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Molecular characterization and heterologous expression of an endo-β-1,6-glucanase gene from the mycoparasitic fungus *Trichoderma harzianum*

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Abstract Hydrolytic enzymes from the filamentous fungus *Trichoderma harzianum* have been described as critical elements of the mycoparasitic action of *Trichoderma* against fungal plant pathogens. In this report we describe the first genomic and cDNA clones encoding a β-1,6-endoglucanase gene. The deduced protein sequence has limited homology with other β-glucanases. Northern experiments show a marked repression of mRNA accumulation by glucose. The protein has been successfully produced in *Saccharomyces cerevisiae* upon construction of a transcriptional fusion of the cDNA with a yeast promoter. This *S. cerevisiae* recombinant strain shows a strong lytic action on agar plates containing β-1,6-glucan.

Key words Glucanase · *Trichoderma* · Mycoparasitism

Introduction

Trichoderma harzianum is a filamentous fungus described as a potential biocontrol agent against several fungal plant pathogens (Papavizas 1985). Mycoparasitism (degradation and assimilation of phytopathogenic fungi) has been described as the main process involved in the antagonistic action of *T. harzianum* against fungal pathogens (Chérif and Benhamou 1990). Degradation of cell walls and further penetration into the host mycelium seems to be a key step in this complex process and it has been extensively documented (Papavizas 1985).

Chitinases (EC 3.2.1.14) and β-1,3-glucanases (EC 3.2.1.39) have been proposed as the main enzymes in cell wall degradation (since chitin and β-1,3-glucans are the major components of most fungal cell walls; Papavizas 1985). However, other cell wall-degrading enzymes may be involved in the effective and complete degradation of mycelial or conidial walls of phytopathogenic fungi by *Trichoderma*. In fact, proteins, β-1,6-glucans and α-1,3-glucans represent relatively minor components of fungal cell walls. β-1,6-glucanase (EC 3.2.1.75) has been described as lytic against yeast and fungal cell walls in filamentous fungi (Shibata and Fujimbara 1973; Yamamoto et al. 1974; De la Cruz 1994) and bacteria (Rombouts and Phaff 1976). However, only recently has an endo-β-1,6-glucanase been purified and extensively characterized (De la Cruz 1994) and so far no cDNA or genomic clone has been isolated and studied. As a result, no information was available up to now regarding enzyme structure, relationship with other proteins or evolutionary inferences.

In this paper, we report the first cDNA and genomic clones encoding a β-1,6-glucanase. This work has been performed with a mycoparasitic strain of *T. harzianum*, described as an agent of biological control (Dawson et al. 1990). The molecular features and structural relationships with other β-glucanases are discussed.

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Materials and methods

Fungal material and growth conditions

T. harzianum CECT 2413 was obtained from Colección Española de Cultivos Tipo (Dawson et al. 1990). All the routine cultures were according to Lora et al. (1994) and De la Cruz et al. (1993). When RNA was to be obtained, *Trichoderma* was cultured for 4 days on Czapek minimal medium (Dawson et al. 1990) supplemented with 10% (w/v) glucose, collected by filtration, washed, resuspended in

the appropriate media and cultured for 48 h (unless other incubation times are indicated). Autoclaved mycelia of *Botrytis cinerea*, *Rhizoctonia solani*, *Gibberella fujikuroi* and *T. harzianum* (used as carbon source of several *T. harzianum* cultures) were prepared as follows. Spores were inoculated into liquid Czapek medium containing 10% glucose as carbon source. After incubation for 96 h, cultures were autoclaved, collected by filtration and washed with 2% (w/v) $MgCl_2$ and H_2O.

Genomic and cDNA library construction

Chromosomal DNA from *T. harzianum* was isolated as described elsewhere (Lora et al. 1994). DNA was partially digested with *Sau*3AI and subjected to sucrose gradient ultracentrifugation. Fragments from 10 to 20 kb were pooled and ligated to *Bam*HI-digested λGEM-11 arms (Promega). RNA from *T. harzianum*, grown for 33 h in minimal medium with chitin as the only carbon source, was used to generate a cDNA library as described (Lora et al. 1994).

Screening of libraries

Libraries were screened with probes prepared by α-[^{32}P]dCTP-labelling using random primer kits and standard hybridization conditions (Lora et al. 1994).

DNA sequencing and data analysis

Nucleotide sequences from cDNA and genomic clones were determined with an A.L.F. automatic DNA sequencer from Pharmacia. Sequence analysis was implemented with programmes developed by the University of Wisconsin Genetics Computer Group (Devereux et al. 1984).

Protein sequence, oligonucleotides and polymerase chain reactions

Amino-terminal and tryptic peptides were sequenced following the Edman degradation method, as described in De la Cruz (1994) and Matsudaira (1989), respectively. Oligonucleotides used (100 pmol/reaction) were: 5′-TTCGA(A/G)CCTGCICTIGCI(A/T)(C/G)IGGI-AA(A/G) AC-3′ (from the amino terminus, sense) and 5′-CCGAT-CATICCIAC(A/G) GTI(C/G)(A/T)(A/G)TAIGCIGG-3′ (from an internal peptide, antisense). PCR conditions were: 95° C, 1 min (denaturation); 55° C, 1 min (annealing); 72° C, 2 min (extension); repeated for 30 cycles.

DNA/RNA manipulations

Standard methods were followed as described (Sambrook et al. 1989; Ausubel et al. 1989; Lora et al. 1994). Northern and Southern blots were done using (per lane) 80 μg of total RNA and 15 μg of chromosomal DNA, respectively. The amount of RNA loaded for Northern blot experiments was monitored by methylene blue staining of the filters and/or ethidium bromide staining of the gel.

Primer extension

Transcription start points were determined according to Ausubel et al. (1989). A 300 ng portion of the oligonucleotide 5′-GCCGG-CGAGAATAGCCGGAGCAACG-3′ (corresponding to amino acids 6–14) was labelled with T4 polynucleotide kinase and γ-[^{32}P]ATP. This labelled oligonucleotide was used to prime reverse transcription from 50 μg of total RNA, using AMV reverse transcriptase (Promega). Sequence reactions were done using a Sequenase kit (USB) and the same primer.

Western blots

Discontinuous SDS-PAGE was performed according to Laemmli (1970), in 0.75 mm gels with 4% acrylamide (stacking gel) and 12% acrylamide (separating gel), in a mini-Protean electrophoresis cell (Bio-Rad). After electrophoresis, proteins (50 mg per lane) were transferred to nitrocellulose filters, blocked with 5% (w/v) skimmed milk and incubated with mouse anti-Bgn16.2 polyclonal antibody (De la Cruz 1994) and the complex detected with peroxidase-conjugated anti-mouse IgG, as described in De la Cruz et al. (1992).

Yeast manipulations

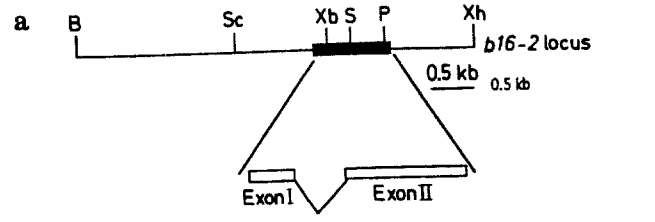
Transformations were performed following the method described in Ito et al. (1983) in the *Saccharomyces cerevisiae* strain GRF167 (*Mata*; *ura3*; *his3*) and all routine manipulations were according to Ausubel et al. (1989). Lytic activity was assayed as follows. Yeast strains under study were plated on minimal medium with galactose or glucose as the sole carbon source. After incubation for 3 days at 30° C, plates were overlaid with 0.5% pustulan (β-1,6-glucan)/0.8% agar (Difco). Both medium and overlay mixture were buffered with 0.1 M phosphate buffer, pH 5.8. Plates were incubated at 37° C for 24 h.

Results

Isolation of genomic and cDNA clones and sequence analysis

The purification of the endo-β-1,6-glucanase II (Bgn16.2) from *T. harzianum* has been described elsewhere (De la Cruz 1994). The protein was purified from culture supernatants of *Trichoderma* grown with chitin as the sole carbon source and further subjected to N-terminal and tryptic peptides sequencing (Rombouts and Phaff 1976; this work). In order to amplify specific sequences corresponding to the Bgn16.2 gene, a polymerase chain reaction (PCR)-based approach was followed. Two degenerate oligonucleotides were designed (see Materials and methods), one from the amino-terminal sequence of the purified protein (FEPALASGKT, sense) and a second one from an internal, tryptic peptide (PAYSTVGMI, antisense). Under the experimental conditions described, a specific band of ca 600 bp was amplified from *T. harzianum* chromosomal DNA (data not shown). This band was then subcloned into the pGEM-T vector (Promega) and checked by sequencing.

The PCR fragment was labelled and used as a probe to screen a *T. harzianum* CECT 2413 genomic library. Upon searching among ca 25 000 phage plaques, three hybridizing clones (λX1, λX2 and λX3) were isolated; λX1 was chosen for further studies. A partial restriction map of this genomic region containing the β-1,6-glucanase gene (*bgn16.2*) is presented in Fig. 1a. The



b

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-633 gagctcctacagctgtcgatattccccgatcctggacctgagattggtccaccctgcatg
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     ttgcctggcgatgtagcagttgtccgcactgacagctggttgattatcatgcgtcaagga
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     agttgcgaattaatagggatgtggcgcgaatcgggccggcgttgtgttttggtacagaca
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     gtcaagcttgtaaatgaaggctcaataaggctcaatttaagtctgtatataagtctgtatg
-33  tttcctgacaagtaggacgaagtttctattatcatcaccgacaactcttgggcaattCCT
+28  ACAGCTACCATCAAGATGAAGTACTCCATCGTTGCTCCGGCTATTCTCGCCGGCACCGCC
                 M  K  Y  S  I  V  A  P  A  I  L  A  G  T  A
    TTTGCATGGCTGCCCCAAGACCGCGACCTGAAGGCCTTCAACCAGACCGCTCGTTTCGAG
     F  A  W  L  P  Q  D  R  D  L  K  A  F  N  Q  T  A  R  F  E
    CAGCTTGGCAAGCGCTTTGAGCCTGCACTTGCTTCTGGTATCACCAAGATCCGTGGTGTC
     Q  L  G  K  R [F  E  P  A  L  A  S  G  I  T  K  I  R  G  V]
    AACTTCGGTGgtaagtgaatgccctattttctagaagattaagagaatcaggctgataga
     N  F  G  G
    ttgattaagGATGGCTTATCAGTGAGCCATGGATGATGTCCAATGAGTGGAACAACAACA
                W  L  I  S  E  P  W  M  M  S  N  E  W  N  N  N  M
    TGGGCTGCAACAATGCTGCCTCTGAGTTCGATTGCATGCGCAACAACTACATGGGCAGCA
      G  C  N  N  A  A  S  E  F  D  C  M  R  N  N  Y  M  G  S  K
    AGCGTGCGACCGGAAACACCAAGTTCCAGAACCACTACAGGGACTGGATCAACCCCCCCA
      R  A  T  G  N  T  K [F  Q  N  H  Y  R] D  W  I  N  P  P  T
    CCGTTCAGTCTGTTCACGATGTCGGCTTGAACACTATCCGTATCCCCATTGGCTACTGGT
      V  Q  S  V  H  D  V  G  L  N  T  I  R  I  P  I  G  Y  W  S
    CCTACGATGCCATTGTCGACACGGCTAGCGAGCCATTTGCCGATGGCAACCTCCAGCTTC
      Y  D  A  I  V  D  T  A  S  E  P  F  A  D  G  N  L  Q  L  P
    CGTATCTTGACGCTGTTGTTCAGAAGGCCGCTGATCTCGGAATCTACGTCATCATTGATC
      Y  L  D  A  V  V  Q  K  A  A  D  L  G  I  Y  V  I  I  D  L
    TTCACGGTGCCCCTGGTGGTCAACAACAGGATGCCTTCACCGGCCAGAACCCCAACCCTG
      H  G  A  P  G  G  Q  Q  Q  Q  D  A  F  T  G  Q  N  P  N  P  A
    CTGGTTTCTACAACTCATACGACTATGGTCGTGCTGAGAAGTGGCTGTCTTGGATGACAA
      G  F  Y  N  S  Y  D  Y  G  R  A  E  K [W  L  S  W  H  T  N]
    ACCGCATCCACACCAACCCTGCCTACTCGACTGTTGGTATGATTGAGGTTCTCAACGAGC
     [R] I  H  T  N  P  A  Y  S  T  V  G  M  I  E  V  L  N  E  P
    CTGTCTCCAGACACGATGGAGGTGGTCGGTACCCTGCTCCTGGGCAGGACCCAAGCATGG
     [V] S  R  H  D  G  G  G  R  Y  P  A  P  G  Q  D  P  S  M  V
    TCCAGACCTACTACCCAGGTGCCCTCAAGGCCGTGCGTGATGCCGAGGCTGCGCTGAACG
      Q  T  Y  Y  P  G  A  L  K  A  V  R  D  A  E  A  A  L  N  V
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      P  S  N  K  K  L  H  V  Q  F  M  S  S  K  W  D  S  G  D  P
    CTCGCAGCAACGCCGCCGTCAAGAACGACCCCATGGTTGGCTTTGACGACCACAACTACA
      R  S  N  A  A  V  K  N  D  P  M  V  G  F  D  D  H  N  Y  I
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      Q  Q  Q  N  V  Y  Q  D  V  C  S  G  Y  R  *
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    ttctttcatatgcgatcaatatgtacgcgagatatgcttccattctccaatgttacgaaa
+1768 ccaaaaacaaaat
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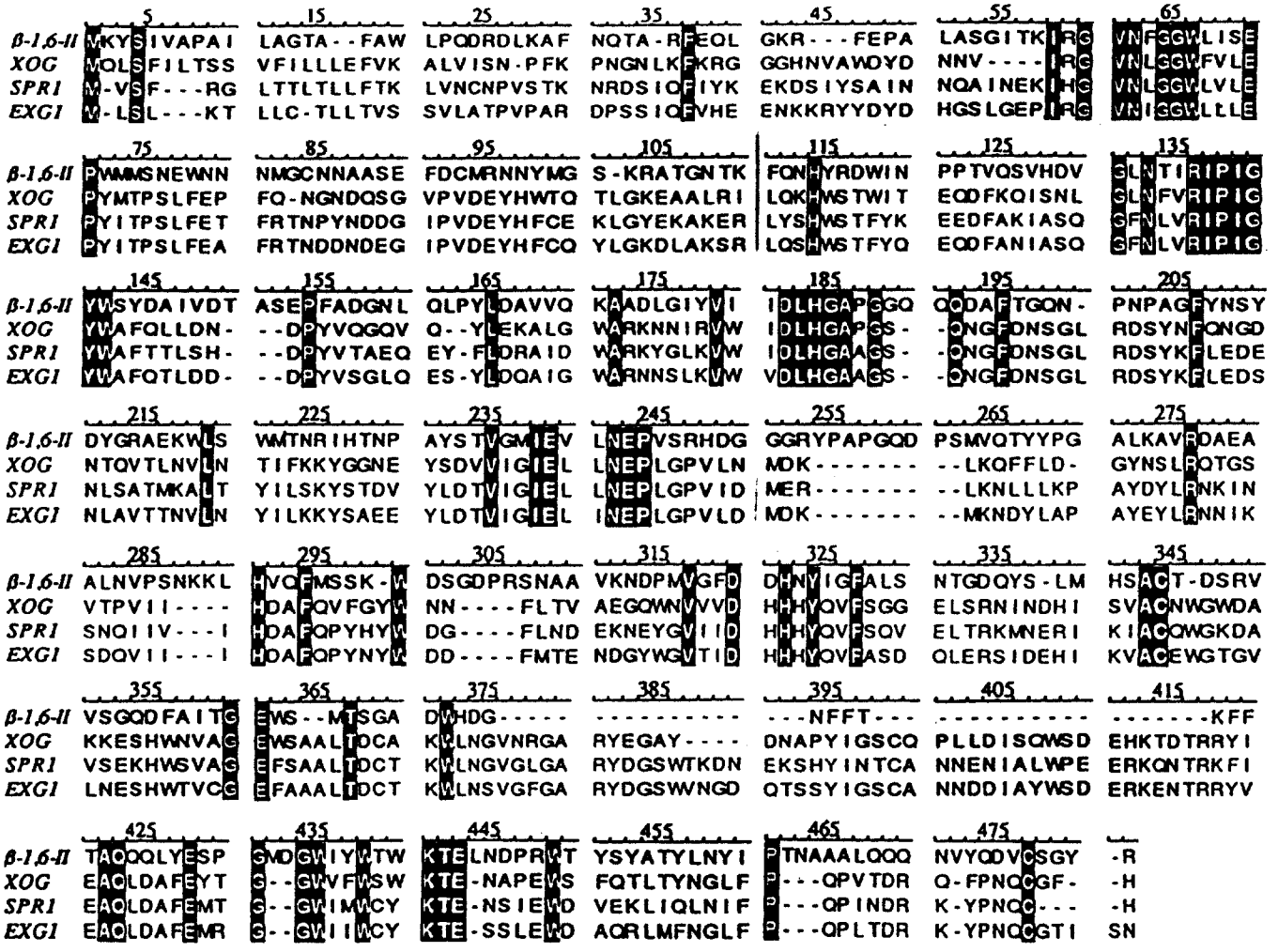
Fig. 1a, b Physical organization of the *bgn16.2* gene. **a** Restriction map of the genomic region containing the β-1,6-glucanase-II gene (*bgn16.2*). *B BamHI, Sc SacI, Xb XbaI, S SalI, P PstI, Xh XhoI.* **b** Nucleotide sequence and deduced protein. Genomic sequence is shown in *lowercase* and cDNA in *uppercase.* Overlapping, putative TATA boxes are *underlined.* + 1 and + 4 are the transcription start points (indicated by *asterisks*) determined by primer extension. Amino acid sequences corresponding to the previously sequenced peptides from the purified protein are *boxed*

1.8 kb *Xba*I-*Xho*I restriction fragment was used as a probe to screen a cDNA library constructed from chitin-induced mycelium (Lora et al. 1994). Only one cDNA clone (cλ16) was obtained out of 80 000 plaques tested. The genomic and cDNA sequences, as well as the deduced protein sequence, are shown in Fig. 1b. A 59 bp intron is present in the 5′ region of the gene. Two transcription start points were identified by primer extension analysis using RNA from mycelium grown with 0.1% glucose as the sole carbon source (adenines in positions + 1 and + 4 in Fig. 1b). These two transcription origins are separated by two nucleotides (see Fig. 1b) and at − 47 and − 49 there are two putative overlapping TATA boxes. Immediately upstream of these TATA elements there are also two CAAT boxes. Both genomic and cDNA sequences were positively identified as Bgn16.2 glucanase by the presence of the partial amino acid sequences previously determined from the purified protein (boxed in Fig. 1b). Some minor disagreements between protein and DNA sequences are likely to be due to artifacts in the HPLC peak identification step during peptide sequencing. The mature protein (as seen by N-terminal sequencing) starts at amino acid 41, and is preceded by a long peptide composed of two modules. The first part (amino acids 1–17) shows the typical structure of a signal peptide with a high hydrophobic index (von Heijne 1983). The second peptide (residues 18–40) ends with the sequence KR which has been described in other proteins as a target sequence for a specific protease, Kex2 (Park et al. 1994; Vázquez de Aldana et al. 1991; Blaiseau and Lafay 1992; Geremía et al. 1993). The protein deduced from the nucleotide sequence has 430 amino acids and a molecular weight of 48 kDa for the unprocessed form. However, if the initial 40 amino acids are excluded, the calculated molecular mass is 43 kDa, in full agreement with the biochemical data (De la Cruz 1994). Likewise, the deduced isoelectric point for the mature protein is around 5, very similar to the value of 5.8 obtained from chromatofocusing and electrofocusing.

Bgn16.2 is a member of a family of β-glucanases

When the Bgn16.2 sequence is compared with those available in the databases, three proteins show acceptable homology scores (20–30% identity at the amino acid level). These proteins are EXG1 (Vázquez de Aldana et al. 1991) and SPR1 (Muthukumar et al. 1993) from *S. cerevisiae*, and XOG (Chambers et al. 1993) from *Candida albicans.* EXG1 codes for an exo-β-1,3-glucanase and belongs to the so-called family A cellulases. SPR1 is a sporulation-specific exo-1,3-β-glucanase, while XOG is an exo-β-1,3-glucanase that shows higher expression during the early, rapid growth phase. Interestingly, when these sequences are aligned with Bgn16.2, structural homologies are evident,



especially at certain conserved domains (Fig. 2). In addition, when the hydropathy profiles are compared, a clear pattern is conserved among these four β-glucanase enzymes (data not shown). The homologies in the conserved domains can be extended to a larger array of β-glucanases, the family A cellulases. Moreover, there is a putative catalytic site in all these proteins, including the cellulases (Chen et al. 1993), that is conserved in Bgn16.2 (Glu^{235}, block 245 in Fig. 2). In order to test if there are any other related genes in the *T. harzianum* genome, high and low stringency Southern blottings were performed, using total chromosomal DNA digested with several restriction enzymes and probed with the *Xho*I-*Xba*I fragment (see Fig. 1a). As shown in Fig. 3, two hybridizing bands are present in the high stringency experiments, indicating the existence of two nearly identical sequences. In fact, genomic clone λX2 contains a second, almost identical gene (not shown).

Fig. 2 Structural similarity of Bgn16.2 to other β-glucanases. Multiple alignment performed to compare the β-1,6-glucanase-II amino acid sequence to other β-glucanases. Only residues identical in all four enzymes have been marked; conservative changes have not been taken into account

Bgn16.2 mRNA and protein accumulation

In order to assess if *bgn16.2* is preferentially transcribed under conditions similar to those involving mycoparasitism, Northern and Western blots were done using RNA and proteins isolated from *T. harzianum* grown on autoclaved mycelia of different fungi and under other growth conditions. As shown in Fig. 4a, maximum mRNA accumulation is detected in the absence of glucose. Strikingly, protein levels (Fig. 4b) and enzymatic activity (data not shown) are low under these conditions. The mRNA was also found when autoclaved *B. cinerea*, *R. solani* or *T. harzianum* mycelia, or chitin, are used as sole carbon sources. In addition to the absence of expression in 10% glucose and on autoclaved *G. fujikuroi* mycelium, the mRNA levels are also below the limits of detection when the fungus is cultured for 48 h on 1% pustulan (β-1,6 glucan). This is puzzling because pustulan is the enzyme substrate and protein is detected under these experimental conditions (see Fig. 4b). However, if incubation time with pustulan is shorter (8 h), a band is visible on Northern blots (data not shown). Therefore,

mRNA accumulation corresponding to Bgn16.2 seems to be subject to strong repression by glucose. It is also worth noting that levels of mRNA accumulation are very low and were only evident after 2 weeks of film exposure and loading 80 mg of total RNA per lane. This is consistent with the low representation of the Bgn16.2 clone in the chitin-based cDNA library (1 out of 80 000 plaques screened).

Generation of a yeast strain expressing active Bgn16.2 enzyme

A transcriptional fusion was made by ligating the Bgn16.2-encoding cDNA to the pEMBL YEX4 vector (Cesarini and Murray 1987). In this construct, the *T. harzianum* sequence is under the control of the *S. cerevisiae GAL10-CYC* hybrid promoter. Thus, transcription of the cDNA is induced by the presence of galactose in the culture medium and repressed by glucose. To test if this recombinant yeast strain (Yβ) actually expresses an active β-1,6-glucanase, the ability to produce a hydrolysis halo on β-1,6-glucan plates was studied. Figure 5 shows that Yβ cells growing with galactose as a carbon source have a strong hydrolyzing ability when pustulan (β-1,6-glucan) is added to the plate and incubated for 24 h at 37° C. Control cells (transformed with the YEX4 vector) or Yβ strain growing with glucose (data not shown) do not produce this halo. This observation indicates that: (i) the clone characterized in this work actually encodes a β-1,6-glucanase; and (ii) it is secreted and processed to yield an active form of the enzyme.

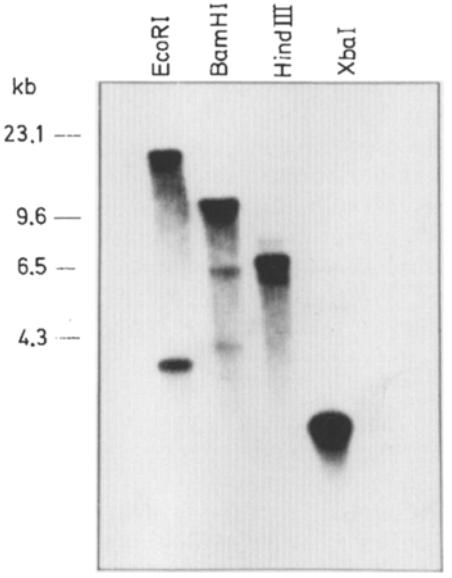


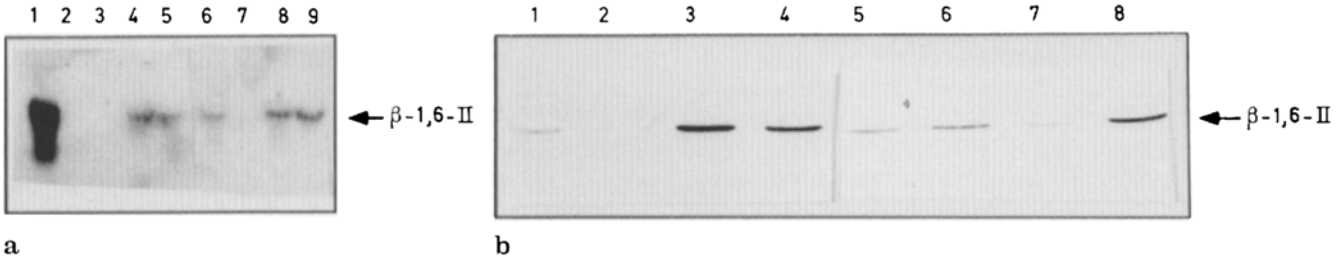
Fig. 3 Southern analysis of the *bgn16.2* gene. The experiment was performed using 15 µg of *Trichoderma harzianum* chromosomal DNA and high stringency conditions

Discussion

In this paper we report for the first time the sequence and preliminary analysis of cDNA and genomic clones encoding a β-1,6-glucanase gene. The cloning strategy took advantage of the information about peptide sequences previously obtained (De la Cruz 1994). This approach has been demonstrated successfully in the isolation of other genes, such as *CHIT42*, encoding a chitinase (García et al. 1994), from *T. harzianum*.

The predicted protein starts with a stretch of 40 amino acids before the first residue determined by N-terminal sequence analysis of the purified protein. The first 17 residues probably form a signal peptide promoting secretion of the enzyme. As a matter of fact, the enzyme was initially purified from culture supernatants. Moreover, enzymatic measurements and Western blots confirm the existence of β-1,6-glucanase activity and Bgn16.2 protein in the culture media of *T. harzianum* (data not shown and Fig. 4b). The segment comprising residues 18–40 is probably involved in proteolytic processing of the Bgn16.2 enzyme. This peptide ends with the sequence Leu-Gly-Lys-Arg. The dibasic moiety Lys-Arg has been described as a recognition site for Kex2 proteinase. Kex2 is a subtilisin-like endoproteinase that processes secreted proteins in mammalian cells, *Xenopus*, *Drosophila*, *Saccharomyces*,

Fig. 4a,b Expression pattern of *bgn16.2*. **a** Northern blot using RNA obtained under the following conditions: lane 1, 0.1% glucose; 2, 10% glucose; 3, 1% pustulan, 4, 1.5% chitin for 24 h; 5, 1.5% chitin for 48 h; 6, *Botrytis cinerea* autoclaved mycelium; 7, *Gibberella fujikuroi* autoclaved mycelium; 8, *Rhizoctonia solani* autoclaved mycelium; 9, *T. harzianum* autoclaved mycelium. **b** Western blot of proteins secreted into the culture medium using anti-Bgn16.2 polyclonal antibodies. Lane 1, 0.1% glucose; 2, 10% glucose; 3, 1% pustulan; 4, 1.5% chitin; 5, *T. harzianum* autoclaved mycelium; 6, *R. solani* autoclaved mycelium; 7, *G. fujikuroi* autoclaved mycelium; 8, *B. cinerea* autoclaved mycelium



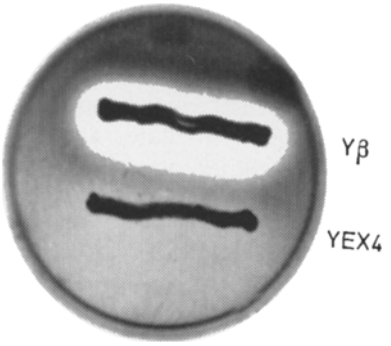


Fig. 5 Expression of *T. harzianum* β-1,6-glucanase in transgenic yeast. A clear hydrolysis halo is evident in cells harbouring the β-1,6-glucanase-II cDNA insert (Yβ) growing in minimal medium with galactose. Control cells (transformed with the vector, YEX4) do not produce this halo

Kluyveromyces and *Ustilago* (Julius et al. 1984; Fuller et al. 1989; Tao et al. 1990; Stark and Boyd 1986; Park et al. 1994). The Kex2 system cleaves after a dibasic sequence preceded at the -2 position by a hydrophobic residue (in Bgn16.2, a leucine) and is extensively found in secreted killer toxins. This Kex2 recognition structure has also been detected in other hydrolases from *Trichoderma*, including the chitinase CHIT42 (García et al. 1994) and the Prb1 protease (Geremía et al. 1993).

The molecular events by which polysaccharide hydrolases cleave at a glycosidic linkage are complex. In a well-established model (Chen et al. 1993), the process involves protonation by the acidic group of an Asp or a Glu and subsequent stablization by a nucleophilic amino acid; after a number of intermediate steps, the reducing terminus of the polymer is regenerated by introducing a molecule of water. From chemical modification studies (Chen et al. 1993; Macarron et al. 1993), some crystallographic data (Keitel et al. 1993) of β-1,3- and β-1,4-glucanases, and directed mutagenesis of a *Bacillus circulans* chitinase (Watanabe et al. 1993), these residues have been shown to be critical for the catalytic breakdown of the glycosidic linkage, regardless of the type (β-1,3 or β-1,4) of linkage. When the amino acid sequences of β-1,3-glucanases and cellulases are aligned, families can be proposed based on structural similarities. For most of the members of these families, the critical sites mentioned above are conserved. Remarkably, the acidic residue (and many others) is also conserved in the sequence we present in this paper. The existence of the putative catalytic structure IEVLNEP (E^{235} in bold type) in β-1,6-glucanase is striking; it would extend the critical role of the acidic group to one more type of glycosidic linkage (β-1,6). Moreover, this active site is independent of the endo- or exo-type of hydrolysis of the glucan, because it is conserved in exo- (EXG1, XOG, SPR1, Vázquez de Aldana et al. 1991; Chambers et al. 1993; Muthukumar et al. 1993) as well as endo-enzymes (Bgn16.2, this work).

β-1,6-Glucanase has been purified and its corresponding gene cloned from a mycoparasitic strain of the filamentous fungus *T. harzianum* (De la Cruz 1994; this work). Our ultimate goal is to understand the mycolytic action of *Trichoderma* against fungal plant pathogens. In this process, hydrolases seem to be key elements, due to their activity as cell wall-degrading enzymes (De la Cruz et al. 1992). β-1,6-Glucan is not a major component of fungal cell walls. However, its nearly universal presence in these structures as a compacting agent, promoting tight packaging of more abundant polymers (such as chitin and β-1,3-glucans), makes it a likely target of the mycolytic system of enzymes produced by *Trichoderma*. β-1,6-Glucanase could act synergistically with chitinases, β-1,3- and α-1,3-glucanases and proteases to attack and digest the pathogen effectively. In fact, this synergistic action has been demonstrated against purified cell walls of fungal plant pathogens (J. De la Cruz, unpublished results) and for plant chitinases and β-1,3-glucanases (Mauch et al. 1988; Sela-Buurlage et al. 1993). As an initial, simple model to test the functional role of Bgn16.2 in mycoparasitism, we have expressed the protein in a heterologous system, *S. cerevisiae*, and investigated its potential as a cell wall-degrading enzyme using pustulan (β-1,6-glucan) in agar plates as substrate. The positive results obtained have prompted us to consider an important function for this enzyme in the interaction between *Trichoderma* and fungal pathogens. In this regard, a saprophytic action of the mycolytic fungus may be of most importance. The fungal biomass, and especially cell walls material, is probably the only carbon source in the soil, since the hydrolases are subject to catabolite repression (see Fig. 4). Maximal mRNA accumulation is observed in cultures with 0.1% glucose as the carbon source (starvation conditions), even though protein levels are low; this fact can be explained in terms of low secretion rates in this situation, or post-transcriptional regulation. The absence of mRNA in cultures grown with pustulan is probably due to repression by the glucose produced after 48 h of pustulan degradation; the presence of the transcript at earlier times supports this idea (data not shown). Accumulation of the protein, as seen in Western blots, indicates a high stability for this enzyme. On the other hand, Bgn16.2 mRNA and protein accumulate in cultures with chitin or autoclaved mycelia as carbon source (Fig. 4), which may represent a good simulation of mycoparasitism. Thus, it is tempting to postulate a fundamental, synergistic role for β-1,6-glucanase in the mycolytic action of *Trichoderma*, provided it is present together with the other secreted hydrolases. Preliminary results show an inhibiting effect of purified Bgn16.2 on growing fungal hyphae, which is synergistically enhanced when chitinase and/or β-1,3-glucanase are added (J. De la Cruz, unpublished results). It will be important to generate transgenic plants that overexpress β-1,6 protein. This

enzyme is absent in plants and β-1,6 linkages are also lacking. Such transgenic lines could be more tolerant to fungal plant pathogens and should trigger a more rapid endogenous response due to the release of elicitors on partial digestion of fungal cell walls by β-1,6-glucanase. Also, transgenic *T. harzianum* with enhanced β-1,6-glucanase activity would be very useful as an integrated biological control agent. These approaches are currently in progress in our laboratory.

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