

## NOTE

# Growth Inhibition of the Yeast Transformant by the Expression of a Chitinase from *Coprinellus congregatus*

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*Coprinellus congregatus* generates several chitinases during its entire life cycle: at the growing hyphal stage and at the mushroom autolysis stage. We have isolated a chitinase gene (*chi1*) from the mushroom tissue at the autolysing stage, and constructed a chitinase expression vector to get large amount of enzyme protein. Chitinase 1 (*chi1*) cDNA was heterologously expressed in *Saccharomyces cerevisiae* by *gall* promoter. The transformants showed no specific change in growth characteristics under normal growth conditions. However the expression of the gene by the *gall* promoter in the yeast transformants resulted in complete growth inhibition, while laccase expression by the *gall* promoter showed normal growth. The chitinase activities from the transformants were also more than 3 times higher than that of the recipient strain, and the chitinase expression by the real time-PCR also showed increased expression of the *chi1* in the yeast transformant. Expression of a chitinase which was produced at the mushroom autolysing stage of *C. congregatus* resulted in yeast growth inhibition.

**Keywords:** chitinase, *C. congregatus*, heterologous expression, inky cap, yeast growth inhibition

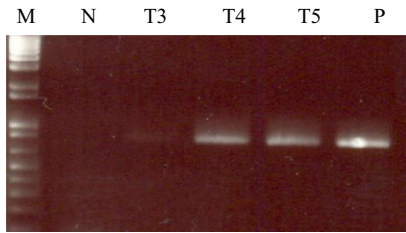
Chitin, a polymer of N-acetylglucosamine is found in many organisms such as insects, crustaceans as well as fungi. The cell wall of filamentous fungi consists of diverse polymers such as  $\alpha$ - and  $\beta$ -glucans, other sugar polymers and chitins. Chitinases (EC 3.2.1.14) hydrolyze the  $\beta$ -1,4-glycosidic linkages of chitin. Chitinases can be grouped in two families, family 18 and family 19 of glycoside hydrolases by their amino acid sequences (Henrissat, 1999). Chitinases in family 19 includes most plant chitinases and *Streptomyces* chitinase, whereas fungal chitinases belong to family 18 (Henrissat, 1999). Most filamentous fungi require chitinase during growth phase to enlarge surface area of their cell wall. ChiA, a glycosylphosphatidylinositol-anchored chitinase is localized at polarized growth site in *Aspergillus nidulans* (Yamazaki *et al.*, 2008). When opportunistically pathogenic fungi infect human body, their cell morphologies showed dimorphic changes to protect themselves from the host defense mechanism and to disseminate their own progenies inside the host (Sundstrom, 2003). Hyphal cells of *Candida albicans* had more chitin than yeast cell during its dimorphic transition (Munro *et al.*, 1998). There are so many anti-bacterial antibiotics which show target specificity to bacterial subcellular structures, while diversity of anti-fungal antibiotics is relatively narrow because fungi consist of eukaryotic cells like human body. Therefore fungal cell wall can be the best target for fungal antibiotics, because human cells do not have any cell wall materials.

*Coprinellus congregatus* is a mushroom forming basidio-

mycete, and it is easy to grow in a complete agar medium. Induction of mushroom generation is also quite simple; incubation at 25°C in a regime of 15 h light/9 h dark cycle is enough to induce mushroom formation (Choi and Cho, 2005). This fungus generates mushrooms which become black ink droplets during their maturation, and this is why this mushroom is designated as an inky cap. When the autolyzed tissue and the liquid droplet were examined by the light and electron microscopy, the cell walls of basidia disintegrated during autolysis and no cell wall was observed in the liquid droplet (Choi and Cho, 2005). A chitinase cDNA and genomic DNA were isolated from the cells of autolyzed mushroom tissues, and reported that the cloned chitinase gene (*chi1*) was most highly expressed at the mushroom maturation/autolysis stage (Lim and Choi, 2009). We would like to analyze biochemical characteristics of the purified chitinase, but it was too hard to get large amount of the autolysing mushroom tissue under lab conditions. We have constructed a chitinase expression vector using *chi1* cDNA, which was regulated by *gall* promoter. This vector was introduced to *Saccharomyces cerevisiae* to generate transformants, and here we report the biological characteristics of the chitinase transformants.

*S. cerevisiae* strain (KM3; MATa, *his3 $\Delta$ 200*, *trp1 $\Delta$ 101*, *ura3-52*) was used, and an YCp vector (GAL1p, URA3, CEN4L, ARS1, Amp<sup>R</sup>) was used for construction of the chitinase expression vector. Chitinase cDNA (*chi1*) with *Bam*HI linker at both ends was ligated into the YCp vector which was digested with *Bam*HI. Genetic transformation of the *S.*

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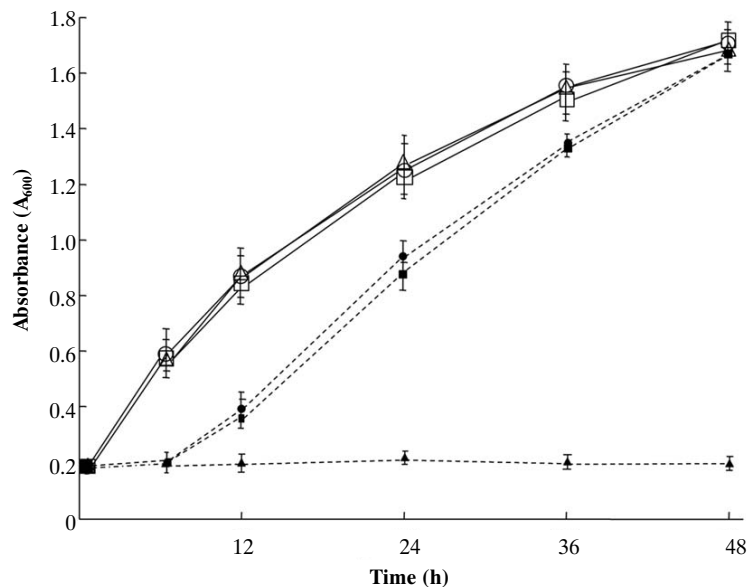
**Fig. 1.** Confirmation of the stable replication of the chitinase expression vector in the transformant cells by PCR using the chitinase specific primers. M, molecular weight marker; N, negative control; P, positive control with the chitinase expression vector; T3-T5, transformants.

*cerevisiae* was performed through the electroporation method (0.2 mm gap; 450 V, 150  $\mu$ F, 99R). The stable replication of the expression vector in the transformants was determined by PCR using chitinase-specific primers: forward primer 5'-ACTTCCTCCTCTACCTGCAAG-3' (chiF1) and reverse primer 5'-GGGGATCCTTGGTAAGTAGAGCAT-3' (chiR1). Several transformants were selected on dextrose agar plate containing basal salts with amino acid and vitamin mixture excluding uracil (C-ura dextrose plate) using the uracil selective marker as the previous report (Kim *et al.*, 2006). They showed very good growth on the selective medium (C-ura) while the recipient strain did not, and genomic PCR was performed to prove the existence of the expression vector in each transformant using chitinase-specific primers. Expected length of amplified DNA fragments appeared only from total DNAs of transformants (T4 and T5), while no amplified DNA band appeared from the recipient strain (Fig. 1). A laccase cDNA of *Phlebia tremellosa* laccase (Yeo *et al.*, 2008) was constructed as the same protocol of above, and one of the laccase transformant examined its growth under galactose

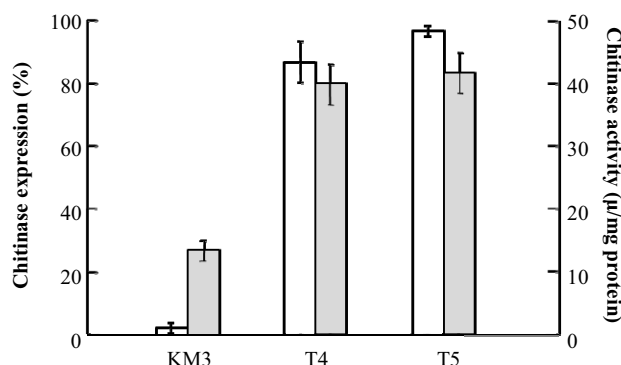
induction to examine whether the growth inhibition of chitinase transformant resulted from the chitinase induction or not.

Two chitinase transformants T4 and T5 and one laccase transformant L1 were grown on C-ura dextrose plate, and then transferred to the same liquid medium. Yeast cells were harvested by centrifugation at 2,650 $\times$ g after 3 day incubation, and then transferred to C-ura galactose liquid medium which had all nutrients but uracil, and galactose (2%) was used instead of dextrose as the carbon source to induce the chitinase cDNA. Yeast cells from T5 strain were harvested to examine growth at the time periods of 12 h, 24 h, 36 h, and 48 h, chitinase activity and chitinase expression were determined from 12 h-grown cells of T4, T5, and L1. Growths of the recipient yeast strain (KM3) and T5 were compared by absorbance at 600 nm. The chitinase transformant (T5) showed good growth in the C-ura dextrose medium at 48 h, while it showed no growth in the C-ura galactose medium (Fig. 2). However, laccase transformant (L1) exhibited normal growth in the C-ura dextrose medium and the C-ura galactose medium, even though a short (6 h) lag phase appeared in the galactose medium (Fig. 2).

Chitinase activity assay was performed by determination of reducing sugar concentration from colloidal chitin. Yeast cell were harvested at the time indicated, and then homogenized by vigorous shaking with glass beads (0.5 mm in diameter) for 10 min on a vortex mixer. The homogenates were centrifuged to get cell extracts for chitinase activity assay. Total RNAs were isolated from yeast cells obtained at 12 h incubation in C-ura with galactose medium, and 3  $\mu$ g of RNA from each chitinase-induced culture was used as the template in the synthesis of cDNA. Real-time PCR was performed using 5  $\mu$ l out of 20  $\mu$ l from the cDNA mix, 12.5  $\mu$ l of iTaq SYBR Green Supermix (Bio-Rad), and 1  $\mu$ l of chitinase-specific forward primer (chiF1) and reverse primer (5'-ATGAGCGCAACATA



**Fig. 2.** Growth inhibition of the chitinase transformant by the chitinase expression in liquid selective medium with galactose as the carbon source. (○) and (●), recipient strain (KM3); (△) and (▲), chitinase transformant (T5); (□) and (■), laccase transformant (L1). Open marks, glucose medium; closed marks, galactose medium.

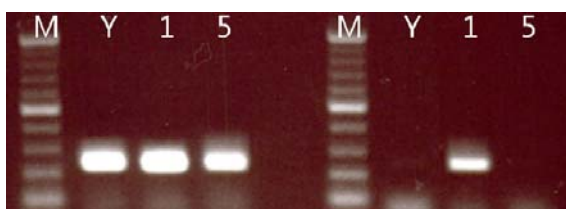


**Fig. 3.** Comparison of chitinase expression and chitinase activity of KM3, T4 and T5 in galactose medium (12 h). White bar, chitinase expression; gray bar, chitinase activity.

GTCCAAGA-3'). The relative chitinase expression level was compared with the expression level of the actin gene. Chitinase activities of the transformant strains were higher than that of recipient strain when they were grown in C-ura galactose medium (Fig. 3). The two yeast transformants showed higher chitinase expression in the galactose medium, which resulted in more than 3 times higher chitinase activities of the transformants (Fig. 3). Even though the recipient strain had its own chitinase expression, it showed very low expression (<5%) when examined by real-time PCR because the *C. congregatus* chitinase-specific primers were used to measure the introduced chitinase gene expression in this analysis. The recipient strain showed about 25% chitinase activity of the transformants, which resulted from its intrinsic chitinase activity.

Total RNAs from dextrose and galactose cultures were isolated and used for RT (reverse transcriptase)-PCR using laccase-specific primers. The laccase transformant which showed good growth in the galactose medium also expressed the laccase gene by the galactose induction when examined by the RT-PCR using the laccase-specific primers (Fig. 4). However, this laccase expression did not inhibit the growth of the yeast transformant.

We have expressed Chi1 in yeast to get enzyme protein for its biochemical characterization. However, the transformed yeast strains showed growth inhibition, which resulted in very low amount of protein. *S. cerevisiae* having chitinase expression from *Trichoderma aureoviride* showed chitinase activity (Jin Zhu *et al.*, 2005), but the authors did not mention about whether



**Fig. 4.** Confirmation of laccase expression in the laccase transformant (1). There were no RT-PCR products in the recipient strain (Y) or chitinase transformant (5). M, molecular weight marker; Y, recipient strain; 1, L1 (laccase transformant); 5, T5 (chitinase transformant). Left 4 lanes represent actin gene expression, and right 4 lanes represent laccase expression.

or not the transformed yeast cells showed decreased growth like our result.

Chitinase has been a candidate for antifungal material for long time. An endochitinase of *Trichoderma atroviride* was reported to have antifungal activity (Deng *et al.*, 2007), and expression of an endochitinase of *T. harzianum* in lemon tree resulted in defense against a fungal pathogen, *Botrytis cinerea* (Disfetano *et al.*, 2008). Mammalian acidic chitinase was reported to have antifungal activity against notorious human pathogens, *Candida albicans*, *Aspergillus fumigatus* and *Trichophyton rubrum* (Chen *et al.*, 2009). We have tried to express chitinase gene in yeast, and the yeast transformants expressing the chitinase resulted in higher chitinase gene expression as well as chitinase activity and growth inhibition. We are not sure yet whether the chitinase expression alone resulted in the yeast growth inhibition or not. In order to confirm this result, purified chitinase (Chi1) must be added to the yeast recipient strain to determine whether Chi1 really showed growth inhibition in the above experiment.

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