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Expression of an α-1,3-glucanase during mycoparasitic interaction of *Trichoderma asperellum*

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Trichoderma species have been investigated as biological control agents for over 70 years owing to their ability to antagonize plant pathogenic fungi. Mycoparasitism, one of the main mechanisms involved in the antagonistic activity of *Trichoderma* strains, depends on the secretion of complex mixtures of hydrolytic enzymes able to degrade the host cell wall. The antifungal activity of an α-1,3-glucanase (EC 3.2.1.59, enzymes able to degrade α-1,3-glucans and also named mutanases) has been described in *T. harzianum* and its role in mycoparasitic processes has been suggested. In this study, we report on the purification, characterization and cloning of an exo-α-1,3-glucanase, namely AGN13.2, from the antagonistic fungus *T. asperellum* T32. Expression at the transcription level in confrontation assays against the strawberry pathogen *Botrytis cinerea* strongly supports the role of AGN13.2 during the antagonistic action of *T. asperellum*.

Trichoderma species have been widely used in agriculture as biocontrol agents [1]. This genus has been extensively studied owing to their ability to rapidly colonize substrates [2], induce systemic acquired resistance in plants [3], their potential for promoting plant growth [4] and their antagonistic activity against a wide range of plant pathogenic fungi [5]. The inhibitory effect of their antibiotics [6,7] and cell wall degrading enzymes (CWDEs) [8] against many plant pathogens is often cited as important aspects of their antagonistic activity. In addition, the role of *Trichoderma* spp. in the interaction with plants has recently been reviewed [9]. The increase in knowledge of *Trichoderma* has supported the use of these microorganisms for biocontrol as whole cells, protein formulations and expressed genes in transgenic plants [10,11].

The highly active nature and diversity of *Trichoderma* enzymatic systems, which include glucanases, chitinases, proteases, lipases, esterases and DNAses [8,12] have led to their successful use in environmental and industrial biodegradation [13], composting [14], textiles [15], food and feed production [16], and pulp and paper treatment [17].

α-1,3-Glucanases (EC 3.2.1.59), also named mutanases, are extracellular enzymes able to degrade polymers of glucose bound by α-1,3-glycosidic links. According to their amino acid sequences, α-1,3-glucanases are grouped inside family 71 of the glycosylhydrolases [18] and are classified as endo-hydrolytic when two or more residues of glucose are released as reaction products, and exo-hydrolytic when glucose monomers are the final reaction products.

The presence of these enzymes has been described in bacteria, such as *Bacillus circulans* [19], *Flavobacterium* sp. [20], *Microbispora rosea* [21] and *Streptomyces chartreusis* [22]; and filamentous fungi, such as *Aspergillus nidulans* [23] and *Penicillium purpurogenum* [18]. However, *Trichoderma* has been the source for the

Abbreviations
CWDE, cell wall degrading enzyme.

purification of a high number of proteins with this activity. To date, there have been described four proteins with α-1,3-glucanase activity in *T. harzianum* [18,24–26] and one in *T. reesei* [27]. Only two have been cloned [18,25] and one has been overexpressed [18]. The comparative study of these two sequences has demonstrated that both are almost identical proteins from two different *Trichoderma* strains.

The function performed by these enzymes in the fungal metabolism is not clear, although it may be connected with the morphogenesis of the cell wall [28], mobilization of α-1,3-glucan from the cell wall in energy starvation conditions [23,29], or the degradation of α-1,3-glucan from other fungi during mycoparasitic interactions [25].

Applications derived from the use of these enzymes are related to their antifungal effect against phytopathogenic fungi containing α-1,3-glucan in their cell wall, like *Fusarium oxysporum* [30], and to the presence of its substrate in the dental plaque as one of the main components, responsible for the accumulation of microorganisms on tooth surfaces and the consequent development of caries [31].

In this article, we report on the purification and molecular characterization of an exo-α-1,3-glucanase (EC 3.2.1.84), named AGN13.2, and the cloning of the corresponding gene, named *agn13.2*, from *T. asperellum*. We also show that expression of the gene and enzyme secretion occur when *T. asperellum* grows under simulated antagonism. The expression of *agn13.2* during *in vivo* assays against the strawberry pathogen *Botrytis cinerea* strongly supports the role of AGN13.2 in the antagonistic action of *T. asperellum*.

Results

Biochemical properties of AGN13.2

Physicochemical and kinetic parameters of AGN13.2 are summarized in Table 1. The release of reducing sugars was detected only when using mutan as substrate. The main product detected was glucose, suggesting an exolytic mode of action for AGN13.2. Low levels of cellotriose, three linked glucose residues, could also be identified after longer incubations, which may represent the lowest of the substrates that AGN13.2 could not degrade due to its obliged linear character.

Protein sequence

The N-terminal peptide of the purified protein was subjected to sequencing and an eight amino acid sequence was obtained. These residues were: ASSADRLV. Degenerate primers were designed according to this sequence and a highly conserved internal sequence present in other mutanases (WNDYGES). AGN13.2 presented an overall protein sequence identity of 79 and 77% to the previously cloned α-1,3-glucanases in *T. harzianum* CECT2413 [25] and *T. harzianum* CBS243.71 [18], respectively. A multiple sequence alignment was carried out with α-1,3-glucanases from *Trichoderma* (Fig. 1).

Regulation of *agn13.2* and *agn13.1* expression

Regulation studies carried out in liquid phases show that the highest transcript levels for *agn13.2* and *agn13.1* were found for *B. cinerea* cell wall inductions. However, no *agn13.2* and *agn13.1* mRNA was detected in conditions of carbon and nitrogen source starvation and chitin induction (Fig. 2). Regulation studies carried out in the solid phase show that *agn13.2* is induced during the interaction of *T. asperellum* and *B. cinerea*, despite the presence of glucose in the culture media; meanwhile, no signal was detected in the *T. asperellum* vs. *T. asperellum* interaction (Fig. 3). No signal was detected during the interaction of *T. harzianum* and *B. cinerea* and *T. harzianum* with itself.

Table 1. Biochemical characteristics of α-1,3-glucanases in *Trichoderma* sp.

Origin	*T. asperellum* CECT20539	*T. harzianum* CECT2413 [25]	*T. harzianum* SP234^R [18]	*T. harzianum* CCM-F470 [26]	*T. harzianum* QMZ779 [24]	*T. reesei* QM6A [27]
M_r SDS/PAGE (kDa)	75	75	75	67	–	47
IP-chromatofocusing	6.1	7.5	6.7–7.5	7.1	7.1	–
Inactivation T (°C)	55	50	–	45	–	–
Optimum T (°C)	45–55	55	50–55	40	–	50
Optimum pH	5	5	3.5–5	5.5	6	4.5
K_m (mg mutant·mL^{-1})	0.8	1.5	–	1.2	–	1.2
Mode of action	Exo	Exo	Exo	Exo	Exo	Endo
N-glycosylation	No	No	No	–	–	–

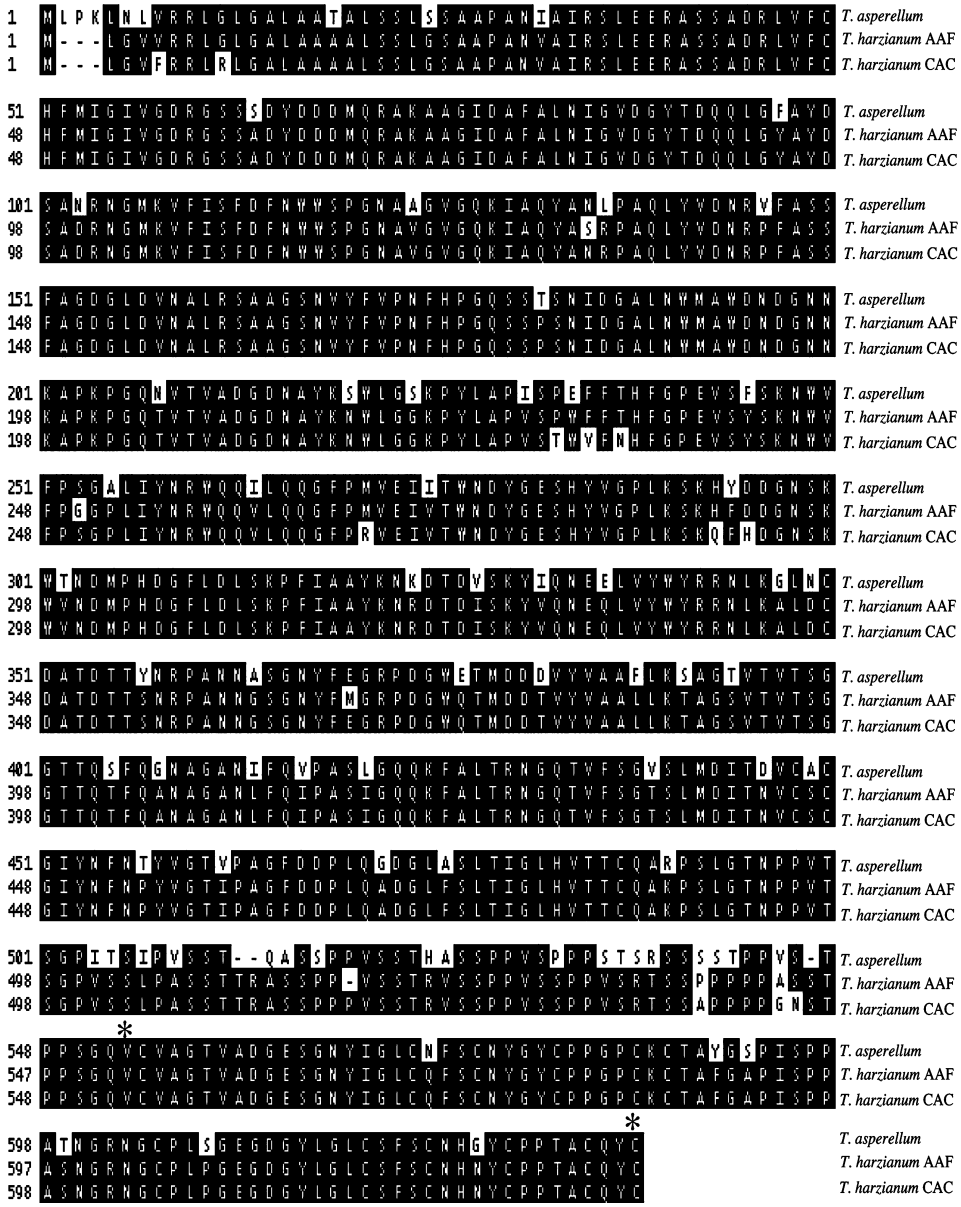

Fig. 1. Alignment of *T. asperellum* AGN13.2 (Accession no. AJ784420) with homologous sequences of *T. harzianum* (Accession nos. AAF27911, CAC80439) [18,25]. Identical amino acids in two or more sequences are shaded. The alignment was carried out with DNASTAR using MEGALIGN (CLUSTAL) with a gap penalty of 10. The putative mutan-binding region is the one comprised between the two residues marked with asterisks [18].

Discussion

The essentially pure mutanase had a molecular mass of ~ 75 kDa after SDS/PAGE and 132 kDa after gel filtration chromatography. This result suggests that AGN13.2 could be a dimeric protein in solution, unlike another purified α-1,3-glucanase from *T. harzianum* CCM-F470, which is probably a tetramer [26]. The kinetic constant K_m for the purified protein was similar and its specific activity was also in the range reported for enzymes isolated from *T. harzianum* CECT2413 [25], *T. reseei* QM6A [27] and *A. nidulans* [29].

Substrate specificity of AGN13.2 was similar to that reported for the enzyme isolated from *T. harzianum* [27], showing a high specific recognition of α-1,3-glucans with α-1,6 branches.

The observed optimum pH and thermostability are in the range obtained for the *T. harzianum* CECT2413 mutanase [25] and other enzymes from *T. harzianum* CCM-F470 (pH 5.5) [26], *T. harzianum* QMZ779

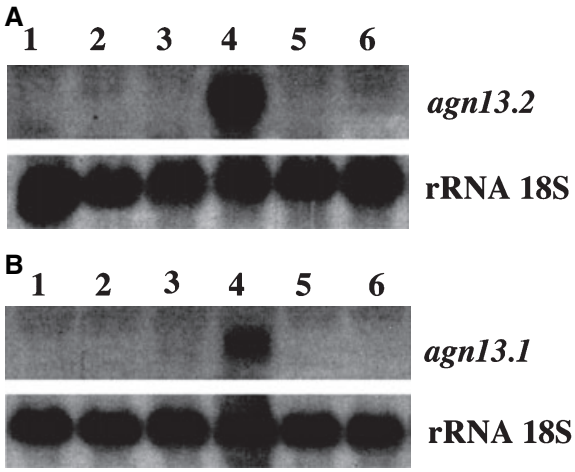

Fig. 2. Expression profile of *agn13* orthologues in *T. asperellum* CECT20539 and *T. harzianum* CECT2413 under different growing conditions. RNAs were extracted from mycelia grown from *T. asperellum* CECT20539 (A) and *T. harzianum* CECT2413 (B) for 8 h without a carbon source (1), on 2% glucose pH 5.5 (2), 2% chitin pH 5.5 (3), 0.5% *B. cinerea* cell walls pH 5.5 (4), 2% chitin pH 3 (5) and under nitrogen starvation (6).

(pH 6) [24], *B. circulans* (pH 5.5) [19] and *Flavobacterium* (pH 6.3–6.9) [20]. Thermostability studies suggest that, as described for other glucanases from *Trichoderma* [32], the binding of the enzyme to its substrate confers a higher thermal stability to the protein.

Previous regulation studies of the different proteins characterized with α-1,3-glucanase activity were carried out in *T. harzianum* [25] and *A. nidulans* [23]. These studies suggested that AGN13.1 from *T. harzianum* is an enzyme involved in mycoparasitism, whereas the protein MutA of *A. nidulans* allows the mobilization of the mutan as energy source from its own fungal cell wall. Regulation studies carried out in liquid phases show that AGN13.2 and AGN13.1 are induced specifically by the presence of *B. cinerea* cell walls. Interestingly, chitin, a polymer commonly used to establish simulated mycoparasitic conditions and reported as inducer of several enzymes related to mycoparasitism [33,34], is not able to induce the expression of either *agn13.2* or *agn13.1*. Regulation studies carried out in confrontation assays in solid phase between *B. cinerea* and *T. asperellum* and *T. harzianum*, respectively, strongly support the involvement of AGN13.2 and not AGN13.1 in the mycoparasitic process under these conditions. The differential expression of the two AGN13 orthologues in two strains representing two different biocontrol biotypes of *Trichoderma* (*asperellum* and *harzianum*) could be related to differences in antagonistic behaviour and/or host range between these strains and perhaps between the two biotypes. In connection with this idea, it is worth mentioning that both strains display clear distinctive host range and antagonistic abilities in controlled assays (unpublished results).

Gene expression in the host–*Trichoderma* interaction area during *in vitro* confrontation assays has already been reported for some other extracellular cell wall degrading enzymes produced by *Trichoderma*, such as endochitinase CHIT42 [35,36]. Interestingly, both enzymes, AGN13.2 and CHIT42, were purified from *T. asperellum* supernatant after mutan affinity purification (data not shown). This indicates a common induction of both antifungal CWDEs in the presence of fungal cell walls as well as either an association between the two proteins or a significant affinity of CHIT42 for mutan.

Experimental procedures

Strains and culture conditions

T. asperellum CECT20539, *T. harzianum* CECT2413 and *Streptococcus mutans* CECT4034 were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain).

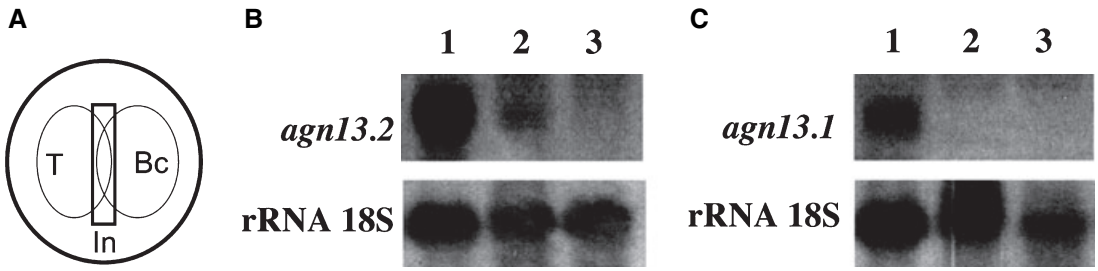

Fig. 3. Expression of *agn13* orthologues in *T. asperellum* CECT20539 and *T. harzianum* CECT2413 during mycoparasitic interaction. (A) Schematic representation of the confrontation assay, samples were taken from the interaction area (In) between *Trichoderma* strains (T) and *B. cinerea* B98 (Bc) grown in PDA plates. RNAs were extracted from mycelia grown of *T. asperellum* CECT20539 (B) and *T. harzianum* CECT2413 (C) during mycoparasitism simulation in liquid culture using fungal cell walls induction (1), during *Trichoderma* vs. *Botrytis* confrontation in plate assay (2) and during *Trichoderma* vs. *Trichoderma* confrontation in plate assay (3).

B. cinerea B98 was isolated in our laboratory from infected strawberries. For protein production a two-step growing method was used: *Trichoderma* was grown in Mandel's minimum medium [37] supplemented with 2% of glucose ($\approx 10^5$ conidia·mL medium^{-1}) in a rotary shaker (150 r.p.m.) at 25 °C. After 48 h the mycelium was filtered, thoroughly washed with 2% magnesium chloride and water, and transferred to a new flask containing Mandel's minimum medium supplemented with different carbon sources (replacement medium). In the purification of AGN13.2, 0.5% of *B. cinerea* cell walls were used as carbon source. Mutan, α-1,3-glucan with some α-1,6-glucan (dextran) side chains, was prepared by growing *Streptococcus mutans* as described in Wiater *et al.* [26].

Protein purification and biochemical properties

The purification of AGN13.2 from *T. asperellum* cultures was based on ammonium sulfate precipitation of the supernatant (90% saturation), its affinity towards mutan, chromatofocusing and gel filtration as main steps, following the same procedure and methodology as described in Ait-Lahsen *et al.* [25]. The purified AGN13.2 activity was tested against several polymers with glycosidic linkages using 0.5 mg·mL^{-1} of each substrate: mutan (α-1,3- and α-1,6-glucan), nigeran (α-1,3- and α-1,4-glucan), soluble starch (α-1,4- and α-1,6-glucan), pustulan (β-1,6-glucan), laminarin (β-1,3-glucan), carboxymethilcelullose (β-1,4-glucan) or chitin (polymer of NAG linked by β-1,4-glycosidic bonds). Activity on these substrates was measured by reducing sugars quantification by Somogyi [38] and Nelson [39] method, except for chitinase activity that was determined as described in De la Cruz *et al.* [40]. The products from hydrolysis by the purified AGN13.2 were applied to a HPLC Aminex HPX-42A column (Bio-Rad, Barcelona, Spain); diffraction index of the eluate was used for the detection of the products. Glucose and cellulose oligosaccharides (2–5 polymerization degree) were used as standards. Substrate controls were considered in each determination. Thermal stability of the enzyme was determined incubating the purified protein at temperatures from 25 to 70 °C in 50 mM sodium acetate for 30 min and then measuring the remaining enzymatic activity adding mutan. For optimum pH determination phosphate buffer was used at pH values between 2 and 3, acetate buffer at pH values between 4 and 5, and phosphate buffer at pH values between 6 and 8. In all cases the concentration of the buffer was 50 mM. We used MALDI-TOF MS combined with PNGase F (New England Biolabs, Herts, UK) treatment to identify its N-glycan structures and their sites of expression.

Protein sequence and degenerate primed PCR

Amino terminal sequencing from the purified AGN13.2 was carried out at the National Center of Biotechnology (CNB, Madrid, Spain) following Edman degradation method. Degenerate primers were designed according to the sequence obtained and an internal region highly conserved in other mutanase proteins. These were AGN1 [5′-GCI WSIWSIGCIGAYMGIYTIGT-3′] and AGN2 [5′-SWYT CICCRTARTCRTTCCA-3′], respectively. *T. asperellum* chromosomal DNA was used as template under the following conditions: 94 °C, 40 s (denaturation); 52 °C, 1 min (annealing); 72 °C, 2 min (extension); repeated for 40 cycles and a final extension step of 2 min at 72 °C. We used 100 pmol of each primer in 25 μL reactions.

RNA extraction and RT-PCR

RNA extractions for RT-PCR and northern blotting were carried out using TRIZOL (Invitrogen, Barcelona, Spain) following manufacturer's directions. To obtain the cDNA sequence encoding AGN13.2, specific primers were designed according to the previous amplified genomic sequence. AGN3 [5′-GCCGTAGTCGTTCCACGTGATAATC-3′] was used to clone the 5′-end of *agn13.2* mRNA using SMART-PCR system (Clontech, Palo Alto, CA USA) and AGN4 [5′-GCAGATCGTCTTGTCTTTTG-3′] was used to clone the 3′-end of *agn13.2* mRNA using RACE-PCR system (Roche Diagnostics, Barcelona, Spain). RNA was extracted from mycelia grown for 8 h with 0.5% *B. cinerea* cell walls.

Regulation of *agn13.2* expression

Regulation of the previous cloned α-1,3-glucanase in *Trichoderma* [25] was also considered. Northern blots were performed using Hybond N+ (Amersham Biosciences, Barcelona, Spain) membranes and Ultrahyb (Ambion, Cambridge, UK) as hybridization buffer following manufacturer's instructions. RNA was extracted from mycelia grown for 8 h in different induction media. During direct confrontation experiments, agar plugs cut from growing colonies of *B. cinerea* were placed in PDA plates covered with sterile cellophane sheets. Mycelia were allowed to grow for 48 h and then plugs from growing colonies of *Trichoderma* were placed 5 cm away from the *B. cinerea* plug. Control plates were inoculated with two *Trichoderma* plugs (*Trichoderma* vs. *Trichoderma*). Mycelia for RNA extractions were collected from the interaction area in a range of 12–24 h after both fungi touched each other. Equivalent zones were harvested from control plates.

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