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Purification, Cloning and Characterization of Two Xylanases from *Magnaporthe grisea*, the Rice Blast Fungus

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***Magnaporthe grisea*, the fungal pathogen that causes rice blast disease, secretes two *endo*-β-1,4-D-xylanases (E. C. 3.2.1.8) when grown on rice cell walls as the only carbon source. One of the xylanases, XYN33, is a 33-kD protein on sodium dodecyl sulfate–polyacrylamide gel and accounts for approximately 70% of the *endo*xylanase activity in the culture filtrate. The second xylanase, XYN22, is a 22-kD protein and accounts for approximately 30% of the xylanase activity. The two proteins were purified, cloned, and sequenced. XYN33 and XYN22 are both basic proteins with calculated isoelectric points of 9.95 and 9.71, respectively. The amino acid sequences of XYN33 and XYN22 are not homologous, but they are similar, respectively, to family F and family G xylanases from other microorganisms. The genes encoding XYN33 and XYN22, designated *XYN33* and *XYN22*, are single-copy in the haploid genome of *M. grisea* and are expressed when *M. grisea* is grown on rice cell walls or on oatspelt xylan, but not when grown on sucrose.**

Additional keywords: enzyme purification, gene expression, molecular cloning, plant cell walls, polymerase chain reaction.

The cell walls of living plants fulfill a variety of functions (reviewed in Darvill et al. 1980; Bacic et al. 1988; Varner and Lin 1989; Hahn et al. 1989). In addition to providing mechanical strength, plant cell walls serve as the primary barrier against physical wounds and microbial infection. It is generally agreed that, in order to penetrate the cell wall and to use its building blocks as nutrients, many, if not all, fungal and bacterial pathogens secrete cell wall hydrolases when attempting to infect plant tissues (Cooper 1983; Cooper et al. 1988; Dean and Timberlake 1989; Bucheli et al. 1990; Holden and Walton 1992; Braun and Rodrigues 1993; Southerton et al. 1993). It has also been hypothesized that, as a consequence of partially depolymerizing cell walls, some of the hydrolases generate signal molecules that activate the defense mechanisms of the host, including the possibility of eliciting hypersensitive cell death (reviewed in Darvill and Albersheim 1984; Hahn et al. 1989; Ryan and Farmer 1992; Darvill et al. 1992).

Our laboratory has selected *Magnaporthe grisea* (*Pyricularia oryzae*), the fungal pathogen that causes the devastating blast disease of rice (Valent and Chumley 1991), to study the roles of fungus-secreted hydrolases in the degradation of plant cell walls and in the release of endogenous elicitors. We have demonstrated previously that heat-stable substances, released from plant cell walls by heat-labile components of the culture filtrate of *M. grisea*, inhibit protein synthesis in maize and rice cells (Yamazaki et al. 1983; Doares et al. 1989; Bucheli et al. 1990). Preliminary chemical analyses indicated that the active fractions have arabinosyl and xylosyl residues as major components (Doares 1990). We fractionated the proteins in the *M. grisea* culture filtrate by cation exchange chromatography and tested all fractions, individually and in combination, for their ability to release bioactive fragments from rice cell walls. By this means and with no knowledge of the nature of the wall fragments that were being solubilized, our laboratory purified to homogeneity two proteins that, in concert, solubilize cell wall fragments that inhibit the ability of cultured plant cells to synthesize proteins (S. Kauffmann et al. unpublished results). The two proteins were found to be a 33-kD *endo*-β-1,4-D-xylanase and a 32.5-kD α-L-arabinofuranosidase (Bucheli et al. 1990; S. Kauffmann et al. unpublished data). The chemical and biochemical data taken together provide considerable evidence that the bioactive cell wall fragments are fragments of arabinoxylan. Arabinoxylans consist of a β-1,4-D-xylopyranosyl backbone to which various side chains containing mostly arabinosyl residues are attached to the O-2 and/or O-3 of xylosyl residues. Arabinoxylans are the most abundant hemicelluloses of graminaceous primary cell walls and account for approximately 40% of the total cell wall polysaccharides (Bacic et al. 1988; Carpita and Gibeaut 1993).

Another line of evidence that supports an important role for xylanases in the pathogenicity of *M. grisea* consists of reports that treatment by commercial xylanase on cultured rice cells causes cell death (Ishii 1988) and that a 21-kD xylanase from *Trichoderma viride* induces defense responses in tobacco plants, including ethylene production, necrosis, and the induction of pathogenesis-related proteins (Bailey et al. 1990; Lotan and Fluhr 1990; Raz and Fluhr 1993).

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Xylanases from two fungal plant pathogens, *Cochliobolus carbonum* and *Gaeumannomyces graminis*, have been previously purified and characterized (Holden and Walton 1992; Apel et al. 1993; Southerton et al. 1993). These studies indicated the existence of several xylanase isozymes, possibly encoded by different genes. An early report also identified two types of xylanases with different pH optima from *M. grisea* (Sumizu et al. 1961). As a step toward analyzing the role of xylanases in the interaction of *M. grisea* with its rice host, we report here on the purification, cloning, and characterization of two unrelated *endo*-β-1,4-D-xylanases from *M. grisea*.

RESULTS

Purification of *endo*xylanases from *Magnaporthe grisea.*

Magnaporthe grisea secretes cell-wall-degrading enzymes when grown on Vogel's medium that includes rice cell walls as the carbon source. With an inoculum of 10^6 conidia, maximum xylanase activity is detected in the medium approximately 9 days after culture initiation, while no activity is detected throughout the culture when sucrose is used as the carbon source (Fig. 1). Thus, xylanases were purified from 9-day-old culture filtrate.

Two fractionations of the culture filtrate by cation exchange chromatography at pH 5.0 resulted in a single xylanase-active peak that is eluted by approximately 0.09 M NaCl (peak II in Figure 2A and peak IIb in Figure 2B). However, the xylanase-active peak is separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) into two proteins, with molecular weights of 22,000 and 33,000 (data not shown). Gel permeation chromatography was used to separate the two proteins that are designated by their molecular weights as XYN22 and XYN33 (Fig. 2C). The purified proteins are apparently homogeneous by SDS-PAGE (Fig. 3A).

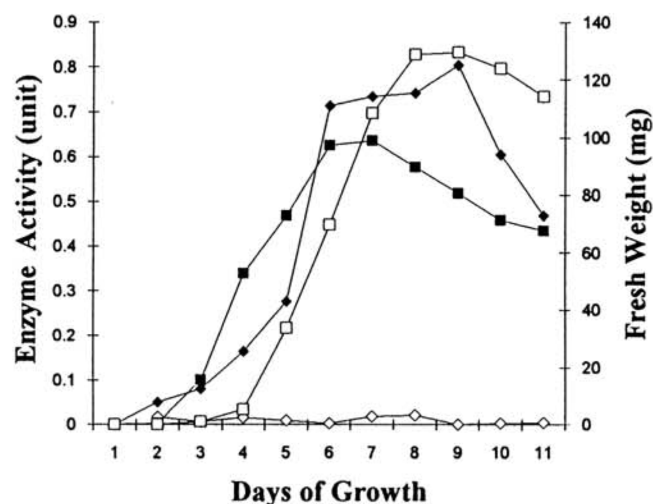

Fig. 1. Fungal growth and *endo*xylanase activity. Fresh weight of mycelia (—■—) and secreted xylanase activity (—♦—) when *Magnaporthe grisea* is grown on rice cell walls as the carbon source. Fresh weight of mycelia (—□—) and secreted xylanase activity (—◇—) when *M. grisea* is grown on sucrose as the carbon source. *Endo*-β-1,4-D-xylanase activity is defined and measured as described in the Methods section.

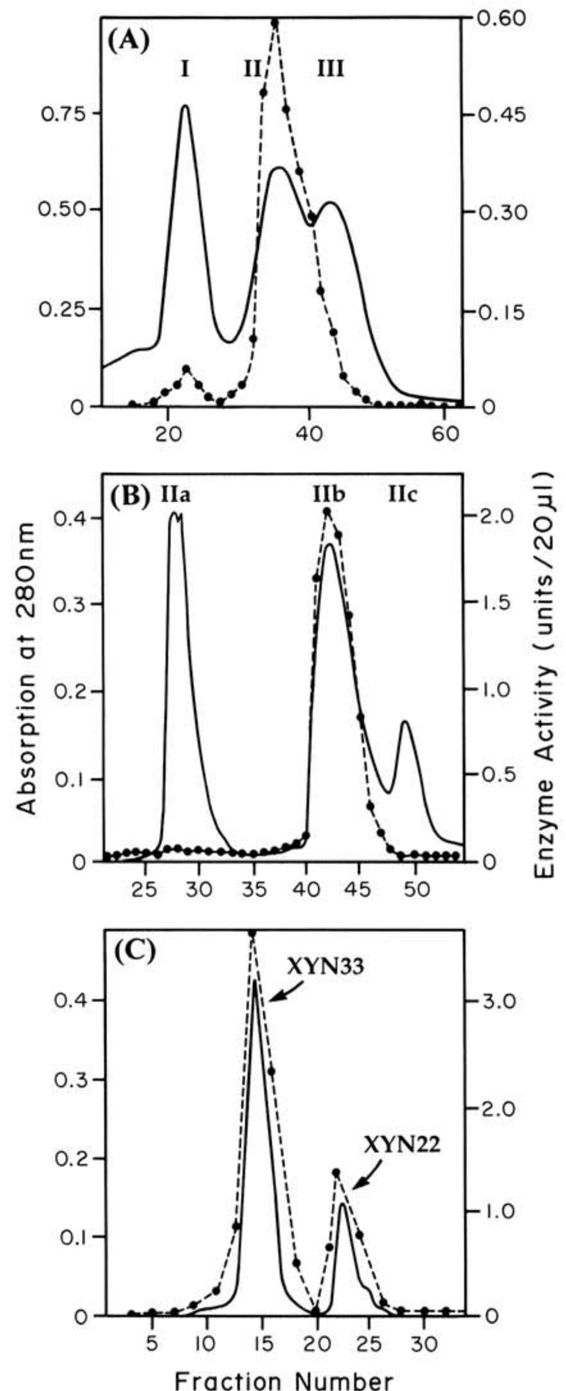

Fig. 2. Purification of *endo*xylanase from *Magnaporthe grisea* culture filtrate. **(A)** Fractionation of *M. grisea* culture filtrate on a CM-Sepharose column. The bound proteins were eluted with a linear gradient of 0 to 0.3 M NaCl (fractions 0 to 82) in 25 mM sodium succinate buffer, pH 5.0, at 4°C. **(B)** Fractionation of peak II from **(A)** on a Mono-S fast protein liquid chromatography (FPLC) column. The bound proteins were eluted at 20°C with a linear gradient of 0 to 0.25 M NaCl (fractions 0 to 120) in 25 mM sodium succinate, pH 5.0. **(C)** Fractionation of peak IIb from **(B)** on a Superose FPLC column. The elution buffer was 100 mM NaCl in 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0. —, absorption at 280 nm; --•--, xylanase activity.

Data from several independent purifications, including the one shown in Figure 2, indicated that XYN33 constituted approximately 70% of the total xylanase activity secreted by *M. grisea* (data not shown).

XYN22 and XYN33 are *endo*-β-1,4-D-xylanases, as each one hydrolyzes 4-*O*-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (Biely et al. 1985). Interestingly, when the proteins in the SDS-PAGE (Fig. 3A) were blotted onto a membrane filter and stained with an antibody raised against a 21-kD xylanase from *Trichoderma viride* (Dean et al. 1989), only XYN22 bound the antibody (Fig. 3B), indicating that XYN22 is antigenically related to the *T. viride* enzyme and XYN33 is not. The isoelectric point of XYN22 was determined to be 9.7 by isoelectric focusing– (IEF-) PAGE. Due to the limitation of the ampholine used (operating pH 3 to 10), the pI of XYN33 was not accurately determined by IEF-PAGE, but it appeared to be nearly 10, as XYN33 was focused in the IEF gel adjacent to the basic edge (data not shown).

The N-terminus of XYN22 and several internal peptides of XYN22 and XYN33 were sequenced (Table 1). Probably due to a blockage in the N-terminal amino acid, XYN33 could not be sequenced from the N-terminus. Protein databases (GenBank, PIR, and Swiss-Prot) were searched for matches with the peptide sequences of XYN22 and XYN33 (Table 1). The N-terminal and internal peptide sequences of XYN22 are homologous to family G glycosyl hydrolases, which include low-molecular-mass alkaline xylanases such as the 21-kD *T. viride* xylanase (Dean et al. 1989; Gilkes et al. 1991). All three internal peptide sequences of XYN33 are homologous to family F glycosyl hydrolases, which include high-molecular-weight acidic xylanases (reviewed by Gilkes et al. 1991).

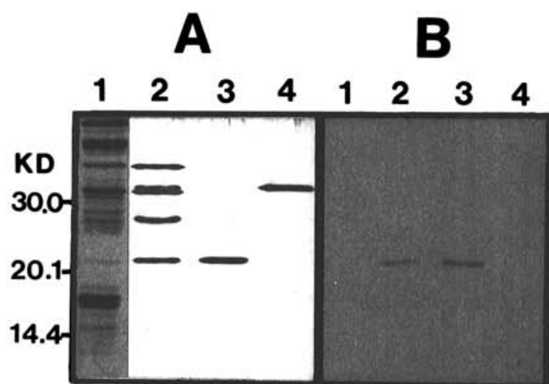

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (**A**) and immunoblot (**B**) analyses of purified xylanases. Lane 1, 5 µg of crude proteins from *Magnaporthe grisea* culture filtrate; lane 2, 1.5 µg of peak II from Figure 2A; lane 3, 0.25 µg of purified XYN22; and lane 4, 0.25 µg of purified XYN33.

Table 1. Sequences of N-terminus and Purified Peptides of XYN22 and XYN33[a]

Peptide	XYN22	XYN33
N-terminus	XSTPSSTGRHNGYYYSXXT	
#1	XNPLVEYYXXEX	XQSVFSXVLGEDFVR
#2	NQPSIA/EGTR	PNAKLYINDYNLDXP
#3		XPQGNPLLFDSNL/YNP

[a] "X" represents undetermined residues, and "/" separates two possible residues at a single site.

Cloning of *XYN22* and *XYN33*.

Cloning of *XYN22* and *XYN33* was achieved by screening a *M. grisea* genomic library using gene-specific probes. The probe for *XYN22* was a 473-bp DNA fragment amplified by polymerase chain reaction (PCR) from the genomic DNA of *M. grisea* using two degenerate oligonucleotide primers (20-mer each) derived, respectively, from HNGYYYS, part of the N-terminal sequence of XYN22 (Table 1), and from TFKQYWS, part of an internal consensus sequence of the family G xylanases (Shareck et al. 1991). The probe for *XYN33* was a 457-bp DNA fragment amplified by PCR from the genomic DNA of *M. grisea* using two degenerate oligonucleotide primers (20-mer each) derived, respectively, from PNAKLYI, part of internal peptide #2, and from FDSNYNP, part of internal peptide #3 of XYN33 (Table 1). Seven positive clones of *XYN22* and five positive clones of *XYN33*, each in a lambda vector, were isolated and mapped with restriction enzymes. The seven genomic DNA inserts that include *XYN22* are overlapping sequences covering a stretch of 28.5 kb (data not shown). Likewise, the five genomic DNA inserts containing *XYN33* are overlapping sequences that could be assembled into a single 21-kb fragment (data not shown). Gel blot analyses of *M. grisea* genomic DNA established that *XYN22* and *XYN33* were both single-copy genes in the haploid genome of *M. grisea* (Fig. 4).

Based on the nucleotide sequences of *XYN22* and *XYN33*, we have also cloned by PCR and sequenced the cDNAs of *XYN22* and *XYN33* transcripts. The sequence data are summarized in Figure 5A and B. The *XYN22* and *XYN33* genes

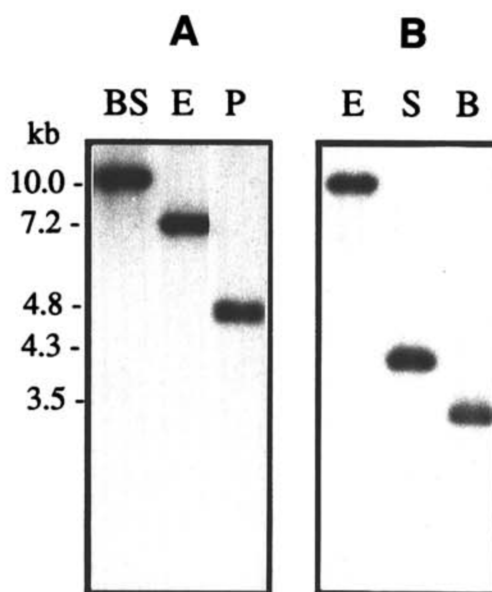

Fig. 4. Gel blot analysis of *Magnaporthe grisea* genomic DNA. Genomic DNA (5 µg samples) was digested with *Bam*HI (B), *Eco*RI (E), *Pst*I (P), *Bam*HI and *Sac*I (BS), or *Sal*I (S), separated by electrophoresis in 1.0% agarose gels, and blotted onto nylon filters. Filter A was hybridized with a 1.2-kb DNA probe of *XYN22* and filter B with a 1.5-kb DNA probe of *XYN33*.

are similar to other described *M. grisea* genes in that they more often use G/C than A/T at the third nucleotide of their codons (Sweigard et al. 1992a; Talbot et al. 1993). *XYN22* has 90.6% of its codons ending with G or C, and *XYN33* has 80.1% ending with G or C (Fig. 5A and B).

Properties of *XYN22*.

XYN22 encodes an 84-bp intron, a putative 5′ untranslated region (UTR) of 148 bp, a 3′ UTR of 194 bp, and a putative polypeptide of 233 amino acids (Fig. 5A). The first 39 amino acids appear to compose a hydrophobic signal peptide that is cleaved from the secreted enzyme (Heijne 1983) since the N-terminus of XYN22 starts at the 40th amino acid residue of the deduced polypeptide (Fig. 5A). This signal peptide is unusually long, but it is not unprecedented for microbial xylanases (Shareck et al. 1991). The calculated molecular mass and isoelectric point of the mature polypeptide are 21.3 kD and 9.71, respectively, which agree with the experimental values of 22 kD by SDS-PAGE (Fig. 3A) and 9.7 by IEF-PAGE (data not shown). *XYN22* encodes no potential Asn-X-Ser glycosylation sites.

Properties of *XYN33*.

XYN33 encodes a putative 5′ UTR of 116 bp, a 3′ UTR of 142 bp, four introns with lengths varying from 78 to 120 bp, and a putative polypeptide of 331 amino acids. The first 28 amino acids are proposed as a signal peptide for protein secretion because (a) they are very hydrophobic, and (b) although unusual (Heijne 1983), an Arg as a cleavage site has been found in the XYN22 (Fig. 5A) and in both xylanase A and xylanase C from *Aspergillus kawachii* (Ito et al. 1992a, 1992b). The calculated molecular mass and isoelectric point of the putative mature polypeptide of 303 amino acid residues are 32.8 kD and 9.95, respectively, which agree with the experimental values of 33 kD by SDS-PAGE (Fig. 3A) and about 10 by IEF-PAGE (data not shown). XYN33 has two potential Asn-X-Ser glycosylation sites (Fig. 5B), but it remains to be determined whether XYN33 is glycosylated.

Comparison with other xylanases.

The deduced amino acid sequences of XYN22 and XYN33 do not share any similarity. Searches in the protein databases (GenBank, PIR, and Swiss-Prot) using the amino acid sequences of XYN22 and XYN33 find many matches for each with other microbial glycosyl hydrolases. Examples of the best matches are presented in Figure 6A and B. XYN22 is homologous to members of the family G glycosyl hydrolases, and XYN33 is homologous to members of the family F glycosyl hydrolases. The identity to family G xylanases ranges from 40 to 60% for XYN22; the identity to family F xylanases ranges from 33 to 58% for XYN33. Xylanases from other fungi are most similar to both XYN22 and XYN33 (Fig. 6A and B). XYN33 also shares 30 to 35% identity with the catalytic domains of the family F cellulases (Gilkes et al. 1991).

Expression of *XYN22* and *XYN33* in *M. grisea*.

The expression of *XYN22* and *XYN33* in *M. grisea* was studied by gel blot analysis with total RNA samples prepared from *M. grisea* mycelia grown on rice cell walls, oatspelt xylan, or sucrose. The probes used for hybridization were a 0.72 kb ^{32}P-labeled DNA derived from the 3′ end of *XYN33* cDNA

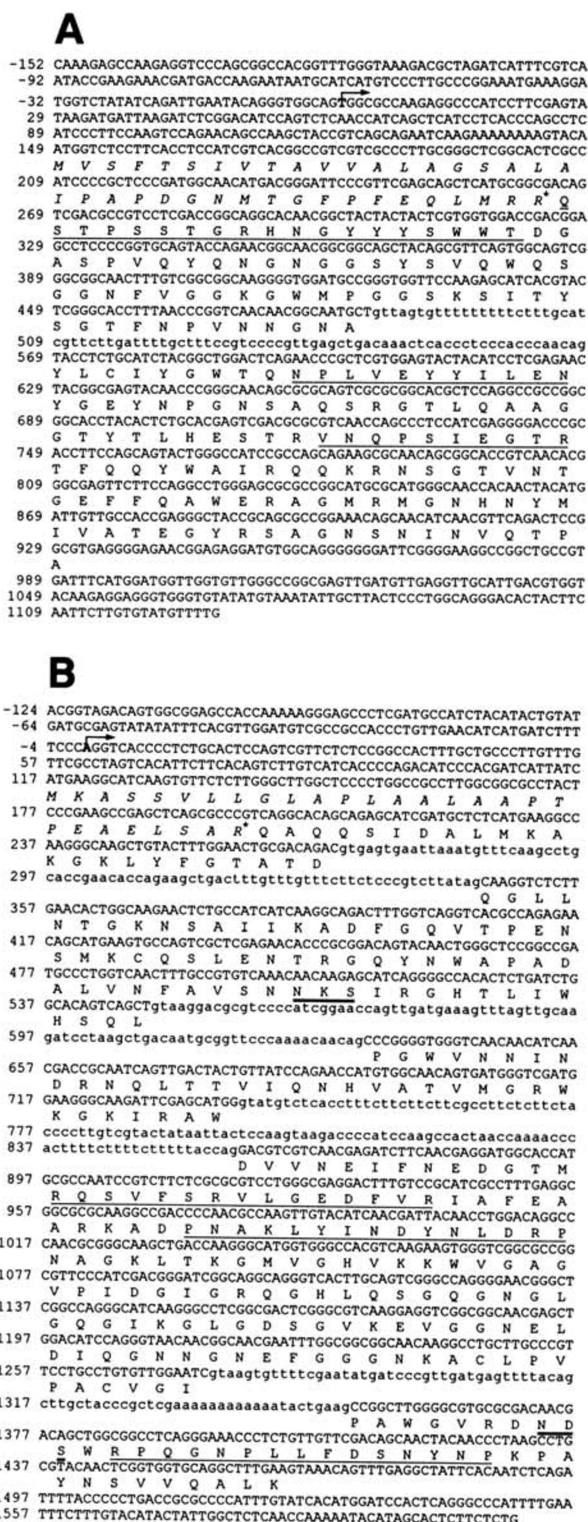

Fig. 5. Nucleotide and deduced amino acid sequences of *XYN22* (**A**) and *XYN33* (**B**). The nucleotide sequences are numbered from the transcription start sites and are terminated by the 3′ ends of the corresponding cDNAs. The intron sequences are printed in lower case letters. The putative signal peptides are shown in italics with their cleavage sites marked by an asterisk. The amino acid sequences that match with the sequenced peptides (Table 1) are underlined. Potential N-glycosylation sites are indicated with double lines. The sequences of *XYN22* and *XYN33* have been deposited in GenBank with the accession numbers of L37529 and L37530, respectively.

(Fig. 5B) and a ^{32}P-labeled RNA transcribed in vitro from the 1.05-kb *XYN22* cDNA (Fig. 5A). The more sensitive antisense riboprobe was chosen for the study of *XYN22* expression because a clear-cut hybridization signal could not be obtained with a conventionally labeled DNA probe. Transcripts of *XYN33* and *XYN22* are present in *M. grisea* grown on rice cell walls. Transcripts of *XYN33* and *XYN22* were also detected in *M. grisea* fed on oatspelt xylan or other xylan-containing carbon sources (data not shown). The abundance of *XYN33* and *XYN22* transcripts is greatest on the fifth post-inoculation day, although the accumulation patterns of *XYN33* and *XYN22* messenger RNAs differ slightly (Fig. 7A and B). No transcript of *XYN33* or *XYN22* was detected in the RNA samples isolated from *M. grisea* grown on sucrose (Fig. 7C). These results are consistent with the observed accumulation of xylanase activity (Fig. 1).

DISCUSSION

Many bacteria and fungi secrete multiple isozymes of *endo*xylanases. These isozymes can be divided into two families: the low molecular weight, basic family G xylanases and the high molecular weight, acidic family F xylanases. Members within each family are highly conserved in primary structure (Gilkes et al. 1991; Shareck et al. 1991; Gilbert and Hazlewood 1993; Thomson 1993). *Magnaporthe grisea*, the rice blast fungus, secretes two *endo*-β-1,4-D-xylanases, the 22-kD XYN22 and the 33-kD XYN33. Based on their characteristics and amino acid sequences, XYN22 belongs to family G and XYN33 to family F xylanases. However, XYN33 has a very basic pI (~9.95), whereas all but two of the approximately 20 family F xylanases present to date in protein databases have a pI below 7.

We observed that when *M. grisea* was grown on rice cell walls as the only carbon source, XYN22 is the minor isozyme secreted, accounting for about 30% of total xylanase activity under our experimental conditions (Figs. 2C, 3A, and 7B). This finding contrasts with other bacterial and fungal systems in which family G isozymes predominate. For example, two fungal plant pathogens, *C. carbonum* and *G. graminis*, secrete at least 80% of the xylanase activity as family G hydrolases

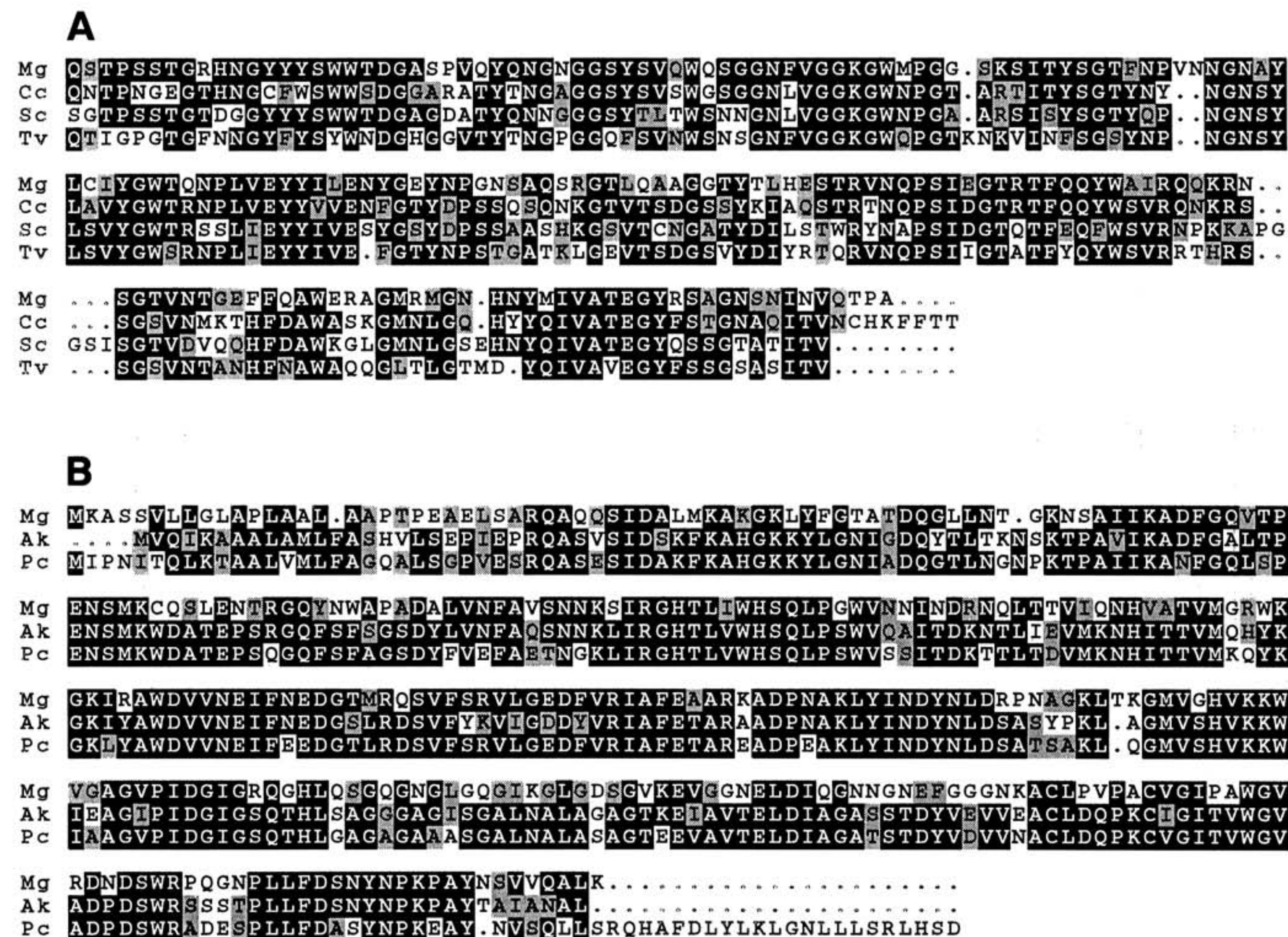

Fig. 6. Comparison of the sequences of XYN22 (**A**) and XYN33 (**B**) with the sequences of other fungal *endo*xylanases. Protein sequences were retrieved, compared, and compiled by software programs in the Wisconsin Package (Genetics Computer Group, Madison, Wis.). Characters in dark and gray backgrounds indicate, respectively, identical and similar amino acid residues among the compared sequences. The putative signal peptides of XYN22 and its counterparts from other fungal species were excluded from the comparison. The abbreviations are: Ak, *Aspergillus kawachii* (Ito et al. 1992a and 1992b); Cc, *Cochliobolus carbonum* (Apel et al., 1993); Mg, *Magnaporthe grisea* (this paper); Pc, *Penicillium chrysogenum* (Haas et al. 1993); Sc, *Schizhophyllum commune* (Oku et al. 1993); and Tv, *Trichoderma viride* (Yaguchi et al. 1992).

(Holden and Walton 1992; Southerton et al. 1993). We do not know whether these differences have any significant effect on the pathogenicity of the pathogens. It is possible that XYN22 might account for a larger proportion of the total xylanase activity if *M. grisea* is grown on other carbon sources, such as pure xylans. It is also possible that the proportion of XYN22 versus XYN33 activity might change if the enzymes are assayed at different pH, since XYN22 and XYN33 are likely to have different pH optima (Sumizu et al. 1961).

It was impossible to detect *XYN22* transcripts with a conventionally labeled DNA probe without producing a high level of background, an indication that the concentration of *XYN22* transcripts was low in *M. grisea* culture. Indeed, we have obtained preliminary results that the amount of *XYN22* transcripts is about 10-fold less than that of *XYN33* transcripts in *M. grisea* culture grown on rice cell walls for 5 days (data not shown). These observations strongly suggest that the composition of xylanase activity secreted by *M. grisea* (Fig. 2C) is regulated at the level of gene expression under the culture conditions used. Furthermore, the expressions of *XYN22* and *XYN33* in *M. grisea* may be independent of each other, as the relative concentration of *XYN22* and *XYN33* transcripts varies considerably from day 4 through day 6 (Fig. 7A and B). The recent discovery (Arhin et al. 1994) that deletion of a family F xylanase in *Streptomyces lividans* does not interfere with the production of a family G xylanase and vice versa supports our speculation.

XYN22 and XYN33 are encoded, respectively, by two unrelated single-copy genes in *M. grisea* (Fig. 4). It is most likely that *XYN22* and *XYN33* are the only xylanase genes in *M. grisea*, since XYN22 and XYN33 represent the only two *endo*xylanase families discovered so far among microorganisms and a third, structurally unrelated *endo*xylanase was not found in the culture filtrate of *M. grisea*. Thus, it is possible that a *M. grisea* mutant that lacks *XYN22* and *XYN33* will no longer be able to utilize xylan as the only carbon source. Since xylan accounts for about 60% of total cell wall carbohydrates in rice cells (Takeuchi et al. 1994), such a mutant may also have reduced virulence towards its rice host. We are attempting targeted disruption (Sweigard et al. 1992b) of the *M. grisea* xylanases to ascertain their role in pathogenesis.

MATERIALS AND METHODS

Sources of fungus and rice cell walls.

Magnaporthe grisea laboratory strain ken60-19 was a gift of Barbara Valent (Central Research & Development, E. I. du Pont de Nemours & Co., Wilmington, Del.). The ken60-19 is maintained on oatmeal agar as described (Valent et al. 1986). Rice cell walls were prepared from a suspension culture according to a standard procedure (Hahn et al. 1992).

Fungal growth.

Magnaporthe grisea conidia (10^6), collected from mycelia grown for 1 month on oatmeal agar, were inoculated in 1 liter of Vogel's basal medium (Vogel 1964) supplemented with 5 μg of biotin, 1.0 mg of thiamine, and either 5 g of rice cell walls, 5 g of oatspelt xylan, or 10 g of sucrose as the carbon source. The culture was incubated in the dark with constant shaking (100 rpm). To monitor fungal growth and xylanase secretion, 5 ml of culture liquid was removed each day and filtered through two layers of 934-AH paper (0.7 μm pore size, Whatman LabSales, Hillsboro, Oreg.). The fresh mycelial pad was weighed and 50 μl of the clear culture filtrate assayed for *endo*-β-1,4-D-xylanase activity.

Protein and enzyme assay.

Protein content was measured with the Quantigold reagent according to the supplier's instruction (Diversified BioTech, Boston, Mass.), using human β-immunoglobin as a standard. *Endo*-β-1,4-xylanase activity was determined by the method of Biely et al. (1985), using 4-*O*-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (RBB-xylan, product of Sigma, St. Louis, Mo.) as substrate. One unit of xylanase is defined as the amount of enzyme that, at 37°C for 60 min, liberates 1.0 absorption unit at 595 nm of ethanol-soluble dye. A more sensitive but less specific method (Lever 1972) was used, as described by Bucheli et al. (1990), to measure the reducing sugar released from oatspelts xylan (Sigma) by xylan-degrading enzymes (*endo*- and *exo*xylanases, xylosidase, arabinosidase, etc.).

Enzyme purification.

Xylanases were purified from the culture filtrate of *M. grisea* that had been grown on rice cell walls for 9 days. The clear culture filtrate (1 liter) was concentrated to about 100 ml in an Amicon ultrafiltration cell by passage through a PM10 membrane (molecular weight cut-off of 10,000). The concentrate was dialyzed extensively against 25 mM sodium succinate, pH 5.0 (buffer A), and loaded onto a CM-Sepharose

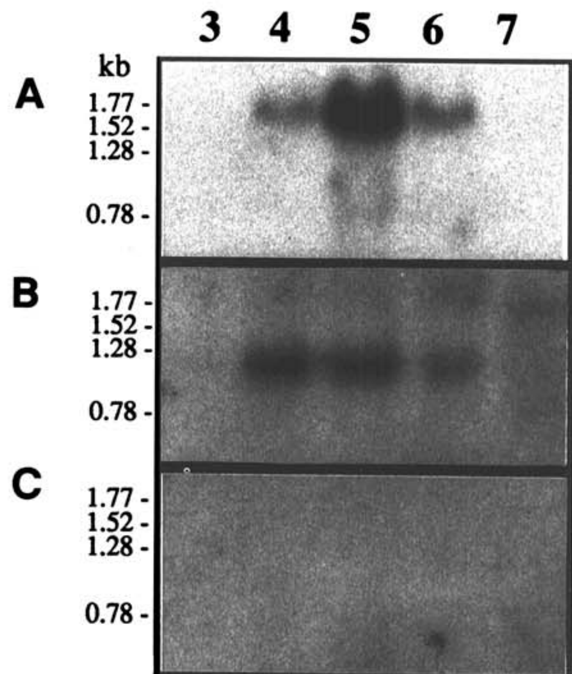

Fig. 7. Gel blot analysis of total RNA from *Magnaporthe grisea*. Total RNA samples (10 μg), isolated from fungal mycelia grown for 3 to 7 days on rice cell walls **(A and B)** or sucrose **(C)**, were separated by electrophoresis in 1.2% agarose gels and blotted onto nylon filters. Filters A and C were hybridized with a 0.72-kb ^{32}P-labeled DNA probe derived from the 3′ end of *XYN33* cDNA. Filters B and C were hybridized with a ^{32}P-labeled antisense riboprobe transcribed in vitro from the full length cDNA of *XYN22* (Fig. 6A). After hybridization, filter A was exposed at –80°C to X-ray film for 2 days and filters B and C for 8 days.

column (40 ml, fast flow, Sigma). The bound proteins were eluted with a linear gradient of 0 to 0.3 M NaCl. Fractions (4 ml) of the eluent were assayed for *endo*-β-1,4-D-xylanase activity. The xylanase-active fractions were combined and dialyzed against buffer A. This sample (50 ml) was divided into two equal portions and each was subjected to cation exchange chromatography on a mono-S column (HR 5/5, Pharmacia BioTech, Inc., Piscataway, N.J.). The bound proteins were eluted from the mono-S column using a linear gradient of 0 to 0.25 M NaCl. Fractions (0.4 ml) containing xylanase activity were combined and further purified by gel permeation chromatography on a Superose 12 column (HR 10/30, Pharmacia). The purified xylanases were stored at –20°C in 100 mM 2-(N-Morpholino)-ethane sulfonic acid (MES) buffer, pH 6.0, 100 mM NaCl, and 40% glycerol.

Electrophoresis and isoelectric focusing.

Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) was used to estimate the purity and molecular weight of xylanases. IEF-PAGE was used to determine the isoelectric point of the xylanases (Sambrook et al. 1989). The native IEF gel contains 2.4% of Biolyte (operating pH 3-10; BioRad Laboratories, Inc., Hercules, Calif.). IEF standard markers with pIs ranging from 4.2 to 9.6 were purchased from BioRad, Inc.

Immunoblotting analysis.

Protein samples were separated by SDS-PAGE, electroblotted onto a ProBlott membrane (Applied BioSystems Inc., Foster City, Calif.), and incubated in a solution containing a rabbit polyclonal antibody raised against a 22-kD ethylene-inducing xylanase from *Trichoderma viride* (Dean et al. 1989). The filter was then treated with an alkaline phosphatase-conjugated anti-rabbit IgG antibody from goat (Sigma). Cross-reacting proteins were visualized by the method of Johnson et al. (1984).

Peptide sequencing.

XYN22 (10 μg) was digested at 37°C for 20 h with 0.5 μg of trypsin in 1 ml of 0.1 M NH_4HCO_3. XYN33 (15 μg) was digested at room temperature for 20 h with CNBr in 70% formic acid. The digested proteins were fractionated by high-pressure liquid chromatography on a reversed-phase column (C-18, Applied BioSystems Inc.). The purified peptides were sequenced on a gas-phase peptide sequencer (Model 476A, Applied BioSystems Inc.) at the Molecular Genetics Instrumentation Facility (Research Services, University of Georgia, Athens).

Molecular cloning.

Except where stated otherwise, all cloning procedures were performed as described by Sambrook et al. (1989). The genomic library of *M. grisea*, strain ken60-19, was constructed by inserting partially digested DNA fragments (13 to 20 kb) into a lambda Fix II vector according to the manufacturer's instructions (Stratagene, San Diego, Calif.). The library was screened separately for genes encoding XYN22 and XYN33 by using gene-specific DNA probes generated by PCR. The DNA inserts of positive clones were mapped by restriction *endo*nucleases and subcloned for DNA sequencing in a pBluescript II phagemid vector (Stratagene).

RNA gel blot analysis.

Total RNA samples were isolated, according to the method of Cathala et al. (1983), from *M. grisea* mycelia grown for 1-10 days. Samples containing 10 μg of total RNA were separated by electrophoresis in a 1.2% agarose gel, blotted onto nylon membrane (Hybon-N filter, Amersham Co., Arlington Heights, Ill.), and hybridized with ^{32}P-labeled DNA or RNA probes according to standard procedures (Sambrook et al. 1989).

PCR procedures.

Conventional PCR was performed as described (Sambrook et al. 1989). The procedure for a reverse transcriptase-mediated PCR (RT-PCR) was modified from the two-tube system of Goblet et al. (1992). First-strand cDNAs were synthesized from a 10-μg total RNA sample with avian myeloblastosis virus (AMV) reverse transcriptase, using a 34-mer oligo(dT) as the extending primer. The total RNA sample was isolated from *M. grisea* mycelia that had been grown on rice cell walls for 5 days. The cDNA products were purified with a Geneclean II kit of Bio101, Inc. (San Diego, Calif.) and eluted into 50 μl of H_2O. Samples (5 μl) of the first strand cDNA were then used as a template for PCR.

Cloning of *XYN22* and *XYN33* transcripts.

The full-length cDNAs of *XYN22* and *XYN33* transcripts were amplified from the first-strand cDNA sample (above) by PCR. The 5′ ends of cDNAs were amplified according to the rapid amplification of cDNA ends (5′ RACE) procedure as outlined in the instructional manual for the 5′-AmpliFinder RACE kit from Clontech Laboratories, Inc. (Palo Alto, Calif.). The gene-specific primers (see below) used in 5′ RACE were primer II for *XYN22* and primer VI for *XYN33*. The internal cDNA fragments were amplified using primer pair I/IV for *XYN22* and primer pair V/VII for *XYN33*. The 3′ ends of cDNAs were amplified using the oligo(dT) as downstream primer and gene-specific oligonucleotides III, for *XYN22*, and VI, for *XYN33*, as the upstream primers. Each PCR product was cloned into pBluescript II vector and analyzed by restriction enzymes and by DNA sequencing. The synthetic oligonucleotides are as follows:

Oligo(dT): 5′ GCTCGAGGGTCGACG$(T)_{20}$
Primer I: 5′ TGGCGCCAAGAGGCCCATCCTTCG
Primer II: 5′ GTTGTGCCTGCCGGTCGAGGACGG
Primer III: 5′ GACCCGCACCTTCCAGCAGTACTG
Primer IV: 5′ GCTCAATGCAACCTCAACATCAAC
Primer V: 5′ CACACTCTGATCTGGCACAGTC
Primer VI: 5′ GACTGTGCCAGATCAGAGTGTGG
Primer VII: 5′ GTAGTTGCTGTCGAACAACAGA

Determination of the 5′ ends of XYN22 and XYN33 transcripts.

Primer extension and the 5′ RACE (see above) were used to determine the 5′ ends of *XYN22* and *XYN33* transcripts. A standard primer extension protocol was performed with slight modifications (Sambrook et al. 1989). A 30-mer oligonucleotide primer complementary to the sequence upstream from the putative start codon was synthesized, labeled at the 5′ end with ^{32}P-ATP, and hybridized, under stringent conditions (0.25 M KCl, 65°C) to 0.5 μg of poly(A) RNA that had been isolated from *M. grisea* mycelia grown on rice cell walls for 5

days. First-strand cDNA was then extended from the annealed primer with AMV reverse transcriptase. The product was sized on a sequencing gel (Sambrook et al. 1989). The extending primer for *XYN22* was 5′ GGATGAGGCTGGGTGAGGATGAGCTGATGG and for *XYN33* 5′ CGTGGGATGTCTGGGGTGATGACAAGACTG. The 5′ transcription start sites of *XYN22* and *XYN33* were identified because the primer extension results matched those obtained by 5′ RACE.

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