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NOTES

Enhanced Expression of Endochitinase in *Trichoderma harzianum* with the *cbh1* Promoter of *Trichoderma reesei*

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Production of extracellular endochitinase could be increased 5-fold in the mycoparasite fungus *Trichoderma harzianum* by using the cellulase promoter *cbh1* of *Trichoderma reesei*, whereas the total endochitinase activity increased 10-fold. The *cbh1* promoter was not expressed on glucose and sucrose in *T. harzianum* and was induced by sophorose and on cellulase-inducing medium. The endogenous endochitinase gene was expressed at a low basal level on glucose and sucrose. No specific induction by crab shell chitin or sophorose was observed.

The filamentous fungus *Trichoderma harzianum* is one of the most potent agents for biocontrol of plant pathogens. The antagonistic mode of action of the fungus is proposed to be due to the production of antibiotics (3, 16) and fungal cell wall-degrading enzymes such as chitinases, glucanases, and proteases (2, 10, 11). The endochitinase of *T. harzianum* has been shown to be the most effective enzyme when tested alone or in combination with β-1,3-glucanase. It appears to be more effective than plant and bacterial chitinases against a wider range of target fungi (10, 11). *T. harzianum* produces only moderate amounts of endochitinase naturally, and overexpression of the endochitinase could generate more-effective biocontrol strains. However, no strong *T. harzianum* promoters are available for strain construction. An efficient expression system has been developed for the related species *Trichoderma reesei*, which utilizes the promoter of the highly expressed cellulase gene *cbh1* (9). Here, we report the use of this *cbh1* promoter in the construction of *T. harzianum* strains overexpressing the *T. harzianum* endochitinase gene *ThEn-42* (5).

We previously constructed the plasmids pCL-9 and pCL-7 for production of the endochitinase in *T. reesei* (see accompanying paper [12]). Plasmid pCL-9 contains the entire coding region of the endochitinase gene linked to the *cbh1* promoter and terminator, including the signal sequence and the propeptide region, and pCL-7 contains only the mature part of the endochitinase linked to the cellulase cellobiohydrolase I (CBHI) signal sequence. pCL-7 and pCL-9 were separately cotransformed into the *T. harzianum* strain P1 (ATCC 74058) as described in the accompanying paper (12). Twenty transformants from both constructs were grown in shake flasks on cellulase-inducing (CI) medium, which was *Trichoderma* minimal medium (pH 6.7) (14), in which glucose was substituted with complex plant material (13). Analysis of culture media by Western blotting (immunoblotting) (12) showed that approximately 90% of the pCL-9 transformants produced amounts of endochitinase higher than those of the host itself (not shown). No significant differences were observed between the transformants. Unexpectedly, none of the pCL-7 transformants overproduced endochitinase, although they contain the expression cassette (see below).

Three random clones transformed with either pCL-7 (P71 to P73) or pCL-9 (P91 to P93) were analyzed by Southern blotting (Fig. 1) essentially as described in the accompanying paper (12). No integration had occurred at the endogenous *ThEn-42* locus. The copy numbers of the expression cassette were greater than 10 in all transformants, on the basis of intensity comparisons of the 1.5-kb *Hin*dIII band (PhosphorImager).

Expression of endochitinase on different carbon sources in 50-ml shake flask cultivations at 28°C (inoculum of 10^7 spores) was investigated. The mineral salt-V8 medium (17) that was used contained either 5% glucose or 0.5% sucrose supplemented with 1% crab shell chitin and/or CI medium. Extracellular endochitinase protein and activity against colloidal chitin and endochitinase mRNA were analyzed as described in the accompanying paper (12). The endochitinase protein and mRNA produced from the transformed expression cassette are indistinguishable from those produced from the endogenous *ThEn-42* gene, and this analysis was based on differences in signal intensities and enzyme activities.

Extracellular endochitinase activity was not detected in any of the strains cultivated on 5% glucose (not shown). However, expression was not totally repressed, since Western (Fig. 2A) and Northern (Fig. 3A) analyses revealed comparable basal levels of expression even when more than 1.5% of glucose was present in the medium (high-performance liquid chromatography [HPLC]; data not shown). No differences were observed between the host strain and the transformants; consequently, the *cbh1* promoter was most likely repressed by glucose, as is the case in *T. reesei* (6, 7, 15), and the endochitinase expressed was a product of the endogenous gene. Cultivations on 5% sucrose as the sole carbon source gave similar results (not shown).

Clones P91 to P93 transformed with pCL-9 overexpressed the endochitinase only in CI medium. No overexpression was detected when crab shell chitin was the only carbon source, and the presence of chitin in the CI medium had no additional effect on endochitinase production by the transformants when it was studied by activity (Fig. 2A), Western (Fig. 2A), and

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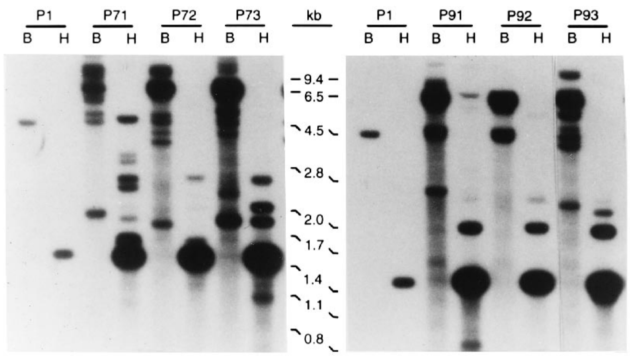

FIG. 1. Southern analysis of genomic DNA (2 μg) isolated from the transformants P71 to P73, P91 to P93, and the host *T. harzianum* P1 digested with *Bgl*II (B) and *Hin*dIII (H). The *Sal*I-*Hin*dIII fragment of the endochitinase gene from plasmid pCL-7 (12) was used as a probe. The positions of molecular weight markers are indicated in the center.

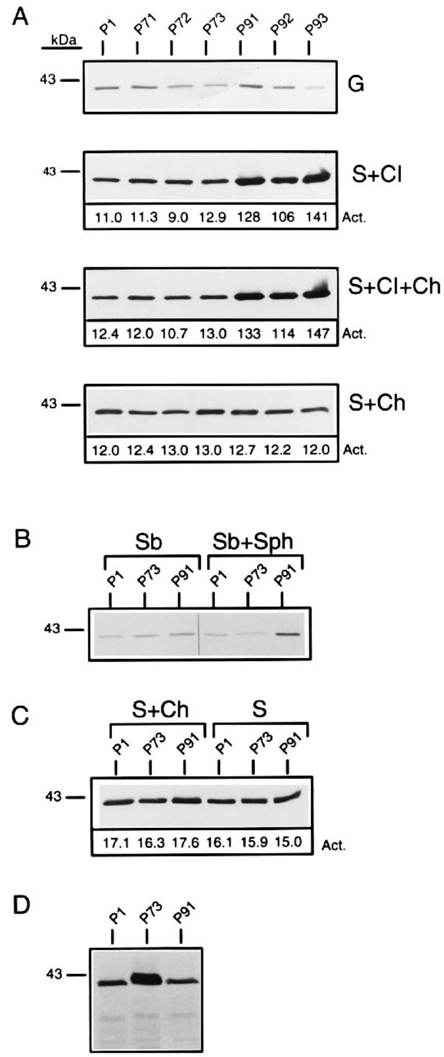

FIG. 2. Endochitinase production under different growth conditions analyzed by Western blotting and enzymatic activity. Endochitinase activity values in the culture medium (in units per milliliter) are given at the bottoms of the panels. (A) Analysis of the endochitinase secreted into growth medium by the transformants P71 to P73, P91 to P93, and the host *T. harzianum* P1 (P1) cultivated for 3 days on glucose (G), on sucrose-CI medium without (S+CI) and with (S+CI+Ch) chitin, and on sucrose-chitin medium (S+Ch). (B and C) Strains P73, P91, and the host P1 were also cultivated for 58 h on sorbitol (Sb) and sorbitol with the addition of sophorose (Sb+Sph) and for 5 days on sucrose-chitin (S+Ch) and sucrose (S), respectively. (D) Analysis of intracellular proteins extracted from mycelia of P73, P91, and the host P1 cultivated for 3 days on sucrose-CI medium. In panels A and C, 1 μg of total extracellular protein was loaded, except on glucose (G), on which 20 μg was loaded. In panels B and D, 50 μg of extracellular protein and 100 μg of intracellular protein were loaded, respectively. The positions of molecular weight markers are indicated on the left.

Northern (Fig. 3A) analyses. Transformants P91 to P93 produced four- to fivefold more endochitinase mRNA (quantified by a PhosphorImager) and protein (High Resolution Color Scanner JX-325, Sharp, ImageMaster program; Pharmacia Biotech) on CI medium than the nontransformed strain did (Fig. 2A). However, the endochitinase activities produced by transformants P91 to P93 were approximately 10-fold higher than that of the nontransformed strain (Fig. 2A).

An increase in the endochitinase mRNA on CI medium was also observed in clone P73 transformed with pCL-7 (Fig. 3A), which did not overproduce extracellular endochitinase (Fig. 2A). To analyze possible intracellular accumulation of the endochitinase, mycelium of strain P73 cultivated in 50 ml of CI medium was washed (three times in 50 mM sodium citrate buffer, [pH 6.0] containing 2% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, and 0.02% NaN_3), homogenized in liquid nitrogen, suspended into 5 ml of buffer (see above), vortexed briefly, and centrifuged (10 min, 5,000 rpm, room temperature), and the supernatant was analyzed. The amounts of endochitinase that accumulated intracellularly in P73 were higher than those in the nontransformed strain and the transformant P91 treated similarly (Fig. 2D). The accumulated endochitinase was most likely produced from the transformed expression cassette. The extracellular endochitinase levels of P73 were comparable to those produced by the nontransformed strain and most probably represent normally secreted endogenous endochitinase. These results contradict those obtained with *T. reesei* transformed with pCL-7 and pCL-9 (12), which showed equal extracellular endochitinase levels with both plasmids. The reasons for this difference are not known.

Cellulase expression is highly induced in *T. reesei* when the disaccharide sophorose is added into cultivations carried out on nonrepressing, noninducing carbon sources such as sorbitol (6, 15). To study the possible sophorose induction of the *cbh1* promoter in *T. harzianum*, the nontransformed strain and the transformants P73 and P91 were cultivated on medium containing 3% sorbitol, and 2 mM α-sophorose (Serva) was added twice (at 48 and 54 h of growth) to provoke prolonged induction. Protein and mRNA analyses were carried out 10 h after the first addition. A basal level of endochitinase expression occurred in all of the strains on sorbitol medium (Fig. 2B and 3B). Sophorose addition increased production of endochitinase mRNA (in P73 and P91 [Fig. 3B]) and extracellular protein (in P91 [Fig. 2B]) by the transformants but did not affect production by the nontransformed strain (Fig. 2B and 3B).

Unexpectedly, the nontransformed *T. harzianum* strain produced similar amounts of endochitinase activity (Fig. 2A), protein (Fig. 2A), and mRNA (Fig. 3A) when it was cultivated for 3 days on the 0.5% sucrose medium supplemented with crab shell chitin, CI medium, or a mixture of both. It is possible that CI medium contains components that fully induce the endogenous endochitinase gene, and a possible additional induction by chitin is not observable in the mixture of both substrates. Alternatively, full expression could also occur under possibly derepressing conditions once sucrose has been consumed. To test the second alternative, the nontransformed strain and the

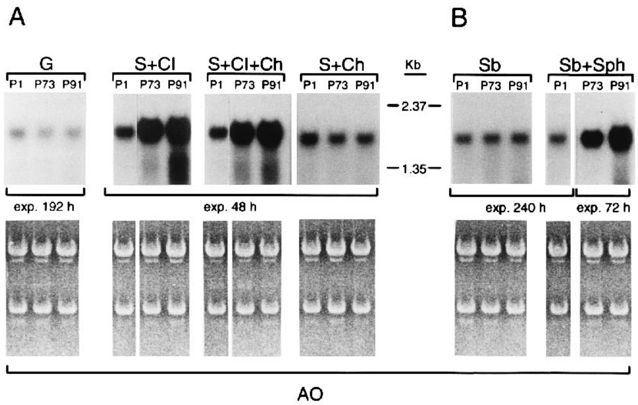

FIG. 3. Northern analysis of total RNA (2 μg) isolated from mycelia of strains P73 and P91 and the host strain *T. harzianum* P1 (P1). (A) Strains cultivated for 3 days on glucose (G), sucrose-CI medium without (S+CI) and with (S+CI+Ch) chitin, and sucrose-chitin (S+Ch); (B) strains cultivated on sorbitol (Sb) and sorbitol with the addition of sophorose (Sb+Sph). RNA was isolated from the same cultivations at the time points described in the legend to Fig. 2A and B. The *Pvu*II-*Sma*I fragment (942 bp) (12) from the endochitinase gene was used as a probe. The exposure times (exp.) of the films are indicated at the bottoms of the gels. No signal or very faint signals were observed when the lanes exposed in this figure for 192 h were exposed for only 48 h or when lanes exposed for 240 h were exposed for only 72 h. The positions of molecular weight markers are indicated in the center. AO, acridine orange-stained gel before blotting.

transformants P73 and P91 were grown for 5 days on medium containing either 1% sucrose or 1% sucrose supplemented with 1% crab shell chitin (Fig. 2C). After 5 days of growth, the sucrose was totally consumed as measured by HPLC. Endochitinase activities that were somewhat higher than those from the previous 3-day cultivation on sucrose-chitin medium were obtained (Fig. 2A), reflecting the longer cultivation time. No difference in endochitinase amounts between the two media (sucrose alone and sucrose supplemented with chitin) was observed (Fig. 2C). This result suggests that endochitinase expression occurred in *T. harzianum* P1 because of sucrose depletion rather than specific induction by chitin. The endochitinase level produced by the transformant P91 was not higher than that of the nontransformed host (Fig. 2C), which indicates that the *cbh1* promoter needs proper induction to function in *T. harzianum*, as is the case for *T. reesei* (6).

Several studies carried out with different *T. harzianum* strains agree that expression of endogenous chitinases is highly induced by chitin and is repressed by glucose (1, 4, 17, 18), with repression also occurring in the presence of chitin (4, 18). On the other hand, other reports demonstrate basal production of endochitinase in cultures containing repressing carbon sources such as glucose and sucrose (18). This has been suggested to also occur in the *T. harzianum* strain P1 (17), and we have now confirmed this at a molecular level. Thus, possible glucose repression is not very strong in this strain. Basal endochitinase expression was also observed when sorbitol was used as a carbon source. In contrast to what has been reported, our results indicate that expression of the endogenous endochitinase, at least in the *T. harzianum* strain P1, is not specifically induced by crab shell chitin. It is possible that endochitinase expression is simply allowed under nonrepressing conditions in the absence of glucose or sucrose. This is supported by other findings which show that significant amounts of chitinases are produced on low amounts of substrates other than chitin and even with no substrate (1, 17, 18). The lack of a clear specific inducer also makes the experiments carried out to study possible glucose repression difficult to interpret. This problem might be overcome now that the glucose repressor gene *cre1* of *T. harzianum* has been cloned (7), and its possible role in expression of chitinases can be studied.

The *T. reesei cbh1* promoter needs induction to be functional in *T. harzianum* and is regulated in a manner similar to that in *T. reesei* (6, 15). The results indicate that cellular mechanisms mediating sophorose induction are present also in *T. harzianum*, and it would be attractive to study whether sophorose also induces endogenous cellulase expression in this fungus. Although the expression levels in *T. harzianum* are not comparable to those of *T. reesei*, the *cbh1* promoter seems to be a good candidate promoter for *T. harzianum*. These regulation characteristics might prove useful, especially when controlled expression of potentially toxic products is needed.

Transformation of pCL-9 and pCL-7 into *T. reesei* resulted in strains which secrete amounts of endochitinase more than 20-fold higher than those normally produced by *T. harzianum* P1 (12), which enables easy purification of large quantities of the enzyme for various application trials. On the other hand, endochitinase overexpression in *T. harzianum* could be more advantageous for biocontrol. Other chitinases and hydrolytic enzymes are coexpressed (17), and these interact synergistically with the endochitinase against plant pathogens (10, 11). Biocontrol strains also have other characteristics contributing to mycoparasitism, such as the capability to produce antibiotics and to form invasion structures (8, 16). Although endochitinase expression was increased only moderately (5-fold) in the *T. harzianum* transformants, total extracellular chitinolytic activity increased by 10-fold. This could be due to the synergistic action of different chitinases and could be more beneficial for biocontrol than the actual amounts of endochitinase produced.

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