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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Received June 23, 1997

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 conidiphore 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, philalides, 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, several chitin synthase-encoding genes were disrupted in *Aspergillus nidulans* and *Neurospora crassa* to show that some of the genes are essential for normal rate of hyphal growth of fungi.1,2 Chitinase (EC 3.2.1.14) 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, mycoparasitism, 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 extracts7 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-accumulating 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 pS51 (containing the argB gene, Motoyama et al.18) derived from ABPU1. ABPU1 and the transformants were cultured in MM medium18 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 (MCM). 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: Tn10 tet' (F' traD36 proAB lacF2ΔZAM15)] was used for construction and amplification of plasmids. For screening of the λ2001 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 midum (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and M 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 was used as the template for polymerase chain reaction (PCR) with the degenerated oligonucleotide primers: #A1, 5'-GAATTCCAT(AC)RARA NTGYCA-3' (corresponding to the sequence IE(K)/TCQ with an EcoRI restriction site, N, R, and Y indicate any one of four nucleotides, purine and pyrimidine, respectively); #A2, 5'-GTGCAGTTR TTTRTARAYTG-3' (corresponding to the sequence QFYNYN with a PstI restriction site); and #3, 5'-GANCNCARTGYCNC(AT) (NCCNGA-3' (corresponding to the sequence APQCPYYPD). The first PCR was done with 200 pm #A1 and 200 pm #A2 as primers and 0.5 μg 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 600 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-[32P]-dATP by T4 DNA ligase and used for screening of the genomic library constructed with λ2001 by plaque hybridization. A 3.4-kb BamHI-PstI 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. 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 24 hours as described. 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 using an Amplifinder 3'-RACE kit (Clontech) with the oligonucleotides #A1, 5'-CCTGGTGTTGGAAGCAGC-3'; #A2, 5'-CCTTTCTACATCGAACCC-3' as the first and the second primer specific primer, respectively.

Plasmid construction. DNA was manipulated by the standard methods. pB3 was constructed by inserting the 2.8 kb BamHI fragment of chia into the BamHI site of pUC119. pCHIA was constructed by ligating the blunt-ended argB fragment, which had been purified from pSS1 as the 1.8 kb SphI-BamHI fragment, with pB3, which had been digested with EcoRV 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 KpnI site followed by lacZ and the terminator sequence of chsB encoding chitin synthase 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'-CCTGGTACCCGACAGATAGTG-3' and 5'-AACAGTGGTAGTTCTGGA-3'. The product was finally digested with KpnI and ligated with KpnI-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 MCM medium. Mycelia were harvested, suspended in the lysis buffer (Mcllvaine 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-indoxyl-β-galactopyranoside) and incubated at 37°C until the blue color was visible (usually for one to two hours), then observed
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 consen-
sus sequences with the introns of A. nidulans. A genomic DNA clone containing this sequence was subsequently cloned by screening the genomic DNA library constructed with λ2001. The 3.8-kb BamHI-PstI 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, C. albicans CHT2, C. albicans CHT3, R. oligosporus chl, and R. niveus chl (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 (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. 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.

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 chia1 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 pCHL1 (Fig. 3A). By Southern blot analysis of the total DNA from one of the transformants named CGT3 using the 2.8-kb BamHI 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. Allosaminide (20 μg/ml) and demethylallosaminide (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 those 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 XhoI (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-
AnCh1A  M-APKLFPT-FVASLGLSLAS--LSAHFAEBASKINVYQQGQRPLPEACRFSTDY
Rch1l  M-LARTFLGMAIATASLASTQGAWSNPSNGVNYWQSSNSQAGSQAFLGTSCEQGVD
Rch1l  M-NCLGTVITTAFFIST--LASYSGSNHGNWQSSNSQAGSQAFLGTSCEQGVD
Sct1a  MSLYIIII-LFQFLPLPT---DAFSDSANNTINAVQNGNSAGT---EQLSTLYCSESSBAD
Cah2t  ML-SFSEL-IAAPVASSA---LSAASNQVA---LYQQNGNQAGG---QRLAQYCQETDUD

AnCh1A  IIN1UGIFS--PRQNLPTGLGPSDFGNCWADTF---VVDQGIAQLYSCHPNIAEDIPIC
Rch1l  AVLCILAFLGNCWADTF---PPEINLLSNC-AGTFQPTQL---CPPCNAVBDYX
Rch1l  VIILFSLKFNMQG---LPEINLADAC-ETQFTPMNLL---HCPTVGSIDYX
Sct1a  IFLSFLKMQP---PTLGLMFANAD-SDF---SDDGL---HCMTQIAEDFT
Cah2t  IVLSSFLKMQP---PTLGLMFANAD-SDF---SDDGL---HCMTQIAEDFT

AnCh1A  QAAGKEVFLGSLGATPTVFDDATSTKIVALDFLWGVPVTDAT-MTVADKPPFPNGAV
Rch1l  QDKGKVVLGSSAGYPSDQFTQDDTAINNFGGSSSD---TRPFDGAV
Rch1l  QCNQVYGLSSAGYPSDQFTQDDTAINNFGGSSSD---TRPFDGAV
Sct1a  QSLGKEVLSSAGGSLYPSDQAFEFQTLWDFGQVTAS---ERBPSDFAV
Cah2t  QSLGKEVLLSSAGGSLYPSDQAFEFQTLWDFGQVTAS---ERBPSDFAV

AnCh1A  TPFDIPIRRPGSKYANMIKRFQKPQVPVPQDYTPS1AASAPCTSEPDRLQFVSLKNHAVIDFF
Rch1l  TPFDIPIRRPGSKYANMIKRFQKPQVPVPQDYTPS1AASAPCTSEPDRLQFVSLKNHAVIDFF
Rch1l  QPNIDGLVYAAPFTLARSK---G---HFLIGAACPQPPFDIAIGSYVLINDPVF
Rch1l  QPNIDGLVYAAPFTLARSK---G---HFLIGAACPQPPFDIAIGSYVLINDPVF
Sct1a  QPNIDGLVYAAPFTLARSK---G---HFLIGAACPQPPFDIAIGSYVLINDPVF
Cah2t  QPNIDGLVYAAPFTLARSK---G---HFLIGAACPQPPFDIAIGSYVLINDPVF

AnCh1A  WQFYNYNPCSARNFVGL-TKGN-YNDSWVEYIK-AGANPBKLVLVPLASDAAALNGY
Rch1l  WQFYNYNPCSARNFVGL-TKGN-YNDSWVEYIK-AGANPBKLVLVPLASDAAALNGY
Rch1l  WQFYNYNPCSARNFVGL-TKGN-YNDSWVEYIK-AGANPBKLVLVPLASDAAALNGY
Rch1l  WQFYNYNPCSARNFVGL-TKGN-YNDSWVEYIK-AGANPBKLVLVPLASDAAALNGY
Sct1a  WQFYNYNPCSARNFVGL-TKGN-YNDSWVEYIK-AGANPBKLVLVPLASDAAALNGY
Cah2t  WQFYNYNPCSARNFVGL-TKGN-YNDSWVEYIK-AGANPBKLVLVPLASDAAALNGY

AnCh1A  -LYTPFSEYLVKVLKYMTP-ETFGOVMMAEATAA---RNQIKDQVSNVKEINLIDYLD
Rch1l  -LYTPFSEYLVKVLKYMTP-ETFGOVMMAEATAA---RNQIKDQVSNVKEINLIDYLD
Rch1l  -LYTPFSEYLVKVLKYMTP-ETFGOVMMAEATAA---RNQIKDQVSNVKEINLIDYLD
Rch1l  -LYTPFSEYLVKVLKYMTP-ETFGOVMMAEATAA---RNQIKDQVSNVKEINLIDYLD
Sct1a  -LYTPFSEYLVKVLKYMTP-ETFGOVMMAEATAA---RNQIKDQVSNVKEINLIDYLD
Cah2t  -LYTPFSEYLVKVLKYMTP-ETFGOVMMAEATAA---RNQIKDQVSNVKEINLIDYLD

AnCh1A  HPPFEPSEPTETSTSTTSSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTST
Rch1l  HPPFEPSEPTETSTSTTSSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTST
Rch1l  HPPFEPSEPTETSTSTTSSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTST
Rch1l  HPPFEPSEPTETSTSTTSSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTST
Sct1a  HPPFEPSEPTETSTSTTSSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTST
Cah2t  HPPFEPSEPTETSTSTTSSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTSTST

AnCh1A  TPSDEPSTASATTSTTVTPEPKDPSEZTSEZTSTETSPSTETPTPSPSTTSPSTTP
Rch1l  TPSDEPSTASATTSTTVTPEPKDPSEZTSEZTSTETSPSTETPTPSPSTTSPSTTP
Rch1l  TPSDEPSTASATTSTTVTPEPKDPSEZTSEZTSTETSPSTETPTPSPSTTSPSTTP
Rch1l  TPSDEPSTASATTSTTVTPEPKDPSEZTSEZTSTETSPSTETPTPSPSTTSPSTTP
Sct1a  TPSDEPSTASATTSTTVTPEPKDPSEZTSEZTSTETSPSTETPTPSPSTTSPSTTP
Cah2t  TPSDEPSTASATTSTTVTPEPKDPSEZTSEZTSTETSPSTETPTPSPSTTSPSTTP

AnCh1A  PLLCSSDPVQGSSTASSSTSTSTSTESTESTESTESTESTESTESTESTESTESTESTESTESTEST
Rch1l  PLLCSSDPVQGSSTASSSTSTSTSTESTESTESTESTESTESTESTESTESTESTESTESTESTEST
Rch1l  PLLCSSDPVQGSSTASSSTSTSTSTESTESTESTESTESTESTESTESTESTESTESTESTESTEST
Rch1l  PLLCSSDPVQGSSTASSSTSTSTSTESTESTESTESTESTESTESTESTESTESTESTESTESTEST
Sct1a  PLLCSSDPVQGSSTASSSTSTSTSTESTESTESTESTESTESTESTESTESTESTESTESTESTEST
Cah2t  PLLCSSDPVQGSSTASSSTSTSTSTESTESTESTESTESTESTESTESTESTESTESTESTESTEST

AnCh1A  VPATSSPVPEASISPSTVTSETETRPVPTVSTSESTESTSESTDSTETSSGALTPHVSE
Rch1l  VPATSSPVPEASISPSTVTSETETRPVPTVSTSESTESTSESTDSTETSSGALTPHVSE
Rch1l  VPATSSPVPEASISPSTVTSETETRPVPTVSTSESTESTSESTDSTETSSGALTPHVSE
Rch1l  VPATSSPVPEASISPSTVTSETETRPVPTVSTSESTESTSESTDSTETSSGALTPHVSE
Sct1a  VPATSSPVPEASISPSTVTSETETRPVPTVSTSESTESTSESTDSTETSSGALTPHVSE
Cah2t  VPATSSPVPEASISPSTVTSETETRPVPTVSTSESTESTSESTDSTETSSGALTPHVSE

AnCh1A  A565EAIASSEPTSAASAPSTTETETLTVPPR8PPECPLEPPRPPLCPALPDGLAVADPQ
Rch1l  A565EAIASSEPTSAASAPSTTETETLTVPPR8PPECPLEPPRPPLCPALPDGLAVADPQ
Rch1l  A565EAIASSEPTSAASAPSTTETETLTVPPR8PPECPLEPPRPPLCPALPDGLAVADPQ
Rch1l  A565EAIASSEPTSAASAPSTTETETLTVPPR8PPECPLEPPRPPLCPALPDGLAVADPQ
Sct1a  A565EAIASSEPTSAASAPSTTETETLTVPPR8PPECPLEPPRPPLCPALPDGLAVADPQ
Cah2t  A565EAIASSEPTSAASAPSTTETETLTVPPR8PPECPLEPPRPPLCPALPDGLAVADPQ

AnCh1A  PCFHFVPPDHAQRAPG
Rch1l  PCFHFVPPDHAQRAPG
Rch1l  PCFHFVPPDHAQRAPG
Rch1l  PCFHFVPPDHAQRAPG
Sct1a  PCFHFVPPDHAQRAPG
Cah2t  PCFHFVPPDHAQRAPG

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: AnCh1A, A. nidulans ChlA; Rch1l, R. oligosporus chitinase I; Rch1l, R. niveus chitinase I; Sct1a, S. cerevisiae Cts1p; Cah2t, C. albicans Chl2p.

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Fig. 3. Construction of chiA Deficient Mutants.
(A) Strategy for the gene disruption by introducing the plasmid pCHL4 after digestion with BamHI. (B) Southern blot analysis of the total DNA of ABPU1 (lane 1) and CGT3 (lane 2) digested with BamHI using the 2.8 kb BamHI fragment as a probe. Abbreviations: B, BamHI; P, PstI; R, EcoRV. Molecular weight markers are shown on the left.

Fig. 5. Construction of CGZ1.
(A) Strategy for homologous recombination between the plasmid pCAZ2 digested with XhoI and the chromosomal chiA locus. (B) Southern blot analysis of the total DNA of ABPU1 (lanes 1, 3) and CGZ1 (chiA(p/I)::lacZ) (lanes 2, 4). Lanes 1 and 2, BamHI digestion; lanes 3 and 4, SalI 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 observed 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
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of Education, Science, and Culture of Japan. This work was done using the facilities of the Biotechnology Research Center, The University of Tokyo.

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Acknowledgments

We thank Dr. S. Sakuda for providing allosaminamide and demethylallosaminide. This study was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan. This work was done using the facilities of the Biotechnology Research Center, The University of Tokyo.

Chitinase Gene of A. nidulans is seen in most chitinases reported and suggested to be O-glycosylated. As the ST domain of CtSlp 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. 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 measured 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, 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 CtSlp is essential for separation between mother and daughter cells after budding. In *A. nidulans*, it is suggested that cells proliferate from a vesicle of the stalk in bud-like growth. 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.