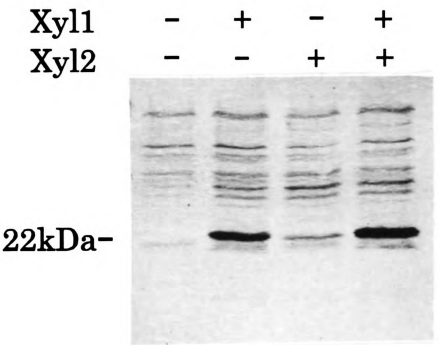

Figure 16. Immunoblot of concentrated culture filtrate of the wild-type and the *XYL1* mutant, *XYL2* mutant, and *XYL1/XYL2* double mutant. Partially purified and concentrated culture filtrate of *Cochliobolus carbonum* was loaded in each lane. The blot was hybridized with the anti-serum raised against a 22 kDa xylanase from *Trichoderma viride* (gift from Dr. James Anderson, USDA Beltsville, MD).

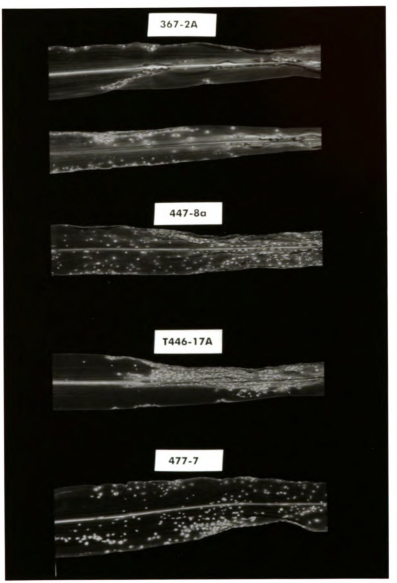

Figure 17. Pathogenicity test of xylanase mutants 447-8a ($XYL1^-$/$XYL2^+$), T446-17A ($XYL1^+$/$XYL2^-$), and 477-7a ($XYL1^-$/$XYL2^-$) and wild type strain 367-2A ($XYL1^+$/$XYL2^+$). Maize leaves were inoculated with 10^4 spores per milliliter. Photograph was taken 5 days after inoculation.

hybridized with *XYL3* and no signal was present. To ensure that the blot was intact, the blot was then hybridized with the glyceraldehyde-3-phosphate dehydrogenase gene from *C. heterostrophus*. Each lane of the gel was loaded with the same amount of RNA but the expression levels of glyceraldehyde-3-phosphate dehydrogenase decline over time and under different growing conditions (Figure 18). This is probably due to the growth state of the culture. To date, *XYL3* mRNA has not been seen in culture when *C. carbonum* is grown on xylan or maize cell walls.

mRNA was isolated from 7-day old infected plant leaves, fractionated on a formaldehyde gel, blotted to Zeta-Probe, and hybridized with *XYL1*. The blot was then stripped and hybridized sequentially with *XYL2*, *XYL3*, and the glyceraldehyde-3-phosphate dehydrogenase gene (*GPD1*) from *C. heterostrophus*. As seen in Figure 19, *XYL1* mRNA is present, *XYL2* mRNA is not present, and *XYL3* mRNA is present.

DISCUSSION

XYL2 and *XYL3* were cloned and sequenced. Transformation-mediated gene disruption created mutants specifically lacking intact copies of *XYL2* and *XYL3*. Immunoblot analysis showed that *XYL2* produces a low level of protein product, but this protein could not be detected enzymatically.

Pathogenicity tests showed that both *XYL2* and *XYL3* individually as well as *XYL1* and *XYL2* in combination are dispensable for pathogenicity on maize. This does not mean that xylanases are not important for

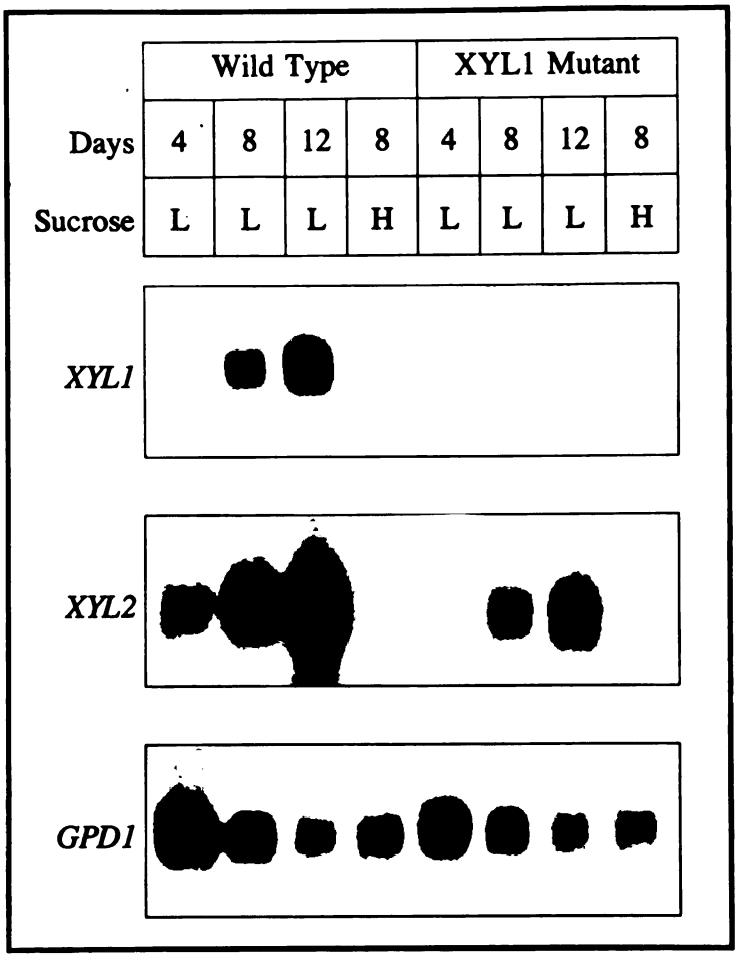

Figure 18. RNA blot hybridized with *XYL1*, *XYL2* and *XYL3*. Total RNA was isolated from fungal mats of the wild type (367-2A) and the *XYL1* mutant (447-8a) grown on maize cell walls for 4, 8, or 12 days with low or high levels of sucrose (L=0.2% sucrose, H=2.0% sucrose). Approximately 10 μg of RNA per lane was fractionated on a 1.2% agarose gel containing formaldehyde (Selden, 1987) and blotted to a nylon membrane. The blot was sequentially hybridized with the *XYL3* KpnI/XbaI fragment, the *XYL2* cDNA, the *XYL1* cDNA, and the glyceraldehyde-3-phosphate dehydrogenase gene (*GPD1*) from *C. heterostrophus*.

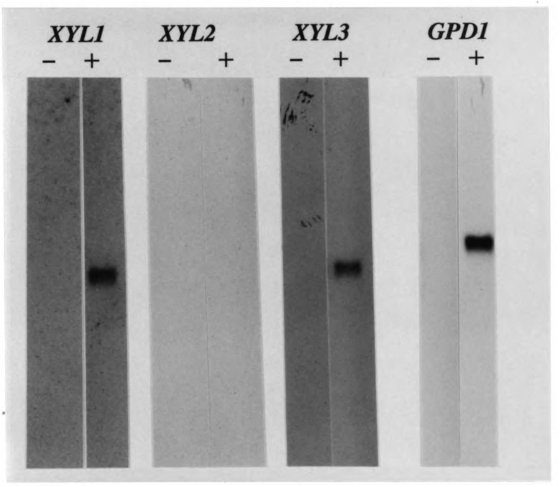

Figure 19. RNA blot of infected maize leaves. mRNA was isolated from infected maize lesions 7 days after inoculation with wild-type strain 367-2A. Approximately 10 μg of the mRNA was loaded per lane on a 1.2% agarose gel containing formaldehyde (Selden, 1987) and blotted to Zeta-Probe. The blot was sequentially hybridized with the *XYL1* cDNA, the *XYL2* cDNA, the *XYL3* KpnI/XbaI fragment, and the glyceraldehyde-3-phosphate dehydrogenase gene (*GPD1*) from *C. heterostrophus*.
- = mRNA isolated from uninfected leaves. + = mRNA isolated from infected leaves.

pathogenicity since *C. carbonum* produces other xylan degrading enzymes such as Xyl4 (xylanase III) and β-xylosidases.

Not only does *C. carbonum* produce several xylan-degrading enzymes, it appears that they are induced by different factors or may be produced during different stages of the life cycle. *XYL1* is expressed in culture as well as in infected leaves. *XYL2* is expressed in culture but not in 7-day infected leaves, and *XYL3* is not expressed in culture but is expressed in infected leaves. Because of the different patterns of expression of these three genes, their promoters will be interesting to study.

CHAPTER 5

TRIPLE XYLANASE MUTANT

INTRODUCTION

Three genes, *XYL1*, *XYL2*, and *XYL3*, encoding endo-β1,4-xylanases were cloned, sequenced, and disrupted. In order to obtain a strain with mutations in all three xylanase genes, strains containing single disruptions were crossed to each other and the resulting double-disrupted (*XYL1*$^-$/*XYL2*$^-$/*XYL3*$^+$) strain was crossed with the third single-disruptant strain (*XYL1*$^+$/*XYL2*$^+$/*XYL3*$^-$). The triple mutant was analyzed for ability to grow on xylan or maize cell walls. mRNA expression levels of the three xylanases and the ability of the triple mutant to infect maize were also studied.

RESULTS

A triple xylanase mutant was obtained by crossing strains 477-3a (*XYL1*$^-$/*XYL2*$^-$/*XYL3*$^+$) and T448-4A (*XYL1*$^+$/*XYL2*$^+$/*XYL3*$^-$). When the progeny were isolated no selection was used so that all eight progeny classes were obtained from the cross. The eight strains from cross 556 are representative of the eight progeny classes from cross #1 (Table 2; Chapter

4). The number of progeny obtained in each progeny class is shown in Table 3. Because no selection was used when the progeny were isolated, all of the progeny were analyzed by Southern analysis to determine their xylanase genotype. A Southern blot showing the representative progeny from each class is shown in Figure 20.

The expression levels of the progeny from the cross were studied by RNA blotting (Figure 21). *XYL3* message is not present as expected from previous experiments (Figure 21). *XYL2* and *XYL1* appear to be slightly up-regulated in mutants of the opposite genotype (Figure 21).

Growth on maize cell walls or xylan was measured by weighing the mats. As seen in Figure 22, no significant difference in growth was found between the wild type (556-2), a *XYL1* mutant (556-11), a *XYL2* mutant (556-8), a *XYL3* mutant (556-1), and a *XYL1/XYL2/XYL3* triple mutant (556-13) as determined by dry weight at 3 or 8 days when grown on maize cell walls or xylan.

No detectable differences in the rate of lesion development, size, and morphology were seen when the wild type and the xylanase mutant strains were inoculated onto susceptible maize leaves (Figure 23).

DISCUSSION

The triple mutant was obtained by crossing a *XYL1/XYL2* double mutant with a *XYL3* mutant and analyzing the progeny by Southern analysis. From work by Joong-Hoon Ahn (personal communication) it is

Table 3. Crosses to obtain a triple xylanase mutant.

Cross #1: T448-4A (*XYL1*$^+$/*XYL2*$^+$/*XYL3*$^-$) X 447-3a (*XYL1*$^-$/*XYL2*$^-$/*XYL3*$^+$)

Progeny genotypes	Number of progeny obtained
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

Cross #2: T448-4A (*XYL1*$^+$/*XYL2*$^+$/*XYL3*$^-$) X 447-7a (*XYL1*$^-$/*XYL2*$^-$/*XYL3*$^+$)

Progeny genotypes	Number of progeny obtained
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

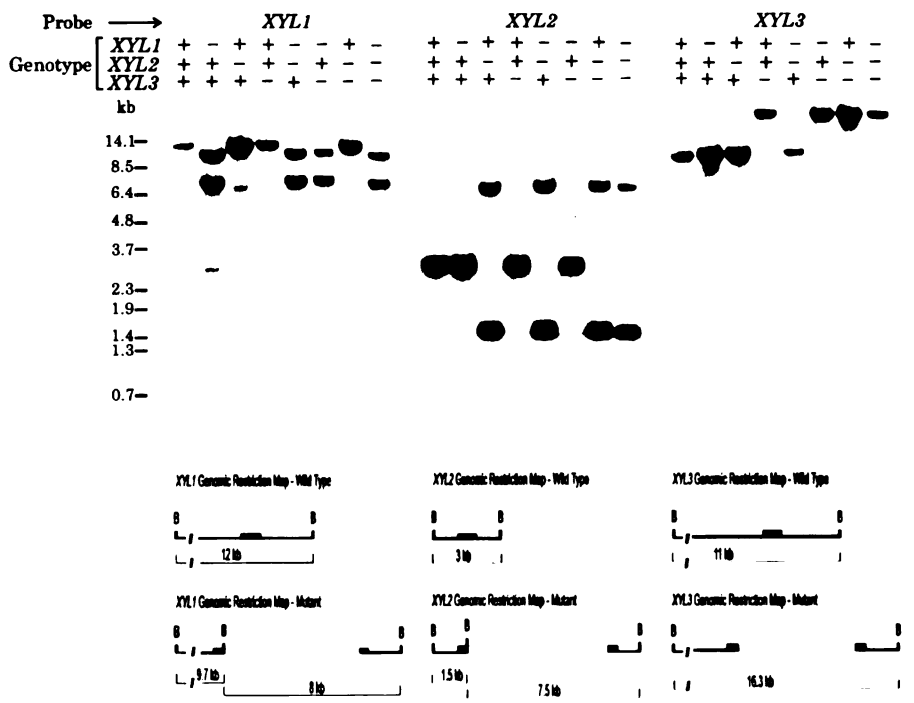

Figure 20. DNA blot analysis of wild-type and disruption mutants. Genomic DNAs from *Cochliobolus carbonum* wild type and disruption mutants were digested with BamHI, fractionated, blotted, and hybridized with either *XYL1*, *XYL2*, or *XYL3*. The predicted restriction map is shown below the blots corresponding to each gene. The black shading indicates the xylanase gene of interest while the light shading indicates the disruption vector. B=BamHI endonuclease restriction sites.

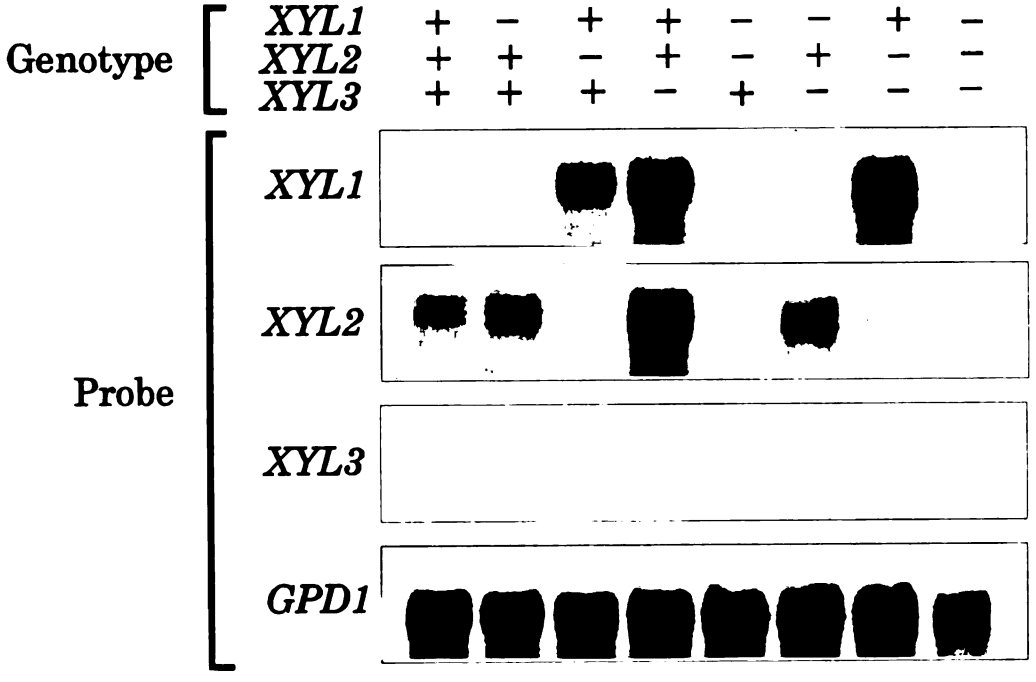

Figure 21. RNA blot of wild-type and disruption mutants. Approximately 10μg of total RNA, isolated from fungal mats grown for 6 days on maize cell walls, was fractionated on a 1.2% agarose gel containing formaldehyde and blotted to a nylon membrane. The blot was hybridized sequentially with the *XYL1* cDNA, *XYL2* cDNA, the KpnI/XbaI fragment of *XYL3*, and the glyceraldehyde-3-phosphate dehydrogenase gene (*GPD1*) from *C. heterostrophus*.

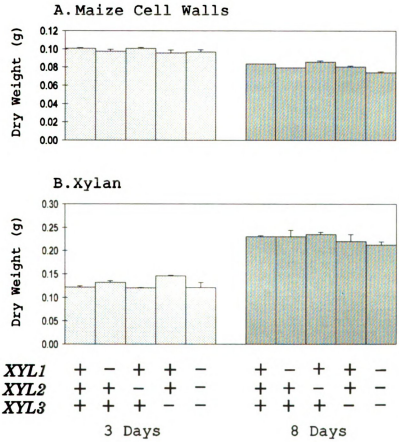

Figure 22. Growth of wild-type and mutant strains. Dry weights of fungal mats (in grams) at 3 and 8 days when grown on maize cell walls or xylan. All treatments were done in duplicate. Error bars indicate the range.

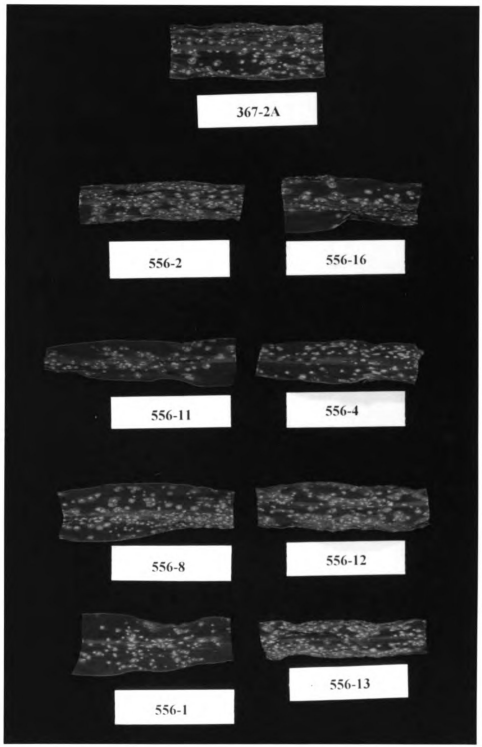

Figure 23. Pathogenicity test of xylanase mutants. Maize leaves were inoculated with 10^4 spores per milliliter of the indicated strain. Photograph was taken 5 days after inoculation.

known that *XYL1* and *XYL2* are definitely on different chromosomes. However, it is not clear if *XYL1* and *XYL3* are on the same or different chromosomes because they hybridize to a location on CHEF gels that contains more than one chromosome. Even if they are on the same chromosome, it is clear that *XYL1* and *XYL3* are not tightly linked (Table 3).

Northern analysis (Figure 21) suggests that *XYL1* may be upregulated when XYL2 and/or XYL3 is disrupted. The expression of these genes needs to be studied further to confirm and extend this observation.

The disruption of all three xylanase genes in the same strain did not affect the growth of the fungus on xylan or maize cell walls. However, the oat spelt xylan used in our growth studies contains only about 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 the growth of the triple mutant on xylan. Alternatively, growth may be due to Xyl4 (xylanase III; Holden and Walton, 1992; Apel *et al.*, 1993) or another unknown xylan degrading activity.

Pathogenicity tests indicate that *XYL1*, *XYL2* and *XYL3* are dispensable for pathogenicity of *C. carbonum* on maize and do not contribute to virulence. From these experiments, it cannot be concluded that xylanases are unimportant for pathogenicity since *C. carbonum* produces other xylan degrading enzymes such as Xyl4.

CHAPTER 6

CONCLUSIONS

Three endo-xylanase genes were cloned from the plant pathogenic fungus *Cochliobolus carbonum*. *XYL1*, *XYL2*, and *XYL3* are all predicted to encode family G, 22-kDa xylanases. Xyl2 and Xyl3 are 60% and 42% identical to Xyl1 at the amino acid level. Xyl2 and Xyl3 are 39% identical. Transformation-mediated gene disruption was used to create mutants in all three genes alone and in combination. A triple *XYL1/XYL2/XYL3* mutant was created by crossing. The mutants were tested for enzyme production in vitro, growth in culture on xylan and maize cell walls, and pathogenicity on maize.

XYL1 has one intron. It encodes two xylanase isoforms that together are responsible for 85-94% of the total extracellular endo-xylanase activity of *C. carbonum* when grown on maize cell walls. Growth of the *XYL1* mutant was indistinguishable from the growth of the wild type in culture. The residual xylanase activity is apparently sufficient to support growth. *XYL1* is also expressed at the mRNA level in planta. The *XYL1* mutant is still fully pathogenic on maize.

XYL2 has two introns, one of which is at the same position as the intron in *XYL1*. It is expressed at the mRNA level in culture but in a *XYL2* mutant the residual xylanase activity seen in the *XYL1* mutant is still present. No enzyme activity can be associated with *XYL2*. However, immunoblot analysis indicates that the protein product of *XYL2* is present in culture filtrates but at a low and enzymatically undetectable level. *XYL2* mRNA is not detected in planta and disruption of *XYL2* has no effect on pathogenicity.

XYL3 has one predicted intron at the same position as the intron in *XYL1*. A cDNA of *XYL3* was not obtained because it is not expressed in culture. *XYL3* is expressed in planta but a *XYL3* mutant grows on xylan and maize cell walls and is still pathogenic.

A triple xylanase mutant grows as well as the wild type on xylan and maize cell walls. The triple mutant is still pathogenic.

The question of whether xylanase activity is necessary for growth on xylan and for pathogenicity is still an open question due to the residual xylanase activity remaining in the triple mutant. This xylanase activity has been purified and is due to a 33-kDa family F endo-xylanase (J. Scott-Craig, personal communication). The gene, *XYL4,* encoding this xylanase is being cloned. Furthermore, *C. carbonum* makes at least one β-xylosidase, which is also in the process of being cloned (R. Ransom, personal communication).

At this time it is important to clone and disrupt *XYL4* alone and in combination with *XYL1*, *XYL2*, and *XYL3*. If this quadruple mutant cannot grow on xylan or is non-pathogenic, then by studying strains with different combinations of the four xylanase genes mutated, it can be determined which enzymes are important for each process.

The xylanases of *C. carbonum* are redundant. *C. carbonum* has multiple xylanase genes and makes multiple xylanases. However, the relationship between the enzymes and the genes is not simple: *XYL1* encodes two of the three enzymes seen in culture, and *XYL4* probably encodes the fourth. Although *C. carbonum* has multiple xylanase genes, the genes are not equivalent in their pattern of expression: *XYL1* and *XYL3* are expressed in planta, and *XYL1* and *XYL2* are expressed in culture. Whether Xyl1, Xyl2, and Xyl3 have similar enzymatic activities could not be determined since only Xyl1 can be assayed.

Redundancy is a common theme in cell wall degrading enzymes from plant pathogenic bacteria and fungi. *Magnaporthe grisea* and *Alternaria brassicicola* each make at least two cutinases (Sweigard et al., 1992; Yao and Koller, 1995). *Glomerella cingulata* has a large pectin lyase gene family (Bowen et al., 1995) and *Erwinia chrysanthemi* makes at least eight pectin lyases (Kelemu and Collmer, 1993). The reasons for this redundancy are not known. One explanation is that the enzymes are so important that the organism protects itself against mutation by having more than one gene.

Another explanation is that facultative pathogens have specialized enzymes for saprophytic versus pathogenic growth. Yao and Koller (1995) proposed that *A. brassicicola* makes one cutinase specialized for saprophytic growth and another for pathogenic growth. A third explanation is that different isozymes are specialized for hydrolyzing polymers that are slightly but significantly different. Perhaps, Xyl1, Xyl2, and Xyl3 are adapted to hydrolyze slightly different xylans. Takenishi and Tsujisaka (1975) found that *Aspergillus niger* secretes three xylanases. Xylanase I most effectively hydrolyses cotton xylan while xylanase II contributes to the hydrolysis of rice straw arabinoxylan by the removal of arabinosyl substituents (Takenishi and Tsujisaka, 1975).

More studies need to be completed before we will know the true purpose of the multiple xylanases in *C. carbonum*. They may be important for pathogenicity, for saprophytic growth, or for another unknown purpose. In order to elucidate the role of xylanases or other cell wall degrading enzymes, all isozymes need to be characterized and all related genes must be disrupted in a single strain.

APPENDIX

APPENDIX

EXPRESSION OF A FUNGAL ENDO-XYLANASE GENE IN TOBACCO

ABSTRACT

There are several potential applications for xylanases but the high cost of xylanases produced by fermentation is a significant commercial barrier. As an approach to producing large quantities of xylanase less expensively, we have expressed the *XYL1* gene of the fungus *Cochliobolus carbonum* in *Nicotiana tabacum*. Several *XYL1* gene constructs were tested. All the *XYL1* gene constructs were driven by the double CaMV 35S promoter from p1079 (DuPont Chemical Company, Wilmington, DE). These include a *XYL1* cDNA with and without the native signal peptide as well as *XYL1* containing an engineered endoplasmic reticulum-retention signal (KDEL) at the carboxyl end. *Agrobacterium*-mediated transformation was used to obtain transgenic tobacco. Transgenic plants were screened for *XYL1* mRNA by tissue printing (Reid and Pont-Lezica, 1992). RNA was isolated from plants that screened positively for *XYL1* message by tissue printing. The RNA was blotted and hybridized with the *XYL1* cDNA. Some plants from each construct group

showed high levels of mRNA expression of *XYL1*. However, not all plants that were positive by tissue printing produced *XYL1* mRNA. Additionally, none of the transgenic plants produced any Xyl1 protein as determined by protein blots reacted with antibody to a *Trichoderma viride* 22-kDa xylanase which cross-reacts with Xyl1. None of the transgenic plants had xylan-degrading activity.

INTRODUCTION

Enzymes that can depolymerize proteins and polysaccharides of plant cell walls are produced by many pathogenic fungi and bacteria. These enzymes have potential usefulness for commercial purposes. Xylanases can be used in food manufacturing to improve baking with high fiber material, to clarify fruit juices and wine, to improve nutritional and product properties of cereal fibers, and to produce food thickeners (Zeikus *et al.*, 1991). Additionally, xylanases can be used in the paper and fiber manufacturing industry for biobleaching of kraft pulps and the biopulping of wood (Senior and Hamilton, 1992; Zeikus *et al.*, 1991).

These uses are advantageous for the consumer and the environment. For example, limited hydrolysis of kraft pulps with xylanases enhances the extractability of lignins by conventional bleaching chemicals, resulting in reduction of chemical bleaching requirements and improvement in viscosity (Senior *et al.*, 1992; Yang *et al*, 1992). The use of xylanases in the treatment of bread dough decreases the dough strength, yielding loaves with an open

structure (Kulp, 1968). Adding xylanases to ensiled herbage improves the rate of lactic acid production and produces a better quality silage (Gilbert and Hazlewood, 1991). Xylanases improve the rate of plant-fiber degradation resulting in increased plant cell wall utilization in ruminant animals. This increases the amount of available nutrients to the animal, which results in increased milk production in dairy cattle (Gilbert and Hazlewood, 1991; Choung and Chamberlain, 1992).

While there are several potential commercial applications for xylanases, the high cost of xylanases produced by fermentation is a significant barrier to large volume production of xylanases (Zeikus *et al.*, 1991). Expression in plants might be a way to produce xylanases and other enzymes less expensively. This was our purpose in expressing *XYL1*, the gene encoding the major endo-xylanase from *Cochliobolus carbonum*, in tobacco.

MATERIALS AND METHODS

Gene constructions

Specific PCR primers were used to amplify the *XYL1* cDNA with and without the signal peptide, and with and without a KDEL endoplasmic reticulum retention signal. Primers used were:

JDW194=AATGGTTTCTTTCACCTCCATCATC,

JDW195=CATGCAGAACACCCCCAACGG,

JDW196=GTTATGGGCAGTTGACGGTGATCT, and

JDW197=GTTAGAGTTCATCTTTTGGGCAGTTGACGG. The product used in pPA5 was from primers JDW194 and JDW196; pPA6 was from primers JDW194 and JDW197; pPA7 was from primers JDW195 and JDW196; and pPA8 was from primers JDW195 and JDW197. The PCR products were cloned into the EcoRV site of pBluescript. After the clones were confirmed by sequencing, the 0.7-kb SmaI/ApaI fragment containing each PCR product was subcloned into the PstI site of p1079 (DuPont Chemical Company, Wilmington, DE) in the proper orientation behind a double CaMV enhancer region. These clones were then digested with XbaI to release a 1.8-kb fragment containing the subcloned PCR fragment of 0.7 kb, with the 35S promoter double enhancer region (0.8 kb) and the nopaline synthase 3' terminator region (0.3 kb). The fragment was blunted with T4 DNA polymerase and subcloned into a blunted HindIII site in the plasmid pBI121 (Clonetech) for *Agrobacterium* transformation. *Agrobacterium* strain LBA4404 was transformed by electroporation under the conditions recommended by the manufacturer (Bio-Rad; 25μF, 2.5kV, 200Ω).

Plant materials, growth and transformation

Growth of *Nicotiana tabacum* SR1 plants and the transformation of the plants was done as described by Newman *et al.* (1993).

Nucleic acid manipulations

Tissue prints were done by squashing a leaf from each tobacco plant onto a nylon membrane using a pestle covered with plastic wrap (Reid and

Pont-Lezica, 1992). Fresh plastic wrap was used for each leaf to avoid cross-contamination. The blot was wetted in 2x SSPE and the RNA was fixed to the membrane using a UV Stratalinker 1800 at 1200 mjoules according to the manufacturer's instructions (Stratagene). The filters were washed for 5 hr in a solution containing 50 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, and 0.1% SDS (Reid and Pont-Lezica, 1992).

RNA was isolated from leaves by the method of Yoder (1988) except cresol and glass beads were omitted. For the RNA blots 10 μg of total RNA was loaded per lane of a 1.2% agarose gel (Sambrook *et al.*, 1989). RNA electrophoresis was done in the presence of formaldehyde at 70V for 4 hr (Selden, 1987). GIBCO-BRL RNA standards (0.24 - 9.5 kb) were used for transcript size estimation.

Routine hybridization of the tissue prints was done with the sub-cloned *XYL1* cDNA labeled with ^{32}P-dCTP by random primer labeling (Sambrook *et al.*, 1989) in 50% formamide, 5X SSPE, 2X Denhardt's solution, 0.1% SDS, and 0.1 mg of denatured salmon sperm DNA per ml at 42°C overnight (Pitkin *et al.*, 1996). The blots were washed in 2X SSPE and 0.1% SDS two times for 15 min at room temperature and in 0.1X SSPE and 0.1% SDS two times for 30 min each at 42°C.

The Northern blot was pre-hybridized for 1 hour at 65°C in 4X SET, (20X SET = 3 M NaCl, 0.6 M Tris-HCl, and 0.04 M EDTA, pH 7.4), 0.1% tetrasodium pyrophosphate, 0.2% SDS, and 100 μg/ml heparin before being

hybridized with the *XYL1* cDNA labeled with ^{32}P-dCTP by random primer labeling (Sambrook *et al.*, 1989) overnight at 65°C in 4X SET, 0.1% tetrasodium pyrophosphate, 0.2% SDS, 10% (w/v) dextran sulfate, and 625 μg/ml heparin. The RNA blot was washed in 2X SSPE and 0.1% SDS two times for 15 min at room temperature and in 0.1X SSPE and 0.1% SDS two times for 20 min each at 65°C (Singh and Jones, 1984).

Xylanase activity assays

Protein extracts were obtained from the leaves of the transgenic plants and were assayed for xylan degrading activity using the Remazol Brilliant Blue Assay as described by Herbers *et al.* (1995), except that the assay was done at 37°C rather than at 60°C.

Leaves from the transgenic plants were harvested and ground in 4 ml 50 mM sodium acetate, pH 5. The solution was centrifuged for 10 min at 3200 rpm (1513 x g) and the supernatant was decanted. The samples were desalted using a BioRad Econo-Pak 10DG desalting column according to the manufacturer's instructions. The samples were then concentrated to 100 μl using an Amicon Centricon 3 microconcentrator and xylanase activity was assayed by a reducing sugar assay (Lever, 1972). Each sample was assayed in a 300 μl reaction volume containing a solution of 1 % oat spelt xylan (Fluka), 50 mM sodium acetate, pH 5.0, at 37°C for 30 min. Twenty five μl aliquots from the reactions were taken at 0 min and 30 min and added to 1.5 ml of p-hydroxybenzoic acid hydrazide working solution which was heated to

100 °C for 10 min prior to measuring the absorption at 410 nm. The p-hydroxybenzoic acid hydrazide working solution was prepared from a 5% (w/v) stock solution in 0.5 N HCl by diluting 1:4 with 0.5 N NaOH. Absorbance of the 0 min sample was subtracted from the 30 min absorbance and xylose was used as a standard.

Immunoblot analysis

The protein from leaf extracts was fractionated on a 17% acrylamide SDS-PAGE gel. The gel was blotted to nitrocellulose and reacted with anti-serum raised in rabbit against the 22-kDa xylanase of *Trichoderma viride* (Dean *et al.*, 1989) at a 1:1000 dilution. The antibody was a gift from Dr. James Anderson (USDA Beltsville, MD). The protein was visualized with a goat anti-rabbit antibody conjugated with alkaline phosphatase (Cooper Biomedical, Inc., Malvern, PA). Markers correspond to the prestained low molecular weight standards from Gibco-BRL.

RESULTS

Nicotiana tabacum plants were transformed with each of four constructs (Table 4). The constructs are various combinations with or without the fungal signal peptide or an endoplasmic reticulum retention signal (KDEL). The transgenic plants were initially screened by tissue printing. The plants which showed *XYL1* mRNA by tissue printing were analyzed by RNA blot analysis. As seen in Figure 24, the wild type control

Table 4. *XYL1* constructs used and transformation results.

Clone Name	Signal Peptide	KDEL	Transformants Recovered	# Transformants with *XYL1* mRNA
pPA5	Yes	No	17	9
pPA6	Yes	Yes	20	7
pPA7	No	No	16	4
pPA8	No	Yes	12	2

plants do not have any *XYL1* mRNA, as expected, while plants 5-6, 5-13, 6-17, 7-5, and 8-2 do have *XYL1* mRNA. Plants 6-6, 7-15, and 8-10 do not have detectable *XYL1* mRNA even though they show positive expression by tissue printing.

All of the transgenic plants were tested for xylan degrading activity by the Remazol Brilliant Blue Assay (Herbers *et al.*, 1995). None of the plants showed any xylan degrading activity above wild type controls (data not shown). Plants WT10, WT11, 5-6, 5-13, 6-6, 6-17, 7-5, 7-15, 8-2, and 8-10 were assayed for xylan degrading activity by the reducing sugar assay which is six times more sensitive than the Remazol Brilliant Blue assay (Biely *et al.*, 1985). Even with this assay no xylan degrading activity was found in any of the plants. Additionally, an immunoblot of protein extracts from these plants showed no evidence of xylanase protein expression in the transgenic plants (data not shown). All proteins that reacted with the anti-xylanase antibody were present in untransformed tobacco controls.

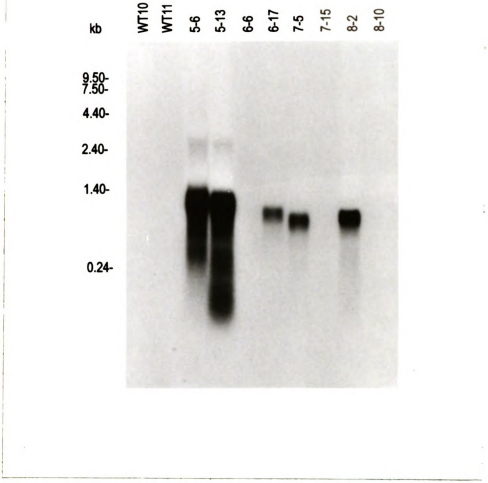

Figure 24. RNA blot of *XYL1* transgenic plants. WT 10 and WT 11 are two untransformed control plants. 5-6 and 5-13 are plants transformed with plasmid pPA5. 6-6 and 6-17 are plants transformed with plasmid pPA6. 7-5 and 7-15 are plants transformed with plasmid pPA7. 8-2 and 8-10 are plants transformed with pPA8.

Total RNA was isolated from a selection of plants that showed *XYL1* expression by the leaf print method. The RNA (30 µg per lane) was run on a 1.2% agarose/formaldehyde gel, blotted to Nytran and probed with a *XYL1* cDNA.

It appears that the transgenic plants are producing *XYL1* transcript but are not producing any Xyl1 protein. One possible reason for this is that the codon bias of *Cochliobolus carbonum* is not compatible with expression in tobacco. As seen in Table 5, however, there appears to be no significant difference in codon usage between *XYL1*, 56 tobacco genes or 20 genes encoding the highly-expressed small subunit of ribulose 1,5 bisphosphate carboxylase.

DISCUSSION

XYL1, the gene encoding the major endo-xylanase from *Cochliobolus carbonum*, was expressed at the mRNA level in tobacco both with and without its native signal peptide and with and without an endoplasmic reticulum retention signal (KDEL). While plants transformed with each construct produce large amounts of transcript none of the plants produce any xylanase protein.

This project was started before it was realized that another group was doing a similar set of experiments with a xylanase from *Clostridium thermocellum* (Herbers *et al.*, 1995). The xylanase used in these studies was originally a 90-kDa protein but only a coding region yielding a 37-kDa protein was expressed in transgenic plants. They also fused their sequence to the signal peptide of the proteinase inhibitor II protein from potato. Herbers *et al.* (1995) did obtain plants that had active xylan degrading protein activity.

Table 5 Codon usage comparison

Amino Acid	Codon	[a] Tobacco 56 genes		*C carbonum* 6 CWDE genes		*XYL1*		[b] RuBP SSU 20 genes	
		#aa x 1000	%	# aa	%	# aa	%	# aa	%
Arg	CGA	4 80	11	1	2	0	0	3	2
	CGC	3,80	9	21	49	3	38	44	31
	CGG	2 20	5	1	2	1	12	1	1
	CGT	7 70	18	11	26	3	38	48	33
	AGA	12 50	29	3	7	0	0	31	21
	AGG	12 10	28	6	14	1	12	17	12
Leu	CTA	7 40	9	1	2	0	0	9	4
	CTC	12 70	16	34	52	2	33	43	20
	CTG	7 10	9	6	9	0	0	27	12
	CTT	21 80	27	19	29	4	67	56	25
	TTA	10 30	13	0	0	0	0	6	3
	TTG	20 70	26	5	8	0	0	79	36
Ser	TCA	15 80	22	3	2	0	0	48	19
	TCC	10 00	14	68	41	9	36	89	34
	TCG	4 30	6	13	8	1	4	10	4
	TCT	20 60	29	35	21	10	40	33	13
	AGC	9 00	12	44	26	5	20	56	22
	AGT	11 90	17	3	2	0	0	21	8
Thr	ACA	16 40	31	8	6	0	0	21	13
	ACC	11 40	22	86	62	19	79	79	48
	ACG	4 00	8	10	7	0	0	5	3
	ACT	20 40	39	34	25	5	21	59	36
Pro	CCA	26 20	43	6	12	2	29	72	34
	CCC	9 00	15	29	58	5	71	66	31
	CCG	3 50	6	2	4	0	0	13	6
	CCT	21 70	36	13	26	0	0	60	29
Ala	GCA	22 30	29	9	7	0	0	42	14
	GCC	16 20	20	69	54	12	67	125	43
	GCG	4 40	6	8	6	0	0	16	5
	GCT	35 20	45	41	33	6	33	110	38
Met	ATG	22 80	100	21	100	3	100	115	100
Trp	TGG	13 70	100	34	100	8	100	86	100
Gly	GGA	31 40	37	36	18	5	18	95	51
	GGC	14 00	16	103	52	12	43	43	23
	GGG	10 00	12	2	1	0	0	16	9
	GGT	30 00	35	56	29	11	39	32	17
Val	GTA	11 50	17	6	6	1	7	7	3
	GTC	13 80	20	68	63	7	46	73	28
	GTG	14 40	21	11	10	1	7	93	36
	GTT	29 40	42	23	21	6	40	87	33
Lys	AAA	23 20	43	2	3	0	0	30	15
	AAG	30 70	57	68	97	6	100	176	85
Asn	AAC	26 00	48	104	96	17	94	102	74
	AAT	27 80	52	4	4	1	6	35	26
Gln	CAA	22 50	62	11	19	0	0	73	49
	CAG	14 10	38	47	81	11	100	75	51
His	CAC	8 90	45	21	95	3	100	18	82
	CAT	11 10	55	1	5	0	0	4	18
Glu	GAA	26 70	51	7	22	0	0	49	26
	GAG	26 20	49	25	78	4	100	139	74
Asp	GAC	17 20	34	55	74	2	33	79	67
	GAT	33 00	66	19	26	4	67	39	33
Tyr	TAC	10 60	34	59	88	14	93	151	90
	TAT	20 60	66	8	12	1	7	17	10
Cys	TGC	8 10	45	32	91	2	100	72	91
	TGT	10 10	55	3	9	0	0	7	9
Phe	TTC	18 10	42	31	66	4	67	125	80
	TTT	25 20	58	16	34	2	33	32	20
Ile	ATA	10 70	20	2	3	0	0	1	1
	ATC	13 70	25	61	79	6	86	83	56
	ATT	29 40	55	14	18	1	14	63	43

a Wada, 1992
b Murray, 1989
RuBP SSU = Ribulose 1,5 bisphosphate carboxylase small subunit.

It is not known why Xyl1 is not present in the transgenic plants. Perhaps the signal peptide or some other portion of the protein is unstable in the plant cell environment and the protein is degraded. Inaccurate polyadenylation may be occurring if there are cryptic polyadenylation signals within the coding sequence of *XYL1*. Also, perhaps the transcript is not translated since we do not see any protein accumulation or even partial breakdown products with an anti-xylanase antibody. The first step toward solving this problem would be to see if the transcript can be translated. To test this, *XYL1* transcript could be in vitro translated with wheat germ extracts (Struhl, 1993). However, it may be better to start over by constructing a xylanase with a signal peptide proven to work in tobacco and perhaps, as a control, putting a different cell wall degrading enzyme in tobacco to see if it is produced and is functional.

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