Deletion of Two *Endo*- β -1,4-Xylanase Genes Reveals Additional Isozymes Secreted by the Rice Blast Fungus

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Fungal pathogens secrete hydrolases during infection of plant tissues capable of fragmenting the primary cell wall polysaccharides of the host. *Magnaporthe grisea*, the fungal pathogen that causes blast disease of graminaceous monocots, secretes two distinct *endo*- β -1,4-D-xylanases when grown on xylan-rich rice cell walls as the carbon source. We have previously reported the cloning of the genes encoding these two xylanases, *XYL1* and *XYL2* (formerly *XYN22* and *XYN33*, respectively; see S.-C. Wu, S. Kauffmann, A. G. Darvill, and P. Albersheim, *Mol. Plant-Microbe Interact.* 8:506-514, 1995). We now present three *M. grisea* mutants created by selective deletion of *XYL1* and/or *XYL2*. The *xyl1* mutant grows as well as the parent in culture medium when rice cell walls or xylan is the sole carbon source. Under the same conditions, the *xyl2* mutant grows slightly slower than the parent, whereas the *xyl1/xyl2* double mutant exhibits a 50% reduction in accumulation of total mycelial mass. Under conditions idealized for infection, all three mutants infect host plants as efficiently as the parent, indicating neither *XYL1* nor *XYL2* is required by *M. grisea* for infection. *Endoxylanase* assays showed that, at the stationary stage of growth in culture when the accumulation of total xylanase activity is at its maximum, the *xyl1* mutant retains $\approx$ 88%, *xyl2*, 39%, and *xyl1/xyl2*, 19% of the *endoxylanase* activity of the parent. Partial protein purification of xylanases secreted by the *xyl1/xyl2* double mutant revealed four distinct *endoxylanase* activities. One of the xylanases, *XYL3*, is present in the culture filtrate of both the parent and the mutant strains, and, like *XYL1*, has been identified as a member of the Family G xylanases. The other three xylanases are not found in the culture filtrates of the parent or of the *xyl1* mutant. Thus, *M. grisea* appears capable of secreting additional xylanases and does so when *XYL2*, a member of the Family F glycanases, has been eliminated.

Additional keywords: electroporation, fungal pathogenesis, gene disruption, hypersensitive response, selection markers.

The cell walls of plants act as a barrier to and as a source of nutrition for pathogens, and as a reservoir of signal molecules that regulate plant defense, growth, and development (Hahn et al. 1989; Darvill et al. 1992; Zabackis et al. 1996). It is generally agreed that, in order to penetrate the cell wall and use the cell wall's building blocks as nutrients, most (and perhaps all) fungal and bacterial pathogens secrete wall polysaccharide-cleaving *endoglycanases* when infecting plant tissues (Cooper et al. 1988; Dean and Timberlake 1989; Lehtinen 1993; Southerton et al. 1993; Walton 1994; Kolattukudy et al. 1995; Peltonen 1995; Howard and Valent 1996; Xu and Mendgen 1997). During their attack on host cell wall polymers, some of the *endoglycanases* generate oligosaccharide signal molecules that activate the defense mechanisms of the host, possibly including hypersensitive cell death (Hahn et al. 1989; Darvill et al. 1992; Ryan and Farmer 1992; Ham et al. 1995; Wu et al. 1995).

Arabinoxylan, the quantitatively predominant hemicellulosic polysaccharide of monocotyledonous plants, is composed of a β -1,4-linked D-xylopyranosyl backbone that is frequently substituted, at the O₂ and/or O₃ positions of xylosyl residues in the backbone, by various mono- or oligosaccharide side chains. The side chains consist of largely arabinosyl, xylosyl, and/or glucosyluronic acid residues (Carpita and Gibeau 1993). In graminaceous crops and grasses, arabinoxylan accounts for up to 60% of total cell wall carbohydrate (Carpita and Gibeau 1993; Takeuchi et al. 1994), making them a likely target for fragmentation by invading pathogens.

There is evidence that microbial *endo*- β -1,4-D-xylanases, which cleave β -1,4 linkages of the xylosyl backbone, play a central role in the breakdown and utilization of the cell walls of *Graminaceae*. For example, bacterial strains of *Erwinia chrysanthemi* that infect maize secrete more xylanase activity than strains that infect only dicotyledonous plants (Braun and Rodrigues 1993), and fungal pathogens, such as *Septoria nodorum*, that infect graminaceous monocots are reported to secrete more xylan-degrading enzymes than pectinases (Cooper et al. 1988; Lehtinen 1993). Other evidence supporting a role for xylanases in the pathogenicity of fungal plant pathogens includes the observations that a fungal xylanase is capable of killing cultured rice cells (Ishii 1988), and that a 21-kDa xylanase from *Trichoderma viride* elicits defense responses in tobacco plants, including tissue necrosis and increased production of both stress ethylene and pathogenesis-related proteins (see review by Walton 1994).

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Magnaporthe grisea (T. T. Hebert) Yaegashi & Udawa (anamorph, *Pyricularia oryzae* (Cooke) Sacc.), a fungal pathogen that causes blast disease in rice and other graminaceous monocots (Valent and Chumley 1991; Howard and Valent 1996), secretes *endo*- β -1,4-D-xylanases and other cell wall hydrolases when grown on isolated rice cell walls as a carbon source (Bucheli et al. 1990; Wu et al. 1995; S.-C. Wu, A. G. Darvill and P. Albersheim, *unpublished observation*). We have purified two xylanases, XYL1 and XYL2 (formerly XYN22 and XYN33, respectively; see Wu et al. 1995), from the culture filtrate of *M. grisea*. XYL1 accounts for less than 30% of the total xylanase activity in the culture fluid, whereas XYL2 accounts for about 70% of the total. Cloning and analysis of the sequences of XYL1 and XYL2 show that they are structurally unrelated. XYL1 is a 22-kDa protein and a member of a family of small, basic, microbial xylanases called Family G, and XYL2 is a 33-kDa protein and a member of a family of large, acidic xylanases called Family F (reviewed by Gilkes et al. 1991). We now report the deletion of *XYL1* and *XYL2* from the genome of *M. grisea* and the effect the loss of these enzymes has on the growth, pathogenicity, and xylanase production of this fungus.

RESULTS

Generation of *M. grisea* xylanase mutants.

Magnaporthe grisea mutants lacking the xylanase genes *XYL1* and *XYL2* were created by gene replacement (Fig. 1). *M. grisea* strain CP987 was selected as the parent, as CP987 is genetically stable and sporulates in large quantities on oatmeal agar (Sweigard et al. 1995; S.-C. Wu, *unpublished observation*). Strain CP987 infects some cultivars of rice (e.g., Sariceltik), barley, and weeping lovegrass, and causes hypersensitive necrotic lesions on others (e.g., rice cv. Marotelli). Protoplasts of strain CP987 were electro-transformed with linearized plasmid pX1HYG or pX2BEN (Fig. 1). The electroporation protocol delivers the linearized plasmids into *M. grisea* protoplasts, yielding approximately 500 transformants per microgram of DNA (see Materials and Methods).

M. grisea transformants resistant to hygromycin B and/or benomyl were screened for gene replacement mutants by polymerase chain reaction (PCR) (Fig. 1). The majority of hygromycin-resistant (*HYG^r*) transformants result from random integration of plasmid pX1HYG, giving rise to a 0.6-kb PCR fragment from the native *XYL1* and a 1.4-kb fragment from the integrated pX1HYG (results not shown). A transformant generated by gene replacement of the *XYL1* locus yields only the 1.4-kb PCR product (see Fig. 2A). Similarly, benomyl-resistant (*BEN^r*) transformants resulting from the random integration of pX2BEN give rise to a 2.8-kb PCR fragment from the native *XYL2* and a 5.1-kb PCR fragment from the integrated pX2BEN (results not shown). A transformant resulting from homologous recombination in the *XYL2* locus yields only the 5.1-kb PCR product (Fig. 2B). The PCR screening allowed the isolation from 100 pX1HYG transformants of one *xyl1::HYG^r* mutant (*xyl1* mutant; strain X7201), and from 200 pX2BEN transformants one *xyl2::BEN^r* mutant (*xyl2* mutant; strain X7301). A *xyl1::HYG^r xyl2::BEN^r* double mutant (*xyl1/xyl2* mutant; strain X7231) was obtained by transforming X7201 with plasmid pX2BEN and screening approximately 250 transformants.

Fig. 1. Xylanase gene replacement vectors. **A**, Construction of plasmid pX1HYG (*xyl1::HYG^r*). Plasmid pX1, which contains the single-copy *XYL1* gene (hatched bar) of *Magnaporthe grisea* (Wu et al. 1995), was digested with *Bgl*II and *Xho*I. Resulting 0.6-kb fragment was replaced by a 1.4-kb *Bam*HI/*Sal*I DNA fragment containing a hygromycin B phosphotransferase gene (*HYG^r*, dotted bar; see Staben et al. 1989). Numbered bars flanking *XYL1* indicate positions of oligonucleotide primers designed for screening of *xyl1* mutants by polymerase chain reaction (PCR) (Fig. 2A). Primer 1 is 5' TGGCGCCAAGAGGCCCA TCCTTCG, and primer 2 is 5' GGGTGTGACTCGCCGTAGTTCTCG. Brackets above pX1, labeled "Probes a/b," represent size and location of DNA probes used to identify homologous recombinants by Southern blot hybridization (Fig. 2C). Plasmid pX1HYG was linearized by restriction enzymes *Bam*HI and *Sac*I before being electroporated into *M. grisea* protoplasts. **B**, Construction of pX2BEN (*xyl2::BEN^r*). Plasmid pX2, which contains the single-copy *XYL2* gene (solid bar) of *M. grisea* (Wu et al. 1995), was digested with *Mlu*I and *Xba*I. Resulting 1.4-kb *Mlu*I/*Xba*I fragment was replaced by a 3.7-kb *Nsi*I/*Xba*I DNA fragment excised from plasmid pCB630 (courtesy of J. Sweigard and B. Valent, Central Research and Development, E. I. DuPont de Nemours, Wilmington, DE), which contains a mutated *M. grisea* β -tubulin gene (*BEN^r*, open bar) that confers benomyl resistance (Orbach et al. 1986). Numbered bars flanking *XYL2* indicate positions of oligonucleotide primers designed for screening of *xyl2* mutants by PCR (Fig. 2B). Primer 3 is 5' CGACGGTAGACAGTGGCGGAGCCAC, and primer 4 is 5' GACTGGCCAGCTCGCACGACAAACC. Brackets above pX2, labeled "Probes c/d," represent size and location of DNA probes used to identify homologous recombinants by Southern blot hybridization (Fig. 2D). Plasmid pX2BEN was linearized by restriction enzyme *Nar*I before being electroporated into *M. grisea* protoplasts. Restriction enzymes abbreviated (**A** and **B**) as follows: B, *Bam*HI; Bg, *Bgl*II; M, *Mlu*I; N, *Nar*I; Ns, *Nsi*I; P, *Pst*I; S, *Sal*I; Sc, *Sac*I; X, *Xba*I; and Xo, *Xho*I.

Gel blot analyses of total genomic DNA and RNA samples were performed to ensure that X7201, X7301, and X7231 are gene replacement mutants. Southern blot hybridization with probe b (Fig. 1A) demonstrated that in the *xyl1* mutants (strains X7210 and X7231) a 4.1-kb fragment from the native *XYL1* locus is replaced by a 3.1-kb *Pst*I-cleaved DNA fragment of the vector pX1HYG (Figs. 1A and 2C). Similarly, Southern blot analysis with probe d established that in *xyl2* mutants (strains X7301 and X7231) a 3.4-kb fragment from the native *XYL2* locus is replaced by a 5.7-kb *Nar*I-cleaved DNA fragment of the vector pX2BEN (Figs. 1B and 2D). Furthermore, Northern (RNA) blot hybridization experiments confirmed that X7201 no longer transcribes detectable amounts of *XYL1* mRNA, X7301 no longer transcribes detectable quantities of *XYL2* mRNA, and X7231 no longer transcribes detectable quantities of *XYL1* and *XYL2* mRNA (Fig. 2E, F).

Growth of *xyl* mutants in media rich in xylan.

M. grisea was grown in a complete medium or in Vogel's basic salt medium (Vogel 1964) with isolated rice cell walls or oat spelt xylan as a carbon source. The growth rates of the parent and each of the xylanase (*xyl*) mutants are linear between day 4 and day 6 post inoculation (S.-C. Wu, unpublished ob-

servation; see Materials and Methods). There is no significant difference in the growth rate of strains CP987, X7201, X7301, and X7231 in the complete medium, i.e., the amount of accumulated mycelium mass during log-phase growth in the complete medium is the same for the parent and each of the mutant strains (Table 1). However, in Vogel's basic salt medium containing isolated rice cell walls or oat spelt xylan as the sole carbon source, the growth and morphology of the *xyl* mutants differ considerably, with the double mutant X7231 accumulating approximately 50% less mycelial mass than the parent at the middle of log-phase growth (Table 1).

The radial expansion of the fungi in agar media is approximately proportional to the mycelial mass accumulation in liquid culture (Fig. 3). Morphologically, a slower radial expansion of fungal hypha correlates with a sharper colony border, deeper pigmentation, and earlier production of conidia (Fig. 3).

Deletion of both *XYL1* and *XYL2* does not affect pathogenicity of *M. grisea*.

The effect of the deletion of both *XYL1* and *XYL2* on the pathogenicity of *M. grisea* was tested with an infection assay (Valent et al. 1991). Seedlings of rice, barley, and weeping lovegrass were inoculated with a suspension of *M. grisea* conidia (2×10^4 conidia per ml) from the parent strain or from one of the *xyl* mutants. As shown in Figure 4 (left panel), the two single mutants X7201 and X7301, the double mutant X7231, and the parent CP987 are all equally able to infect the fourth leaf of rice cv. Sariceltik; the size and color of the disease lesions generated by the parent and by each of the single mutants and the double mutant are indistinguishable. The same result is obtained when either barley or weeping lovegrass is the host (results not shown). These experiments demonstrate that neither *XYL1* nor *XYL2* is needed for pathogenicity of *M. grisea* under optimized growth chamber conditions.

There is evidence that xylanases may be involved in eliciting a plant's hypersensitive response (reviews in Ryan and Farmer 1992; Walton 1994; S.-C. Wu, S. Kauffmann, A. G. Darvill, and P. Albersheim, unpublished results). The rice cv. Marotelli, which is resistant to *M. grisea* strain CP987 (Sweigard et al. 1995), was used as a host to test this hypothesis. Marotelli was inoculated with conidia of CP987 or one of the three *xyl* mutants—X7201, X7301, and X7231. Marotelli responds to each of the three *xyl* mutants by forming hypersensitive flecks, just as it responds to the parent strain (Fig. 4, right panel). Thus, under optimized growth chamber condi-

Fig. 2. Identification of *xyl* mutants. **A**, Polymerase chain reaction (PCR) amplification of *XYL1* (0.6 kb) and *xyl1::HYG⁺* (1.4 kb) gene fragments from pX1HYG transformants with primers 1 and 2 (see Fig. 1A). **B**, PCR amplification of *XYL2* (2.8 kb) and *xyl2::BEN⁺* (5.1 kb) gene fragments from pX2BEN transformants with primers 3 and 4 (see Fig. 1B). **C**, Southern blot analysis with probe b (Fig. 1A). Approximately 10 ng of plasmid DNA of pX1 and pX1HYG (Fig. 1A) or 2 µg of fungal DNA from each of CP987, X7201, X7301, and X7231 strains was digested with *Pst*I prior to agarose gel electrophoresis. **D**, Southern blot analysis with probe d (Fig. 1B). Approximately 10 ng of plasmid DNA of pX2 and pX2BEN (Fig. 1B) or 2 µg of fungal DNA from each of CP987, X7201, X7301, and X7231 strains was digested with *Nar*I prior to agarose gel electrophoresis. **E**, Northern (RNA) blot analysis of total fungal RNA samples (5 µg) with a riboprobe transcribed from *XYL1* cDNA (Wu et al. 1995). **F**, Northern blot analysis of total fungal RNA samples (5 µg) with a riboprobe transcribed from *XYL2* cDNA (Wu et al. 1995). Total RNA samples were prepared from fungal mycelia of the *xyl* mutants and their parent that had been grown for 5 days in Vogel/RCW medium (see Table 1 footnotes and Wu et al. [1995]).

Table 1. Total mycelium mass in liquid cultures of *Magnaporthe grisea* strains used in this paper

Strain	Genotype	Mycelium fresh weight (mg) ^a		
		CM	Vogel/RCW	Vogel/OSX
CP987	<i>XYL1 XYL2</i>	99 ± 6 ^b	78 ± 5	70 ± 3
X7201	<i>xyl1::HYG⁺ XYL2</i>	98 ± 6	79 ± 8	67 ± 5
X7301	<i>XYL1 xyl2::BEN⁺</i>	100 ± 8	71 ± 4	59 ± 5
X7231	<i>xyl1::HYG⁺ xyl2::BEN⁺</i>	96 ± 4	44 ± 5	34 ± 3

^a Measurement taken on fifth day of culture in liquid media. Media are CM (0.6% yeast extract, 0.6 casein hydrolysate, 1% sucrose), Vogel/RCW (Vogel's basic medium with 0.5% of rice cell walls as the sole carbon source) (Vogel 1964), and Vogel/OSX (Vogel's basic medium with 0.5% oat spelt xylan), as described in Materials and Methods.

^b Average and standard deviation of three independent measurements.

Fig. 3. Growth of *xyl* mutants on agar media. Three different media (described in Table 1) were modified by addition of 1.5% (wt/vol) agar. Each medium was inoculated with a $2 \times 2 \times 1$ mm³ gel block from a 20-day-old culture of the parent or from the mutant strains growing on CM agar. After 14 days at room temperature, the cultures were stained for 3 h with a diluted solution of Celltiter 96 (10% vol/vol; Promega, Madison, WI). The parent and all three mutants that grow on CM agar exhibit similar morphology (data not shown). Radial growth and pigmentation of the *M. grisea* parent and its three *xyl* mutant derivatives: growing on either (A) rice cell walls or (B) oat spelt xylan as the sole carbon source.

tions, neither *XYL1*, *XYL2*, nor their oligosaccharide products is required for Marotelli plants to respond hypersensitively to *M. grisea* strain CP987.

Additional xylanases secreted by *M. grisea*.

The observation that the *xyl1/xyl2* double mutant X7231 grows in medium containing xylan as the sole carbon source, albeit at a reduced rate (Table 1; Fig. 3), is evidence that the deletion of both *XYL1* and *XYL2* does not abolish xylanase activity in *M. grisea*. We have measured *endo*- β -1,4-xylanase activity in *M. grisea* culture with 4-*O*-methyl-D-glucurono-D-xylan-remazol brilliant blue R (RBB-xylan) as a substrate (Biely et al. 1985). After 8 days of culture under defined conditions (see Materials and Methods) in 1 liter of Vogel's basic medium (Vogel 1964) containing isolated rice cell walls as the sole carbon source, the parent strain CP987 and the three *xyl* mutant strains have all just entered stationary growth, and the accumulated xylanase activity is at its maximum (S.-C. Wu, A. G. Darvill, and P. Albersheim, unpublished observation). At this stage, the total accumulated xylanase activity in the 1-liter culture filtrate of the parent strain CP987 is 5.9×10^4 units. Under the same conditions, strain X7201 (*xyl1* mutant) secretes a total of 5.2×10^4 units of xylanase activity or 88% of the amount secreted by the parent, strain X7301 (*xyl2* mutant) secretes 2.3×10^4 units or 39% of the amount secreted by the parent, and strain X7231 (*xyl1/xyl2* double mutant) secretes 1.1×10^4 units or 19% of the amount secreted by the parent. We conclude from these results that *M. grisea* secretes in culture at least one xylanase in addition to *XYL1* and *XYL2*.

Western blot (immunoblot) analysis was used to determine whether the residual xylanase activity was structurally related to *XYL1* or *XYL2*. A polyclonal antiserum raised against a Family G ethylene-inducing xylanase (EIX) of *Trichoderma viride* (Dean et al. 1989) was used to detect Family G xyla-

Fig. 4. Infection of rice seedlings. Suspension of *Magnaporthe grisea* conidia (6×10^4 conidia in 3 ml of 0.25% gelatin solution) from the parent CP987, the *xyl1* mutant X7201, the *xyl2* mutant X7301, or the *xyl1/xyl2* double mutant X7231 was used to inoculate 14-day-old seedlings of rice cvs. Sariceltik (compatible with CP987, left panel) and Marotelli (incompatible with CP987, right panel). Disease symptoms were evaluated 5 days after inoculation by the method of Valent et al. (1991). Sariceltik responds to each of the four strains with large, light-colored disease lesions; Marotelli responds with tiny, dark brown, hypersensitive flecks.

nases of *M. grisea* (Wu et al. 1995), and a polyclonal antiserum raised against XYL2 of *M. grisea* was used to monitor XYL2 secretion by *M. grisea*. The XYL2 antibodies did not detect proteins secreted by the *xyl2* mutants X7301 and X7231 (Fig. 5A). However, the EIX antibodies continued to detect Family G xylanase(s) in the *xyl1* mutants X7201 and X7231 (Fig. 5B). Since, by molecular biology criteria, the gene encoding XYL1 has been deleted from the genome of X7201 and X7231 (Figs. 1 and 2), the band of the 22-kDa protein that cross-reacts with the EIX antibodies is likely to be the product of a Family G xylanase gene other than *XYL1*.

Experiments were carried out to determine whether *M. grisea* secretes one or more xylanases in addition to XYL1 and XYL2 (Wu et al. 1995). The proteins secreted by the three *xyl* mutants and their parent were separately subjected to cation exchange chromatography (Fig. 6). A xylanase activity (designated as XYL3 in Figure 6) present in the parent and in the three xylanase mutants was eluted by ≈ 0.85 M NaCl from the Mono-S column (Pharmacia Biotech, Piscataway, NJ). This xylanase has been purified to apparent homogeneity (data not shown). XYL3 cross-reacts with the EIX antibody and has a molecular weight similar to that of XYL1 (Fig. 5B). The N-terminal sequence of XYL3 has been determined as GTPSSTGTHNGFYYSHW, which is 83% identical to the corresponding portion of the N terminus of XYL1 (Wu et al. 1995). We conclude that XYL3, similar to XYL1, is a member of the Family G xylanases.

We unexpectedly observed three additional xylanase activities (peaks *a*, *b*, and *c* of Figure 6) in the culture filtrate of the *xyl1/xyl2* double mutant (strain X7231). Fractions *a*, *b*, and probably *c* are also detectable in strain X7301 (*xyl2* mutant, see Figure 6). Based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), each of the three fractions contains up to 10 individual proteins (data not shown). Western blot analyses with antisera raised against EIX or XYL2 failed to detect convincing cross-reactivity between any of the protein bands and either of the antisera, suggesting that the three addi-

Fig. 5. Immunoblot analysis of proteins secreted by *xyl* mutants. CM-sepharose-bound proteins were prepared, as described by Wu et al. (1995), from the culture filtrate of the parent (CP987) and of each of the mutant strains (X7201, X7301, and X7231). A 10- μ g sample of these proteins was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto a nitrocellulose membrane, and stained, as described by Wu et al. (1995), with primary antibodies and an alkaline phosphatase-conjugated secondary antibody (Sigma). Two previously purified xylanases (200 ng each), XYL1 and XYL2 (Wu et al. 1995), were included in the gel as controls. XYL3 was purified from the peak-labeled XYL3 in the chromatogram of X7231 (Fig. 6). A 500-ng sample of the purified XYL3 was subjected to SDS-PAGE and immunoblotted as described above. **A**, The membrane blot was incubated with primary antibodies raised against the XYL2 from *M. grisea* (Wu et al. 1995). **B**, The membrane blot was incubated with primary antibodies raised against a Family G xylanase (EIX) obtained from *Trichoderma viride* (Dean et al. 1989).

tional xylanases are not serologically related to either XYL1 or XYL2 (data not shown). Further studies are required to determine the structure and function of these newly discovered xylanases. The formal nomenclature of the xylanases in peaks *a*, *b*, and *c* will not be assigned until these xylanases are purified or their genes are cloned.

DISCUSSION

Gene disruption has become the method of choice to elucidate the functions of genes in fungal-plant interactions (Sweigard et al. 1992; Apel et al. 1993; Talbot et al. 1993; Hwang et al. 1995; Mitchell and Dean 1995; Apel-Birkhold and Walton 1996; Froeliger and Carpenter 1996; Lau and Hamer 1996). We have used this method to study the role of two xylanases, XYL1 and XYL2, during saprophytic and pathogenic growth

Fig. 6. Cation-exchange chromatograms of *xyl* mutants. A 17-mg protein sample from culture medium of the parent and of each *xyl* mutant strain was individually subjected to fast protein liquid chromatography on a Mono-S column (Pharmacia Biotech, Piscataway, NJ). Proteins were eluted with a two-step linear salt gradient from 0.02 to 0.25 M NaCl (fractions 1 to 95) and from 0.25 to 1.0 M (fractions 95 to 125). Xylanase activity in peak *a* was eluted by ≈ 0.040 M NaCl; xylanase activity in peak *b* was eluted by ≈ 0.043 M NaCl; xylanase activity in peak *c* were eluted by ≈ 0.1 M NaCl; and xylanase XYL3 was eluted by ≈ 0.85 M NaCl.

of *M. grisea*. We define *M. grisea* growth in culture with isolated rice cell walls or xylan as the sole carbon source as saprophytic growth, and growth in planta on a compatible host as pathogenic growth.

The anticipated phenotype of *M. grisea xyl* mutants grown in liquid culture when deprived of XYL1 and/or XYL2 was characterized by markedly reduced xylanase activity in the culture filtrates. In fact, deletion of the *XYL1* gene reduces secreted xylanase activity by only 11%. Deletion of *XYL2* reduces secreted xylanase activity by 61%, while deletion of both *XYL1* and *XYL2* reduces secreted xylanase activity by 81% (see Results section). These results are consistent with our earlier observation that XYL2 is the quantitatively dominant xylanase secreted by *M. grisea* in culture with rice cell walls as the sole carbon source. Our earlier estimation also put the activity of XYL1 at about 30% of the total (Wu et al. 1995), a figure probably overestimated because we did not take into account the activity of XYL3. The activity of XYL3 accounts for approximately 10% of the total xylanase activity secreted by strain CP987 (data not shown).

Deletion of the genes encoding XYL1 and XYL2 results in a double mutant (*xyl1/xyl2*) that produces on day 5 postinoculation 50% less mycelial mass than its parent grown on either xylan or xylan-rich rice cell walls as the sole carbon source (Table 1 and Fig. 3). It is noteworthy that the single mutants that lack either *XYL1* or *XYL2* retain a much higher amount of accumulated hypha than the *xyl1/xyl2* double mutant does when xylan is the sole carbon source: after 5 days in liquid culture the *xyl1* mutant (strain X7201) retains about 94% of the growth capability of the parent strain CP987, the *xyl2* mutant about 84%, and the *xyl1/xyl2* double mutant only 48% (Table 1; Fig. 3). This finding indicates that XYL1 and XYL2 are both required for *M. grisea* to grow effectively as a saprophyte. Probably the Family G XYL1 and the Family F XYL2 cooperate in the degradation of xylan polysaccharide. Apel-Birkhold and Walton (1996) reported that deletion of three Family G xylanase genes from *Cochliobolus carbonum* had no impact on fungal growth in medium containing xylan as the sole carbon source, even though deletion of these three Family G xylanases from *C. carbonum* reduced by 90% the xylanase activity secreted by *C. carbonum* into the culture fluid (Apel et al. 1993; Apel-Birkhold and Walton 1996). It is not known why this triple *xyl* mutant of *C. carbonum* grows on xylan at the same rate as its parent. However, it is known that this triple mutant secretes at least one Family F xylanase (Apel-Birkhold and Walton 1996).

The primary goal of our work is to determine whether the endoxylanases XYL1 and XYL2 are involved in pathogenic growth of *M. grisea* in plant tissues. Our results demonstrate that the loss of XYL1 and/or XYL2 does not have a detectable impact on the pathogenicity of *M. grisea* (Fig. 4), indicating that these two xylanases are more likely involved with saprophytic rather than pathogenic growth of *M. grisea*. Also, the loss of XYL1 and XYL2 does not affect the hypersensitive response of the incompatible rice cv. Marotelli to *M. grisea* strain CP987. Thus, XYL1 and XYL2 or the xylan oligosaccharide fragments they generate are unlikely to serve as signal molecules in the interaction between *M. grisea* strain CP987 and rice cv. Marotelli. However, since all the infection experiments were carried out under optimized growth chamber conditions, we do not know whether the deletion of *XYL1* and

XYL2 has a significant effect on *M. grisea*'s ability to infect its hosts in a natural environment. There are examples of deletion of a gene from a microorganism exhibiting an effect on the vitality of the microbe only when the microbe is grown under challenging growth conditions (Keen 1995; Oliver 1996).

The most interesting result from the knockout of the *XYL1* and *XYL2* genes is that the *M. grisea xyl2* mutants (X7301 and X7231) secrete three xylanase activities that are not present in the parent strain (Fig. 6). These xylanases found in chromatographic fractions *a*, *b*, and *c* in Figure 6 become detectable in the culture medium after the Family F xylanase gene *XYL2* is deleted. Since these three xylanases do not appear to cross-react with the antibodies raised against either a Family F or a Family G xylanase, they may be divergent members of the Family F or G xylanases or members of a new family of xylanases (Keen et al. 1996). Perhaps these xylanases are the ones associated with pathogenicity. Purification and cloning of these xylanases should lead to answers to these and other questions, such as their substrate specificity and the nature of the oligosaccharides generated when rice cell walls are subjected to the different xylanases.

A number of publications report the secretion of previously undetected cell wall-degrading enzymes following inactivation of related enzymes. Examples of this phenomenon include deletion from *Erwinia chrysanthemi* of one set of pectate lyase genes leading to the secretion of pectate lyases from a second set of genes (Kelemu and Collmer 1993) and antisense inhibition in tobacco of a pathogenesis-related *endo*- β -1,3-glucanase resulting in the production of an isozyme (Beffa et al. 1993). The detection of previously unknown xylanases in the culture fluid of *M. grisea xyl2* mutants provides another example of the biological complexity of cell wall-degrading enzymes (Sweigard et al. 1992; Apel et al. 1993; Kelemu and Collmer 1993; Arhin et al. 1994; Yao and Köller 1995; Apel-Birkhold and Walton 1996; Keen et al. 1996). It remains to be determined whether the newly emerged xylanases *a*, *b*, and *c* (Fig. 6) are "induced" or "released" from suppression as a result of the absence of XYL2.

Many microbes secrete into their culture medium multiple endoxylanases each of which fragments the backbone of arabinoxylan (Gilbert and Hazlewood 1993; Arhin et al. 1994). Fungal pathogens of plants are no exception. Two Family G xylanases were purified from the culture of *Gaeumannomyces graminis* (Southerton et al. 1993), and at least three xylanases are secreted by *C. carbonum* (Apel et al. 1993). *M. grisea* strain CP987 secretes into the culture medium at least two 22-kDa Family G xylanases (XYL1 and XYL3, shown in Figures 5 and 6) and a 33-kDa Family F xylanase (XYL2; see Wu et al. 1995). We have now shown that the *M. grisea* mutants that lack XYL2 secrete three other xylanases into the culture medium (Fig. 6). Thus, *M. grisea* most likely encodes at least six different endoxylanase genes. The secretion by *M. grisea* of a variety of endoxylanases supports the hypothesis that different sets of endoxylanases function in saprophytic and pathogenic growth.

MATERIALS AND METHODS

Fungal strains.

Magnaporthe grisea strain CP987 described by Sweigard et al. (1995) was used as the parent strain in this study. Condi-

tions for growing CP987 have been described (Valent et al. 1986). The genotypes of CP987 and of several derivative mutant strains used in this paper are listed in Table 1.

Source and growth of plants.

The rice (*Oryza sativa* L.) cultivars used in this study were Sariceltik and Marotelli, which are susceptible and resistant, respectively, to *M. grisea* strain CP987 (Sweigard et al. 1995). Barley (*Hordeum vulgare* L.) and weeping lovegrass (*Eragrostis curvula* (Schrader) Nees), two other hosts, and goosegrass (*Eleusine indica* (L.) Gaertn.), a nonhost plant, were also used (Valent et al. 1986; Sweigard et al. 1995). Seeds of the above plants were kindly provided by B. Valent (Central Research and Development, E. I. DuPont de Nemours, Wilmington, DE).

Rice seedlings were grown for approximately 16 days in a vermiculite medium (Verlite, Tampa, FL) in a growth chamber with 14 h of light at 27°C, 10 h of dark at 22°C, and a relative humidity of 70%. The plant roots were submerged continuously in a solution of 0.5 g of Peters professional fertilizer 20-20-20 (Scotts-Sierra Horticultural Products, Marysville, OH) per liter and 0.05 g of Sprint 330 iron chelate micronutrient (Ciba-Geigy Corporation, Greensboro, NC) per liter. Weeping lovegrass and goosegrass were grown for 10 and 20 days, respectively, in the same growth chamber in Fafard soil Mix No. 3 (Conrad Fafard, Agawam, MA) without additional fertilizer. Barley seedlings were grown for 8 days in Fafard soil Mix No. 3 in a growth chamber with 14 h of light at 24°C, 10 h of dark at 20°C, and a relative humidity of 60%.

Protoplast preparation.

The following protocols were modified from those described by Sweigard et al. (1992). *M. grisea* conidia (10^6) were inoculated into 500 ml of a complete medium (CM, 0.6% yeast extract, 0.6% casein hydrolysate, and 1% sucrose) and grown, with constant gyratory shaking (100 rpm; G10 Gyratory Shaker, New Brunswick Scientific, Edison, NJ), for approximately 4 days at 23°C in the dark. Fungal mycelia were harvested by filtration through a single layer of a glass microfiber filter 934-AH (Whatman, Hillsboro, OR).

The fungal mycelia (≈ 10 grams wet weight) were resuspended in 150 ml of 20% tissue culture grade sucrose (Sigma, St. Louis, MO). Four milliliters of a 100 mg/ml “lysing enzymes” solution (Sigma) was added to digest the fungal cell walls. The suspension was maintained for approximately 2 h at room temperature with constant gyratory shaking (100 rpm; G2 Gyratory Shaker, New Brunswick Scientific). The digest was filtered through a polyester screen (21 μ m, Tetko, Lancaster, NY) to remove mycelial debris. Protoplasts in the filtrate were collected by centrifugation for 5 min at 4°C at 5,000 rpm in a JA-14 rotor (Beckman, Ontario, CA). This was followed by three washes with 200 ml of ice-cold 20% sucrose. The protoplast pellet was finally resuspended in 20% sucrose at a final concentration of approximately 3×10^9 protoplasts/ml. Aliquots (100 μ l) of the protoplast suspension were frozen in liquid nitrogen and stored at -80°C . The protoplasts can be held under these conditions for about a year without significant loss of transformation efficiency.

Electro-transformation of *M. grisea* protoplasts.

One 100- μ l aliquot of the protoplasts was thawed at 0°C.

One microliter of a 100 $\times$ concentrated electroporation buffer (1.0 M Tris-Cl, pH 7.5, 0.5 M MgCl₂ and 1.0 M CaCl₂) and 5 μ g of pX1HYG or pX2BEN DNA (approximately 5×10^{-13} mol), linearized by appropriate restriction enzymes (Fig. 1), were mixed with the protoplasts. Following a 15-min incubation at 0°C, the mixture was transferred to a 0.2-cm electroporation cuvette (BioRad, Hercules, CA). Electroporation was performed on a BioRad GenePulser by pulsing once at a capacitance of 25 μ F and a voltage of 600 V, which resulted in a “time constant” of approximately 2.5 milliseconds (see BioRad manual). The electro-transformed protoplasts were immediately mixed with 2 ml of a TB3 liquid medium (CM plus 20% sucrose) and 100 μ l of a 1.0 M glucose solution, and incubated at room temperature for 2 h with constant shaking (200 rpm; G2 Gyratory Shaker, New Brunswick Scientific). A 0.2-ml aliquot of the transformed protoplasts was mixed with 3 ml of a 50°C regeneration medium (TB3 plus 1% low temperature gelling agarose) containing a selection drug (see below). The mixture was quickly poured onto a disk (8.2 cm in diameter) of the 21- μ m polyester screen lying on the agar surface of a 9-cm TB3 agar plate. Two selection drugs were used in these experiments: hygromycin B (Sigma) at a concentration of 700 μ g/ml and benomyl (Dragon Chemical, Roanoke, VA) at a concentration of 1 μ g/ml. After an incubation period of 4 to 5 days at 26°C, the polyester screen with attached fungal colonies (100 to 300) was transferred, to induce sporulation, to a plate containing water agar medium (1.5% agar and 1% sucrose). The water agar plate was maintained for about 20 h at room temperature. Single conidia were isolated from each sporulated colony and grown in complete agar medium (CM plus 1.5% agar).

Preparation of DNA templates for PCR.

Single transformants were grown in 150 μ l of CM in the wells of a 96-well microtiter plate. After incubating for approximately 3 days at 26°C, 30 μ l of a 10 mg/ml solution of “lysing enzymes” was added to each well and the suspension incubated for 30 min at room temperature. The digest, containing suspensions of aggregated protoplasts and spheroplasts, was dispersed by repeated pipetting with a P200 Pipetman and a 200- μ l yellow tip. The protoplast suspension was transferred to a 1.5-ml microcentrifuge tube and mixed with 150 μ l of a lysis buffer (50 mM Tris-Cl, 100 mM EDTA, pH 8.0, and 1% SDS). Following incubation at 70°C for 15 min, 150 μ l of a 5 M potassium acetate solution (pH 7.5) was added to the tube. The mixture was vortexed briefly and incubated at 0°C for 5 min, followed by centrifugation for 10 min at $10,000 \times g$ in a microcentrifuge. The DNA in the supernatant was recovered by precipitation with 0.8 vol of isopropanol. The DNA pellet was washed once with 1 ml of 70% ethanol, air dried, and then dissolved in 20 μ l of water. One microliter of the fungal DNA preparation was used as a template for PCR with oligonucleotide primers 1 and 2 or 3 and 4 (see Figure 1).

Infection assay.

Fungal conidia were harvested from a 20-day-old *M. grisea* culture grown on oatmeal agar plates as described (Sweigard et al. 1992; Wu et al. 1995). Collected conidia were diluted in a 0.25% gelatin solution to a concentration of 2×10^4 conidia per ml. A 3-ml aliquot of the conidial suspension was sprayed evenly with an airbrush (type H-1, Paasche Airbrush, Har-

wood Heights, IL) onto plant seedlings grown in a 4-in. pot (8 to 10 seedlings per pot for rice, barley, or goosegrass, and 50 to 60 seedlings per pot for weeping lovegrass). Each pot of inoculated seedlings was individually sealed in a 12 × 15 in. Ziploc bag to keep 100% humidity for 24 h in the dark. The inoculated seedlings were then removed from the Ziploc bags and placed in appropriate growth chambers with optimized growth conditions for the plants (see above). Disease symptoms were scored 4 to 6 days postinoculation according to the infection standards described by Valent et al. (1991).

Molecular biology.

Unless otherwise described, isolation and manipulation of nucleic acid, molecular cloning, gel blot analyses, PCR procedures, and other relevant techniques were the same as those presented by Wu et al. (1995, 1996).

Enzyme purification and activity assay.

Experiments were performed as described by Wu et al. (1995) except that the succinate buffer used during the purification of xylanases was replaced by a 50 mM sodium acetate buffer, pH 5.0, and *endo*- β -1,4-xylanase activity was assayed in 100 mM sodium phosphate buffer, pH 7.2, instead of in 50 mM sodium acetate, pH 5.2.

Measurement of fungal mycelial weight.

The fresh weight of *M. grisea* mycelia in liquid culture was determined as described by Wu et al. (1995). An inoculum of 10⁶ conidia was added to 1 liter of either a complete medium (CM), Vogel's basic medium (Vogel 1964) supplemented with 5 g of rice cell walls (Vogel/RCW), or Vogel's basic medium supplemented with 5 g of oat spelt xylan (Vogel/OSX). After 5 days of growth at 23°C in the dark with constant shaking (100 rpm; G10 Gyrotory Shaker, New Brunswick Scientific), three 5-ml samples were removed with a wide-tipped (≈6 mm in diameter) pipette from each fungal culture. The 5-ml samples were each filtered through a layer of glass microfiber filter (Type GF/F, Whatman International, Maidstone, England), and the wet weight of mycelial pads on the filters was determined.

Antibodies and immunoblot analysis.

The polyclonal antiserum raised against a Family G xylanase (EIX) from *T. viride* was a generous gift of J. F. D. Dean (School of Forestry, University of Georgia, Athens, GA; see Dean et al. 1989). The antiserum against XYL2 was raised in a rabbit by the University of Georgia Animal Resources (Athens, GA). Immunoblot analyses were performed as described (Wu et al. 1995). The EIX and the XYL2 antisera were diluted 500 and 2,000 times, respectively, before being used.

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