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Identification of an *endo*-β-1,4-D-Xylanase from *Magnaporthe grisea* by Gene Knockout Analysis, Purification, and Heterologous Expression

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***Magnaporthe grisea*, a destructive ascomycetous pathogen of rice, secretes cell wall-degrading enzymes into a culture medium containing purified rice cell walls as the sole carbon source. From *M. grisea* grown under the culture conditions described here, we have identified an expressed sequenced tag, *XYL-6*, a gene that is also expressed in *M. grisea*-infected rice leaves 24 h postinoculation with conidia. This gene encodes a protein about 65% similar to *endo*-β-1,4-D-glycanases within glycoside hydrolase family GH10. A *M. grisea* knockout mutant for *XYL-6* was created, and it was shown to be as virulent as the parent strain in infecting the rice host. The proteins secreted by the parent strain and by the *xyl-6*Δ mutant were each fractionated by liquid chromatography, and the collected fractions were assayed for *endo*-β-1,4-D-glucanase or *endo*-β-1,4-D-xylanase activities. Two protein-containing peaks with *endo*-β-1,4-D-xylanase activity secreted by the parent strain are not detectable in the column eluant of the proteins secreted by the mutant. The two endoxylanases (XYL-6α and XYL-6β) from the parent were each purified to homogeneity. N-terminal amino acid sequencing indicated that XYL-6α is a fragment of XYL-6β and that XYL-6β is identical to the deduced protein sequence encoded by the *XYL-6* gene. Finally, *XYL-6* was introduced into *Pichia pastoris* for heterologous expression, which resulted in the purification of a fusion protein, XYL-6H, from the *Pichia pastoris* culture filtrate. XYL-6H is active in cleaving arabinoxylan. These experiments unequivocally established that the *XYL-6* gene encodes a secreted *endo*-β-1,4-D-xylanase.**

Magnaporthe grisea (Hebert) Barr (anamorph, *Pyricularia oryzae* Cav. or *Pyricularia grisea*) is the causal agent of the devastating rice blast disease (31). *M. grisea* primarily infects rice but also infects other Poaceae such as wheat and barley. There have been extensive studies of the cellular and molecular basis underlying *M. grisea*-plant interactions, particularly of the signal transduction pathways leading to early events of fungal infection (for a review, see reference 31). The recent publication of the *M. grisea* genome sequence is a cornerstone upon which the molecular interactions between a major crop killer and its plant hosts may be elucidated (9). Also, the ongoing accumulation and increased accessibility of large numbers of expressed sequence tags (ESTs) is greatly expediting the identification of individual proteins and the elucidation of gene expression profiles acquired under various environmental and culture conditions (14, 22, 23). We now describe the identification of an endoglycanase gene from a pool of ESTs of *M. grisea* grown on rice cell walls (RCWs) as its carbon source.

The primary walls of plant cells (6) are pivotal battlegrounds between microbial pathogens and their plant hosts (8, 13, 19, 32–34, 38). Microbial pathogens secrete an array of cell wall-degrading enzymes (CWDEs) capable of depolymerizing the noncellulosic polysaccharides of primary cell walls (2, 10, 11, 18, 19, 37). For example, the recently published *M. grisea* genome sequence unveiled the possible presence of as many as 20 xylanase genes, which encode six glycoside hydrolase family 10 (GH10), five GH11, and nine GH43 members (reference 9 and unpublished genome-mining data of this laboratory). This high level of redundancy is an indication that xylanase activity is essential for the vitality of *M. grisea*, either saprophytically or pathogenetically or both.

We previously described the purification, cloning, and gene knockout analyses of two *endo*-β-1,4-D-xylanases (EC 3.2.1.8) secreted by *M. grisea* (35, 37). We also provided evidence of the presence of at least three other xylanases encoded by *M. grisea* (37). PCR using degenerated oligonucleotide primers also allowed amplification and cloning of three putative xylanase genes, *XYL-3*, *XYL-4*, and *XYL-5* (GenBank accession numbers AY144348 to 144350) (manuscript in preparation). We now show that one of the *M. grisea* ESTs encodes a hitherto-undiscovered family GH10 *endo*-β-1,4-D-xylanase that also possesses a class III (fungal) carbohydrate-binding domain (fCBD) (13, 20, 27).

MATERIALS AND METHODS

Fungal strain and host plant. The protocols for growth, maintenance, and infection assays of both the rice BLAST fungus, *M. grisea* (Herbert) Barr strain CP987, and its host plant, *Oryza sativa* variety Sariceltik, were the same as previously described (37).

Construction of a cDNA library. *M. grisea* (strain CP987) was grown in a basic medium containing purified rice cell walls as the sole carbon source, as described by Wu et al. (35). Fungal mycelia were harvested after 5 days of culture and used to extract a total RNA sample as previously described (35, 37). Polyadenylated mRNA species were purified using the Oligotex mRNA kit from QIAGEN, Inc.

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TABLE 1. Oligonucleotide primers[a]

Primer	Nucleotide sequence	Expt
Oligo62	CACACTCTGATCTGGCACAGTC	*XYL-2* RT-PCR
Oligo65	GTAGTTGCTGTCGAACAACAGA	*XYL-2* RT-PCR
Oligo100	CTCATGCGCGGTAGCTTCAAGTCG	*Ces1* RT-PCR
Oligo167	CACAAATTTACACCGTCGCGGAC	*Ces1* RT-PCR
Oligo102	GCATGAGCTTGCCGTTGGCCGTG	*XYL-6* RT-PCR
Oligo168	GGTGCCAAGACTTGCGTCTCTGG	*XYL-6* RT-PCR
Oligo169	CTGAACGTTGATGTTGCTGTTTCC	*XYL-1* RT-PCR
Oligo170	CACGTACTCGGGCACCTTTAACC	*XYL-1* RT-PCR
Oligo103	*p*TCGACATG[b]	*pX6Sur* construction
Oligo116	CACCCAACCTATCACACACC	Mutant screening
Oligo117	CGATTGACGAGTTGTATTGCC	Mutant screening
Oligo350	*p*CTAGGGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCG	*pPicH* construction
Oligo351	*p*GGCCGCTACTTACTCAATGATGATGATGATGATGGTCGACGGCGC	*pPicH* construction
Oligo352	*p*TCGACCATCATCATCATCATTGAGTAAGTAGC	*pPicH* construction
Oligo227	TTTACGTAACCACCATGCGTACTCCCGC	*pHX6* cloning
Oligo359	TTTCCTAGGCAAAGCGTTCATGATGGCGGTG	*pHX6* cloning

[a] All primers were synthesized by the Integrated Biotechnology Laboratories of the University of Georgia.
[b] The italic lowercase *p* preceding the nucleotide sequence represents a 5′ phosphorylation.

(Valencia, CA) according to the manufacturer's instructions. A cDNA library was constructed from 2 μg of the fungal mRNA using the λ bacteriophage vector Uni-Zap XR according to the manufacturer's manual (Stratagene Corp., La Jolla, CA). The packaged λ phage was amplified once on agar medium plates and stored at −80°C in 7% dimethyl sulfoxide as described in the manual.

Phagemids containing the cloned *M. grisea* cDNA were excised from randomly selected λ phages in the cDNA library as described in the manufacturer's manual (http://www.stratagene.com/manuals/211204.pdf). The *M. grisea* cDNA species cloned in the phagemids were subjected to nucleotide sequencing using the T3 and/or T7 primers (see the Uni-Zap XR manual) by the University of Georgia Integrated Biotechnology Laboratories. DNA sequence data were assembled using the software programs in the Wisconsin Package (Genetics Computer Group, Madison, WI) and deposited in GenBank.

Generation of a *XYL-6* knockout mutant. The strategy for creating a *XYL-6* knockout mutant was the same as previously described (37). The *XYL-6* gene was isolated by screening a *M. grisea* genomic library using EST RCW105 as a probe (see Table 2). A 5-kb Hind III/XhoI DNA fragment containing *XYL-6* was subcloned from the genomic clone to the phagemid pBluescript II SK(+) (Stratagene). The resulting plasmid (*pX6*) was digested with restriction enzyme SphI to remove a 2-kb DNA fragment that encodes the entire transcript of *XYL-6*. This SphI-cleaved plasmid was then ligated in the presence of an oligonucleotide gap-filler, pTCGA CATG (Oligo103 in Table 1), to a 3-kb SalI fragment encoding a mutated *M. grisea* acetolactate synthase gene (30). This mutated acetolactate synthase gene was named *Sur* for its ability to confer sulfonylurea resistance (plasmid pCB1637 that carries *Sur* was a generous gift from Jim Sweigard of The DuPont Company, Wilmington, DE). The resulting knockout vector, *pX6Sur*, was transformed into protoplasts of the *M. grisea* strain CP987 as described by Wu et al. (37). Sulfonylurea-resistant *M. grisea* transformants were screened by PCR for knockout mutants using oligonucleotide primers Oligo116 and Oligo117 (Table 1). These two primers are located immediately upstream and downstream, respectively, of the 2.0-kb *XYL-6* gene being deleted. Therefore, PCR on DNA samples of the *M. grisea* transformants using Oligo116 and Oligo117 will amplify both the 2.0-kb *XYL-6* gene and the 3.0-kb *Sur* gene if construct *pX6Sur* were integrated into the *M. grisea* chromosome via nonhomologous recombination (resulting in "ectopic" transformants). If the integration of *pX6Sur* into the chromosome occurs via homologous recombination that results in a "knockout" mutant, Oligo116 and Oligo117 will only amplify the 3.0-kb *Sur* gene. One *xyl-6ΔSur* mutant, designated strain X7601, was isolated out of a total of 73 transformants. Southern blot analyses were then performed using the 2-kb *XYL-6* and the 3-kb *Sur* gene as probes to confirm the gene replacement as described previously (37).

Enzyme purification and activity assay. The extracellular proteins present in the culture media of the parent *M. grisea* strain (CP987) and the mutant strain (X7601) were fractionated as described by Wu et al. (37), except that the Mono-S column (Pharmacia Biotech, Piscataway, NJ) was replaced by a 1-ml HiTrap SP column (Pharmacia Biotech). The final purification step utilized hydrophobic interaction chromatography to purify the target *endo*-β-1,4-xylanases to apparent homogeneity. The enzyme-containing fractions that were collected following the HiTrap SP chromatography were diluted with 1 volume of a 2× ammonium sulfate buffer (3.4 M ammonium sulfate in 50 mM sodium acetate, pH 5.0). The sample (2 ml) was injected onto a Phenyl-Superose HR 10/10 column (Pharmacia Biotech) that was irrigated with buffer A (1.7 M ammonium sulfate in 50 mM sodium acetate, pH 5.0) until UV absorption was reduced to that of the baseline. Column-bound proteins were eluted with a linear gradient from 0 to 50% of buffer B (50 mM sodium acetate, pH 5.0) in 100 min with a flow rate of 0.5 ml/min.

An aliquot (5 μl) of each fraction (1 ml from the HiTrap SP column and 0.5 ml from the Phenyl-Superose column) was assayed for *endo*-β-1,4-xylanase as described by Wu et al. (37), except that the assay was conducted with 100 mM 2-(*N*-morpholino)-ethane sulfonic acid buffer at pH 6.0, the optimum pH for XYL-6 (data not shown). *endo*-β-1,4-Glucanase activity was determined by the PAHPAH method (35), which measures the reducing sugar formed from the carboxymethyl cellulose substrate (Sigma). The assay buffer for the *endo*-β-1,4-glucanase was 50 mM sodium acetate at pH 5.0.

Construction of a *Pichia* expression vector. Plasmid *pPicH* was constructed by ligation of oligonucleotide primers Oligo350, Oligo351, and Oligo352 (Table 1) with Avr II- and NotI-cleaved pPIC3.5k vector (Multi-Copy *Pichia* Expression Vector, catalogue no. K1750-01; Invitrogen Corp., Carlsbad, CA). After transformation of the ligated product into *Escherichia coli*, plasmids harboring the correctly inserted DNA sequences were screened by SalI digestion (Oligo351 includes a SalI restriction site that is not present in vector pPIC3.5k) and confirmed by DNA sequencing. The cloning resulted in a 74-bp insertion encoding a c-*myc* epitope and a His_6 tag. Therefore, a protein expressed in *Pichia pastoris* (7) using *pPicH* is a fusion protein with the c-*myc* epitope and the His_6 tag at its C terminus (16, 25).

Heterologous expression of *XYL-6*. Expression of *XYL-6* in *Pichia pastoris* was performed according to the Invitrogen manual *Multi-Copy Pichia Expression* (7). A 1.2-kb cDNA fragment containing the entire coding region of the *XYL-6* gene was amplified by RT-PCR (3, 36) from an mRNA sample using oligonucleotide primers Oligo227 and Oligo359 (Table 1). The mRNA sample was the same as the one used for the construction of the cDNA library. The PCR fragment was then digested with SnaBI and AvrII and inserted into SnaBI/AvrII-cleaved *pPicH*. The resulting plasmid, *pHXyl6*, was multiplied in *E. coli* strain Top10 (Invitrogen), and determined by DNA sequencing to be error free. The plasmid was then linearized by SacI digestion and electrotransformed into *Pichia* cells. Transformants resistant to a minimum of 1.0 mg of antibiotic G418 sulfate (Geneticin)/ml were selected for protein expression in 10 ml buffered complex methanol medium according to the Invitrogen manual. Aliquots (each, 1 ml) of the culture were taken every 24 h following induction and centrifuged in a microcentrifuge for 5 min at full speed to remove the *Pichia* cells. Samples (each, 10 μl) of the cell-free supernatant were assayed for *endo*-β-1,4-xylanase activity as described above with blue-dyed RBB-xylan used as a substrate (37). For large-scale induction, *Pichia* cells at a density of 2 U of optical density at 600 nm per ml were grown in 1 liter of buffered complex methanol medium at 30°C for 3 days. After induction, the culture medium that contained secreted proteins was concentrated to about 100 ml and subjected to nickel-chelate affinity column chromatography (25) using HisLink resin (catalogue no. V8821; Promega Corp., Madison WI), according to the manufacturer's manual. Western blot analysis

TABLE 2. Putative functions of *M. grisea* ESTs

Type and sequence	Accession no.	Putative function[a]	Match[b]
Cell wall-degrading enzymes and sugar transportation			
RCW5	AA415060	α-L-Arabinofuranosidase (*Afs1*)	AL021411
RCW20 [2][c]	AA415086	β-Glucosidase (EC 3.2.1.21)	S52771
RCW22	L37530	*endo*-β-1,4-Xylanase (EC 3.2.1.8) (*XYL-2*)	L37530
RCW105[d]	AA415141	Group 10 glycohydrolase (*XYL-6*)	P46239
RCW109	L81126	*endo*-β-1,4-Xylanase (EC 3.2.1.8) (*XYL-3*)	L81126
RCW18	AA415084	Sugar transporter protein (*STP1*)	P18631
RCW69	AA415111	Sugar transporter protein (*STP2*)	P108708
RCW79	AA415117	Sugar transporter protein (*STP3*)	U64903
Filamentous fungus-specific extracellular proteins			
RCW8 [4]	AA415063	Unknown	BI190440
RCW58	AA415101	Phytotoxin	AL513410
RCW59	AA415102	Unknown	BI189031
Development			
RCW100	AA415137	Sporulation-specific protein SPS2 (*Ces1*)	S54039
RCW108	AA415143	Yeast meiosis-specific SPO14 (phospholipase D)	P36126
Metabolism			
RCW6	AA415061	NADP-dependent mannitol dehydrogenase	AF387300
RCW7	AA415062	tRNA-guanine transglycosylase (EC 2.4.2.29)	P44594
RCW11	AA415064	Eukaryotic initiation factor 4A	Z74132
RCW19 [8]	AA415085	Ergosterol synthase (C-4 sterol methyl oxidase)	U31885
RCW26	AA415091	Triacylglycerol lipase (EC 3.1.1.3)	S49236
RCW35	AA415071	Ubiquinol-cytochrome *c* reductase	P07056
RCW37	AA415072	Stearoyl-CoA desaturase (EC 1.14.99.5)	S52745
RCW50	AA415081	26S protease regulator subunit 8 (factor SUGI)	S24016
RCW55	AA415099	Nuclear control of ATPase mRNA expression (NCA3)	D63817
RCW65 [6]	AA415108	NAD-dependent formate dehydrogenase (EC 1.2.1.2)	Q07103
RCW88	AA415125	Aspartate aminotransferase (EC 2.6.1.1)	P23542
RCW91	AA415128	Multicatalytic endopeptidase complex, subunit PRE2	S43739
RCW92	AA415129	D-2-Hydroxy acid dehydrogenase (EC 1.1.99.6)	P30799
RCW112	AA415145	Mitochondial $^{+}$H transporting ATP synthase (EC 3.6.1.34)	S28794
Housekeeping			
RCW16	AA415082	30-kDa heat shock protein 1	P19752
RCW25	AA415090	Clathrin assembly protein	U44890
RCW110	AA415144	Flavohemoprotein	P39662
RCW94	AA415131	60S ribosomal protein LI 6	Q10157
RCW96	AA415133	Frequency clock protein	S44457
RCW101	AA415138	Histone H3	P07041
RCW60	AA415103	Cross-pathway control protein 1	A30208
RCW68	AA415110	60S ribosomal protein LI 7	P04451
RCW82	AA415120	30-kDa heat shock protein 2	P40920
RCW83 [2]	AA415121	Ubiquitin precursor	X13140
Hypothetical			
RCW12 [2]	AA415065	*Schizosaccharomyces pombe* 35.9-kDa hypothetical protein	Q10256
RCW23	AA415088	Probable membrane protein (*Neurospora crassa*)	BF072630
RCW49	AA415073	*Erysiphe graminis* gEghL-6 clone homolog	L40637
RCW54	AA415098	*Emericella nidulans* hypothetical protein homolog	M59935
RCW63	AA415106	*Schizosaccharomyces pombe* hypothetical 8.2-kDa protein	Z69240
RCW102	AA415139	Yeast hypothetical protein (AMDI 5′ region)	S49741

[a] BLAST similarity search against GenBank databases (1). EC numbers and encoding genes are listed in parentheses.
[b] Accession number of a matched entry with the highest probability or smallest E value (1).
[c] Numbers in brackets indicate the number of redundancies in the EST pool.
[d] Subject of this paper; its corresponding genomic sequence, *XYL-6*, has been deposited in GenBank (AY124591).

using the alkaline phosphatase-conjugated anti-myc antibody (catalogue no. R950-25; Invitrogen Corp.) was performed on the purified protein according to the accompanying instructional manual.

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequences described in this paper are AA415057 to AA415145 and AY124591.

RESULTS

Analyses of ESTs from *M. grisea*. The fungus was grown in a basal medium containing RCWs as the sole carbon source. An mRNA sample isolated from the culture was used to prepare a

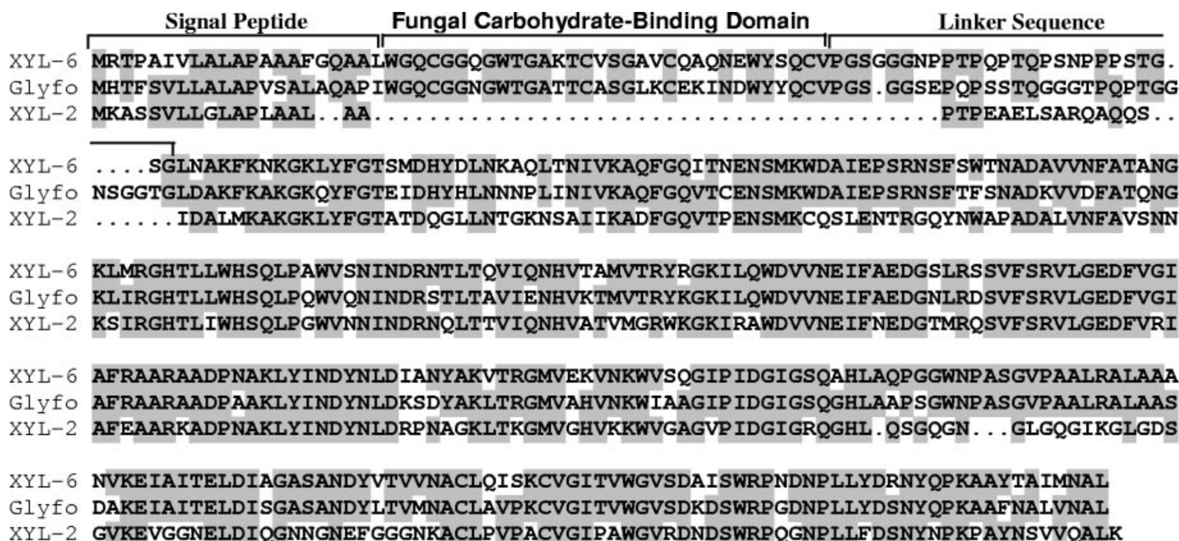

FIG. 1. Comparison of family GH10 endoglycanases. The amino acid sequence of XYL-6p was deduced from the five exons encoded by the *XYL-6* gene (GenBank accession number AY124591) and compared, using software programs included in the Wisconsin Package (Genetics Computer Group, Madison, WI), to *M. grisea* XYL-2 (formerly XYN33) (35) and a putative *endo*-β-1,4-glycanase from *Fusarium oxysporum* (Glyfo) (28, 29). Identical amino acid residues among the compared sequences are highlighted.

cDNA library. Partial or complete nucleotide sequences of 112 random cDNA clones, or ESTs, were determined and subjected to similarity comparison with the BLAST software to all entries in publicly available sequence databases (1). It was assumed that the similarity between two sequences was significant if the E value resulting from the comparison was $<1e - 10$, i.e., 10^{-10} (1). Based on this criterion, this 112-EST pool contained 86 singletons. Only half of these singletons encode proteins with significant matches in the databases (Table 2). The other 43 singletons did not have significant similarity to any entries in public protein databases. In agreement with the growth conditions in which polysaccharides (the major component of RCWs) are used as the carbon source, eight ESTs (~10% of all singletons) encoded polypeptides involved in polysaccharide catabolism and sugar transport. This statistic was similar to that derived from a larger EST pool (14).

***XYL-6* encodes a member of family GH10 endoglycanases.** Among the ESTs, RCW105 is a partial transcript that encodes a protein that is 55 to 80% similar to family GH10 glycohydrolases (13, 21, 27–29). The gene, *XYL-6*, was isolated from a genomic library of *M. grisea* (35) using RCW105 as a probe. The nucleotide sequence of *XYL-6* (GenBank accession number AY124591) includes four introns and five exons that encode a polypeptide of 380 amino acids (Fig. 1). Excluding the putative signal peptide, XYL-6p had a molecular mass of 38.8 kDa and a theoretical pI of 8.5. The mature peptide starts with a typical class III fCBD (21, 27), which is connected through a proline-glycine-rich linker sequence to the catalytic domain (20). XYL-6p does not contain any putative N-glycosylation sites. In comparison to other family GH10 endoglycanases, the amino acid sequence of XYL-6p is 79.2% similar to a putative *endo*-β-1,4-glycanase from *Fusarium oxysporum* (28, 29) but is only 60.3% similar to XYL-2p from *M. grisea* (35).

***XYL-6* is expressed in culture and in infected rice leaves.** Gene expression of *XYL-6* was analyzed by Northern blot analysis and reverse transcriptase-mediated PCR (RT-PCR) (Fig. 2) (35–37). The detected *XYL-6* mRNA signal was strong in *M. grisea* cells growing in basal medium with RCW as the only carbon source; it was about 10 times stronger than that for *XYL-1* mRNA and about 8 times stronger than that for *XYL-2* mRNA (Fig. 2A). In infected rice seedlings, *XYL-6* transcripts were detectable as early as 24 h postinoculation with *M. grisea*

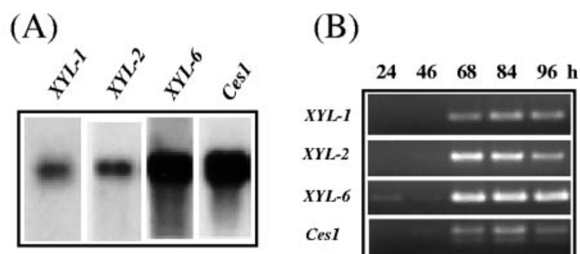

FIG. 2. Northern blot and RT-PCR analysis of *XYL-6* transcripts. (A) The total RNA sample for construction of the cDNA library was subjected to Northern blot analysis using digoxigenin-labeled cDNA fragments as probes according to the manufacturer's instructions (Roche Applied Sciences catalogue no. 1636090, 1363514, and 1603558). Probes *XYL-1* (transcript = 1.1 kb) and *XYL-2* (transcript = 1.3 kb) have been described previously (35, 37); probe *XYL-6* is EST RCW105 (transcript = 1.5 kb); and probe *Ces1* is EST RCW100 (transcript = 1.7 kb). The *Ces1* gene encodes a homolog of a yeast sporulation-related gene, *SPS2* (26). *Ces1* is constitutively transcribed in *M. grisea* culture (unpublished observations). (B) RT-PCR measurement of gene transcripts in infected rice leaves. Seedlings of rice cultivar Sariceltik were grown for 14 days in a growth chamber and inoculated with an aqueous conidial suspension (10^7/ml) of *M. grisea* strain CP987 as previously described (37). Total RNA samples were isolated from the infected seedlings 24, 46, 68, 84, or 96 h postinoculation. The RNA samples were treated with DNase I prior to RT-PCR as previously described (36). RT-PCR was performed using gene-specific primers (Table 1) designed for fragments of *XYL-1* (397 bp), *XYL-2* (621 bp), *XYL-6* (325 bp), and *Ces1* (349 bp), respectively.

TABLE 3. Saprophytic growth and pathogenicity of *M. grisea* xylanase mutants

Growth and pathogenicity	Strain and genotype		
	CP987, wild-type	X7231,[a] *xyl-1*ΔHyg[r] *xyl-2*ΔBen[r]	X7601, *xyl-6*Δ*sur*
Wild-type			
Fresh wt (mg)[b] on:			
CM	107 ± 8	94 ± 9	111 ± 12
Vogel/RCW	86 ± 9	48 ± 4	83 ± 7
Vogel/OSX	71 ± 7	29 ± 6	69 ± 6
Pathogenicity[c]			
No. of lesions	166	158	149
Lesion length (mm)	3.1 ± 1.0	2.7 ± 0.8	3.2 ± 1.2

[a] X7231 is a double-knockout strain for the *XYL-1* and *XYL-2* genes (37).

[b] Mycelia (fresh weight) were taken from 5-day-old cultures as described by Wu et al. (37). Growth media are complete media (CM) and Vogel's basic salt medium with rice cell walls (RCW) or oat spelt xylan (OSX; Sigma) as the sole carbon source. Medium recipes and sampling methodology were the same as described previously (37).

[c] An infection assay was performed on 17-day-old rice seedlings (compatible cultivar Sariceltik) as described previously (37). The number of lesions was the total from 10 fourth-folial leaves, and lesion length was the average of 30 randomly selected lesions from the 10 fourth-folial leaves 5 days postinoculation of fungal conidial suspension (2×10^4/ml).

conidia and accumulated to a PCR-saturated level 68 h postinoculation. In comparison, *XYL-1* and *XYL-2* transcripts were barely detectable at 46 h, accumulated to maximum at 84 h, and declined in amount by 96 h postinoculation.

Generation of a *xyl-6*Δ knockout mutant. In an attempt to identify any biological roles *XYL-6* may play in *M. grisea*, a knockout mutant, X7601, was generated from the *M. grisea* parent strain, CP987, by the same strategy as previously described (see Materials and Methods for screening details) (37). In X7601, the entire coding sequence of *XYL-6* was replaced by a selection marker gene that encodes a sulfonylurea-resistant acetolactate synthase (Sur). In comparison with the wild-type strain, the *xyl-6*Δ*Sur* mutant did not appear to exhibit any morphological abnormality. For example, it grew normally in medium containing either RCW or xylan as the sole carbon source and infected rice hosts nearly as efficiently as the parent strain (Table 3). Thus, under the defined experimental conditions (37), *XYL-6* is not required for either saprophytic or pathogenic growth of *M. grisea*.

Two Xylanases are absent from the *xyl-6*Δ*Sur* mutant. It is known that family GH10 glycanases may be *endo*-β-1,4-xylanase, *endo*-β-1,4-glucanase, or both (20, 21). To determine the enzymatic activity of XYL-6p, secreted proteins in the culture filtrates of the RCW-grown (35, 37) parent strain (CP987) and the *xyl-6*Δ*Sur* mutant (strain X7601) were separately subjected to cation-exchange chromatography. The collected fractions were assayed for both *endo*-β-1,4-glucanase and *endo*-β-1,4-xylanase activities. The results, summarized in Fig. 3, showed glucanase activity in fractions eluting in a single peak (Fig. 3A, peak γ) present in the proteins secreted by both CP987 and X7601. Fractions containing xylanase activities were separated into three peaks (α, β, and δ) from the culture filtrate of CP987. Peak δ, which contained both XYL-1 and XYL-2, has been previously described by Wu et al. (35, 37). Peaks α and β, however, were both missing from strain X7601. Therefore, the

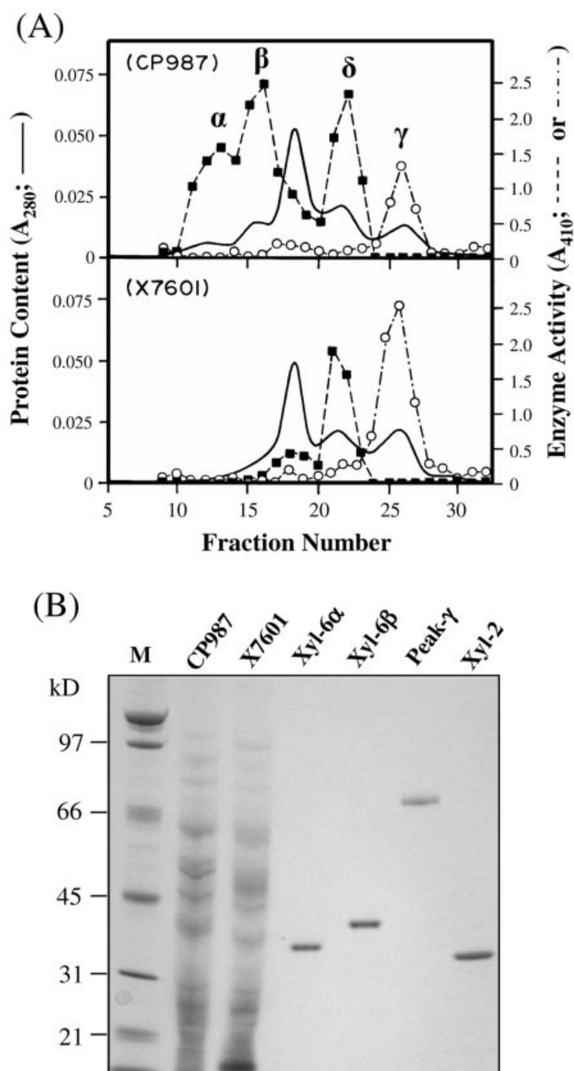

FIG. 3. Purification of XYL-6p. (A) Cation-exchange chromatograms of secreted proteins. Secreted protein samples from 200 ml of fungal cultures were processed as previously described (35). The protein samples (~2 mg each) were subjected to cation-exchange chromatography using a HiTrap SP column (Pharmacia Biotech) at pH 5.0. The column-bound proteins were eluted with a linear salt gradient from 0 to 1.0 m sodium chloride (fractions 1 to 120). Peak α was eluted by ~0.045 M NaCl; peak β was eluted by ~0.065 M NaCl; peak δ was eluted by ~0.09 M NaCl, and peak λ was eluted by ~0.11 M NaCl. A fifth peak eluted by ~0.6 M NaCl and present in both CP987 and X7601 is not shown in this figure. This fifth peak contained an *endo*-β-1,4-xylanase, XYL-3, that has been described previously (37). Each fraction was assayed for *endo*-β-1,4-xylanase (■) and *endo*-β-1,4-glucanase (○) activities using oat spelt xylan (Sigma catalog no. X-0627) dissolved in 100 mM 2-(*N*-morpholino)-ethane sulfonic acid buffer (pH 6.0) and carboxymethylcellulose (Sigma catalog no. C-0806) dissolved in 50 mM sodium acetate buffer (pH 5.0), respectively, as the substrates (see Materials and Methods) (35). (B) Gel analysis of the purified enzymes. Samples CP987 (2 μg) and X7601 (2 μg) are total extracellular proteins from the culture filtrate of *M. grisea* strains CP987 and X7601, respectively. XYL-6α (100 ng) and XYL-6β (100 ng) were purified, respectively, from peaks α and β shown in panel A by hydrophobic interaction chromatography (see Materials and Methods). Peak γ (40 ng) is the *endo*-β-1,4-glucanase-containing peak shown in panel A. XYL-2 (100 ng) is a 33-kDa xylanase purified previously (35). The protein samples were separated by SDS-PAGE in a 4 to 12% NuPAGE Bis-Tris precast gel (Invitrogen) as previously described (35, 37) and stained with a Colloidal Blue Staining kit (Invitrogen). Lane M, molecular mass ladder.

deletion of the *XYL-6* gene from *M. grisea* eliminates the secretion of two xylanases, but not the glucanase activity.

The xylanases eluting in the fractions of peaks α and β were purified to apparent homogeneity by two rounds of hydrophobic interaction chromatography as previously described (37). The yield was low, with about 4 μg of XYL-6α and 7 μg of XYL-6β obtained from 200 ml of culture (Fig. 3). Denaturing gel analysis showed that the purified XYL-6α had a molecular mass of about 34 kDa and that XYL-6β had a molecular mass of about 40 kDa (Fig. 3B). The latter agreed with the calculated molecular weight of mature XYL-6p based on its amino acid sequence (Fig. 1). The purified XYL-6α and XYL-6β were subjected to amino acid sequencing. The N-terminal amino acid sequences of XYL-6α and XYL-6β were determined to be WGQCGGXGWT and WXQCGGQXXTGA, respectively, where X represents a single, unidentified amino acid residue. Except for the unidentified X residues, these two N-terminal sequences were identical to the deduced N terminus of XYL-6p (Fig. 1). Thus, XYL-6α and XYL-6β are most likely two isoforms encoded by the same *XYL-6* gene, with XYL-6α being a fragment of XYL-6β.

Heterologous expression of *XYL*-6 generated *endo*-β-1,4-xylanase activity. Heterologous expression is another approach employed to unequivocally confirm the enzyme activity of XYL-6p. A full-length *XYL-6* cDNA was amplified by PCR from an mRNA preparation of the RCW-grown CP987 mycelia (Table 1). The amplified *XYL-6* cDNA was ligated into the SnaBI and AvrII restriction sites of a *Pichia pastoris* expression vector, pPicH, which was created by inserting a 74-bp DNA fragment into the AvrII and NotI restriction sites of the commercial vector pPIC3.5K (see Materials and Methods). Since the 74-bp DNA sequence encodes a c-*myc* epitope and a His_6 tag, a protein expressed in *Pichia pastoris* using pPicH will have the tandem c-*myc*–His_6 tag fused at its C terminus (16, 25).

The resulting construct, *pHXyl-6*, was transformed into *Pichia pastoris* cells, followed by selection of two independent transformants for protein induction. The culture media of both *Pichia* clones contained *endo*-β-1,4-xylanase activity and a unique protein band of approximately 47 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 4, the enzyme activity and the intensity of the 47-kDa protein band of clone 141 were both slightly weaker than those from clone 173. These results were consistent with the measured level of secretion for clone 141 (75 mg/liter), clone 173 (100 mg/liter), and the vector-transformed clone (12 mg/liter). Thus, XYL-6H was the predominant component of the secreted proteins (Fig. 4). The expressed fusion protein (XYL-6H) was purified from the 1 liter of induction medium of clone 173 by nickel-chelate affinity chromatography (25) with a recovery of approximately 2 mg and a specific *endo*-β-1,4-D-xylanase activity of 301 U/mg on RBB-xylan substrate (for a definition of the unit, see reference 37). The purified XYL-6H bound specifically to the c-*myc* antibody by a Western blot analysis (Fig. 4). It is noteworthy that XYL-6H is, by SDS-PAGE, approximately 6 kDa larger than the predicted molecular mass of 41.3. Therefore, it is possible that XYL-6H is posttranslationally modified. Consistent with the data shown in Fig. 3, XYL-6H had no detectable *endo*-β-1,4-D-glucanase (cellulase) activity (data not shown).

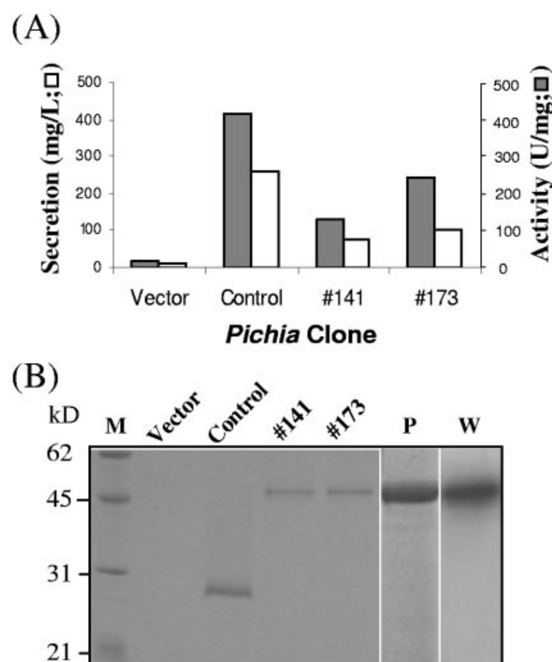

FIG. 4. *Pichia* expression of XYL-6H. (A) *XYL-6*-transformed *Pichia* clones (clones 141 and 173), a positive control-expressing *M. grisea* xylanase XYL-4 (GenBank no. AY144349; manuscript in preparation), and a clone carrying the empty vector pPic3.5K were induced for protein expression in 1 liter of induction medium for 80 h. Specific *endo*-β-1,4-xylanase activity in the culture filtrate was assayed with 100 mM phosphate, pH 6.0, using RBB-xylan (Sigma) as the substrate according to Wu et al. (37). Total protein content was measured using Protein Assay Reagent purchased from Bio-Rad Laboratories (Hercules, CA). (B) SDS-PAGE and Western blot analysis of secreted proteins in the culture filtrate (30 μl). Lane M, molecular mass markers; lane P, 0.5 μg of HisLink-purified XYL-6H from the culture filtrate of *Pichia* clone 173; lane W, Western blot analysis of lane P using an alkaline phosphatase-conjugated anti-myc antibody.

DISCUSSION

Recent progress in genomics has resulted in exponential expansion of the number of identified genes (9). The pilot analysis of 112 ESTs that are unique to *M. grisea* growing on RCWs as the sole carbon source revealed expression profiles similar to those of a larger study (14). Among the sequences analyzed, five encode plant CWDEs and another three are responsible for sugar transport across the plasma membrane (Table 2). It is most likely that the CWDEs and sugar transporters are expressed in response to the culture environment of the RCW carbon source and are needed for depolymerizing the RCW into monosaccharides that can be utilized by the fungus as nutrients. Among the identified ESTs that encode CWDEs is *XYL-6*, whose function is the focus of this study.

XYL-6 is expressed more strongly than *XYL-1* and *XYL-2* (35, 37) both in culture with RCW as a carbon source and in infected rice leaves. Nonetheless, we noticed that the accumulation pattern of *XYL-1*, *XYL-2*, and *XYL-6* transcripts in planta was similar to that in culture. In other words, under both conditions, *XYL-6* mRNA had the highest level of accumulation, *XYL-2* mRNA had a lower level, and *XYL-1* mRNA had the lowest level. In addition, transcripts of these three genes were not detectable by RT-PCR in culture using sucrose as the

carbon source (data not shown). Thus, although each xylanase gene is uniquely expressed, they may be regulated by a coordinated mechanism (10, 11, 18, 28). In line with this thought, the *M. grisea* genome (9) encodes two homologs (MG01414 and MG02880) of XlnR, a transcription factor that regulates most xylan-degrading enzymes in *Aspergillus niger* (for a review, see reference 11).

The protein fractionation profiles in Fig. 3 indicate that the biochemical properties of XYL-6 are different from those of XYL-1, XYL-2, and XYL-3. For example, XYL-6 was eluted by low-salt buffer (XYL-6α by 0.045 M and XYL-6β by 0.065 M NaCl), whereas XYL-1 and XYL-2 (Fig. 3, peak δ) were eluted by 0.09 M NaCl, and XYL-3 (not shown in Fig. 3) was eluted by 0.6 M NaCl. Also, XYL-6 appears to have an optimal pH of 6 instead of 7.2 for XYL-1, XYL-2, and XYL-3 (37). Structurally, XYL-6 includes an N-terminal fCBD, while the other characterized xylanases do not (35). The successful purification and/or heterologous expression makes it possible to investigate and compare the enzymatic mode of action and substrate specificity (5, 12, 24, 27) of these various isoforms of *M. grisea* xylanases.

The conclusion that the 34-kDa XYL-6α and the 40-kDa XYL-6β are the same gene product of *XYL-6* arises from the following two facts. (i) Deletion of the *XYL-6* gene eliminates both α and β activities. (ii) Both XYL-6α and XYL-6β have exactly the same N terminus as the one predicted from the nucleotide sequence of *XYL-6*. Analysis of protein purification results also indicated that a good portion of XYL-6 was fragmented into the smaller 34-kDa polypeptide that is still active at hydrolyzing xylan substrate. Therefore, the proteolytic degradation must be rather specific, and the cleaved C terminus (mass of 6 kDa or ~55 amino acid residues) must not be required for xylanase activity. It has been predicted (and in some cases shown) that the catalytic site of group 10 xylanases from other microbial species involves conserved glutamic acid (Glu) residues (5, 20, 25). There are no Glu residues within the ~55 C-terminal amino acids, as shown in Fig. 1. However, it remains to be determined whether the 34-kDa and the 40-kDa XYL-6 differ in their mode of enzymatic action and/or substrate specificity.

Heterologous expression of *XYL-6* unequivocally confirmed that XYL-6 is an *endo*-β-1,4-xylanase (Fig. 4). This experiment also indicated for the first time that the native XYL-6 signal peptide (Fig. 1) directs protein secretion from *Pichia* cells into the culture medium (Fig. 4). The secretion level of 75 to 100 mg/liter is similar to that of an *Aspergillus niger* xylanase directed by the built-in *Saccharomyces cerevisiae* α-factor (4). Using the same strategy, we also successfully expressed a number of genes in *Pichia* encoding putative secreted proteins from *M. grisea* (unpublished data). Therefore, it is most likely that *M. grisea* signal peptides in general are recognized by the *Pichia* secretory machinery.

The inclusion of an His_6 tag for heterologous protein expression greatly facilitates purification of the expressed protein by a one-column affinity chromatography. However, the yield of 2 mg of pure XYL-6H by nickel-chelate chromatography out of an estimated 100 mg secreted into the culture media is extremely low. It is possible that the C-terminal fusion of the His_6 tag leads to an XYL-6H conformation that limits exposure of the tandem histidine residues to the nickel ligand, resulting in low binding of XYL-6H to the HisLink resins. It is also possible that the purification process will have to be optimized for maximum yield, which could include tests of buffer conditions and various affinity media.

endo-β-1,4-Xylanases from various microbial sources are being intensively investigated. Most of these studies focus on xylanase's potential in industrial applications, such as in paper pulping and bleaching (27). A few studies attempted to elucidate the role of xylanases in microbial pathogenesis (2, 10, 17, 18, 33, 37), as arabinoxylan is the quantitatively predominant hemicellulosic component of the cell walls of the Poaceae (6, 15). These studies have established that plant pathogenic fungi secrete multiple xylanases when infecting plant tissues as well as when growing in pure culture with arabinoxylan as the carbon source. The strong transcription of *XYL-6* in both the culture and rice leaves during the early infection stage further supports these claims, although irrefutable evidence that xylanases are pathogenicity factors has yet to be obtained. The *xyL-6*Δ mutant, like previously investigated *xyl-1*Δ and *xyl-2*Δ mutants, is not required for pathogenicity under the defined growth chamber conditions. However, our investigation of the *M. grisea* genome sequence indicates the presence of as many as 20 xylanase genes, including at least genes encoding six family GH10 members, five family GH11 members, and nine family GH43 members (9, 29, 37; unpublished data). It is possible that any of the xylanases, other than XYL-1, XYL-2 and XYL-6, is required for pathogenicity, or a member of the xylanases lost is complemented by the others. Alternatively, pathogenicity may partially depend on the fungus's ability to depolymerize cell wall xylan, which could require two or more xylanases working in concert during infection growth in host tissues. Xylanases, working alone or with other inhibiting proteins, may also be indirectly involved in fungus-plant interactions by generating structure-specific xylan oligosaccharide fragments that are recognized by the plant host as elicitor signal molecules (8, 13, 19). Our growing collection of purified or *Pichia*-expressed *M. grisea* xylanases, as well as their knockout mutants, allows us to continue investigation of these possibilities.

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