The Endo- β -1,4-Xylanase Xyn11A Is Required for Virulence in *Botrytis cinerea*

Nélida Brito, José Juan Espino, and Celedonio González

Departamento de Bioquímica y Biología Molecular, Universidad de La Laguna. E-38206 La Laguna (Tenerife), Spain

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Phytopathogenic fungi can degrade xylan, an abundant hemicellulose in plant cell walls, by the coordinate action of a group of extracellular enzymes. Among these, endo- β -1,4-xylanases carry out the initial breakdown by cleaving internal bonds in the polymer backbone. We have isolated and characterized a gene, *xyn11A*, coding for an endo- β -1,4-xylanase belonging to family 11 of glycosyl hydrolases. *xyn11A* was shown to be induced by xylan and repressed by glucose and to be expressed in planta. The disruption of *xyn11A* caused only a moderate decrease, about 30%, in the level of extracellular endo- β -1,4-xylanase activity and in the growth rate, with beechwood xylan as the only carbon source. However, deletion of the gene had a more pronounced effect on virulence, delaying the appearance of secondary lesions and reducing the average lesion size by more than 70%. Reintroducing the wild-type gene into the mutant strains reversed this phenotype back to wild type.

Additional keyword: pathogenicity.

Botrytis cinerea is a fungal plant pathogen with a wide range of hosts. It infects a large number of plant species, including many commercially important vegetables, fruits, and ornamentals, both in the field or greenhouse and during storage, causing important economic losses worldwide (Elad et al. 2004).

B. cinerea, like other phytopathogenic fungi, secretes a high number of enzymes to the extracellular medium during the infection process to degrade the various plant tissues and defensive barriers. These include cell-wall-degrading enzymes (CWDE), such as pectinases, cellulases, and xylanases, as well as cutinases, lipases, and proteases (Prins et al. 2000). Extracellular enzymes have the presumably important role of both facilitating the growth of the hyphae by softening the plant tissues and converting complex plant material into readily assimilable nutrients and have, therefore, been singled out as potential virulence factors (Staples and Mayer 1995).

The genes for some of these enzymes have been cloned and disrupted in order to study their specific role in the infection process (Tudzynski and Siewers 2004). A partial effect on

virulence has been observed for the disruption of the genes for endopolygalacturonases 1 and 2, but pathogenicity was not affected by the mutation of cutinase, lipase, and five different aspartic proteases. Contradictory results have been obtained in the case of pectin methyl-esterase, and we have shown recently that endo- β -1,4-glucanase is not required for pathogenicity (Espino et al., in press). However, the role on virulence of enzymes involved in the degradation of hemicellulose, which comprises about one third of the plant cell wall for most plants, has not been studied in *B. cinerea*.

Xylan is the major hemicellulosic component of the plant cell wall and the most abundant renewable hemicellulose (Collins et al. 2005). The actual structure of this polymer depends on its origin. It consists of a backbone of 1,4-linked β -D-xylose residues and side chains composed of a variety of substituents in carbons 2 or 3 of the xylose units. The side groups are linked to the main chain by either glycosidic (L-arabinofuranose, 4-O-methyl-D-glucuronic acid) or ester (acetic acid) bonds (Kulkarni et al. 1999). The degradation of this complex polysaccharide requires the synergistic action of several hydrolytic enzymes for efficient and complete hydrolysis (Collins et al. 2005), of which endo- β -1,4-xylanase (EC 3.2.1.8) is a crucial component that carries out the initial breakdown of the xylan backbone producing xylo-oligomers of different lengths. This enzyme has received much attention because of its industrial applications (Collins et al. 2005), such as its use by the paper industry as an aid in the bleaching process, and two main groups have been identified (Collins et al. 2005), acidic high-molecular-mass endoxylanases belonging to family 10 of glycosyl hydrolases and low-molecular-mass basic endoxylanases belonging to family 11. Apart from their role in the degradation of xylan, family 11 fungal endo- β -1,4-xylanases are well-known proteinaceous elicitors of defense response reactions in plants in a way that is independent of its enzymatic activity (Enkerli et al. 1999).

Although endo- β -1,4-xylanase is clearly involved in the degradation of plant cell walls and the suggestion that it may therefore play an important role in pathogenesis (Walton 1994), the xylanase knockout mutants that have been constructed so far with three fungal species (Apel et al. 1993; Apel-Birkhold and Walton 1996; Gomez-Gomez et al. 2002; Wu et al. 1997) all retain full virulence. Nevertheless, in all three fungi the existence of additional endo- β -1,4-xylanase genes could not be ruled out, so the question still remains whether endo- β -1,4-xylanase activity is really dispensable in the infection process.

Here, we report the cloning, characterization, and disruption of the *xyn11A* gene from *B. cinerea*, which codes for an endo- β -1,4-xylanase. This same gene has recently been identified by Schulze Gronover and associates (2004) in a screening for genes under the control of the *B. cinerea* *Ga* subunit of a het-

Corresponding author: C. Gonzalez; E-mail: cglez@ull.es; Telephone: +1 (34) 922 318 422; Fax: +1 (34) 922 318 35.

Nucleotide sequence data for *xyn11A* is available in the GenBank database under accession number DQ057980.

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erotrimeric G protein, Bcg1, a protein required for virulence. One of the two endoxylanase clones that appeared in the study is identical in sequence to *xyn11A* (J. Schumacher and B. Tudzynski, *personal communication*) and was shown to be expressed in planta in the wild type but not in the *bcg1* mutant or in a mutant for the adenylate cyclase gene *bac*. In this paper, we provide evidence that *xyn11A* is required for pathogenesis.

RESULTS

Isolation of *xyn11A*.

Analysis of Genoscope's publicly available *B. cinerea* expressed sequence tag (EST) database using the stand-alone BLAST (ver. 2.0.11) program (Altschul et al. 1990) allowed us to identify only one clone (Genoscope sequence ID W0AA061ZB01C1) homologous to previously identified fungal endo- β -1,4-xylanase genes. This clone contained a full open reading frame (ORF) of 681 bp, coding for a protein highly similar to fungal endo- β -1,4-xylanases belonging to family 11 of the glycosyl hydrolases, plus 11 bp before the ATG codon. The gene was named *xyn11A*, following recommendations by Henrissat and associates (1998), and a 6.3-kb genomic fragment containing it was isolated and sequenced. The sequence of the coding region was identical to the clone from the EST database, except for the presence of a single 65-bp intron that follows the GT/AG rule (Shapiro and Senapathy 1987) at the boundaries between introns and exons. The presence of the intron was confirmed by polymerase chain reaction (PCR) amplification and sequencing of the whole *xyn11A* cDNA with primers XYLFBGL and XYLRECO. Analysis of the sequence of the promoter region shows a possible TATA box at -99 and several putative binding sites for transcriptional factors; within the first 1,000 bp upstream of the translation start site, we could find three sites for the carbon catabolite repressor CREA from *A. nidulans* (de Vries and Visser 2001), one for the repressor of cellulase and xylanase ACEI from *Trichoderma reesei* (Aro et al. 2003), as well as five CCAAT sites that have been shown to play a role in the regulation of cellulase and xylanase in *T. reesei* by an Hap-like protein complex (Zeilinger et al. 1998).

The *xyn11A* gene contains an ORF of 681 bp, encoding a putative protein consisting of 227 amino acids. The N-terminal amino acid sequence shows features of a signal peptide for the first 19 residues, according to SignalP 3.0 (Bendtsen et al. 2004) and PSORT II (Nakai and Kanehisa 1992). Residues 20 to 35 in the predicted protein are similar to the propeptide of *T.*

reesei endoxylanase II (Fig. 1), which is of unknown function but probably involved in either the targeting to the extracellular medium, folding, or both (Conesa et al. 2001). The putative mature protein (residues 36 to 227) has a calculated molecular mass of 20.6 kDa and an isoelectric point (pI) of 9.1. It contains the two, family 11, active site signatures from PROSITE (Falquet et al. 2002) and was identified as a family 11 glycosyl hydrolase by running a Conserved Domain Search at the National Center for Biotechnology Information (NCBI) website (Marchler-Bauer et al. 2003). The alignment of Xyn11A with other fungal and bacterial xylanases (Fig. 1) reveals a 50% amino acid sequence identity between the *B. cinerea*, *T. reesei*, and *Streptomyces lividans* enzymes. The similarity is especially high with endoxylanase II from *T. reesei*, a protein whose structure is very well known (Havukainen et al. 1996), and all amino acids that have been implicated in substrate binding or catalysis in the *T. reesei* enzyme are conserved at similar positions in Xyn11A.

xyn11A expression.

Expression of *xyn11A* was determined by quantitative PCR for mycelia of *B. cinerea* that were grown in glucose and were then induced for 12 h in minimal medium supplemented with different carbon sources (Fig. 2). Levels of the *xyn11A* mRNA were maximal when xylan was the only carbon source and decreased to about 5% of that value in all other situations. The expression of *xyn11A* is thus clearly regulated by the carbon source such that the enzyme is secreted at high levels only when its substrate is available and only when cells lack the more easily assimilable carbon source glucose. This pattern of expression suggests the existence of regulatory pathways for the induction by xylan and for the repression by monomeric sugars. Surprisingly, when induction was carried out with whole tomato fruit extract, a condition set to resemble the in planta environment, the level of *xyn11A* transcript observed was similar to the uninduced or repressed samples. This is probably not an indication of lack of expression in planta but a consequence of the high concentration of reducing sugars in this medium (20 mM xylose equivalents) at the time of harvesting, which could be enough to repress transcription of *xyn11A*.

This pattern of regulation is exactly the same as that observed for the endo- β -1,4-xylanase activity determined in the extracellular medium (not shown). The activity was also maximal when beechwood xylan was the only carbon source and was almost undetectable (below 2% of the value in xylan) for

Fig. 1. Alignment of *Botrytis cinerea* Xyn11A (B.c.) with *Trichoderma reesei* endoxylanase II (T.r.) (Saarelainen et al. 1993) and *Streptomyces lividans* endoxylanase B (S.l.) (Shareck et al. 1991). Signal peptides have been omitted, as well as region 191 to 293 of the *S. lividans* enzyme. Identical (*), conserved (:), and semiconserved (.) amino acids are marked. The glycosyl hydrolases family 11 active site signatures are indicated in bold on a gray background, and the residues involved in catalysis (two Glu) or substrate binding (two Tyr) in *T. reesei* (Havukainen et al. 1996) are marked with downward-pointing arrows (↓). The propeptide of the *T. reesei* enzyme is indicated with a black background and was aligned only to the *B. cinerea* enzyme. GenBank accession numbers for the sequences are as follows: DQ057980 (B.c.), S39883 (T.r.), and JS0590 (S.l.).

the cultures in glucose or glucose plus xylan. In cultures induced with tomato extract, endo- β -1,4-xylanase activity could also not be detected at 12 h after induction, but it rose quickly around 24 to 36 h after induction, when reducing sugars were depleted from the medium (not shown), reaching levels similar to the culture induced with xylan.

Quantitative PCR was also used to reveal the presence of the *xyn11A* transcript in tomato leaves infected with *B. cinerea* (Fig. 2). Expression of the gene was detected from the beginning of the infection in the just-inoculated leaves, and the level of transcript increased between 24 and 48 h postinfection (hpi) simultaneously with the appearance of visible lesions on the leaves.

Role of *xyn11A* on virulence.

In order to study the role of *xyn11A* in pathogenesis, we have generated two *xyn11A* mutants (XM4 and XM31), in which the hygromycin resistance cassette was inserted into the *xyn11A* ORF, and two rescued strains (XM4R2 and XM31R11), in which the wild-type *xyn11A* gene was introduced back into the mutant strains. Both mutants showed a reduced ability to infect tomato leaves (Fig. 3A) or grape berries (Fig. 3B), and this phenotype was reversed by transformation with a wild-type copy of the *xyn11A* gene. The deletion of *xyn11A* had two clear effects on the development of the lesions caused by the fungus on detached tomato leaves. First, it delayed the expansion of the infections from the primary necrotic lesions (Fig. 3C). Second, it resulted in a reduction of more than 70% in the average lesion size for those infections that were actually spreading (Fig. 4D). The reintroduction of the *xyn11A* gene in the mutants almost completely rescued this phenotype, reversing the delay in the appearance of secondary lesions and restoring the average lesion size.

Ability of *xyn11A* mutants to use xylan.

To analyze whether the mutation of *xyn11A* affects the ability of *B. cinerea* to use xylan, we compared the growth of the wild-type, mutant, and rescued strains on minimal solid medium with 1% beechwood xylan as the only carbon source (Fig. 4). Growth on xylan was only slightly affected by the deletion of

xyn11A; both mutants grew about 30% slower than did the wild type. The two rescued strains, XM4R2 and XM31R11, did however, grow at about the same rate as the wild type, showing that it was indeed the lack of the Xyn11A protein that was causing the phenotype. Corresponding growth tests on

Fig. 2. In culture and in planta expression of *xyn11A*. Levels of *xyn11A* mRNA, determined by quantitative real-time polymerase chain reaction and normalized to the levels of actin mRNA, are shown as a percentage of the highest value. X, G, XG, and T represent the expression in mycelia induced for 12 h in minimal media with xylan, glucose, xylan+glucose, and tomato extract as carbon sources, respectively. ip0, ip24, and ip48 represent expression in planta at 0, 24, and 48 h postinfection. Error bars are not the standard deviation (SD) but the range of variation derived from the SD in threshold cycle.

Fig. 3. Effect of the *xyn11A* mutation on virulence. **A** and **B**, Growth of the wild-type (B05.10), mutant (XM4 and XM31), and rescued strains (XM4R2 and XM31R11) on grape berries or on tomato leaves, at the indicated times. Growth of XM31 and XM31R11 on grapes (not shown) was similar to XM4 and XM4R2, respectively. **C**, Percentage of the inoculations on tomato leaves that had begun to spread, generating a clearer infection halo (secondary lesion) around the black inoculation spot, at the indicated times for each strain. **D**, Mean radius of the secondary lesions produced by each strain on tomato leaves at different times, calculated only for lesions actually spreading beyond the black inoculation spot. A minimum of 20 inoculations were carried out for each strain.

rich media, such as whole tomato fruit extract or malt extract, yielded no significant differences in growth rate among the five strains (not shown).

Effect of the *xyn11A* mutation on endo- β -1,4-xylanase activity.

Endo- β -1,4-xylanase activity in culture filtrates was determined for both *xyn11A* mutants, for their respective rescued strains, and for wild-type strain B05.10. The secretion of endo- β -1,4-xylanase was induced with beechwood xylan as described below. In this case also, the mutation produced a slight decrease, about 30%, in the level of endo- β -1,4-xylanase activity detected in the medium, and the retransformation with the native gene resulted in the recovery of the activity to levels similar to those of the wild type (Fig. 5A and B).

The lack of a functional Xyn11A protein also affected the accumulation of reducing sugars in the medium, resulting from the hydrolysis of xylan (Fig. 5C and D). A transient extracellular accumulation of reducing sugars was observed, typically not only with xylan as the only carbon source (Fig. 5) but also with other polysaccharides, such as carboxymethylcellulose (Espino et al., in press), or disaccharides, such as sucrose (not shown). The exact cause of this phenomenon is unknown but may be due to a delay in the induction of the monosaccharide uptake systems with respect to the polysaccharide degrading enzymes. In both *xyn11A* mutants, reducing sugars accumulated in the medium to an approximately 30% lower level than in the wild type, and again, this phenotype was reversed by transformation with the native gene.

These results, which are consistent with the effect of the *xyn11A* mutation on the use of xylan as carbon source, indicate that Xyn11A is not responsible for the whole amount of endo- β -1,4-xylanase activity. Therefore, additional endo- β -1,4-xylanase genes are likely to be found in the *B. cinerea* genome.

Fig. 4. Growth of the *xyn11A* mutant strains with xylan as the only carbon source. Growth in petri dishes was followed by measuring the colony radius daily for the indicated strains. The growth rate (inset) was calculated from the slopes obtained for each strain. Values plotted represent the mean and standard error from three replicate plates. B05.10 = wild-type *B. cinerea*, XM4 and XM31 = *xyn11A* mutants, and XM4R2 and XM31R11 = their respective rescued strains.

DISCUSSION

In order to study the role of the extracellular endo- β -1,4-xylanase in the pathogenesis of *B. cinerea*, we have isolated the endo- β -1,4-xylanase-encoding gene *xyn11A*. This was the only endoxylanase gene that was found by systematically analyzing a *B. cinerea* EST database (Viaud et al. 2005), and the result of Southern blot analysis of the *B. cinerea* genome with a *xyn11A* probe under moderate stringency (not shown) was also consistent with a single-copy gene. However, two different *B. cinerea* endo- β -1,4-xylanase genes have recently been identified in a suppression subtractive hybridization study (Schulze Gronover et al. 2004) as genes not expressed in a knockout mutant of the gene *bcg1*. One of these genes is indeed *xyn11A* (J. Schumacher and B. Tudzynski, *personal communication*) and the other is an endoxylanase belonging to family 10 of the glycosyl hydrolases. It is clear, therefore, that *B. cinerea* has at least one more enzyme able to degrade xylan besides Xyn11A, explaining why the *xyn11A* mutant strains are still able to use xylan as carbon source and to secrete endo- β -1,4-xylanase activity. Redundancy in extracellular enzymes is common in fungi (ten Have et al. 2002), and there are several reports regarding fungi and bacteria secreting multiple xylanase isoforms (Collins et al. 2005). In fact, using the NCBI Genomic BLAST interface, we have found more than one endoxylanase homolog, belonging to either family 10 or family 11, in the sequenced genomes of *Neurospora crassa*, *Aspergillus nidulans*, *Ustilago maydis*, *Gibberella zeae*, and *Magnaporthe grisea*.

The putative mature protein Xyn11A shows all the characteristics of family 11 endo- β -1,4-xylanases, including the basic pI and low molecular weight that are common for this group (Biely et al. 1997). Similarity with other family 11 members is actually so high that it enabled the prediction of a modeled structure for Xyn11A by comparison with other family 11 endo- β -1,4-xylanases of known structure, using the web-based 3D modeling tool SWISS-MODEL (Schwede et al. 2003). This structure (not shown) shows the typical jelly-roll topology

Fig. 5. Effect of *xyn11A* disruption on the extracellular β -1,4-xylanase activity. **A**, and **B**, Activity as well as **C**, and **D**, total reducing sugar concentration was determined in the culture filtrates for wild-type, mutant, and rescued strains. Induction was carried out with beechwood xylan as the only carbon source in every case. Values are means $\pm$ standard error from three independent experiments. B05.10 = wild-type *B. cinerea*, XM4 and XM31 = *xyn11A* mutants, and XM4R2 and XM31R11 = their respective rescued strains.

of family 11 glycosyl hydrolases (Hakulinen et al. 2003), with two twisted β -sheets enclosing a substrate-binding cleft.

The expression pattern of *xyn11A* was as expected for a gene involved in converting a cell-wall component into low-molecular-weight easily-assimilable sugars, since it was subjected to induction by the availability of the substrate and repression by glucose. Interestingly, the promoter of *xyn11A* shows several binding sites for the *A. nidulans* transcription factor CREA, which mediates repression by glucose in other fungi (de Vries and Visser 2001). The ortholog of *creA* in *B. cinerea* has been cloned and was shown to complement an *A. nidulans creA* null mutant, indicating that both proteins most probably bind to the same motif (Tudzynski et al. 2000). The three CREA-binding sites in the *xyn11A* promoter could therefore have a role in its repression by glucose.

xyn11A is also expressed in the *B. cinerea*–tomato interaction, which agrees with a role for this gene as a pathogenicity factor. The expression of *xyn11A* in planta was also observed for the *B. cinerea*–bean interaction by Schulze Gronover and associates (2004) in their suppression subtractive hybridization study. The expression pattern observed was identical to the one reported here for the first 48 h, and the levels of *xyn11A* transcript were shown to remain stable between 44 and 68 hpi. *xyn11A* is therefore expressed by the fungus throughout the whole infection process but at higher levels at the second and following days after inoculation.

We were able to disrupt the native *xyn11A* gene in *B. cinerea* using the plasmid pDXC-7, which contains the hygromycin resistance cassette inserted into the *xyn11A* ORF. Two *xyn11A* knockout strains showed no detectable intact copies of the gene and showed no ectopic integrations of the transforming DNA. The two mutants exhibited a diminished ability to produce endoxylanase or to use xylan as carbon source, approximately 30% lower in both cases, indicating that Xyn11A is responsible for about one third of the endoxylanase production by *B. cinerea*. The rest of the activity would be contributed by the other endoxylanase already reported (Schulze Gronover et al. 2004) or by additional endoxylanase genes yet to be discovered.

Strikingly, the mutation of *xyn11A* had a strong effect on pathogenicity, reducing the average lesion size by more than 70%. Such an effect of the deletion of an endo- β -1,4-xylanase gene in *B. cinerea* contrasts with previous results with other phytopathogenic fungi. So far, gene knockout experiments in *Magnaporthe grisea* (Wu et al. 1997), *Cochliobolus carbonum* (Apel et al. 1993; Apel-Birkhold and Walton 1996), and *Fusarium oxysporum* (Gomez-Gomez et al. 2002) did not support an essential role for endo- β -1,4-xylanases in fungal pathogenesis. In all three fungi, more than one xylanase were simultaneously disrupted and the resulting strains retained full virulence. It is noteworthy that these three fungi secrete in culture at least one more xylanase in addition to those disrupted, which could explain the virulence of the knockout strains. *F. oxysporum* has at least two family 10, *xyl2* and *xyl3*, and two family 11, *xyl4* and *xyl5*, xylanase genes, of which *xyl3*, *xyl4*, and *xyl5* were disrupted. Two and three endoxylanase genes were cloned and disrupted in *M. grisea* and *C. carbonum*, respectively, but in both cases, the authors hypothesize the existence of additional genes, since the double or triple mutant still retained endoxylanase activity. In the case of *C. carbonum*, even a 90% reduction in the level of endoxylanase activity for the triple mutant did not affect pathogenicity, which makes more striking the fact that the mutation of *B. cinerea xyn11A* strongly affected pathogenicity while having a modest effect on the activity.

In our view, three explanations are possible for the effect on pathogenicity of *xyn11A* disruption: i) a 30% decrease in activity may be enough to prevent pathogenicity in this particular organism, similar to what has been observed for polygalacturonases,

in which mutation of one of at least six genes produced a significant reduction in pathogenicity (ten Have et al. 1998); ii) expression of the two or more endoxylanase genes may be differently regulated either temporally or spatially, so that the *xyn11A* disruption could result in a lack of endoxylanase activity at a certain time, e.g., the beginning of colonization, or place, e.g., the hyphal tip, thus reducing virulence; or iii), Xyn11A may have some additional role in pathogenicity apart from its enzymatic activity whose lack in the mutant would explain its phenotype. In this respect, it is interesting that at least some endoxylanases are able to elicit the hypersensitive response in plants in a manner that is independent of its enzymatic activity through direct physical interaction with a specific receptor in the plant plasma membrane (Ron and Avni 2004). It is thus conceivable that Xyn11A could contribute to virulence by inducing in the host the hypersensitive necrosis of the area surrounding the infection. It has been previously reported that the hypersensitive response of the host, an effective defense against biotrophic pathogens, facilitates infection by the necrotrophic pathogen *B. cinerea* (Govrin and Levine 2000).

An important role for xylanase in the infection process is further supported by the existence of proteinaceous xylanase inhibitors as a defense mechanism in plants. Two different types of proteinaceous xylanase inhibitors, TAXI type (Gebruers et al. 2004) and XIP type (Juge et al. 2004), have been described, and there is increasing evidence of their involvement in defense against pathogens (Bellincampi et al. 2003; Igawa et al. 2004). Both types of inhibitors are relatively large proteins that interact physically with endoxylanases, preventing enzymatic activity and probably also elicitor ability. Although these inhibitors have been described only in cereals, which are not infected by *B. cinerea*, recent data suggest that these proteins may be widespread within the plant kingdom (Durand et al. 2005; York et al. 2004).

MATERIALS AND METHODS

Fungal strains and culture conditions.

B. cinerea SAS56 was obtained from E. Perez-Benito (Universidad de Salamanca, Spain) and the *B. cinerea* haploid strain B05.10 was obtained from P. Tudzynski (Westfaelische Wilhelms-Universitaet Muenster, Germany). All fungal strains were kept as conidial suspensions in 15% glycerol at -80°C for long storage and were grown on 3% malt extract (Difco, Detroit) plates at 22°C for routine use. Conidia were prepared as described by Benito and associates (1998) from cultures on tomato-PDA plates (39 g of potato dextrose agar plus 250 g of homogenized tomato fruits per liter). For the induction of CWDE, conidial suspensions were inoculated into Gamborg's B5 medium (Duchefa Biochemie BV, Haarlem, The Netherlands) supplemented with 10 mM KH_2PO_4 and 1% glucose at a density of 10^6 conidia per milliliter. After three days of growth at 22°C , mycelia were filtered, washed with sterile water, and resuspended in fresh medium containing 1% glucose, 1% beechwood xylan, or 25% tomato fruit extract as carbon source. Growth rate of the different strains was determined by measuring the growth of the colony radius on solid medium (1.5% agarose). Growth media used in solid culture were either 3% malt extract or the minimal medium described above containing 1% beechwood xylan or 25% tomato fruit extract as carbon sources.

Standard molecular techniques.

Recombinant DNA methods were performed as described by Sambrook and Russell (Sambrook and Russell 2001). Genomic DNA from *B. cinerea* was extracted as described by Möller and associates (1992). The high-fidelity thermostable DNA polymerase *Pwo* (Roche, Basel, Switzerland) was used in PCR

for cloning purposes and *Taq* DNA polymerase was used in all other PCR assays. Oligonucleotides were from Invitrogen (Paisley, Scotland). DNA sequencing was performed by Sistemas Genómicos S.A. (Valencia, Spain). Total RNA was isolated with the RNeasy plant mini kit (Qiagen Inc., Valencia, CA, U.S.A.), and mRNA was prepared from total RNA with the Oligotex mRNA mini kit (Qiagen). cDNA synthesis was carried out with the First Strand cDNA synthesis kit for RT-PCR (AMV) (Roche). *B. cinerea* transformations were carried out according to the protocol of Hamada and associates (1994), modified by van Kan and associates (1997), and transformants were then purified by single-conidia isolation on hygromycin-containing plates to ensure homokaryosis. Oligonucleotides used in this work are listed in Table 1.

Cloning of *xyn11A*.

The EMBL3 *Bam*HI arms cloning system plus packagene system (Promega Corp., Madison, WI, U.S.A.) was used to construct a gene library from *B. cinerea* SAS56 genomic DNA. The library (3×10^4 phages) was then screened with a PCR-amplified *xyn11A* fragment using the DIG DNA labeling and detection kit (Roche). The *xyn11A* probe was amplified from *B. cinerea* genomic DNA with primers XYL-FW and XYL-RV. One of the positive recombinant phages was characterized by restriction digestion and Southern blot, and a 6.3-kb *Eco*RI-*Eco*RI fragment hybridizing to the probe was cloned into pBluescript KS+ and was sequenced. This fragment contained the whole *xyn11A* ORF plus 4,500 and 1,065 bp of the 5' and 3' untranslated regions, respectively.

Construction of plasmids pDXC-7 and pRXM.

Plasmid pDXC-7, which was used in the disruption of *xyn11A*, was constructed as follows. i) The original *Bam*HI site from pBluescriptKS+ (Stratagene, La Jolla, CA, U.S.A.) was deleted by cutting with the enzyme, filling-in with Klenow, and religating to generate the plasmid pBS-Bam. ii) A *Sall*-*Eco*RI fragment (2,146 bp) from *xyn11A* was then cloned in pBS-Bam to generate plasmid pSBB-4. And iii), the hygromycin resistance cassette (2.7 kb) from plasmid pLOB1 was amplified by PCR with primers HYGF and HYGR and was inserted into the unique *Bam*HI site of *xyn11A*, to generate plasmid pDXC-7. Plasmid pLOB1 was provided by J. van Kan (Wageningen Agricultural University, The Netherlands) and carries the *Escherichia coli* hygromycin phosphotransferase-encoding gene *hph* under the control of the *A. nidulans* *oliC* promoter and the *B. cinerea* β -tubulin transcription terminator.

Plasmid pRXM contains the wild-type *xyn11A* gene and was used to introduce it back into the disruption mutants. It was constructed in pBluescriptKS+ and contains the entire 6,311-bp *xyn11A* gene plus a 3,292-bp *Apa*I-*Nde*I fragment from

plasmid pUT737 (Cayla, Toulouse, France) carrying the *Streptoalloteichus hindustanus* *Shble* gene (phleomycin resistance) under the control of the *T. reesei* *TRI* promoter and *A. nidulans* *trpC* terminator.

xyn11A gene disruption.

In order to construct a mutant defective in Xyn11A, spheroplasts from the *B. cinerea* haploid strain B05.10 were transformed with the vector pDXC-7, which contains the *xyn11A* gene interrupted by the insertion of the hygromycin cassette in opposite orientation, linearized at the *Mlu*I site. Seventeen of the transformants obtained were purified by the isolation of single germinating conidia, in order to obtain homokaryotic transformants, and two PCR reactions were carried out with genomic DNA from all of them to check i) for the insertion of the transforming DNA in the correct place, with primers that bind to the hygromycin resistance cassette and a *xyn11A* region not included in the transforming DNA (primers MUT-X-H and MUT-X-XY), and ii) for the absence of the intact *xyn11A* gene, with primers that bind to *xyn11A* at both sides of the integration site for the hygromycin cassette (primers XYL-Ba-FW and XYL-Ba-RV). Two bona fide mutants, XM4 and XM31, showed only the expected bands (not shown). The disruption of *xyn11A* was also confirmed by Southern blot analysis, and no additional ectopic integrations were detected by hybridization with probes homologous to either the *xyn11A* gene itself or the *E. coli* *hph* gene contained in the hygromycin resistance cassette. Moreover, both mutants were shown to lack expression of the *xyn11A* mRNA by quantitative PCR (not shown) when induced with xylan.

As an additional control to study the effect of Xyn11A on pathogenesis, complementation of the *xyn11A* mutants with the wild-type gene was performed. Both xylanase mutants, XM4 and XM31, were transformed with plasmid pRXM, which confers resistance to phleomycin and contains the whole 6.3-kb *xyn11A* gene. Phleomycin-resistant transformants were checked by PCR for the presence of an intact copy of *xyn11A*, using a pair of primers located at opposite ends of the gene, XYL-FW and XYL-RV. Two transformants, XM4R2 and XM31R11, that showed the expected PCR product were selected, the first rescuing the mutant strain XM4 and the second rescuing strain XM31. Although the 757-bp fragment corresponding to the reintroduced wild-type allele was obtained by PCR for both strains, the 3,467-bp fragment corresponding to the disrupted allele was only amplified in XM31R11. This difference can be explained by assuming different integration patterns for the transforming plasmid in the two strains, a double-crossover homologous recombination at the *xyn11A* locus for XM4R2 and a single crossover for XM31R11, either at the *xyn11A* locus or at a different genomic location. The former integration pattern should result in a strain not resistant to either hygromycin or phleomycin, but XM4R2, although not resistant to hygromycin, is clearly resistant to phleomycin (not shown), so we suspect that this transformant may have a second copy of the transforming DNA integrated elsewhere in the genome.

Real-time quantitative PCR.

cDNA prepared from total RNA was used as template for the PCR reactions. Amplification was carried out in a Bio-Rad iCycler iQ real-time PCR detection system (Hercules, CA, U.S.A.) with the Bio-Rad iQ SYBR Green supermix, using primers XYN11A-FW and XYN11A-RV, which bind to *xyn11A* cDNA. The *B. cinerea* actin gene *actA* was used to correct for sample-to-sample variation in the amount of RNA, and the primers used for its amplification were ACTA-FW and ACTA-RV. In both primer pairs, one of the two oligonucleotide binding sites spans the junction between two exons to

Table 1. Oligonucleotide primers used in this work

Name	Sequence
ACTAFW	5'-GGTAACATTGTTATGTCTGG-3'
ACTARV	5'-CTTGACCTTCATCGACG-3'
HYGF	5'-CAATTTTCACACAGGAAACAGC-3'
HYGR	5'-AAACGACGGCCAGTAAGC-3'
MUT-X-H	5'-TCGATGCGACGCAATC-3'
MUT-X-XY	5'-AATCCGCGAGTCTGGATC-3'
XYL-Ba-FW	5'-ACTCTCTTCGGACCG-3'
XYL-Ba-RV	5'-TCTCGAGGATCTTGTATG-3'
XYL-FW	5'-TCTTCACAATGGTTTCTGCATC-3'
XYL-RV	5'-TTAAGAAACAGTGATGGAAGCG-3'
XYN11AFW	5'-TGTTGGTTCCGAGCGCTCC-3'
XYN11ARV	5'-GGGAGGTAGTCCAACCATAGACGG-3'
XYLFBGL	5'-AGAAGATCTATGGTTTCTGCATCTTCC-3'
XYLRECO	5'-AGAATTCCCCAGATTTAAGAAACAGTG-3'

avoid amplification from genomic DNA, and we checked experimentally that both pairs were unable to generate any PCR product from genomic DNA template. The amplification of a single fragment was verified for every PCR reaction by running the final product on a 20% polyacrylamide gel electrophoresis. The relative mRNA amounts were calculated by the $\Delta\Delta C_t$ method from the mean of three independent determinations of the threshold cycle (Applied Biosystems 2004). Deviation from the mean was calculated from the standard deviation (SD) in the $\Delta\Delta C_t$ value, using the expression $2^{-(\Delta\Delta C_t \pm SD)}$, which results in the asymmetric ranges of variation observed in Figure 2.

For the in planta expression studies, tomato leaves were inoculated by immersion in a conidial suspension (10^7 conidia/ml) and were incubated in a closed container on water-soaked filter paper. At the indicated times, several leaves were taken and RNA was extracted.

Pathogenicity tests.

Detached tomato (cv. Moneymaker) leaves were inoculated with 5- μ l droplets of a conidial suspension (2×10^5 conidia/ml) in 60 mM KH_2PO_4 , 10 mM glycine, 0.01% Tween 20, 0.1 M glucose. Grape berries (commercially obtained) were washed under running tap water and were surface-sterilized by immersion for 5 min in ethanol. After air drying, fruits were inoculated in small punctures with 5 μ l of the above conidial suspension. Leaves and fruits were incubated at 22°C under conditions of high humidity on water-soaked filter paper in closed containers. At different timepoints after inoculation, lesions on leaves were photographed and their radii were measured.

Endo- β -1,4-xylanase assay.

Endo- β -1,4-xylanase activity was assayed by the method of Biely and associates (1988) using 4-O-methyl-D-glucurono-D-xylan-Remazol brilliant blue R (Sigma, St. Louis) as substrate at 40°C. Activity is expressed as the increase in absorbance at 595 nm per minute and per milligram of protein in the culture filtrates. Protein concentrations were determined as described by Bradford (1976), using bovine serum albumin as a standard. The amount of reducing sugars released from xylan was measured by the dinitrosalicylic acid procedure (Wood and Bhat 1988), using xylose as a standard.

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