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Cloning and Characterization of a Chitinase-encoding Gene (*chiA*) from *Aspergillus nidulans*, Disruption of Which Decreases Germination Frequency and Hyphal Growth

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We cloned a chitinase-encoding gene from *Aspergillus nidulans* by polymerase chain reaction using degenerated oligonucleotide primers designed from the conserved amino acid sequences among chitinases from yeasts and *Rhizopus spp*. The cloned gene, named *chiA*, encoded a polypeptide consisting of 660 amino acids. Disruption of *chiA* had no effect on hyphal or conidiophore morphology, but germination frequency and hyphal growth rate decreased substantially. Expression of *chiA* was investigated using *Escherichia coli* β-galactosidase as a reporter enzyme. The β-galactosidase activity was present during hyphal growth and increased twice as the conidiophores developed. *In situ* staining of β-galactosidase activity found high expression in metulae, phialides, and conidia during conidiophore development, indicating that the expression of *chiA* is developmentally regulated. This is the first report to isolate a chitinase gene from A. *nidulans* and investigate its functions using the gene disruption technique and gene fusion methods in filamentous fungi.

Key words: chitinase; *Aspergillus nidulans*; gene cloning; hyphal growth

Chitin, a homopolymer of β-(1,4)-linked *N*-acetyl-D-glucosamine, is one of the major components of fungal cell walls and thought to be important for maintaining the cell wall integrity. It is synthesized by the activity of chitin synthases *in vivo*. Recently, each of several chitin synthase-encoding genes was disrupted in *Aspergillus nidulans and Neurospora crassa* to show that some of the genes are essential for a normal rate of hyphal growth of fungi.[1,2]

Chitinase (EC 3.2.1.1.14) is an enzyme that hydrolyzes chitin at β-1,4 bonds between *N*-acetylglucosamine residues. The enzyme is widely distributed in bacteria, plants, insects, humans, and fungi.[3,4] In filamentous fungi, chitinase is supposed to have roles in the processes requiring cell wall digestion,[5] such as germination of spores, tip growth of hyphae,[6] branching of hyphae, mycoparatisism, and hyphal autolysis. Although much research has been done on chitin synthases, there are only a limited number of reports on chitinases of filamentous fungi. In *M. rouxii*, multiple chitinase activities have been observed in mycelial extracts[7] and germinating spores.[8] Their roles in hyphal growth and their regulation are almost totally unknown.

Chitinase genes have been cloned from several fungi such as *Saccharomyces cerevisiae*,[9] *Candida albicans*,[10] *Rhizopus oligosporus*,[11] *Aphanocladium album*,[12] *Trichoderma harzianum*,[13–15] and *Coccidioides immitis*.[16] All of these gene products belong to Family 18 chitinases according to the classification of Henrissat and Bairoch.[17] Recent analysis showed that these gene products of fungi can be divided into two subclasses based on their primary structures, the fungal (plant)-type chitinases and the bacterial-type chitinases. The fungal-type chitinases are present in all of the fungi so far studied except for *A. album*, and have in most cases a C-terminal extension sequence following a catalytic domain. In *S. cerevisiae*, the gene encoding this type of chitinase (*CTS1*) was cloned and its disruption prevented cell separation between mother and daughter cells.[9] The bacterial-type chitinases are named as such because they have sequence similarity to chitinases from chitin-assimilating bacteria. Among eukaryotic cells, chitinases of this type have been reported only in filamentous fungi, but not in yeasts. Expression of these chitinases were studied in *A. album*[12] and *T. harzianum*[13,14] and it was suggested that they are induced by chitin or the cell wall fraction of other fungi.

Here we report cloning and characterization of a gene encoding a fungal-type chitinase from *A. nidulans*.

Materials and Methods

Strains, cultures, and media. A. nidulans strain FGSC89 (*biA1*; *argB2*) (Fungal Genetic Stock Center, KS) was used for preparation of total DNA. *A. nidulans* strain ABPU1 (*biA1*, *pyrG89*; *wA3*; *argB2*; *pyroA4*)[18] was used as a host for transformation. ABPU1/A was a transformant obtained with pSS1 (containing the *argB* gene, Motoyama *et al.*[18]) derived from ABPU1. ABPU1 and the transformants were cultured in MM medium[18] appropriately supplied with the required elements. When β-galactosidase activity was assayed, the concentration of glucose was increased to 4% to avoid production of endogenous β-galactosidase. In some ex-

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periments, colloidal chitin solution (10%) was used as a carbon source in MM medium (MMC). Incubation was usually done at 37°C with a rotary shaker (120 r.p.m.). When conidia formation was induced, mycelia cultured under the standard conditions were transferred to the MM agar plate with a spatula. *E. coli* strain MV1190 [*Δ*(*lac-proAB*) *thi supE Δ*(*srl-recA*)306::Tn*10* tet^r (F′ *traD36 proAB lacI^{q}ZΔ*M15)] was used for construction and amplification of plasmids. For screening of the *λ*2001 genomic DNA library, *E. coli* strain PLK17 (*lac mcrA mcrB hsdR gal supE*) was used as a host. *E. coli* MV1190 and PLK17 were grown in LB midium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and *λ* broth (1% tryptone, 0.25% yeast extract, 0.25% NaCl), respectively.

Isolation of the chiA *gene.* Total DNA of *A. nidulans* FGSC89 prepared as described[18] was used as the template for polymerase chain reaction (PCR) with the degenerated oligonucleotide primers: #A1, 5′-GAATTCAT(ACT)RARACNTGYCA-3′ (corresponding to the sequence I(E/K)TCQ with a *Eco*RI restriction site, N, R, and Y indicate any one of four nucleotides, purine and pyrimidine, respectively); #A2, 5′-CTGCAGTTRTTRTARAAYTG-3′ (corresponding to the sequence QFYNNY with a *Pst*I restriction site) and #3,5′-GANCCNCARTGYCCNT(AT)NCCNGA-3′ (corresponding to the sequence APQCPYPD). The first PCR was done with 200 pM #A1 and 200 pM #A2 as primers and 0.5 *μ*g total DNA as the template under the following conditions: 94°C; 10 min for denaturation; thirty cycles of 94°C, 0.5 min, 42°C, 1 min, 72°C; 2 min for amplification; then 72°C, 10 min for extension. Reaction products with approximately 300 to 400 bp were purified by agarose gel electrophoresis and used as the template for the second PCR. The second PCR was done under the same conditions except that #A2 and #A3 primers were used. A DNA band of the expected size was obtained, which was then labeled with *γ*-[^{32}P]-dATP by T4 DNA kinase and used for screening of the genomic library constructed with *λ*2001[1] by plaque hybridization.[19] A 3.4-kb *Bam*HI-*Pst*I hybridizable fragment obtained from one of the positive clones was isolated and cloned into pUC119 to obtain pBP4.

Nucleotide Sequencing. Sets of deletions were generated from pBP4 by using exonuclease III and mung bean nuclease. These plasmids were sequenced by the dideoxy chain termination method.[20] In some cases, restriction fragments of the *chiA* gene were cloned into pUC119 and sequenced. The nucleotide sequence of *chiA* has been submitted to DDBJ/EMBL/GenBank databases with the accession number D87895.

Isolation of cDNA of chiA. Total RNA was purified from *A. nidulans* FGSC89 cultured in YG medium at 37°C for 18 hours as described.[18] Poly (A)$^+$ RNA fraction was purified with an mRNA purification kit (Pharmacia). The 3′-terminus of *chiA* cDNA was cloned by the 3′-RACE method[21] using an Amplifinder 3′-RACE kit (Clontech) with the oligonucleotides #A1, 5′-CCTGTTGGTGGAAGCAGC-3′; #A2, 5′-CCTTCTACATCGAGCACCC-3′ as the first and the second *chiA* specific primer, respectively.

Plasmid construction. DNA was manipulated by the standard methods.[19] pB3 was constructed by inserting the 2.8 kb *Bam*HI fragment of *chiA* into the *Bam*HI site of pUC119. pCHI*Δ* was constructed by ligating the blunt-ended *argB* fragment, which had been purified from pSS1[18] as the 1.8 kb *Sph*I-*Bam*HI fragment, with pB3, which had been digested with *Eco*RV and treated with bacterial alkaline phosphatase (Fig. 3).

pCAZ2 was constructed as follows: pSS-TB-LZ was prepared by ligating pUC119 and the DNA fragments, which consisted of a unique *Kpn*I site followed by *lacZ* and the terminator sequence of *chsB* encoding chitin synthase[1] in this order. The promoter region of *chiA* (approximately 600 bp in length) was isolated from total DNA of *A. nidulans* with PCR using primers, 5′-CCGGTACCCGACAGAACAGAAGATG-3′ and 5′-AACAGTTTAGGTACCATTGTCTGAA-3′. The product was finally digested with *Kpn*I and ligated with *Kpn*I-digested pSS-TB-LZ to give pCAZ2, in which the *lacZ* gene was under the control of the *chiA* promoter (Fig. 4).

Genetic transformation of A. nidulans. Transformation of *A. nidulans* was done by the method of Rasmussen *et al.*[23]

Southern blot analysis. Total DNA of *A. nidulans* digested with restriction enzymes was analyzed by Southern blotting, which was done with ECL nucleic acid labeling and detection system (Amersham) according to the manufacturer's instructions.

Enzyme activity assay. Transformants were cultured in appropriately supplemented MM medium for 48 hours at 37°C, then mycelia were transferred to the fresh MM medium or MMC medium. Mycelia were harvested, suspended in the lysis buffer (McIlvaine buffer (pH 4.0), 1% Triton X-100) and broken by a Sonicator (Branson). The homogenates were centrifuged at 15000 × g at 4°C and the supernatant was assayed as follows. Chitinase activity was measured as described by Yanai *et al.*[11] using colloidal chitin as substrate. *β*-Galactosidase activity was measured by the method of Miller *et al.*[24] Protein was measured with BCA protein assay reagent (Pierce) according to the manufacturer's instructions.

In situ *staining of β-galactosidase activity.* Strains were cultured on MM agar plates (about 1 mm thick) with 4% glucose and a piece of agar (about 1 cm × 1 cm wide) was cut out and stained. The agar piece was placed on a slide glass and treated with chloroform vapor at room temperature for 10 min. The slide glass was covered with the staining buffer (50 mM sodium phosphate buffer (pH 7.5), 20 mg/ml 5-bromo-3-indoryl-D-galactopyranoside) and incubated at 37°C until the blue color was visible (usually for one to two hours), then observed

by microscopy.

Results

Cloning and sequencing of chiA

Amino acid sequences of fungal-type chitinases from yeasts and filamentous fungi so far reported were aligned and several conserved regions were identified. Three peptide sequences, I(E/K)TCQ, QFYNNY, and APQCPYPD, were chosen to design three degenerated oligonucleotide primers. By PCR using these, one specifically amplified fragment was obtained, the sequence of which was similar to parts of fungal-type chitinases. This fragment had one open reading frame (ORF) with a putative intron that had matched consensus sequences with the introns of *A. nidulans*.[25] A genomic DNA clone containing this sequence was subsequently cloned by screening the genomic DNA library constructed with λ2001. The 3.8-kb *Bam*HI-*Pst*I fragment (Fig. 1) of this clone, which was hybridizable with the PCR product, was cloned into pUC119 giving rise to pBP4 and the complete nucleotide sequence of the insert was analyzed. One ORF was identified by assuming one intron, the presence of which was confirmed by sequencing the cDNA clone of this gene (Fig. 2).

The predicted amino acid sequence consists of 660 amino acids with the calculated molecular mass of 69 kDa and was similar to the gene products of *S. cerevisiae CTS1*,[9] *C. albicans CHT2*, *C. albicans CHT3*,[10] *R. oligosporus chi1*,[11] and *R. niveus chi1* (DDBJ/EMBL/GenBank accession No. D10154) (The similarity is 38%, 38%, 40%, 34%, and 30%, respectively). The encoded polypeptide has a region with eight amino acid residues, +168 DGFDFDIE +175, which is suggested to be critical for chitinase activity[26] (Fig. 2). The amino terminal 21 residues are hydrophobic and thought to compose a secretory signal sequence. It follows a catalytic domain similar to the other chitinases. The carboxy terminal domain (+344 to +660) is rich in serine, threonine, and proline. Serine and threonine residues in this domain could be glycosylated by O-linked glycosyl chains as previously reported for *S. cerevisiae* Cts1p.[9] From extensive similarity in the primary sequences and domain organization, it is suggested that the gene codes for a chitinase similar to fungal-type chitinases, and it was designated *chiA*.

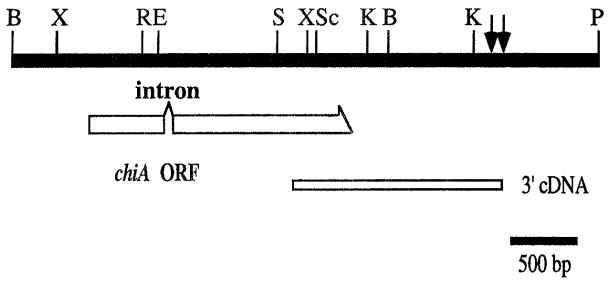

Fig. 1. Restriction Map of the *chiA* Gene.

An open arrow and an open box represent the predicted open reading frame separated by an intron and a cloned 3′-cDNA fragment of *chiA*, respectively. Vertical arrows represent polyadenylation sites.

Abbreviations: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; R, *Eco*RV; S, *Sal*I; Sc, *Sac*I; X, *Xho*I.

chiA was transcribed in the actively growing mycelia of *A. nidulans* and the transcript was found to be 3 kb long by Northern blot analysis (data not shown). The 3′-part of cDNA was cloned by 3′-RACE method. The nucleotide sequence of the clone indicated that a polyadenylation tail is added to the thymidine downstream of the predicted termination codon at either +2967 or +3061.

Characterization of the chiA *defective strain*

For the functional analysis of *chiA*, the ORF of *chiA* in the genome was disrupted by the *argB* gene by *in vivo* recombination between the genomic *chiA* locus and the introduced linearized plasmid pCHIΔ (Fig. 3A). By Southern blot analysis of the total DNA from one of the transformants named CGT3 using the 2.8-kb *Bam*HI fragment of the *chiA* gene as a probe, a signal was detected at 4.6 kb, while it was detected at 2.8 kb in the wild-type strain ABPU1, indicating that the expected homologous recombination occurred in CGT3 (Fig. 3B). The time courses of germ tube formation and the frequency of germination between ABPU1 and CGT3 on MM agar plate were compared. In the wild-type cells, germ tubes emerged 5 hr after inoculation and more than 95% of conidia germinated after 8 hr. In contrast, the frequency of germination in CGT3 was significantly lower, indicating that the *chiA* gene product (ChiA) is essential for normal germination (Fig. 4A). Although the morphology of hyphae of CGT3 was almost the same as that of wild-type cells by microscopy (data not shown), the average size of colonies was slightly smaller in CGT3 than in ABPU1 (Fig. 4B). Thus, it is suggested that ChiA is not essential but required for the normal rate of hyphal growth in *A. nidulans*.

On the other hand, CGT3 was normal in hyphal and conidiophore morphology even at high (42°C) or low (25°C) temperatures. Allosamidine (20 μg/ml) and demethylallosamidine (20 μg/ml), competitive inhibitors for various chitinases, had no effects on the growth. The intra- and extra-cellular chitinase activities were not so much different from these of the wild-type strain (data not shown), possibly, due to the residual redundant activities of the other chitinases in *A. nidulans* (see Discussion). These phenotypes of CGT3 were also observed in another transformant named CGT5.

Regulation of chiA *gene expression*

In the plasmid pCAZ2, the *chiA* promoter/leader (*p*/*l*) sequence was fused to the *E. coli lacZ* gene to monitor the expression of *chiA* by assaying β-galactosidase activity as a reporter enzyme. It was integrated into the chromosomal DNA by introducing it into ABPU1 after digesting with *Xho*I (Fig. 5). Southern blot analysis of the transformants indicated that homologous recombination occurred in one of the transformants, CGZ1. Significant β-galactosidase activity was detected in the mycelial extract of CGZ1 (See the legend for Fig. 6), suggesting that *chiA* was transcribed in the hyphae of *A. nidulans*. When conidiophores developed, β-galactosi-

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AnChiA     M-APKLFT-FVSALSGLAS--LASAFHAEAKSNIAVYYGQGVN---QPRLAEFCAETSYD
RoChi1     M-LARTFLGMAISAFLASTGVQAAWSSNGPNVMYYWGQNSAGGSNTQASLGTYCESGQVD
RnChi1     M-ISCNILGITIAAFITST--LAAYSSNGVNVMYYWGQNSAGGSNTQGSLVSYCQSGQVD
ScCts1     MSLLYIIL-LFTQFLLPPT--DAFDSSANTNIAVYWGQNSAGT---QESLATYCESSDAD
CaCht2     M-LSFKSL-LAAAVVASSA--LASASNQVA---LYWGQNGAGG---QERLAQYCQETDVD
           *                    *                      *  *   *     *

AnChiA     IINIGFINSF--PEQNPLTGLPGSDFGNQCWADTF--VVDGIASQLYSHCPNIAEDIPKC
RoChi1     AVLLSFLHVFNVGG------TPEINLSSAC-AGTYFPNTQLL------SCPAVGADIKKC
RnChi1     VIILSFLNKFNMGG------LPEINLASAC-EQTFFPNTNLL------HCPTVGSDIKTC
ScCts1     IFLLSFLNQF--P-------TLGLNFANAC-SDTF--SDGLL------HCTQIAEDIETC
CaCht2     IVLLSFLNLF--PD------PLNVNFANQC-GNTF--ESGLL------HCSQIGADIKTC
                *   *                  *   *               *     **  *

AnChiA     QAAGKKVFLSLGGATPTYWFDTIDASTKLADFLWGAFGPVTDA-WTVADKPRPFGNAVVD
RoChi1     QDKGVKVILSLGGAAGVYGFTSDAQGQQFAQTIWNLFGGGSSD-------TRPFGDAVID
RnChi1     QSNGVKVLLSLGGAAGSYGFSSDSEGQTFAETIWNLFGGGTSD-------TRPFDDAVID
ScCts1     QSLGKKVLLSLGGASGSYLFSDDSQAETFAQTLWDTFGEGTGAS------ERPFDSAVVD
CaCht2     QSLGKTVLLSLGGGVGDYGFSDVASATKFADTLWNKFGAGEDP-------ERPFDDAVVD
           *  *  * *****    * *          *   *  **             ***  ** *

AnChiA     GFDFDIEFFGSKGYANMIKRFRQRFGEVPDQTFYISAAPQCSIPDEQLSVAIKNAVIDFF
RoChi1     GVDLDIEGGASTGYAAFVNALRQKFSS----NFLIGAAPQCPFPDAILGSVLNSASFDYV
RnChi1     GIDLDIEGGSSTGYAAFVTALRSK--G----HFLIGAAPQCPFPDAILGSVIDAVGLDFV
ScCts1     GFDFDIENNNEVGYSALRTKLRTLFAEGT-KQYYLSAAPQCPYPDASVGDLLENADIDFA
CaCht2     GFDFDIEHGGATGYPELATALRGKFAKDTSKNYFLSAAPQCPYPDASLGDLLSKVPLDFA
           * * ***     **       *              *****  **           *

AnChiA     WVQFYNTPGCSARNFVLG-TKNG-FNYDSWVEVIK-AGANPNAKLYVGLPASGAAANLGY
RoChi1     NVQFYNN--------YCS-ATGSSFNFDTWDNWAKTTSPNKNVKIMFTIPGSPTAAGSGY
RnChi1     NVQFYNN--------VCSVASGSSFNFDVWNDWAKNKSPNKNIKVMLTVPGSSTAAGSGY
ScCts1     FIQFYNN--------YCS-VSGQ-FNWDTWLTYAQTVSPNKNIKLFLGLPGSASAAGSGY
CaCht2     FIQFYNN--------YCS-INGQ-FNYDTWSKFAD-SAPNKNIKLFVGVPATSNIA--GY
             ****           ** * *        * * *     *     *  **

AnChiA     -YLTPEEVKPLVKKYMDKYP-ETFGGVMLWEATQA-RNNQIDGVGYNEKIREILYDLDPN
RoChi1     -VPMSTLQTIVPSL---ASEYSSYGGVSVWDASQA-WNNG----DFSSQLYSLV-----H
RnChi1     -ASIAELGPIVSSV---ISQYSSFGGVSVWDASQA-WNNN----GFHSELYSIV-----H
ScCts1     ISDTSLLESTIADI---ASS-SSFGGIALWDASQA-FSNELNGEPYVEILKNLL-----T
CaCht2     -VDTSKLSSAIEEI---KCD-SHFAGVSLWDASGAWLNTDEKGENFVVQVKNVL-----N
                             *   *    *

AnChiA     HPPPTTSPTPTPTPSTTTTSTTSTTSTTSATSTTSTTSTTSTTSTTPTTSTTSTTTTP
RoChi1     SGGSTPPPSSSTIKTTTKATTTSTKTTTTAAPTATSAPGSCPVA----------NQSCS
RnChi1     GSSAALGQA-----ARKTPFPNTSSITTTSLATASSALFPLLYA----------GRSCS
ScCts1     SASQTATTTVASSKTSAASTSSASTSQKKTTQSTTSTQSKSKVTLSPTASSAIKTSITQT
CaCht2     QNACVAPSSSATTQSTTTTSSAVTQSTTTTSAAITQSATTTSAAVTTKSNQIVTSSSSSS

AnChiA     TPSPSPSTASSSTTETVTPSPKPSPSESSTTSETSSLPSTSTPVVSETPSETKTPTSSSA
RoChi1     TQNQYACTADGKYAVCDHGKWVVSSCPSGTVCIPTTDGTSIYCGYATGSGSTCPSASALE
RnChi1     SQSKVSCTSTGSYTICNYGKWVTAPCPPGVICLSSAQSNN--------------------
ScCts1     TKTLTSSTKTKSS--LGTTTTESTLNAVAITSMKTTLSSQITSAALVTPQTTTTSIVSSA
CaCht2     SSIFYGNSTTESSTGIATGTVLPTGSNENAATTGSGSNTKLAISTVTDVQKTVITITSCS

AnChiA     PPLSSSSPVGGSSSTASSSTSTPSETPSTSSTRAVSETSTHISTSTSSGPETSLTGSSTS
RoChi1     ----------IAAASFGSKNGPVPRPYKASK----VAAQLAVTSTDKNSFEAVINARRT
RnChi1     ----------------------------TDLT----VSAQFFITSFAGGSFKAIINARRT
ScCts1     ----------SLQTAITSTLSPATKSSSVVSLQTATTSKLSPTTTSTSSGSTSSDSTAR
CaCht2     EHKCVATPVTTGVVVVTDIDTVYTTYCPLTNSQVYVPVQTVVCTEETCVPSPTSTAQKPK
                                                    *

AnChiA     VPATSSSVPSSAISPSSTPVISETPRPPVTSSSSSTFVSSTSTSTDCSESSTAIGTHSSS
RoChi1     TLTPFEKSVTIEFTTPSNIKFTESDMGPVRQVGNKVRIQAKNDYNESM-TLVVKVKGSIN
RnChi1     TLTPFEKMKTIEFTVPDHVRLSQCNLGKIEQVGRSVRIRLKD--HPNM-AFVLNLSGNVS
ScCts1     TLAKELNAQYAAGKLNGKSTCTEGEIACSADGKFAVCDHSAWVYMECA-SGTTCYAYDSG
CaCht2     ASTTIKGVEKGQTTSYPVVGTTEGVKKIVTTSAQTVGSSTKYVTIELT-STITPVTYPTS

AnChiA     SIWETPSASTPAASPSTSPETTKTLTVFPRRAPPCLPEPPPRPLCPALFPQLAVATRKPQ
RoChi1     SGVFVAPSTS----------------------AWK----------------------
RnChi1     SDAFVAPDPS----------------------SWR----------------------
ScCts1     DSVYTQCNFS----------------------YLE----------------------
CaCht2     VASNGTNTTVPVFTFEGGAAVANSLNSVWFPVPFLLA--------------------

AnChiA     PCQPPVPTRHPAPRPASL
RoChi1     ----------------FK
RnChi1     ----------------FS
ScCts1     --------------SNYF
CaCht2     --------------AFAF
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Fig. 2. Comparison of the Deduced Amino Acid Sequences among Fungal Type Chitinases.

Identical and conserved residues are indicated by asterisks, while gaps are indicated by dash (-). The conserved region supposed to be an active site is boxed. A serine/threonine/proline rich-domain is underlined.

Abbreviations: AnChiA, *A. nidulans* ChiA; RoChi1, *R. oligosporus* chitinase I; RnChi1, *R. niveus* chitinase I; ScCts1, *S. cerevisiae* Cts1p; CaCht2, *C. albicans* Cht2p.

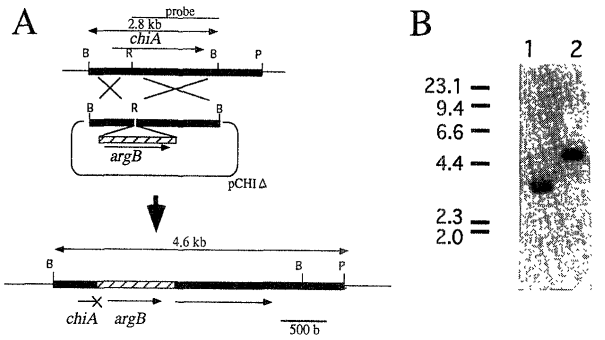

Fig. 3. Construction of *chiA* Deficient Mutants.

(A) Strategy for the gene disruption by introducing the plasmid pCHIΔ after digestion with *Bam*HI. (B) Southern blot analysis of the total DNA of ABPU1 (lane 1) and CGT3 (lane 2) digested with *Bam*HI using the 2.8 kb *Bam*HI fragment as a probe. Abbreviations: B, *Bam*HI; P, *Pst*I; R, *Eco*RV. Molecular weight markers are shown on the left.

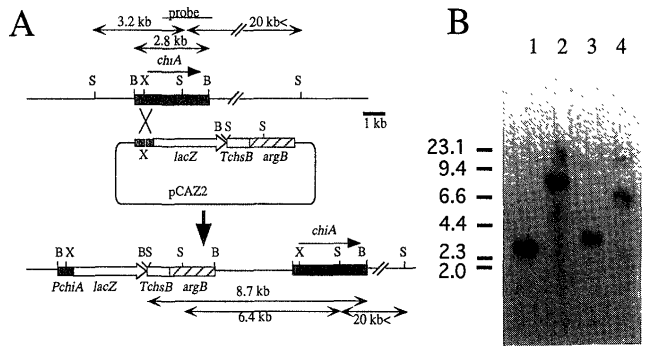

Fig. 5. Construction of CGZ1.

(A) Strategy for homologous recombination between the plasmid pCAZ2 digested with *Xho*I and the chromosomal *chiA* locus. (B) Southern blot analysis of the total DNA of ABPU1 (lanes 1, 3) and CGZ1 (*chiA*(*p*/*l*)::*lacZ*) (lanes 2, 4). Lanes 1 and 2, *Bam*HI digestion; lanes 3 and 4, *Sal*I digestion. Abbreviations are as in Fig. 1. Molecular weight markers are shown on the left.

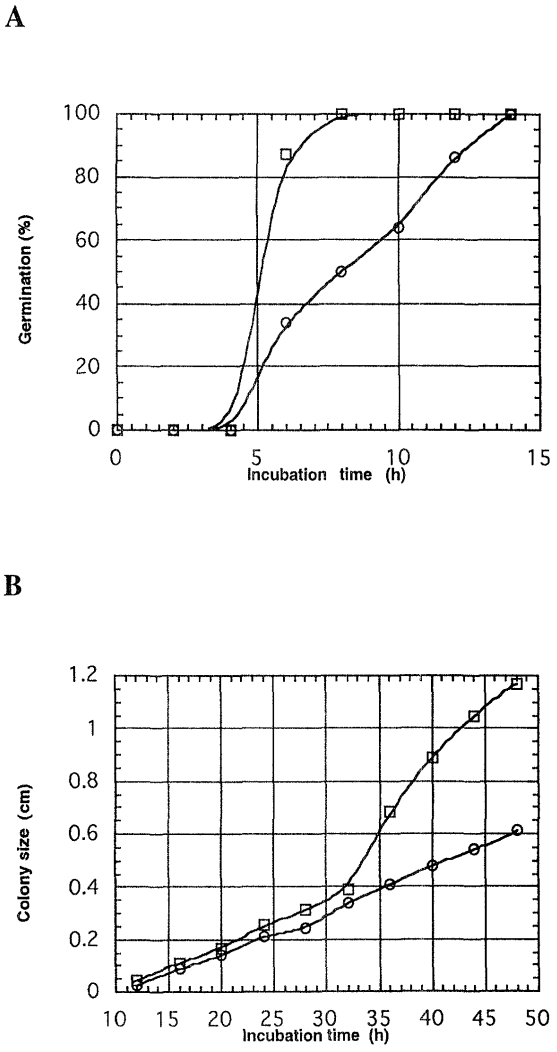

Fig. 4. Germination Frequency and Colony Size of the Wild Type (square) and *chiA*-deficient (circle) strains on MM agar plate.

(A) Relative frequency of germinated conidia obserbed under a microscope. Germination frequency was calculated from about 300 conidia of each strain. (B) Colony size after germination counted with a microscope. Colony size was measured by the average radii of 10 colonies.

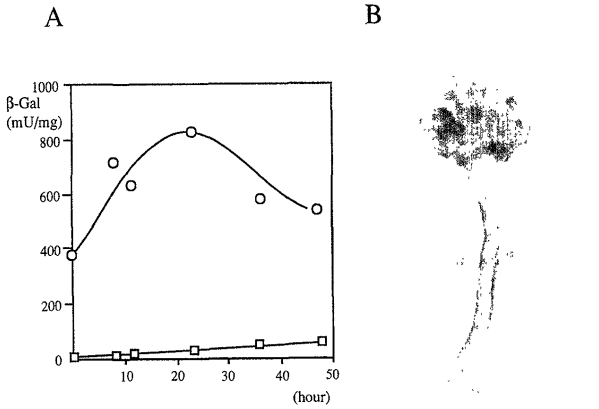

Fig. 6. Expression of the β-Galactosidase Gene under the Control of the *chiA* Gene Promoter. (A) β-Galactosidase activity after the induction of conidiophore development in the wild type (square) and CGZ1 (circle) strains. Note that chitinase activity without induction of conidiophore formation (the chitinase activity in mycelia) corresponds to the activity at zero hour. (B) *In situ* staining for β-galactosidase activity of conidiophore of *A. nidulans*.

dase activity increased approximately two-fold (Fig. 6A). *In situ* staining of the conidiophores for β-galactosidase activity showed that the expression of *chiA* was induced during conidiophore development (Fig. 6B). The non-transformed control strain ABPU1 had negligible β-galactosidase activity and was not stainable. These results suggest that *chiA* functions not only in growing hyphae but also in developing conidiophores.

Discussion

In this study we cloned and sequenced the *chiA* gene encoding a fungal-type chitinase from *A. nidulans*. The N-terminal region of ChiA shows overall similarity to the catalytic domain of the other fungal-type chitinases, although the similarity in the C-terminal region, the function of which is unknown, is quite low. A serine/threonine/proline rich (STP) domain is present in ChiA. The amino acids sequence in this domain shows no significant similarity to the known sequences in the

databases. A serine/threonine rich (ST) domain is seen in most chitinases reported[9-11] and suggested to be O-glycosylated. As the ST domain of Cts1p from *S. cerevisiae* contributes to efficient binding to chitin, the STP domain of ChiA might also act for high-level affinity to chitin. In plant cells, extensin, which is rich in proline and serine residues is found in the cell wall with most of the proline residues hydroxylated.[27] It is possible that the STP domain has a role to localize ChiA to the cell wall in *A. nidulans*.

Although the total chitinase activity did not decrease very much upon disrupting *chiA*, its disruption had significant effects on germination of conidia and hyphal growth. It is strongly suggested that the chitinase activity in the cells we mesured does not reflect precisely the chitinase-related activities required for conidia germination and hyphal growth. Chitinase activity is thought to be involved in the pathways that require cell wall degradation. Germination of conidia is one of such pathways because the rigid cell walls of conidia must first be loosened to form germ tubes. Our results suggest that ChiA would hydrolyze chitin before germ tube formation. The high expression of *chiA* in conidia also supports this possibility. The decrease in hyphal length in the *chiA* mutant suggests that ChiA is concerned also in tip growth of hyphae. By the Bartnicki-Garcia model,[14] hyphal tip extension proceeds with cell wall synthesis and partial cell wall degradation at the tips. Possibly, in the *chiA* mutant, chitin could not sufficiently be hydrolyzed at the hyphal tips, so that the hyphal tips may remain too rigid to extend at the normal rate.

Loss of ChiA did not cause complete loss of germination and hyphal growth, suggesting that the function of ChiA could partially be substituted by other chitinase(s) in the hyphae. In support of this suggestion, we recently cloned another chitinase gene (*chiB*) from *A. nidulans*.

Although our results suggest that transcription of *chiA* occurs in hyphae, higher expression in metulae, phialides, and conidia implies that ChiA functions in these cells. In *S. cerevisiae*, the fungal-type chitinase Cts1p is essential for separation between mother and daughter cells after budding.[9] In *A. nidulans*, it is suggested that cells proliferate from a vesicle of the stalk in bud-like growth.[28] The spatially regulated expression of *chiA* shown here is consistent with this suggestion. ChiA may be involved in bud-like growth of the developing cells in the filamentous fungi having a role in lysing chitin present in the bud neck between conidia.

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