European Journal of Plant Pathology **107:** 457–465, 2001.

Cloning, sequencing and expression of a xylanase gene from the maize pathogen *Helminthosporium turcicum*

Yeshitila Degefu[1], Lars Paulin[2] and Peter Stephensen Lübeck[3]
[1]*Department of Applied Biology, Plant and Forest Pathology, University of Helsinki, P.O. Box 27, Helsinki, FIN-00014, Finland (Fax: +358919158727; E-mail: Yeshitila.degefu@helsinki.fi);* [2]*Institute of Biotechnology, University of Helsinki, P.O. Box 56, Helsinki, FIN-00014, Finland;* [3]*Department of Microbiology, Danish Veterinary Laboratory, Bülowsvej 27, DK-1790 Copenhagen V, Copenhagen, Denmark*

Accepted 6 March 2001

Key words: Endoxylanase, *Helminthosporium turcicum, Zea mays*

Abstract

A gene encoding an endoxylanase from the phytopathogenic fungus *Helminthosporium turcicum* Pass. was cloned and sequenced. The entire nucleotide sequence of a 1991 bp genomic fragment containing an endoxylanase gene was determined. The xylanase gene of 795 bp, interrupted by two introns of 52 and 62 bp, encoded a protein of 227 amino acids showing up to 95% amino acid homology with other fungal xylanases. The precise splicing site of the introns was identified by sequencing the corresponding cDNA. A northern blot showed that the gene is expressed when the fungus is grown in a medium containing xylan as a sole carbon source. The cloned xylanase gene was expressed in maize plants during infection.

Introduction

The cell walls of plants act as a barrier to and as a source of nutrition for pathogens. The cell wall is also a dynamic and metabolically active structure that functions as a reservoir of signal molecules which regulate or activate plant defense responses to microbial attack (Fry et al., 1993).

Arabinoxylan, the predominant hemicellulosic polysaccharide of monocotyledonous plants, is structurally complex, comprising a β-1,4-linked D-xylopyranosyl backbone frequently substituted by arabinosyl, xylosyl and glucosyluronic residues (Biely, 1985). Endo-1,4-β-xylanase (1,4-D-xylan xylohydrolase; EC 3.2.1.8) cleaves the internal glycosidic linkages of the 1,4-β-D-xylan backbone and plays a crucial role in the hydrolysis of xylan. Xylanases are produced by a number of saprophytic and pathogenic fungi (Sunna and Antranikian, 1997). In graminaceous crops and grasses, arabinoxylan accounts for up to 40% of the primary walls (Carpita, 1996) making it a target for breakdown by potential pathogens. The enzymatic adaptation of cereal pathogens to production of predominantly xylanases (Braun and Kelman, 1987; Cooper et al., 1988) might also be a refelection of their basic compatibility established with their monocotyledonous hosts.

Other evidence suggest that fungal xylanases might be key biochemical components in pathogenesis. In rice, fungal xylanases killed cultured cells (Ishi, 1988), and in tobacco they elicited defense responses such as tissue necrosis, electrolyte leakage and ethylene biosynthesis (Bailey et al., 1990).

However, these evidence are circumstantial and the demonstration that xylanases are important factors in fungal pathogenesis has not been accomplished. Of all methods that have been employed to demonstrate this, targeted gene disruption is the most direct and stringent approach. The method has been used to characterize xylanase genes from at least the two important cereal pathogens, *Cochliobolus carbonum* and *Magnaporthe grisea* (Apel-Birkhold and Walton, 1996; Apel et al., 1993; Wu et al., 1997). Disruption of the xylanase genes from these fungi caused significant loss in xylanase activity but failed to produce non-pathogenic

phenotypes. Fungal cell wall degrading enzymes form complex systems where several of such enzymes are secreted during infection. Thus, it is assumed that the disruption of one enzyme might be complemented by the activity of other secreted enzymes (Annis and Goodwin, 1997; Hamer and Holden, 1997). However, new insight on the role of cell wall degrading enzymes is emerging in a concerted approach of using gene disruption and cytological analysis together with gene expression studies. Have and co-workers (1998), found that disruption of *Bcpg*1, one of the genes encoding endopolygalacturonase of *Botrytis cinerea*, reduced its colonization by 22–50% and concluded that PG is required for full virulence in *B. cinerea*. Detection of the expression of cloned putative pathogenicity genes during penetration and invasion has been possible using sensitive techniques such as reverse transcription polymerase chain reaction, even when the gene expression level appears to be low to be detected by northern blots (Di Pietro and Roncero, 1998; Giesbert et al., 1998).

Helminthosporium turcicum Pass (*Exserohilum turcicum* Pass, Leonard and Suggs), is an important pathogen of maize causing significant losses in warm and humid maize growing areas of the world. The pathogen produces high xylanase activity when cultured on isolated maize cell walls as a sole carbon source, and we have previously purified (Degefu et al., 1995) an approximately 22.5 kDa xylanase which belongs to the family 11 xylanases (Lübeck et al., 1997). We now report the cloning, sequencing and expression both *in vitro* and *in planta*, of an endoxylanase gene from the fungus as a step towards the molecular characterization of its role in pathogenicity.

Materials and methods

Organisms, plasmids, chemicals, media and culture conditions

Helminthosporium turcicum isolate H-2 (Awassa, Ethiopia) isolated from infected maize leaf, was stored as a conidial suspension in 20% glycerol at −20 °C. Liquid cultures of *H. turcicum* were grown in minimal salt medium (Degefu et al., 1995) supplemented with 1% glucose or 1.5% birchwood xylan (Sigma) as carbon source. For DNA isolation the fungus was grown in Potato Dextrose Broth (Difco). Cloning, plasmid amplification and preparation, using the *Escherichia coli* strain DH5α (Life Technologies Inc. Gaithersberg, MD, USA) followed routine protocols (Sambrook et al., 1989). The *E. coli* strain LE392 (Stratagene, La Jolla, California, USA) was used for production of genomic library constructed in Lambda DASH II/*Bam* HI cloning kit (Stratagene) and lambda phage infection for plaque lifting. All subclonings were carried out in the vector pBluescript II SK(+) (Stratagene). Except where stated otherwise, all restriction and modifying enzymes were from Promega (Madison, WI).

Nucleic acid isolation and analysis

Genomic DNA from *H. turcicum* was isolated according to Yoder (1988). For Southern hybridization, about 3 μg of genomic DNA was digested with appropriate restriction enzymes, size separated on a 0.7% agarose/Tris-Acetate (TAE) gel and capillary blotted to a positively charged nylon membrane (Boehringer Mannheim). The membranes were hybridized at 68 °C for at least 18 h in the presence of non-isotopic digoxigenin labeled cDNA probe (Boehringer Mannheim) in a modified Church and Gilbert solution (Vahala et al., 1998), and later exposed to Fuji X-ray films.

Total RNA was isolated from fresh mycelium of *H. turcicum* cultured in minimal medium supplemented with 1.5% birchwood xylan or 1% glucose following the protocol by Chang et al. (1993). For analysis of gene expression *in vitro*, RNA blotting was carried out following standard procedures (Sambrook et al., 1989). Equal loading of RNA was confirmed by ethidium bromide staining. Hybridization and washings were done in a modified Church buffer as in Southern blotting.

Isolation and characterization of the htxyl1 *gene*

PCR amplification of a xylanase gene fragment from the genomic DNA and cDNA template was carried out as described by (Lübeck et al., 1997). PCR products were purified by Prep-A-Gene DNA purification systems (BioRad). The fragments were cloned into pBluescript II SK(+) at the *Eco*RV site. The cloned fragments were sequenced and used as a probe for screening the genomic library to isolate the corresponding xylanase gene.

The genomic library of *H. turcicum* was constructed following standard procedures as described in Lübeck et al., 1997. From the genomic library, recombinant phages were plated and replica membranes (Boehringer, Mannheim) were made. Hybridization and washings were performed as in Southern blotting.

Table 1. Primers used in the study

Primer	Sequence
3475	5′GGITGGAA (C/T) CCIGGIAC (A/C/G/T) GC (A/C/G/T) (A/C) G 3′
3476	5′TTI (C/G) (A/T) IGCCCAIGC (A/G) TC (A/G) AA (A/G) TG 3′
YD-2	5′GCCCGCGCTACCTACACCAA (C/T) GG 3′
YD-6b	5′TTTCTGGATCCTTACGGGCAGTTGACAGTGAT 3′
YD-7	5′TTTCTTCCATGGTTTCTTTCACCTCCATCAT 3′

Positive plaques were purified by three rounds of plaque lifting and hybridization. DNA from selected phage of the genomic library was purified using Qiagen columns. Isolation of putative xylanase lambda clones, subclonings, and other routine procedures were performed essentially as described in standard protocols (Sambrook et al., 1989). Specific primers YD6b and YD7 (Table 1) were designed based on the nucleotide sequence of the cloned xylanase gene. The full length cDNA of the xylanase transcript was amplified by PCR, cloned and sequenced.

The DNA sequencing was done on both strands using the BigDye terminator kit and analysed on an ABI 377XL automated DNA sequencer (Perkin-Elmer). The obtained sequences were assembled using xgap in the Staden Package program on a SUN workstation (Bonfield et al., 1995).

RT-PCR and Southern hybridization in infected maize plants

To determine whether *htxyl1* is expressed by *H. turcicum* during infection, two-week-old maize plants were inoculated with conidia of the pathogen. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the primers 3475 and 3476 (Table 1) to detect the presence of *htxyl1* transcript in infected leaves collected 2, 3, 4, 5 and 6 days after inoculation. One microgram of total RNA (treated with RNases free DNases, Promega, Madison, WI) isolated from infected and control maize plants was used and reverse transcribed into cDNA using ALV reverse transcriptase (Reverse transcription system, Promega, Madison, WI) with the antisense primer. The RT reaction product was used for the PCR amplification using the primers 3475 and 3476. The PCR conditions were as follows: one cycle of denaturation at 95 °C for 2 min, annealing at 60 °C for 2 min, extension at 72 °C for 3 min, and 34 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 2 min. As a positive control, RT-PCR was performed on total RNA isolated from mycelium grown for 7 days on 1.5% (w/v) birch wood xylan whereas genomic DNA of *H. turcicum* was used as a template for PCR reaction to compare the size of the amplified fragment with or without the intron.

The amplified fragments were cloned into pGEM T (Promega, Madison, WI) and sequenced in order to check their identity. For Southern analysis, electrophoresis of the amplified PCR products was done on 1.6% agarose gel followed by transferring to a nylon membrane and hybridization with the digoxigenin labelled cDNA of *htxyl1*. Hybridization and post-hybridization washes were done at stringent conditions as described above.

Results and discussion

PCR amplification of a xylanase fragment

As a first step in the cloning of the xylanase gene from *H. turcicum*, the degenerate primers 3475 and 3476 (Table 1) were used in PCR to amplify a xylanase gene fragment from both genomic DNA and cDNA. Fragments of 398 and 336 bp, respectively, were obtained and cloned. The fragments were sequenced and homology search from the international DNA database indicated that the cloned fragment had a high degree of similarity to other fungal xylanases. Genomic Southern blot analysis using high stringency conditions (Figure 1A) confirmed that the cloned xylanase gene fragment originated from *H. turcicum* DNA. The results also suggest that there are no xylanase sequences related to the cloned xylanase gene in *H. turcicum* genome because hybridization and washings even at less stringent conditions (42 °C) did not reveal additional cross hybridizing sequences (data not shown). This is consistent with previous findings of a single protein with such activity (Degefu et al., 1995). It seems apparent that *H. turcicum* does not share the multiplicity of xylanase which is observed in most other fungi where isozymes differing in isoelectric point, molecular weight and often in their regulation have been reported (Apel et al., 1993; Wong et al., 1988; Wu et al., 1995). Although, we cannot exclude the possibility that (1) there might be other xylanase(s) in *H. turcicum* which are more weakly expressed in

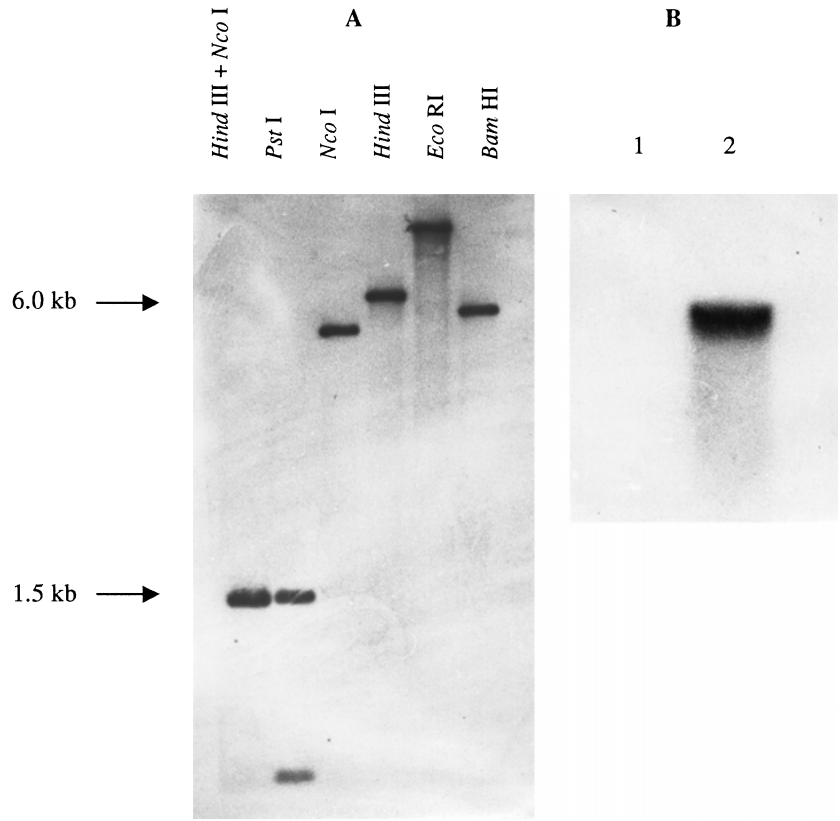

Figure 1. (A) Southern blotting of genomic DNA from *H. turcicum* using a digoxigenin labelled cDNA of *htxyl1* and showing the presence of a xylanase gene in the genome. The cDNA probe was amplified from total RNA isolated from xylanase induced culture using primers YD-2 and 3476. (B) Northern blotting showing xylanase gene expression when *H. turcicum* was grown on birchwood xylan as a sole carbon source (lane 2) and the lack of xylanase gene expression when the fungus was cultured on glucose (lane 1). The same probe was used for both Southern and northern blots.

culture under the tested conditions, and thus difficult to purify and (2) there might be a totally unrelated sequence to the cloned xylanase gene, this study suggests the existence of only one xylanase gene in the *H. turcicum* genome.

Cloning of the htxyl1 *from H. turcicum*

Cloning of the full length xylanase gene was achieved by screening *H. turcicum* genomic library using the gene specific probes generated above. Approximately, 1.5×10^5 plaques were screened and two recombinant clones (plaques) were identified. One of the two positive plaques was selected for further characterization by restriction enzyme and Southern blot analyses. The xylanase gene was identified as two *Pst* I fragments of about 1500 and 500 bp fragment from the N- and C-terminal part of the xylanase gene, respectively. The fragments were cloned and sequenced in both directions. The nucleotide and deduced amino acid sequences of the entire xylanase gene from *H. turcicum* are shown in Figure 2.

Sequence analysis of htxyl1

The sequenced genomic clone of 1991 bp (Figure 2) covers the entire putative endo-1,4-β-xylanase gene of 795 bp including two introns of 52 bp (intron I) and 62 bp (intron II). A non-coding region of 1147 bp precedes the structural gene, and another non-coding region of 46 bp follows the TAA stop codon. No typical polyadenylation site for termination (Gruber et al., 1998) was identified in this region. A typical TATA box is located proximal to the start codon (ATG) at position no. 1054, which also includes a typical transcription start point (TSP) pyAAG. The only CCAAT motif is

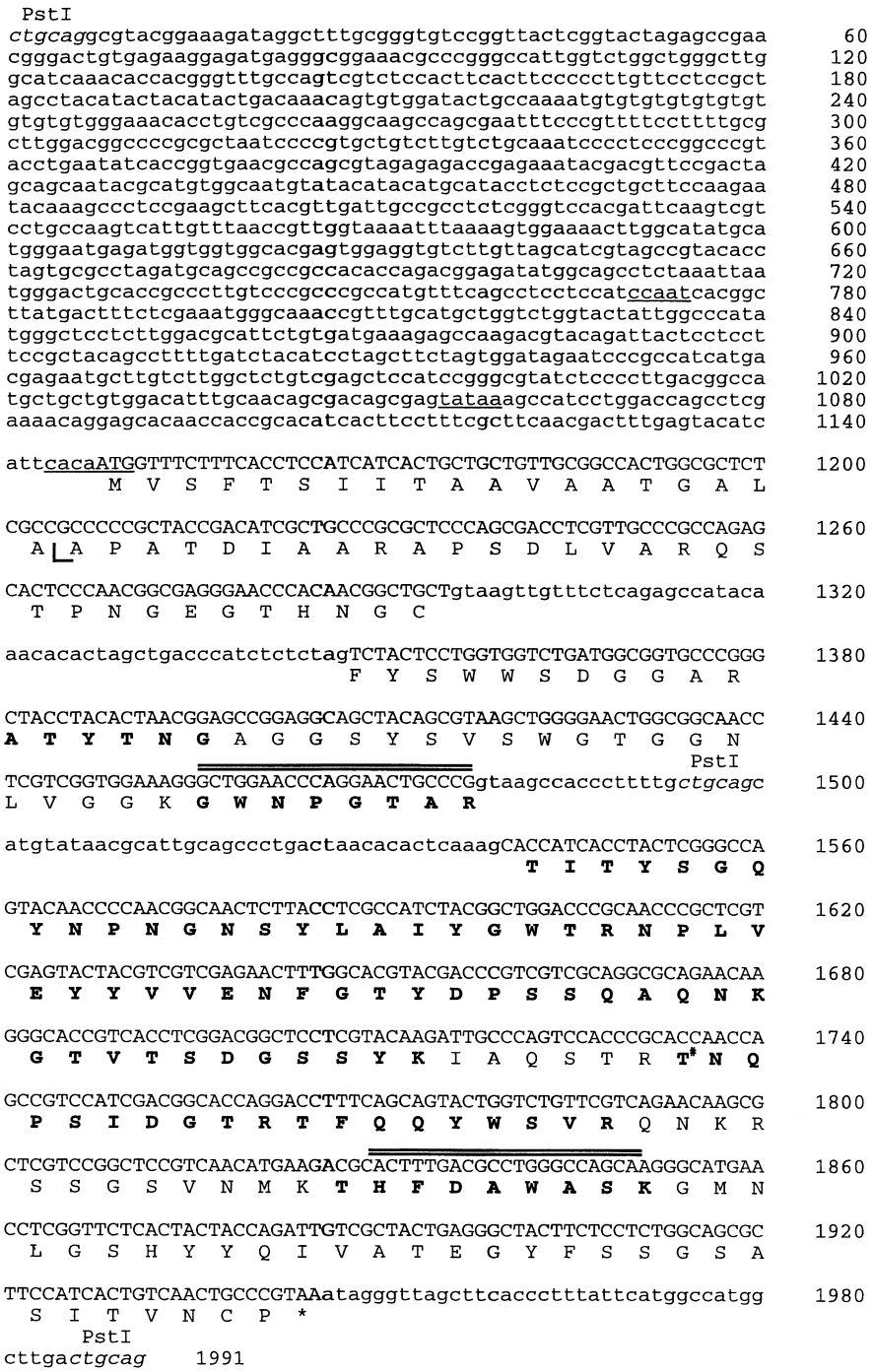

Figure 2. The nucleotide and deduced amino acid sequence of xylanase from *H. turcicum*. The non-coding sequences including the two introns are indicated by lower case letters. Determined amino acid sequences of tryptic peptides derived from *H. turcicum* xylanase (Degefu et al., 1995) are shown in bold. The TATA boxes, putative CAAT motifs and the translation initiation sites are underlined. The L-sign at position 1204 (amino acid 19) indicates the putative cleavage site for the signal peptidase. The sequences which match the PCR degenerate primers are indicated with double lines over the sequence. The # indicates the amino acid discrepancy. The sequence has EMBL accession no. AJ238895.

located at position 770, which is atypical for filamentous fungi (Gruber et al., 1998) where the motif is normally situated between 60 and 120 bp proximal to the cap site. The sequence around position 1148 is a typical translation initiation site, 5′-CACAATG-3′, and agrees with the consensus sequence (5′-CA C/A A/C ATG-3′) found in higher eukaryotes and filamentous fungi (Heinemeyer et al., 1999; Perez-Gonzale et al., 1996).

The derived amino acid sequence is 227 amino acids for the deduced pre-protein which is the same size as an analogous amino acid sequence from *A. pisi* (Lübeck et al., 1997). The data obtained from amino acid sequencing (Degefu et al., 1995) is identical with the amino acid sequence deduced from the cloned genomic DNA and cDNA sequences. This confirms that the cloned xylanase gene codes for the identified mRNA and protein. The eight earlier sequenced tryptic peptides from the *H. turcium* xylanase (Degefu et al., 1995) were identified; but with one discrepancy; threonine instead of glycine at amino acid number 158 (Figure 2). The discrepancy was due to an error in the amino acid determination of peptide number 2 (Degefu et al., 1995). Sequence data from the cDNA showed that the xylanase gene contains two introns which is less common for family 11 xylanases. To our knowledge this is the second xylanase gene, the *XYL2* from *C. carbonum* (Apel-Birkhold and Walton, 1996) being the other xylanase in the family 11, which contains two introns. Both putative introns contain typical intron signals (Gruber et al., 1998; Lübeck et al., 1997) (Figure 2). Comparison of the deduced amino acid sequence of the endoxylanase gene with those of other endoxylanases (Figure 3) compiled in the GenBank/EMBL Data Bank revealed high similarity to the endoxylanase (*XYLl*) of *C. carbonum* (95%) (Apel-Birkhold and Walton, 1996) and *A. Pisi* (85%) (Lübeck et al., 1997) and xylanases from other filamentous fungi (Figure 3). A typical signal peptide cleavage site (Yaguchi et al., 1992) after amino acid number 19 (nucleotide number 1204) gives a hypothetical mature protein of 208 amino acids (Figure 2). The structural gene has a base composition of 21.0% A, 35.6% C, 24.3% G and 19.1% T, which is nearly the same as the base composition of the xylanase gene from *A. pisi*

```
                 1                                                                                80
H.turcicum       MVSFTSIITA AVAATG-ALA APATDIAAR- ---APSDLVA RQSTPNGEGT HNGCFYSWWS DGGARATYTN GAGGSYSVSW
C.carbonum[1]    .......... ......-... .....VS--- -------... ..N....... .....W.... .......... ..........
A.pisi           .......F.. ......-... V.V..L.T.- ---SLGA.T. .AG..SSQ.. .........T ....Q..... ........N.
T.lanuginosus    ..G..PVAL. .L....-... F..GNAT--- ------E.EK ..T...S..W .D.YY..... ....Q..... LE..T.EI..
C.carbonum[2]    ....K.LLL. ...T.S-V.. ..FDFLRE.D DVN.TAL.EK .....SA..Y ...Y.....T ...GS.Q..M .E.SR...T.
H.insolens       ...LK.VLA. .T.VSS-.I. ..FDFV-P.D --NSTA-.Q. ..V...A..W ...Y...... ...GQVQ... LE.SR.Q.R.
E.nidulans       ....K.LLVL CC..L.-.F. T.---VGSED LAAREAS.LE .-...SST.W S..YY..F.T ...GDV.... ......T.Q.
M.grisea         .......V.. V..LA.S... I..P.--GNM TGFPFEQ.MR .....SST.R ...YY....T ..ASPVQ.Q. .N......Q.
T.reesei         ......LLA. --SPPSR.SC R..AEVES-- -----VAVEK ..TIQPGT.Y N..Y...Y.N ..HGGV.... .P..QF..N.
C.carbonum[3]    ..A...VLLG -LS.I.S.F. ..VA.V-PDF EFSG.KH.A. ..D------- Y.QNYKT--- --.GNIQ.-. PTSNG..VTF

                 81                                                                              160
H.turcicum       GTGGNLVGGK GWNPG-TART ITYSGQYNP- -NGNSYLAIY GWTR-NPLVE YYVVENFGTY DPSSQAQNKG TVTSDGSSYK
C.carbonum[1]    .S........ .....-.... .....T..Y- -.......V. ....-..... .......... .....S.... ..........
A.pisi           K......... .....-A... .....T.S.- -S......V. ....-...I. .......... ......TV.. S..A......
T.lanuginosus    .D........ .....LN..A .HFE.V.Q.- -.......V. ....-..... ..I....... ....G.TDL. .VEC...I.R
C.carbonum[2]    RNT..F.... .....-SG.V .N.G.AF..- -Q..G...V. ....-..... ...I.SY... N...G..I.. SFQT..GT.N
H.insolens       RNT..F.... .....-.G.. .N.G.YF..- -Q..G...V. ....-..... ...I.SY... N.G....Y.. .FYT..DQ.D
E.nidulans       SNV..F.... .....-ST.. .N.G.SF..- -S..G...V. ...Q-...I. ..I..SY... N.G.GG.HR. ..Y...AT.D
M.grisea         QS...F.... ..M..GS-KS .....TF..V N...A..C.. ...Q-..... ..IL..Y.EY N.GNS..SR. .LQAA.GT.T
T.reesei         SNS..F.... ..Q..TKNKV .NF..S...- -......SV. ..S.-...I. ..I....... N..TG.TKL. E......V.D
C.carbonum[3]    SGAQDF.L.. .WKQ.T.-.. VK.T.S-TQA QA.TVLV.L. ....GSK... ..IQDFTSGG SG.A.G.KM. Q..C...V.D

                 161                                                                             240
H.turcicum       IAQSTRTNQP SIDGTRTFQQ YWSVRQNKRS -SGSVNMKTH FDAWASKGMN L-GSHYYQIV ATEGY-FSSG SASITVNCP
C.carbonum[1]    .......... .......... .......... -......... .......... .-.Q...... ......-..T. N.Q......
A.pisi           ...TQ..... .....Q.... .......... -......... .....A...K .-.T.N.... ......-.... ..Q.....A
T.lanuginosus    LGKT..V.A. .....Q..D. .......D..T -..T.QTGC. .....RA.L. VN.D...... ......-.... Y.R...ADVG
C.carbonum[2]    V.V...Y... .......... .....TQ..V -G.....QN. .N..SRY.L. .-.Q...... ......-Q... .SD.Y.QTQ
H.insolens       .FV...Y... .......... ...I.K...V -G.....QN. .N..QQH..P .-.Q....V. ......-Q... ESD.Y.QTH
E.nidulans       .YTA..Y.A. ..E..A..E. F.....S..T -G.T.TTAN. .N...AL..R .-.T.N.... ......-Q... ......Y
M.grisea         LHE...V... ..E....... ..AI..Q..N -..T..TGEF .Q..ERA..R M-.N.N.M.. ......-R.A. NSN.N.QT.A
T.reesei         .YRTQ.V... ..I..A..Y. .....R.H.. -.....TAN. .N...QQ.LT .-.TMD.... .V...-.... ......S
C.carbonum[3]    .W.H.QV... ..V..T..V. .I.N.VS... TG.TITT.C. .....KL... .GNQWD..TI S...WGNAA. KSQY..SAA
```

Figure 3. Amino acid sequence alignment of closely related fungal xylanases. *H. turcicum* (this report) (AJ238895); *Cochliobolus carbonum (*Apel-Birkhold et al., 1996; Apel et al., 1993): (L13596)[1], (U58915)[2], (U58916)[3]; *Ascochyta pisi* (Lübeck et al., 1997) (Z68891); *Thermomyces lanuginosus* (Gruber et al., 1998) (O40397); *Humicola insolens* (Dalbøge et al., 1994) (P55334); *Emericella nidulans* (Perez-Gonzalez et al., 1996) (P55332); *Magnaporthe grisea* (Wu et al., L997) (L37529); *Trichoderma reesei* (Törrönen et al., 1992) (P36217).

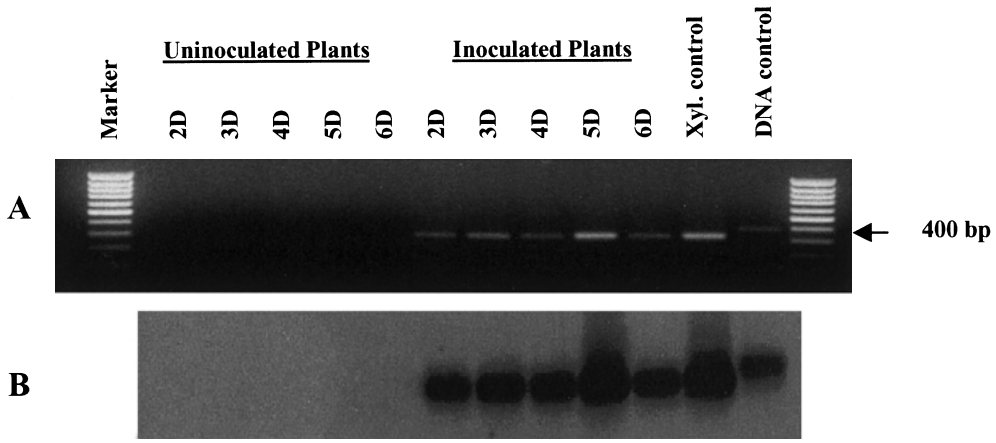

Figure 4. Reverse transcription polymerase reaction (A) Southern hybridization (B) based expression analysis of *htxyl1 in planta*. RNA from different infection stages (2–6 days) and mycelium grown in xylan culture (Xyl control) reverse transcribed into cDNA by ALV served as template for RT-PCR reaction with primers specific to *htxyl1*. Total genomic DNA of *H. turcicum* (DNA control) was used in the PCR to compare the size of the amplified product with or without the intron (398 and 336, respectively). Aliquot of the PCR products were run on 1.6% agarose gel with GeneRuler 100 bp DNA ladder marker (MBI Fermentas, Vilnius, Lithuania). Southern hybridization was done with digoxigenin labelled cDNA of *htxyl1*.

(Lübeck et al., 1997). The amino acid composition of the whole coding region is 33% hydrophobic, 54% hydrophilic, 5% acidic and 8% basic. The use of nucleotides in the third position of the codons are 3.5% of A, 57.3% of C, 20.7% of G and 16.3% of T. There is a clear preference for a pyrimidine and especially a C in the third position of the codons (data not shown). Where a purine is found in the third position, G is used in preference to A (all situations except one), and the AGN codons for Argenine and Serine are very infrequently used. All these conditions are characteristic of highly expressed genes in filamentous fungi (Gruber et al., 1998; Teeri et al., 1987).

Expression of htxyl1 in vitro

In vitro expression of *htxyl1* was determined by northern blot analysis of total RNA from mycelia of *H. turcicum* grown for seven days in a minimal medium (Degefu et al., 1995) supplemented with 1.5% birchwood xylan or 1% glucose. The cloned xylanase gene was strongly expressed in a medium containing xylan as the sole carbon source but no transcript of the gene was detected in the RNA sample isolated from *H. turcicum* cultured on glucose as sole carbon source (Figure 1B). This is consistent with our previous findings of the enzyme induction under culture condition (Degefu et al., 1995). Many xylanases (Royer and Nakas, 1989) and other microbial wall depolymerases (Dean and Timberlake, 1989), are subject to catabolite repression and substrate induction. The lack of expression of xylanase when *H. turcicum* was grown in a media containing glucose as a sole carbon source suggests that this fungus is also under regulation of catabolite repression and substrate induction.

Expression of htxyl1 *in infected maize plant*

RT-PCR with gene specific primers used to reveal the presence of *htxyl1* transcript in infected maize leaves at different times after inoculation showed fragment of the expected size (336 bp) which was absent in uninoculated control plants (Figure 4A). Expression of *htxyl1* was detected in all infection stages described above. As expected the DNA control gave a product of 398 bp fragment which included the 62 bp intron. Southern hybridization (Figure 4B) carried out at high stringent conditions confirmed that the amplified fragment was derived from *htxyl1*. Cloning and sequencing of the amplified fragment proved that the sequence matched exactly with the genomic clone except for the absence of the intron.

Conclusions

The cloned xylanase gene is very highly induced when *H. turcicum* is cultured on isolated maize cell

walls. Previous results (Degefu et al., 1995) and this study suggest that *H. turcicum* does not share xylanase multiplicity observed in other cereal fungal pathogens (Apel-Birkhold and Walton, 1996; Apel et al., 1993; Southerton et al., 1993; Wong et al., 1988; Wu et al., 1995). As multiplicity of xylanase has been regarded as one factor which complicated earlier xylanase gene disruption studies (Apel-Birkhold and Walton, 1996; Apel et al., 1993; Wu et al., 1997), the likely occurrence of a single xylanase gene in *H. turcicum* genome offers a unique opportunity to evaluate the role of xylanase in pathogenesis. The results indicate that the cloned xylanase gene was expressed during infection of maize by *H. turcicum*. By definition, a pre-requisite for pathogenicity factors is that they are expressed during penetration and invasion of the host plant. Hence, the presence of the transcript for the xylanase enzyme at all stages of infection under the tested conditions suggests that xylan degradation may be involved in the disease process. Molecular genetic work for a transformation-mediated gene disruption and a more detailed characterization of the gene to determine its involvement in pathogenesis will be carried out.

Acknowledgements

We wish to thank Mette Lübeck for her help in construction of the genomic library of *H. turcicum* and Dr. Johan Peränen for helpful discussion. One of the authors (Y.D) was financed by the Finnish International Development Agency (FINNIDA).

References

Annis SL and Goodwin PH (1997) Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. European Journal of Plant Pathology 103: 1–14

Apel-Birkhold PC and Walton JD (1996) Cloning, disruption and expression of two Endo-β 1,4-xylanase genes, *XYL2* and *XYL3*, from *Cochliobolus carbonum*. Applied and Environmental Microbiology 62: 4129–4135

Apel PC, Panaccione DG, Holden FR and Walton JD (1993) Cloning and targeted gene disruption of *XYL1*, a β-1,4-xylanase gene from the maize pathogen *Cochliobolus carbonum*. Molecular Plant–Microbe Interactions 6: 467–473

Bailey BA, Dean JFD and Anderson JD (1990) An ethylene biosynthesis inducing xylanase elicits electerolyte leakage and necrosis in *Nicotiana tabaccum cv xanthi* leaves. Plant Physiology 94: 1849–1854

Biely P (1985) Microbial xylanolytic systems. Trends in Biotechnology 3: 286–290

Bonfield JK, Smith KF and Staden R (1995) A new DNA sequence assembly program. Nucleic Acids Research 23: 4992–4999

Braun EJ and Kelman A (1987) Production of cell wall degrading enzymes by corn stalk rot strains of *Erwinia chrysanthemi*. In: Civerolo E, Collmer A, Davis RE and Gillaspe AG (eds) Plant Pathogenic Bacteria (pp 206–211) M. Nijhoff Pub. Dordrecht, The Netherlands

Carpita NC (1996) Structure and biogenesis of the cell wall of grasses. Annual Review of Plant Physiology 47: 445–476

Chang S, Puryear J and Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11: 113–116

Cooper RM, Longman D, Campbell A, Henry M and Lees PE (1988) Enzymic adaptation of cereal pathogens to the monocotyledonous primary wall. Physiological and Molecular Plant Pathology 32: 33–47

Dalbøge H and Hansen HPH (1994) A novel method for efficient expression cloning of fungal enzyme genes. Molecular and General Genetics 243: 253–260

Dean RA and Timberlake WE (1989) Production of cell wall degrading enzymes by *Aspergillus nidulans*: a model system for fungal pathogenesis of plants. The Plant Cell 1: 265–273

Degefu Y, Fagerström R and Kalkkinen N (1995) Purification and partial characterization of xylanase from the fungal maize pathogen *Helminthosporium turcicum* Pass. European Journal of Plant Pathology 101: 291–299

Di Pietro A and Roncero IG (1998) Cloning, expression, and role in pathogenicity of pg1 encoding the major extracellular endopolygalcturonase of the vascular wilt pathogen *Fusarium oxysporum*. Molecular Plant–Microbe Interactions 11: 91–98

Fry SC, Aldington S, Hethrington PR and Aitken J (1993) Oligosaccharides as signals and substrates in the plant cell wall. Plant Physiology 103: 1–5

Giesbert S, Lepping H-B, Tenberge KB and Tudzynski P (1998) The xylanolytic systems of *Claviceps purpurea*: Cytological evidences for the secretion of xylanases in infected rye tissue and molecular characterization of two xylanases. Phytopathology 88: 1020–1030

Gruber K, Klintschar G, Hayn M, Schlacher A, Steiner W and Kratky C (1998) Thermophilic xylanase from *Thermomyces lanuginosus*: high-resolution X-ray structure and modeling studies. Biochemistry 37: 13475–13485

Hamer JE and Holden DW (1997) Linking approaches in the study of fungal pathogenesis: a commentary. Fungal Genetics and Biology 21: 11–16

Have AT, Mulder W, Visser J and Kan JAL (1998) Endopolygalcturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Molecular Plant–Microbe Interactions 11: 1009–1016

Heinemeyer T, Chen X, Karas H, Kel AE, Kel OV, Liebich I, Meinhardt T, Reuter I, Schacherer F and Wingender E (1999) Expanding the TRANSFAC database towards an expert system of regulatory molecular mechanisms. Nucleic Acids Research 27: 318–322

Holden F and Walton JD (1992) Xylanase from the fungal maize pathogen *Cochliobolus carbonum*. Physiological and Molecular Plant Pathology 40: 39–47

Ishii S (1988) Factors influencing protoplast viability of suspension cultured rice cells during isolation process. Plant Physiology 88: 26–29

Lübeck PS, Paulin L, Degefu Y, Lübeck M, Alekhina I, Bulat SA and Collinge DB (1997) PCR cloning, DNA sequencing and phylogenetic analysis of a xylanase gene from the phytopathogenic fungus *Ascochyta pisi* Lib. Physiological and Molecular Plant Pathology 51: 377–389

Perez-Gonzalez JA, De Graaff LH, Visser J and Ramond D (1996) Molecular cloning and expression in *Saccharomyces cervisiae* of two *Aspergillus nidulans* xylanase genes. Applied and Environmental Microbiology 62: 2179–2182

Royer JC and Nakas JP (1989) Xylanase production by *Trichoderma longibrachiatum*. Enzyme and Microbial Technology 11: 405–410

Sambrook J, Fritsch EF and Maniatis TA (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbour Press, Cold Spring Harbour, NY

Southerton SG, Osbourn AE, Dow JM and Daniels MJ (1993) Two xylanases from *Gaeumannomyces graminis* with identical N-terminal amino acid sequence. Physiological and Molecular Plant Pathology 42: 97–107

Sunna A and Antranikian G (1997) Xylanolytic enzymes from fungi and bacteria. Critical Review of Biotechnology 17: 39–67

Teeri TT, Lehtovaara P, Kauppinen S, Salovuori I and Knowels J (1987) Homologous domain in *Trichoderma reesei* celluloytic enzymes: gene sequence and expression of cellobiohydrolase II. Gene 51: 43–52

Törrönen A, Mach RL, Messner R, Gonzalez R, Kalkkinen N, Harkki A and Kubicek CP (1992) The two major xylanases from *Trichoderma reesei*: characterization of both enzymes and genes. Biotechnology 10: 1461–1465

Vahala J, Schlagnhaufer CD and Pell EJ (1998) Induction of an ACC synthase cDNA by ozone in light grown *Arabidopsis thaliana* leaves. Physiological Plantarum 103: 45–50

Wong KKY, Tan LUL and Saddler JN (1988) Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. Microbiological Reviews 52: 305–317

Wu S-C, Kauffmann S, Darvil AG and Albersheim P (1995) Purification, cloning and characterization of two xylanases from *Magnaporthe grisea*, the rice blast fungus. Molecular Plant–Microbe Interactions 8: 506–514

Wu S-C, Ham K-S, Darvill AG and Albersheim P (1997) Deletion of two endo-xylanase genes reveals additional isozymes secreted by the rice blast fungus. Molecular Plant–Microbe Interactions 10: 700–708

Yaguchi M, Roy C, Watson C, Rollin F, Tan LUL, Senior DJ and Saddler JN (1992) Amino acid sequence of 20 kD xylanase from *Trichoderma harzianum*. In: Visser J, Beldman G, Kusters-van Someren MA, Voragen AGJ (eds) Xylans and xylanases Progress in Biotechnonology Vol 7 (pp 435–438) Amsterdam, Elsevier

Yoder OC (1988) *Cochliobolus heterotrophus*, the cause of Southern corn leaf blight. Advances in Plant Pathology 6: 95–112