The Xylanolytic System of *Claviceps purpurea*: Cytological Evidence for Secretion of Xylanases in Infected Rye Tissue and Molecular Characterization of Two Xylanase Genes

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ABSTRACT

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Claviceps purpurea is a common phytopathogenic fungus that colonizes ovarian tissue of grasses. A concerted approach involving cytological and molecular techniques was initiated to investigate the role of the fungus' xylanolytic system in the interaction. Using enzyme-gold and immunogold electron-microscopic techniques, the presence of arabinoxylans in cell walls of rye ovarian tissues (i.e., along the usual path of infection of *C. purpurea*) was confirmed; tissue-print and immunostaining analyses indicated the presence of xylanase(s) exclusively in ovaries infected with *C. purpurea*. This strongly suggests that *C. purpurea* secretes xylanase while colonizing its host. Two xylanase genes (*cpxy1* and *cpxy2*) were isolated from a genomic library of *C. purpurea* using genes from *Cochliobolus carbonum* (*xyl1*) and *Magnaporthe grisea* (*xyn33*) as heterologous

probes. *cpxy1* of *C. purpurea* had an open reading frame (ORF) of 832 bp interrupted by a 181-bp intron. The derived gene product (CPXYL1) had a molecular mass of 21.5 kDa and an pI of 8.88; it showed significant homology to family G endo- β -1,4-xylanases. The *cpxy2* ORF (1,144 bp) contained two introns (76 and 90 bp) and coded for a polypeptide of 33.8 kDa with an pI of 7.01; CPXYL2 belonged to family F xylanases. Southern analyses with genomic DNA demonstrated that both genes were single-copy genes. Using reverse transcription polymerase chain reaction, it could be shown that both genes were expressed in vitro and in planta (during all infection stages). Inactivation of *cpxy1* was achieved by a gene-replacement approach. The mutant strain (Δ *cpxy1*) had significantly reduced xylanase activity; Western analyses confirmed that it lacked a polypeptide of approximately 23 kDa.

Additional keywords: cell wall-degrading enzymes, gene disruption, host-parasite interaction.

Claviceps purpurea (Fr.:Fr.) Tul. is a common flower pathogen of a wide range of grasses and cereals. Wind-borne ascospores, produced by sclerotia in spring, attach and germinate on the pistil surfaces of blooming host florets and initiate a specific pathogenesis pattern: within 1 week, hyphae invade and colonize the ovary, grow down to the tip of the ovary axis, and establish a specific, persisting, host-parasite frontier after having tapped the host vascular system. The highly organ-specific, biotrophic fungus never invades any part further down in the host, but proliferates above this site. A sphacelial stroma grows profusely in the ovary, producing masses of anamorphic spores that are exuded into a syrupy fluid. With this honeydew, the conidiospores are transferred to other florets. Thereby, *C. purpurea* spreads spatially in the field having used the plant gynoeceia for its own proliferation. Next, honeydew production and conidiation usually cease when the formation of sclerotia starts. Sclerotia mature in about 5 weeks and typically contain ergot alkaloids. Finally, during autumn, instead of a kernel, a ripe sclerotium leaves the spike, making ergot a replacement-tissue disease. The hard compact ergot consists of special storage cells and a typically pigmented outer cortex. It serves for sexual reproduction and as a resting structure to survive unfavorable conditions.

During the initial colonization process, the fungus grows intra- and intercellularly, as it is able to penetrate (and completely dissolve) the host's cell walls (37,40). In accordance with its overall biotrophic mode of growth, *C. purpurea* secretes only limited amounts of hydrolytic enzymes in axenic culture. Among the few

enzymatic activities detected until now are pectinases (31), a β -1,3-glucanase (6), and at least two xylanases (S. Giesbert, *unpublished data*). To elucidate the role of extracellular hydrolytic enzymes during the colonization of host tissue by *C. purpurea*, we initiated an integrated molecular approach that encompasses target studies (identification of cell wall polymers degraded during the interaction), cloning and characterization of genes coding for cell wall-degrading enzymes (CWDEs), expression studies (via reverse transcription polymerase chain reaction [RT-PCR], in situ hybridization, and immunogold electron-microscopic [EM] analyses), and targeted gene disruption. We demonstrated that cellulose is efficiently degraded in host cell walls that are in contact with fungal hyphae, and we cloned and characterized a gene (*cel1*), probably coding for a cellobiohydrolase (lacking a substrate-binding domain), that is only expressed during the first days postinfection (28). During intracellular growth, the fungus efficiently degrades pectin/pectate. We cloned two closely linked putative endo-polygalacturonase genes that are expressed throughout the entire infection process (37). Functional analyses of the role these genes play during colonization of host tissues are under way, including creation of deletion mutants.

It has been reported that cell walls of grass leaves consist of approximately 40% glucuronoarabinoxylans (GAX) (8). Evidence for the presence of both β -1,4-xylan and arabinose side groups in cell walls of rye ovarian tissue, attained using enzyme-gold and immunogold techniques, is presented here for the first time. These data indicate that especially xylanases, as well as pectinases and cellulases, are important for the weakening of the host cell walls prior to penetration. As a basis for functional in planta studies, we cloned and characterized two putative xylanase genes, studied their expression and secretion both in planta and in axenic culture, and successfully inactivated one of them by a gene-replacement approach.

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MATERIALS AND METHODS

Strains and culture conditions. A standard field isolate of *C. purpurea*, strain T5 isolated from rye (*Secale cereale* L.) in Hohenheim, Germany (13), and a haploid derivative strain 20.1 obtained after benomyl treatment (27) were used for all studies. Mycelia were cultivated in 100 ml of complete medium BII (14) and modified complete medium according to Mantle and Nisbet (26) with reduced sugar content (Mantle B: 1% [wt/vol] glucose) in 300-ml Erlenmeyer flasks at 28°C on a rotary shaker at 150 rpm or for 14 days on solid medium plates containing 1.5% (wt/vol) agar. *Escherichia coli* DH5 α (18) was used routinely for cloning and propagation of plasmids. λ clones were propagated in *E. coli* strain K 803 (45).

Parasitic culture on rye plants. Rye plants were cultivated in growth chambers as described by Smit and Tudzynski (34) and inoculated with conidia from axenic cultures of *C. purpurea* as described elsewhere (37). Inoculated florets of defined infection stages of ergot were used together with corresponding noninoculated controls. The selected stages depicted in Müller et al. (28) were as follows: infected ovaries before honeydew production, 1 to 7 days post-inoculation (dpi); young sphacelia with honeydew production, 8 to 10 dpi; old sphacelia, about 15 dpi; and young sclerotia, about 20 dpi.

Transmission electron microscopy (TEM) and affinity-gold cytochemistry. For the processing of specimens for on-grid labeling, whole pistils including axes were excised from rye florets. They were fixed in sectioned parts of appropriate size for 1.5 h at room temperature in a mixture of 4% (wt/vol) formaldehyde generated from paraformaldehyde (E. Merck AG, Darmstadt, Germany) and 1% (vol/vol) glutaraldehyde in 25 mM sodium phosphate buffer, pH 7.0. Further processing of the nonosmicated samples via dehydration in ethanol, embedding in London resin white (TAAB Laboratories, Munich) at 60°C, ultramicrotomy with diamond knives, handling of nickel grids, and washing steps during labeling and electron microscopy was performed as previously described (36). The various labeling experiments were repeated several times, each with at least two grids carrying at least two sections and with additional control grids.

For immunogold localization of terminal arabinofuranosyl residues in arabinoxylan, previously described protocols were modified (1,37). Sections were treated for 30 min with normal goat serum diluted 1:100 in 150 mM phosphate-buffered saline (PBS), pH 7.4, containing 0.2% (wt/vol) gelatin and 50 mM glycine. They were incubated with a monoclonal antibody (MAb) from mouse, an α -L-arabinofuranose-specific immunoglobulin G (IgG) (Biosupplies Australia, Parkville, Victoria, Australia), diluted 1:10 in PBS containing 0.5% (wt/vol) bovine serum albumin (BSA) (Sigma-Aldrich, Deisenhofen, Germany) for 14 h at 4°C. Grids were treated for 45 min at room temperature with goat anti-mouse IgG + IgM linked to 20 nm of colloidal gold particles (EM.GAF20; BioCell Research Laboratories, Cardiff, England) diluted 1:30 in PBS plus 0.5% (wt/vol) BSA. Controls for labeling specificity were run as a parallel set of sections incubated either with an inappropriate MAb, without a primary MAb, or with an anti- α -L-arabinofuranosyl solution immunoprecipitated with a corresponding antigen, 1 mg of arabinoxylan (Sigma-Aldrich) per ml or 1 mg of pectin from citrus (Sigma-Aldrich) per ml, for 17 h at 4°C before section treatment. Sections were treated with 2% (wt/vol) periodic acid to alter glycosyl epitopes by oxidation.

For enzyme-gold localization of β -1,4-xylan, an endo- β -1,4-D-xylan-xylano-hydrolase (EC 3.2.1.8) purified from *Trichoderma viride* (Sigma-Aldrich) was linked to colloidal gold according to a previously described protocol (35). Colloidal gold sol was prepared by adding 2 ml of 1% (wt/vol) dihydrate sodium citrate and 0.45 ml of 1% (wt/vol) tannic acid to 100 ml of 0.01% (wt/vol) tetrachloroauric acid (19) and adjusted to pH 5.5. The xylanase was dissolved in and dialyzed against double-distilled water. Eighty milligrams of xylanase per ml of gold sol was added, and the gold

sol was further stabilized with 1% (wt/vol) BSA. After centrifugation at $17,000 \times g$ for 30 min, the pellet was resuspended in double-distilled water, centrifuged again, and recovered in 50 mM acetate buffer, pH 5.0. Before coating, the activity of the xylanase was tested in the xylanase assay, and the purity of the xylanase was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. For labeling of xylan, ultrathin sections were quenched for 15 min with 50 mM acetate buffer, pH 5.0, containing 1% BSA and 50 mM glycine for 20 min and floated on xylanase-gold sol (1:100 diluted). Several labeling controls were performed including preincubation of the gold sol with 1% (wt/vol) xylan (Sigma-Aldrich), 1% (wt/vol) carboxymethyl-cellulose (Serva, Heidelberg, Germany), or the polyclonal anti-xylanase. Sections were treated with uncoated colloidal gold or nonenzymatic protein A-gold suspension.

Sections were contrasted with uranyl acetate and lead citrate and observed with a Siemens Elmiskop 102 electron microscope (Siemens AG, Berlin).

Tissue and mycelium printing. Immunomycelium and immunotissue blots were performed at room temperature, adapting tissue-printing protocols (29,43). Whole mycelia grown for 2.5, 5, or 10 days on solid medium were used. Either infected or healthy ovaries were excised from the selected florets, rapidly plunge-frozen in liquid nitrogen, and divided longitudinally using a new razor blade in each case. The samples were placed on nitrocellulose membranes (0.45 μ m; Schleicher & Schuell, Inc., Dassel, Germany) and pressed gently for up to 120 s, carefully avoiding any displacement while printing. After short-washing with running double-distilled water, the membranes were treated for 1 h with 20 mM Tris-HCl, pH 7.8, containing 2% (vol/vol) Tween 80 and 180 mM NaCl (TBS-Tween). They were incubated for 1 h in either the MAb anti-XYNI, the MAb anti-XYNII, or the polyclonal anti-xylanase anti-XYN, diluted 1:15,000 with TBS-Tween, and washed with running double-distilled water for 1 to 2 min. Detection of immunoreactive peptides was achieved by incubation for 1 h in alkaline phosphatase linked to goat anti-mouse IgG and to goat anti-rabbit IgG, respectively, diluted 1:40,000 with TBS-Tween, mediating colorimetric reactions of naphthol AS-biphosphate and fast red-gg-salt (Sigma-Aldrich). Finally, the membranes were washed with running double-distilled water for 2 min. Photographs were taken with a stereomicroscope Wild M3Z (Wild, Heerbrugg, Switzerland) using the camera device Wild-Leitz MPSS2 (Leica, Bensheim, Germany) with Photoautomat MPS46 (Leica) and Kodak Ektachrome EPY 64T (Eastman-Kodak, Rochester, NY) film. In addition to the analyses of noninfected rye ovaries, control experiments were performed with primary antisera that were incubated with an excess of pure xylanase from *T. viride* (Sigma-Aldrich) prior to its use.

Protein techniques. To obtain protein extracts, the mycelium was separated from the culture fluid by filtration. Extracellular proteins were precipitated from the culture fluid by ammonium sulfate (90% saturation), desalted by gel filtration (Sephadex G 25) in sodium-succinate buffer (0.05 M, pH 5.2), and concentrated with Centricon microconcentrators (Amicon, Heidelberg, Germany). Protein content was measured according to Bradford (5).

Xylanase activity was determined by a modification of the method described by Kombrink et al. (22) using 0.5% xylan (oatspelts; Serva) in sodium-succinate buffer (0.05 M, pH 5.2) as a substrate. SDS-PAGE was performed as described by Lämmli (24). Proteins were stained with Coomassie brilliant blue. Proteins separated by SDS-PAGE were electroblotted onto a Cellulosenitrate-Membrane (Schleicher & Schuell, Inc.) and incubated in a solution containing MAbs raised in rabbit against *T. reesei* xylanase MAb anti-XYNI (23). The filter was afterwards treated with goat anti-mouse IgG (whole molecule; Sigma-Aldrich)-conjugated alkaline phosphatase. Cross-reacting proteins were visualized as described above.

Molecular genetic techniques. Standard molecular techniques such as cloning, gel electrophoresis, DNA and RNA blotting, la-

beling of DNA, and DNA preparation from *E. coli* were performed according to Sambrook et al. (30) and Ausubel et al. (4). Isolation of genomic DNA from *C. purpurea* for Southern and PCR analyses was done as described by Cenis (9). For expression analysis,

RNA was prepared from infected ovaries collected at various stages according to Müller et al. (28). For expression analysis in axenic culture, RNA was extracted from mycelia grown on cellophane according to Chambers and Russo (10).

Fig. 1. Electron-microscopic localization of arabinoxylan in noninfected ovaries of rye. **A to E**, Enzyme-gold labeling of β -1,4-xylan with an endo-xylanase-gold sol in cell walls of different ovary tissues as shown for **A**, the outer epidermal wall; **B**, a three-way junction of the carpel mesophyll; **C**, thick-walled parenchyma; **D**, an intercellular space between young mesophyll cells; and **E**, a thick-walled cell with two pits in the rachilla cortical parenchyma. **F and G**, Immunogold labeling with a monoclonal antibody specific for α -L-arabinofuranosyl residues in xylan in cell walls of **F**, the carpel mesophyll and **G**, phloem elements. cm = Cuticular membrane, is = intercellular space, and ml = middle lamella zone. All samples were fixed in formaldehyde and glutaraldehyde, there was no osmication, samples were embedded in London resin white, and uranyl and lead salts were used for section staining. Scale bars = 1 μ m.

Fig. 2. **A to D**, In situ localization of xylanases using tissue printing and anti-XYNI-mediated immunostaining of infected rye ovaries inoculated with *Claviceps purpurea* T5a. Four progressive stages of ergot infection: **A**, young infected ovary before honeydew production, 4 days postinoculation (dpi); **B**, young honeydew producing sphaecium, 8 dpi; **C**, young sclerotium (sc) developing below an old sphaecium (sp), 16 dpi; and **D**, sclerotium, 28 dpi. **E**, In situ localization of xylanases using mycelium printing and anti-XYNI-mediated immunostaining of *C. purpurea* 20.1 after 5 days of axenic culture on solid medium with reduced sugar content. Tissue printing controls: **F**, noninfected rye ovary at anthesis; and **G**, young healthy caryopsis, 20 days postanthesis. o = Ovule, ra = rachilla, and arrows indicate the persistent host-pathogen boundary in the rachilla. Scale bars = 0.5 mm.

Screening of a genomic EMBL3 library of strain T5 (34) was performed by plaque filter hybridization according to Sambrook et al. (30). Nucleotide sequences of DNA fragments subcloned in pUC19 (48) or pBluescriptIIISK- (33) were determined in an automated sequencer (LI-COR 4000; MWG, Ebersberg, Germany) using the Thermo Sequenase fluorescent-labeled cycle sequencing kit (Amersham International, Braunschweig, Germany). HUSAR (EMBL, Heidelberg, Germany) was used for computer analysis of DNA sequences.

RT-PCR was performed as described by Müller et al. (28); PCR analysis of transformants was done according to Sambrook et al. (30). Primers used for PCR analyses were XYL1a, 5' CAGGTA-CAGTTGTGGTGCC 3'; XYL1b, 5' GTCCCGAGCGGCATGCC 3'; XYL2a, 5' GGGTCAAGGCCATCAAG 3'; and XYL2b, 5' GTGGACGGGCCAGCCGGC 3'.

RESULTS

In situ localization of xylan in cell walls of ovary tissues of rye. To detect the presence of xylans within the polysaccharide network constituting the host cell walls, an endo- β -1,4-xylanase purified from *T. viride* was linked to a gold sol and characterized for its specificity and labeling properties in TEM. The xylan-

degrading activity of the protein preparation was confirmed in a spectrophotometrical xylanase assay. SDS-PAGE revealed one single band that was immunoreactive with the MAb anti-XYNI in Western blotting experiments, confirming the purity of the xylanase preparation intended for the probing of xylan. All on-grid labeling controls verified the specificity of the xylanase-gold marker molecules for xylan localization; the preincubation of the enzyme-gold sol with a polyclonal xylanase antibody or with a potential xylan substrate reduced the labeling density to below that of the background level, while preincubation with carboxymethyl-cellulose had no effect (data not shown).

Treatment of healthy ovaries with the xylanase-gold sol resulted in a specific gold labeling of all cell walls in the different tissues along the usual infection path of the ergot fungus (Fig. 1). In outer epidermal cell walls, no labeling was found over the plant cuticle, while the labeling density increased towards the plant plasma membrane (Fig. 1A). Distinct gold label was present over the cell walls of young and elder mesophyll cells (Figs. 1B to D). In growing cell walls, the innermost wall layers facing the cytoplasm exhibited the highest labeling density. The middle lamella zone between two neighboring cells and the cell wall material that fills three-way cellular junctions (Fig. 1B and C) as well as the outer wall layer facing intercellular spaces (Fig. 1D) was free from xylanase-gold

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-662 cagtcacgggctcagcatgaggtatattcgccgtaacctgtccactgggtctctctcccg -603
                                     PacC
-602 agccgtcaatttgcatgtgtagctgcgcgctgttagtccgcgagtttggtacgcgtggca -543
                                     AREA
-542 tgggctcggttaattttgcgtgatcgcttggtcacaggaagcggaagcatgcgactctatc -483
          AREA                      CREA
-482 tgagatacccggtgtaggctaagccttctttccggagctgaattttctgtgaagatgctt -423
          CREA
-422 cgcacgggtttatgcggttttccggaggtctcggccacagaaagccgctggaagtggaaacag -363
-362 acgaggatttgttttcaggtacctcgttcatcaactgggtaaagtctgagctgcagcgccg -303
          AREA                      AREA
-302 atcaaggaggaggactattgccgttcaatgtgtatcaggagagaagagctagacttggata -243
-242 cgaagaggactgaggagaggtgatcctaatacaattttacctgcctgggacatgaaattgt -183
          AREA                      CREA
-182 tgaagataaaaagagatgccaggtctccagtgccaactgaaagcccttcttggatgagcagc -123
-122 cattcatcactttctctaacaaaaataaaaaaggagcggtccacgcgaacagacttttta -63
          AREA
-62 caccactatccataataaccttttcccttgatcattttcgataaacccgttaataagagcga -3
-2 tcATGTTTCTTACCTCAGTTGTCTCGTCTCGTCTCGGGCCATCAGCTGCGTCTCGGCCG 58
      M F L T S V V S L V V G A I S C V S A A
      ↑
59 CGCCAGCCGAGCCAGCCAGCGAGCTCATGCAAATGACGCCAGGAATTCGTGCTACGGAGGCG 118
      P A A A S E L M Q M T P R N S C Y G G G
          1a
119 GCCTCTATTCTCATATTGGGCGGACTACGGAATAACAGGTACAGTTGTGGTGCCGGAG 178
      L Y S S Y W A D Y G N T R Y S C G A G G
179 GGCATTATGACTTGTCTGTTGGGGGAACGGCGGCAACGTCGTTTCGGGGCAGAGGGTGGAAAC 238
      H Y D L S W G N G N V V A G R G W K P
239 CCGCCAGTCCCAGgtaagcggtctcttctctcattcatttcatgaggttccatatacagg 298
      A S P R
299 tcagagagcggtgctctttgcaatcaccttgttcttaagaatactctcgagttattttctcc 358
359 ccaatatccgtatcacctcgaaatcgctcgcttagtctcggtgctaactagttacgtctctc 418
419 tttccacaattcagAGCTGTAACTACTCCGGCTCCTGGCAGTGCAACGGAACTGCTAT 478
          A V T Y S G S W Q C N G N C Y
479 CTATCTGTCTACGGCTGGACCATAAATCCTCTCGTTCGAGTACTACATTGTCGAGAATTAC 538
      L S V Y G W T I N P L V E Y Y I V E N Y
539 GGCAACTACAACCCATCTGCTGGGGCAGAGACGCGGTCAAGTCACGGCCGATGGGTCC 598
      G N Y N P S A G A Q R R G Q V T A D G S
599 ATCTACGACATCTACATCAGCACGCAACACAACAGCCCTCTATTCTCGGCACGAACACG 658
      I Y D I Y I S T Q H N Q P S I L G T N T
659 TTCCATCAGTACTGGTCTATTTCGGCGTAACAAGCGGTGGGGCGGCACTGTGTCGACGGGC 718
      F H Q Y W S I R R N K R V G G T V S T G
          1b
719 GTACACTTCAATGCTTGGAGGTCGCTTGGCATGCCGCTCGGGACATATGACTACATGATT 778
180 V H F N A W R S L G M P L G T Y D Y M I 199
779 GTTGCAGACAGAGGGTTCCGCGAGCTCTGGAAGTGCTTCTATTACTGTAAGTTAGacaagg 838
200 V A T E G F R S S G S A S I T V S * 216
839 ggctcaacgttcaagttaggcgcaaagcgtggccttgctgcgttaatatctctctttttt 898
899 ggttctctacaccgtgctacctcgcaagattcgatgtagtgggtgctgtatcatgta 958

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Fig. 3. Nucleotide and derived amino acid sequence of *cpyl1* of *Claviceps purpurea* including 5'- and 3'-noncoding sequences. Start and stop codons and putative promoter elements (AREA: N-metabolism [7]; CREA: C-metabolism [11]; and PacC: pH control [38]) are printed in bold letters; binding sites for primers used in reverse transcription polymerase chain reaction experiments (1a, 1b) and putative polyadenylation sites are underlined. Coding sequences are printed in capital letters; the putative signal-peptidase cleavage site is indicated by an arrow. Accession number of *cpyl1*: Y16969.

particles. Especially strong labeling signals were detected over the wall thickenings of xylem tracheary elements and over secondary cell wall layers of the rachilla cortical parenchyma (Fig. 1E). These observations show that all the cell walls from different tissues contained sites that bound the gold-linked fungal xylanase, indicating the presence of β -1,4-xylan oligomers, the potential substrate of ergot xylanases.

To identify possible side groups linked to the β -1,4-xylan backbone, arabinose units were visualized in the polysaccharide network of the ovary wall with immunogold TEM and a MAb specific for terminal α -L-arabinofuranosyl epitopes. Low but specific gold labeling was present in several different tissues of the ovary; the labeling was more intense in the region of the carpel mesophyll (Fig. 1F) and over the phloem elements (Fig. 1G) in the innermost cell wall layer facing the plasma membrane. No arabinosyl epitopes were detected in other cell wall regions. The labeling controls lacking primary antibody or treated with an antibody solution that was immunoprecipitated with arabinoxylan prior to application were devoid of gold particles, while preincubation of the antibody solution with pectin did not alter the labeling density (data not shown). The spatial pattern of the detected α -L-arabinofuranosyl epitopes corresponded with that of the β -1,4-xylan epitopes detected in the enzyme-gold labeling, suggesting the occurrence of arabinoxylan in these wall layers.

Immunodetection of secreted xylanases in rye ovaries infected with *C. purpurea*. For the analysis of the spatio-temporal secretion of xylanase in infected rye ovaries, three different heterologous anti-xylanase antibodies, one polyclonal antibody, and

two MAbs (described in Material and Methods) were utilized in immunolocalization experiments. Western analysis after SDS-PAGE indicated that all three antibodies were immunoreactive with (at least) one xylanase of *C. purpurea* (described below). Immunogold labeling was not successful, neither after London resin white embedding nor with use of Lowicryl sections (Lowi, Chemische Werke GmbH, Waldkraiburg, Germany). This appeared to be due to a fixation-induced alteration of the epitopes, since aldehyde treatment was found to prevent the immunostaining of blots. Taking advantage of the immunoreactivity on blots, we adopted the immunoprinting technique to spatially localize the native proteins in relation to the physical imprints in nitrocellulose membranes. The experiments resulted in a positive staining for the presence of xylanases in all of the four major infection phases (Fig. 2). The red staining was visible throughout the colonized ovary wall. As is known from EM analysis (40), hyphae are regularly scattered throughout the ovary wall mesophyll during the early colonization phase (Fig. 2A); the staining intensity increased in the subsequent sphacelial phase following the proliferation of the fungus in the host tissue (Fig. 2B). The typically noncolonized ovule zone did not stain (Fig. 2A and B). The young sclerotial plectenchyma, which develops intercalary between the fungal foot in the rachilla and the sphacelium, showed intensive staining, whereas the immunoreaction in the elderly sphacelium was only slight (Fig. 2C). Likewise, the staining intensity decreased while the sclerotium matured basipetally, and fully developed sclerotial hyphae gave no immunoreaction (Fig. 2D). Notably, at the persistent host-pathogen

Fig. 4: Alignment of CPXYL1 to derived amino acid sequences of family G-type xylanase genes of *Cochliobolus carbonum* (xyl1 [2]), *Trichoderma reesei* (xynII [39]), *Aspergillus nidulans* (xyn2, P55333), *A. niger* (xynII [42]), *Humicola insolens* (xyl1 [12]), and *T. harzianum* (47). Residues identical in all polypeptides are marked by asterisks. Putative catalytic residues (E) are printed in bold letters.

boundary in the host rachilla, a strong red staining for xylanases could be continuously detected and remained visible in the sclerotial phase (Fig. 2D, arrows). Tissue-printing experiments, carried out with healthy rye ovaries (Fig. 2F) and developing rye caryopses (Fig. 2G) as noninfected controls showed no immunoreaction, whereas tissue prints of axenically cultivated wild-type *C. purpurea* gave positive staining (Fig. 2E). The same was true for *T. reesei* cultures (data not shown). All three different antisera resulted in identical positive staining for xylanase on prints.

Cloning and characterization of two putative xylanase genes.

Two different fungal xylanase genes were used as heterologous probes to isolate putative xylanase genes from a genomic EMBL3 library of *C. purpurea* strain T5: *xyl1* from *Cochliobolus carbonum*, coding for a family G-type xylanase (2), and *xyl2* from *Magnaporthe grisea*, coding for a family F-type xylanase (46). Both genes were first checked in Southern experiments for significant homology to genomic DNA of *C. purpurea* (data not shown).

From 50,000 plaques screened with the *Cochliobolus carbonum* probe, 21 λ clones were selected that showed strong homology to the probe (moderately stringent hybridization conditions). The homologous region was subcloned and sequenced; it was shown to contain an open reading frame (ORF) with significant homology to fungal xylanase genes and was named *cpxyll*. The *cpxyll* sequence comprised an ORF of 832 bp and was interrupted by an intron of 181 bp (Fig. 3), the presence of which was confirmed by cDNA sequencing. Assuming that the first 19 amino acids probably represented a signal peptide, the derived gene product, CPXYL1, had an estimated molecular mass of 21.47 kDa and a predicted pI of 8.88. The *cpxyll* sequence showed significant homology to other xylanases on both the nucleic acid and amino acid levels (e.g., 60% to Xyl1 from *C. carbonum* [2]). A comparison at the amino acid level (Fig. 4) indicated that *cpxyll* coded for a family G xylanase according to the classification of Gilkes et al. (16). Evaluation of the noncoding upstream sequence of *cpxyll* revealed typical

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CREA
-690 cccccggggcttgagtttccggaacacggcatggcaccggaccgcaagccgagaatctg -631
PacC
-630 cccttggcgggtctcgcgacaaatctgcgacaagttatgtaggtaacaagattgaagaca -571
CREA
-570 ccggtatttaccgacaagccgaggggtggagatggacggggatggcggcggggatggagaa -511
-510 tggagtcgcaggctaaagcaattagccatcaagcgagactttagcctggtagtacatact -451
PacC
-450 ccgatgcggtgtctcaaataaaggattttgcctgcgttacttttgcgtggccctgtga -391
STRE
-390 ggccctgcgagggccctgcgtgttgccgttgagaaacgagccaggggctgaagcgcaagag -331
AREA
-330 acgcagagacaaggaagagtgcgagctatcgcgacatccatttcatttaaaagcgaccgtct -271
-270 ttccgcccgtctcattactggttcttcttctctctgtagttataacaatcggtgtgccc -211
-210 attctcttctcttctgtcttttccagactcaaatacttcgctttcttctcacttcaccactga -151
-150 aaccttctcttccaacactacacgagaagcactcttttattctcgggttttgcataaac -91
STRE
-90 cctcactctcatttgtttttatctctggaacttattttttattcgatttatattttttgt -31
-30 ctacggcaaaacctcaataactatacaccATGTTGTACACCTCCATCTTGTGCGGCT 30
M L Y T S I F A A A
31 ATGGCCGCCAGCGGCGCCATGGCTGCTCCCAACCTCTCACGGTGCCAGCAACTGCACC 90
M A A S G A M A A P T T S H G A S N C T
↑
91 ACGCTCGACTCTTTCTTCAAGTCCACGGCAAGCTGTACTGGGGTACCGCCGCCGACAAG 150
T L D S F F K S H G K L Y W G T A A D K
151 AACACCCCTCATGAAGCCCGGTGTGGCTGACTTCATCGCCAAGGAGTTCGGTCAAGTCACC 210
N T L M K P G V A D F I A K E F G Q V T
211 CCGGAGAACAGCATGAAGTTCGACGCCACTGAGCCTTCCCGAGGCCAGTTCCTACTTCGAC 270
P E N S M K F D A T E P S R G Q F H F D
271 GCCGCCGACTACCTCGTCGACTATGCTGAGAAGCACGACCTGTTGATCCGTGGACACACC 330
A A D Y L V D Y A E K H D L L I R G H T
2a
331 TTCCTCTGGTGTCTCAGATGCCCGCTGGGTCAAGGCCATCAAGGACAAGGACACCTTG 390
F L W W S Q M P A W V K A I K D K D T L
391 ATCGATGTTATCCAGACCCACATCTCTACCGTCGCTGGACGATACAAGGGCAAGATTAC 450
I D V I Q T H I S T V A G R Y K G K I Y
451 GCTTGGGtaagacgaaggcctctccagaatcgattgtttaatcgacgccgacatactgac 510
A W
511 ctgtcttttcttctctctatagGATGTCGTCACGAGATCTTCGAGCAGGACGGCTCTTT 570
D V V N E I F E Q D G S F
571 CCGAAAGACCGTCTACTACAACCTCCTCGCGGAGGACTATGTCGGCATTGCTTTTCGAGGC 630
R K T V Y Y N L L G E D Y V R I A F E A
631 TGCCACAAAGGCTgttagtgctctctctctcgaccgcgccccttgctgctctgtcca 690
A H K A
691 ccatactcttcttcttcttctctgtgacaaaaaaccttccagGACCCCAAGGCCAAGCT 750
D P K A K L
751 CTACATCAACGACTTCAACCTCGACGACCCCAACGCCCAAGTTGAAGGCCATGATCAA 810
Y I N D F N L D D P N A A K L K A M I K
2b
811 GTACGTCACCAAGTGGAGAGCTGCCGGCTGGCGCTCCACGGAATCGGCAGCCAGTCTCA 870
Y V T K W R A A G W P V H G I G S Q S H
871 CCTGTTCTGCTGGCATGGCGGAGAAGAGCGCTGCTGCCATCAAGATGCTTGGTCCGCTGC 930
L F A G M G E K S A A A I K M L G A A A
931 CGACGAAGTTGCCATCACCAGGCTCGACATCACTGGCGCCCCCAGGCCGACTACGAAGC 990
D E V A I T E L D I T G A P Q A D Y E A
991 CGTCAACCAAGGGCTGCATTGACGTCGAAGAACTGCGTTGGTATTACCAGCTGGGGTGCTCG 1050
V T K G C I D V K N C V G I T S W G A R
1051 TGACACCGACAGCTGGTTGGCCAGCAAGTCCCTCTTCTCTCGATGGCAACTTCAAGCC 1110
L T D S W L A S K S P L L F D G N F K P
1111 CAAGGCTGCCGTCAAGGCCATCATGGCCATCTAAgcaaaaaattccatggacaaaagaa 1170
326
316 K A A V K A I M A I *
1171 gcatatcttgttttgactcgtcttttggcgctgcatgtacccccaccggtgtttcctctg 1230
1231 tatggttttgactgtctgagatgacgttgggggtatctcttttctgtatgacagcatctt 1290

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Fig. 5. Nucleotide and derived amino acid sequence of *cpxyll* of *Claviceps purpurea* including 5'- and 3'-noncoding sequences. Start and stop codons, putative promoter elements (Fig. 3), and N-glycosylation sites are printed in bold letters; binding sites for primers used in reverse transcription polymerase chain reaction experiments (2a, 2b) are underlined. Coding sequences are printed in capital letters; the putative signal-peptidase cleavage site is indicated by an arrow. Accession number of *cpxyll*: Y16970.

fungus promoter elements such as CT-rich regions, a TATAA box, and two CAAT boxes; the presence of several putative binding sites for CREA- and AREA-like transcription factors (Fig. 3) indicated that the expression of *cpxy1* might be regulated by the major C and N catabolite repression systems.

Using the *M. grisea* gene as a probe, 28 positive plaques were selected (from 50,000 screened). Subcloning and sequencing yielded a second putative xylanase gene termed *cpxy2* (Fig. 5). The gene had a coding sequence of 1,144 bp that was interrupted by two introns of 76 and 90 bp (confirmed by sequencing of cDNA clones). Assuming that here (as in *cpxy1*) the first 19 amino acids represented a signal peptide, the estimated gene product (CPXYL2) had an expected molecular mass of 33.8 kDa and an pI of about 7.01. Comparison of the derived amino acid sequence showed significant homology to family F glycosyl hydrolases from *Aspergillus awamori*, *Penicillium chrysogenum*, and *Fusarium oxysporum* (Fig. 6), strongly suggesting that CPXYL2 was a family F xylanase. As in *cpxy1*, the promoter sequence of *cpxy2* contained several potential CREA and AREA binding sites; however, the large distance between the GATA-boxes made AREA control of this gene unlikely.

Southern analyses with genomic DNA of strain T5 showed that *cpxy1* and *cpxy2* were single-copy genes. The probes hybridized to one or two genomic fragments, depending on the enzyme used;

as the bands hybridizing with the two probes were different, there was obviously no cross hybridization (data not shown).

Expression of *cpxy1* and *cpxy2* in axenic and parasitic culture. The expression of the two xylanase genes in axenic culture was subjected to Northern analysis. However, as had been the case with the cellulase and polygalacturonase genes of *C. purpurea* (28,37), the signals obtained were very weak, indicating a low expression level of these genes under the tested conditions. Therefore, the far more sensitive method of RT-PCR was used. To differentiate between RNA-derived PCR products (426 and 326 bp for *cpxy1* and *cpxy2*, respectively) and (larger) PCR products caused by DNA contaminations (607 and 492 bp), primers spanning the intron-containing regions of both genes (Figs. 3 and 5) were used. We detected transcripts of both genes under all the conditions we tested in axenic culture (high/low C and N content, data not shown). As we were especially interested in the in planta expression of the genes, we performed RT-PCR for *cpxy1* and *cpxy2* using RNA from infected rye ovaries collected from all four infection stages (described above). The results are presented in Figure 7; both primer sets of PCR fragments of the expected sizes (426 and 326 bp) were detectable in all samples. As expected, the corresponding DNA controls yielded PCR fragments of 607 and 492 bp, respectively. Southern hybridization confirmed that the 426-bp PCR fragment was derived from *cpxy1* (Fig. 7A). The identity of the

					150
<i>A. awamori</i>	IGDQYTLTKN	SKTPAVIKAD	FGALTPENSM	KWDATEPSRG	QFSFSGSDYL
<i>P. chrysogenum</i>	IADQGTNGN	PKTPAIKAN	FGQLSPENSM	KWDATEPSQG	QFSFAGSDYF
<i>F. oxysporum</i>	EIDHYHLNNN	PLI.NIVKAQ	FGQVTCENSM	KWDAIEPSRN	SFTFSNADKV
<i>C. purpurea</i>	AADKNTLMK.	PGVADFAKE	FGQVTPENSM	KFDATEPSRG	QHFHDAADYL
	* *		** **** * *		* * *
					200
<i>A. awamori</i>	VNFAQSNNKL	IRGHTLVVHS	QLPSWVQAIT	DKNTLIEVMK	NHITTVMQHY
<i>P. chrysogenum</i>	VEFAETNGKL	IRGHTLVVHS	QLPSWVSSIT	DKTTLTDVMK	NHITTVMKQY
<i>F. oxysporum</i>	VDFATQNGKL	IRGHTLVVHS	QLPQWVQNIN	DRSTLTAVIE	NHVKTMTVTRY
<i>C. purpurea</i>	VDYAEKHDL	IRGHTLVVHS	QMPAWVKAIA	DKDTLIDVIQ	THISTVAGRY
	* * *	*****	* * * * *	* * * *	* * *
					250
<i>A. awamori</i>	KGKIYAWDVV	.NEIFNEDGS	LRDSVFYKVI	GDDYVRIAFE	TARAADPNK
<i>P. chrysogenum</i>	KGKLYAWDVV	.NEIFEEDGT	LRDSVFSRVL	GEDFVRIAFE	TAREADPEAK
<i>F. oxysporum</i>	KGKILQWVV	NNEIFAEEDGN	LRDSVFSRVL	GEDFVGIAFR	AARAADPAK
<i>C. purpurea</i>	KGKIYAWDVV	.NEIFEQDGS	FRKTVYNNLL	GEDYVRIAFE	AAHKADPKAK
	***	****	****	* * * * *	* * * *
					300
<i>A. awamori</i>	LYINDYNLDS	ASYPKLA.GM	VSHVKKWIEA	GIPIDGIGSQ	THLSAGGG..
<i>P. chrysogenum</i>	LYINDYNLDS	ATSAKLQ.GM	VSHVKKWIAA	GVPIDGIGSQ	THLGAGAG..
<i>F. oxysporum</i>	LYINDYNLDS	SDYAKVTRGM	VAHVKNWIAA	GIPIDGIGSQ	GHLAAPSGWN
<i>C. purpurea</i>	LYINDFNLDD	PNAAKL.KAM	IKYVTKWRAA	GWPVHGIGSQ	SHLFAGMGKEK
	*****	***	* *	* * * * *	***
					350
<i>A. awamori</i>	..AGISGALN	ALAGAGTKEI	AVTELDIAGA	SSTDYVEVVE	ACLDQPKCIG
<i>P. chrysogenum</i>	..AAASGALN	ALASAGTEEV	AVTELDIAGA	TSTDYVDVNV	ACLDQPKCVG
<i>F. oxysporum</i>	PASGVPAALR	ALAASDAKEI	AITELDIAGA	SANDYLTVMN	ACLAVPKCVG
<i>C. purpurea</i>	SAAAIK....	.MLGAAADEV	AITELDITGA	PQADYEAVTK	GCIDVKNCVG
			* * **** *	** *	* *
					400
<i>A. awamori</i>	ITVWGVAD..	.PDSWRSSST	PLLFDSENYN	KPAYTAIANA	L.....
<i>P. chrysogenum</i>	ITVWGVAD..	.PDSWRADSE	PLLFDSENYN	KEAY.NVSQL	LSRQHAFDLY
<i>F. oxysporum</i>	ITVWGVSD..	.KDSWRPGDN	PLLFDSENYQ	KAFAFALANA	L.....
<i>C. purpurea</i>	ITSWGARD..	.TDSWLASKS	PLLFDGNFKP	KAFAKAIMAI	*.....
	** * *	***	*** *	* *	
					414
<i>A. awamori</i>					
<i>P. chrysogenum</i>	LKLGNNLLSR	LHSD			
<i>F. oxysporum</i>					
<i>C. purpurea</i>					

Fig. 6. Alignment of derived amino acid sequences of family F-type glycosyl hydrolase genes of *Aspergillus awamori* (*xynA* [20]), *Penicillium chrysogenum* (*xynA* [17]), and *Fusarium oxysporum* (cellulase [32]) with CPXYL2. Residues identical in all four polypeptides are marked by asterisks. A glutamic acid residue that is probably involved in catalytic activity is printed in bold letters.

cpxy2 fragment (326 bp) was verified by cloning and sequencing of the PCR product. These data confirm that both genes were expressed throughout the entire infection process.

Targeted inactivation of *cpxy1*. To explore the role of CPXYL1 during the infection process, a gene-replacement approach was used to inactivate the gene. As shown in Figure 8, the phleomycin resistance cassette from vector pAN8-1 was used to construct a disrupted gene version (vector p Δ *xyl1*phleo^R). The haploid derivative 20.1 of strain T5 (27) was transformed with the purified *Hind*III fragment of the vector containing the disrupted *cpxy1* copy. Phleomycin-resistant transformants were obtained at a frequency of about two transformants per μ g. The DNA of 38 transformants, obtained from single-spore cultures, was digested with *Hind*III and subjected to Southern analysis using the labeled *cpxy1* gene as a probe. Transformants that carry the disrupted gene version from p Δ *xyl1*phleo^R as a result of a double-crossover event should contain a 9.7-kb *Hind*III fragment instead of the wild-type 6.3-kb fragment that hybridizes to *cpxy1*. As documented in Figure 9, the *cpxy1*-homologous band of one of the 38 transformants (X12) showed the expected shift in molecular weight. This homologous transformant was named Δ *cpxy1-1* and analyzed biochemically. Activity tests showed that it had a significantly reduced xylanase activity in axenic culture (Mantle B medium; strain 20.1: 133.3 ± 5.8 μ kat/kg; transformant X12: 43.3 ± 7.1 μ kat/kg), indicating that CPXYL1 was responsible for at least about 60% of the total xylanase activity under these conditions.

Using the *cpxy1* deletion strain it was possible to show that a 23-kDa protein that showed a cross-reaction with MAb (MAb

XynI) raised against the xylanase XynI from *T. reesei* was indeed the *cpxy1* gene product. As shown in Figure 10, the 23-kDa band (the only one detected by the heterologous antibodies) present in strain 20.1 is missing in strain Δ *cpxy1-1*, confirming both that this band represented CPXYL1 and that *cpxy1* was indeed disrupted in the transformant. Likewise, in contrast to experiments with the wild-type strain, mycelium printings of the mutant strain axenically cultured on solid plates gave no immunostaining with MAb XynI.

DISCUSSION

Cytological aspects. According to the model of plant cell wall architecture outlined by Carpita and Gibeaut (8), grasses possess a type II cell wall containing a high percentage of GAX. The cytological data presented here confirm this model for the first time for the ovarian tissue of *Secale cereale*; the enzyme-gold analyses clearly show the presence of an oligoxylan with β -1,4-linkages, and the immunogold labeling using a MAb indicates the presence of arabinofuranosyl side chain, although the reaction of the antibody with arabinosyl units linked to other polysaccharides such as arabinogalactan or pectin cannot be excluded. The diversity of the enzyme-gold and immunogold labeling suggests a complex, tissue-specific distribution of diversely structured arabinoxylans in the ovarian tissues of rye. These data clearly demonstrate the presence of xylans along the infection pathway of *C. purpurea*. Together with the complete degradation of host cell walls at the host-fungus interface (40,27), this indicates that xylan-degrading enzymes are

Fig. 7. Reverse transcription polymerase chain reaction (RT-PCR)-based expression analysis of *cpxy1* and *cpxy2* in planta. RNA from different infection stages and DNA (as a control) served as templates for the RT-PCR reaction. **A**, Primer 1b was used for reverse transcription and in combination with primer 1a for the amplification (Fig. 3). Expected sizes: 606 and 426 bp for the DNA- and RNA-derived products, respectively. Left, ethidium bromide-stained agarose gel; and right, Southern hybridization using *cpxy1* as a probe. **B**, RT-PCR products that were obtained using primers 2b and 2a (Fig. 5); expected sizes: 491 and 324 bp. Identity of the PCR fragment was verified by sequence analysis.

Fig. 8. Construction of the *xyl1* gene-replacement vector p Δ *xyl1*phleo^R. Plasmid pH_{xyl1} that contains the *xyl1* gene of *Claviceps purpurea* was digested with *Xho*I. The *Eco*RI/*Xba*I phleomycin cassette (comprised of the *Streptomyces verticillium* phleomycin resistance gene *phleo*^R, the *gpd* promoter from *Aspergillus niger* and the *A. niger* terminator sequence *trp*^C) was cloned into the *Xho*I site of the *xyl1* gene. For performing transformation experiments the *Hind*III fragment of p Δ *xyl1*phleo^R was used. The *xyl1* intron is represented by a lightly shaded box, noncoding sequences by narrow open bars.

important for the colonization of the host tissue. The tissue-printing and immunostaining analyses present evidence for xylanase activity in ovaries infected with *C. purpurea* (35,40), indicating that the fungus secretes xylanases in both saprophytic culture and infected tissue.

These indications of the importance of xylanases in the pathogenesis of *C. purpurea* were convincing enough to initiate a molecular characterization of the xylanolytic system including the cloning of xylanase genes, analysis of their expression, and the creation of deletion mutants that would serve as a basis for a functional analysis.

Structural aspects of *cpxy11* and *cpxy12*. The endo-xylanases characterized until now have been grouped into the families F and G of glycosyl-hydrolases according to the classification of Gilkes et al. (16). As shown in Figures 4 and 6, the two putative xylanase genes isolated from a genomic library of *C. purpurea* by heterologous probing are obviously members of those groups: CPXYL1 shows significant homologies to glycosyl hydrolases of the G family, CPXYL2 to those of family F. Accordingly, both genes (and the derived polypeptides) show no significant homology to each other. Both polypeptides contain the conserved residues characteristic of their family, e.g., CPXYL1: two glutamic acid residues (E112 and E203) of the catalytic center (21) and two tyrosine residues that are probably involved in substrate binding (Y103 and Y113) (44). Since the two enzymes have not yet been purified, it is not known if they have different substrate specificities.

The promoter sequences of both genes contain potential binding sites for (homologues of) the major regulatory proteins: AREA (7), CREA (11,25), and PACC (38), indicating that these global regulators of N- and C-metabolism and pH control, respectively, may be involved in the expression of *cpxy11* and *cpxy12*.

Expression of *cpxy11* and *cpxy12*. The RT-PCR experiments outlined above allowed detection of the transcripts of both genes in planta in all stages of infection, which correspond to the results from the tissue printings. These data do not allow conclusions about the level of gene expression in the different infection phases, but they are interesting when compared with RT-PCR data obtained with other CWDEs of *C. purpurea* (27,37); transcripts of the *cell* gene (coding for a cellobiohydrolase) are detectable by RT-PCR only in stage I, and those of *pg1* and *pg2* (coding for polygalacturonases) are detectable only in stages I to III. In contrast, *cpxy11* and *cpxy12* are expressed in all stages, although detailed analyses such as quantitative PCR might show a phase-specific variation in their expression level. Immunolocalization using homologous antibodies raised against the purified *C. purpurea* xylanases and in situ hybridization experiments with specific gene probes will give a detailed view of the temporal and spatial distribution of the *cpxy11* and *cpxy12* expression.

Fig. 9. Southern analysis of pΔxylphleo^R transformants of strain 20.1. Genomic DNA from *Claviceps purpurea* wild type (20.1) and the putative mutants were digested with *Hind*III, fractionated, blotted, and hybridized with the *Hind*III fragment of plasmid pXylI (Fig. 8).

Functional aspects and perspectives. The presence of at least two xylanase genes in *C. purpurea* is not unique; several phytopathogenic fungi secrete more than one xylanase, e.g., *Cochliobolus carbonum* probably has at least four xylanase genes (3) and *M. grisea* six (46). This could be important for the efficient degradation of the complex group of natural xylans; the various enzymes could complement each other with respect to stability, pH optimum, and substrate specificity. Especially in pathogens of cereals and grasses (which have a type II cell wall with a high xylan content), this enzyme system could be very important in leading the initial attack on the host's cell wall, widening the pores, and rendering the other cell wall components (cellulose and pectin) more accessible to degrading enzymes. The technique of targeted gene inactivation methods enables the analysis of the specific function of the different enzymes. As of yet, dramatic effects resulting from the deletion of xylanase genes have not been reported (41). However, most cases lack a detailed cytological analysis, and most of the fungi that have been analyzed in this respect are necrotrophic. The situation might be quite different in a biotrophic fungus such as *C. purpurea*, which secretes only very limited amounts of CWDEs and must establish a stable interaction with its living host. Therefore, a functional analysis using a knock-out approach especially in combination with detailed cytological studies is feasible in *C. purpurea*, in spite of the negative results in other systems. We report here the deletion of *cpxy11* by a gene-replacement approach; the successful inactivation of the gene is documented by the loss of the wild-type genomic DNA fragment in Southern experiments, by the loss of a 23-kDa protein cross-reacting with heterologous xylanase-specific antibodies, and by a significant reduction in overall xylanase activity in the mutant. This is only the third example for a successful targeted gene inactivation in *C. purpurea*, the others include a cellobiohydrolase gene, *cpcell* (27), and a catalase gene, *cpcat1* (15). In all these cases, we used a gene-replacement approach, which has the advantage over a gene-disruption strategy that restoration of the locus via recombination is impossible. Knock-out mutants were obtained in all these systems at a frequency of about 1 to 3%. Thus, the methods are now available for a detailed functional analysis of the xylanolytic system in the interaction of *C. purpurea* and rye. For this purpose, it will be necessary to construct *cpxy12* deletion mutants, *cpxy11/cpxyl2* double mutants, and mutant strains complemented with the corresponding wild-type genes and to monitor the effects of these

Fig. 10. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of proteins secreted by strain 20.1 and the Δ*cpxy11* mutant strain. **A**, Protein from culture filtrate of the wild-type strain (20.1) and the Δ*cpxy11* mutant (X12) were separated by SDS-PAGE and stained with Coomassie brilliant blue. **B**, Proteins were blotted onto a nitrocellulose membrane and stained with antibodies raised against *Trichoderma reesei* xylanase (monoclonal antibody XynI).

deletions/complementations by cytological and biochemical analyses in vitro and in planta.

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