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Aspergillus nidulans ChiA is a glycosylphosphatidylinositol (GPI)-anchored chitinase specifically localized at polarized growth sites

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ABSTRACT

It is believed that chitinases play important physiological roles in filamentous fungi since chitin is one of the major cell wall components in these organisms. In this paper we investigated a chitinase gene, *chiA*, of *Aspergillus nidulans* and found that the gene product of *chiA* consists of a signal sequence, a region including chitinase consensus motifs, a Ser/Thr/Pro-rich region and a glycosylphosphatidylinositol (GPI)-anchor attachment motif. Phosphatidylinositol-specific phospholipase C treatment of the fusion protein of ChiA and enhanced green fluorescent protein (EGFP)—ChiA–EGFP—caused a change in its hydrophobicity, indicating that ChiA is a GPI-anchored protein. ChiA–EGFP localized at the germ tubes of conidia, at hyphal branching sites and hyphal tips. *chiA* expression was specifically high during conidia germination and in the marginal growth regions of colonies. These results suggest that ChiA functions as a GPI-anchored chitinase at the sites where cell wall remodeling and/or cell wall maturation actively take place.

1. Introduction

The cell wall of filamentous fungi is a complex structure mainly composed of polysaccharides, chitin and glucans. It is very important in fungal morphogenesis and in protection from diverse environmental stresses. Although the fungal cell wall has been considered to be an inert organelle, recent analyses revealed that it is a dynamic organelle in which constituent polymers are continuously synthesized, degraded, and chemically modified, and their structures are rearranged (Bernard and Latgé, 2001; Popolo et al., 2001). In the model fungus *Aspergillus nidulans*, chitin—a homopolymer of β-1,4-linked *N*-acetyl-D-glucosamine—is one of the major cell wall components. Six chitin synthase-encoding genes have been cloned and characterized in *A. nidulans*, demonstrating that chitin synthases play critical roles in hyphal growth and conidiophore development (Borgia et al., 1996; Fujiwara et al., 2000; Horiuchi et al., 1999; Ichinomiya et al., 2005; Motoyama et al., 1997; Takeshita et al., 2005, 2006; Yanai et al., 1994).

Chitinases (EC 3.2.1.14), which hydrolyze the β-1,4-glycosidic linkage of chitin, are widely distributed in living organisms. Recent analyses of the whole genome sequence of ascomycete filamentous fungi revealed that there are more than 10 putative chitinase-encoding genes in the genomes of these organisms (Pusztahelyi et al., 2006; Seidl et al., 2005; Taib et al., 2005).

However, the functions of most of these chitinases are currently not well understood. All of the chitinase genes analyzed thus far from yeast and fungi encode chitinases belonging to glycosyl hydrolase family 18 (Henrissat, 1991). These chitinases are further divided into two subclasses, class III and class V. In filamentous fungi, chitinases are considered to function in processes that include cell-wall degradation and modification, such as spore germination, tip growth and branching of hyphae, spore differentiation, autolysis and mycoparasitism (Adams, 2004; Gooday et al., 1992). Deletion of the genes *chiB1* of *Aspergillus fumigatus* or the *Cts1* of *Coccidioides immitis* that encode class V chitinases had no effect on the growth or morphogenesis of these organisms (Jaques et al., 2003; Reichard et al., 2000). A class V chitinase from the zygomycete *Rhizopus oligosporus* was suggested to function in the growth and morphogenesis of this fungus (Takaya et al., 1998b). Recently, we showed that a class V chitinase, ChiB, was induced by carbon starvation and functioned in the autolytic process of *A. nidulans* (Yamazaki et al., 2007). Induction of *chiB* expression under these conditions was also reported (Emri et al., 2006; Pusztahelyi et al., 2006). Class III chitinases are required for cell separation in *Saccharomyces cerevisiae* and *Candida albicans* (Dünkler et al., 2005; Kuranda and Robbins, 1991). In *R. oligosporus*, two class III chitinases were suggested to be primarily involved in autolysis (Takaya et al., 1998b; Yanai et al., 1992). We have previously reported that an *A. nidulans* deletion mutant of *chiA*—a class III chitinase-encoding gene—showed defects in spore germination and hyphal growth (Takaya et al., 1998a). However,

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these defective phenotypes were probably caused by a cryptic mutation(s) at a site other than the *chiA* locus because newly generated *chiA* deletion mutants did not show any phenotypic defects (Yamazaki et al., 2007).

In the present study, we show that (i) ChiA is synthesized as a GPI-anchored protein and modified by *O*-linked glycosylation, (ii) the expression level of *chiA* is very high during germination and in the marginal growth regions of colonieis and (iii) ChiA tagged with EGFP (enhanced green fluorescent protein) is specifically localized at polarized growth sites.

2. Materials and methods

2.1. Strains, cultures and media

Aspergillus nidulans strains used in this study are shown in Table 1. Minimal medium containing 1% glucose as a sole carbon source (MMG) and YG medium (0.5% yeast extract, 1% glucose, and 0.1% trace elements) for *A. nidulans* were used (Rowlands and Turner, 1973). To induce expression of genes under the control of *alcA* promoter (*alcA*(p)), we used 100 mM threonine and 0.1% fructose as carbon sources instead of glucose (MMTF or YTF). Media were supplemented with 0.2 mg/ml arginine, 0.02 mg/ml biotin, 0.5 mg/ml pyridoxine, 2.44 mg/ml uridine and 1.22 mg/ml uracil when necessary, and each supplement added was indicated by a single lowercase letter after the medium name. Strains were grown at 37 °C. Genetic transformation of *A. nidulans* was done by the method of Rasmussen et al. (1990).

2.2. Constructions of plasmids and A. nidulans strains

The 1.4-kb EcoRV–SacI fragment of pBP4 containing the *chiA* gene of *A. nidulans* (Takaya et al., 1998a) was ligated into HincII site of pUC19, to yield pES. For insertion of NotI site just upstream of the coding sequence of the Ser/Thr/Pro-rich region, polymerase chain reaction (PCR) was done for pES by using TaKaRa LA PCR in vitro Mutagenesis Kit (TaKaRa) with the primer 5′-AGGGGAGGTGGTGCGGCCGCGATGGTTGGGGTC-3′ (the under lined sequence represents the NotI recognition site), and the resulting DNA fragment digested with BamHI–SphI was ligated into pUC19 digested with BamHI–SphI, to yield pS13. A primer set of 5′-GGGCGGCCGCATGGTGAGCAAGGGCGAG-3′ and 5′-GGGCGGCCGCTCTTGTACAGCTCGTCCATG-3′ (the under lined sequences represent the NotI recognition sites) was used to amplify an EGFP-encoding sequence of pEGFP (Clontech). The 0.7-kb PCR-amplified product digested with NotI was ligated into pS13 digested with NotI, to yield pS13EGFP. The 1.8-kb StuI–SphI fragment of pBP4 and pS13EGFP digested with StuI–SmaI were ligated, blunted and ligated, to yield pS13EGFPC. The 1.7-kb XbaI–SphI fragment containing the *argB* gene of *A. nidulans* form pSS1 (Motoyama et al., 1994) was ligated into pS13EGFPC digested with SphI, to yield pCHIAEGFPARGB. The ChiA–EGFP strain was constructed by transformation of the strain ABPUS14 (Yamada et al., 2005) with NruI-digested pCHIAEGFPARGB. Arginine-prototrophic transformants were selected, and their total DNAs were used for Southern blot analysis to obtain clones in which *chiA* was replaced by *chiA–egfp* (Fig. 1A).

A primer set of GATGCGGCCGCGTGAGCAAGGGCGAGG and GTCGCGGCCGCTTTAC (the under lined sequences represent the NotI recognition sites) was used to amplify an EGFP-encoding sequence of pEGFP. The 0.7-kb PCR-amplified product digested with NotI was ligated into NotI-digested pBluescript SK$^+$, to yield pEGFP-Not. The 3.2-kb PstI–XbaI fragment containing the *sC* gene of *A. nidulans* from pUSC (Yamada et al., 1997) was ligated into pEGFP-Not digested with BamHI–PstI, to yield pEGFP–sC. pEGFP–sC was partially digested with NotI and self-ligated for deletion of the NotI site between *egfp* and *sC*, to yield pEGFP–sCNX. The 3.9-kb NotI–BssHII fragment of pEGFP–sCNX containing *egfp* and *sC* and pS13 digested with NotI were ligated, blunted and ligated, to yield pSTPEGFPsC. The ChiAn–EGFP strain was constructed by transformation of the strain ABPUS14 with the 5.3-kb PstI–StuI fragment of pSTPEGFPsC. Methionine-prototrophic transformants were selected, and their total DNAs were used for Southern blot analysis to obtain clones in which *chiA* was replaced by *chiAn–egfp* (Fig. 1A).

The 0.4-kb EcoRI–KpnI fragment containing the alcohol dehydrogenase gene promoter (*alcA*(p)) of *A. nidulans* from pAL3 (Waring et al., 1989) was ligated into pBluescript II digested with XbaI, to yield pBSalc. The 0.6-kb BamHI–PflMI fragment of pB3 containing the 5′-region of *chiA* gene of *A. nidulans* (Takaya et al., 1998a) and pBSalc digested with BamHI were ligated, blunted and ligated, to yield pNalc. The 3.2-kb PstI–XbaI fragment of pUSC containing *sC* and the 1.7-kb XbaI–SphI fragment of pSS1 containing *argB* were ligated into pNalc digested with SpeI, to yield pNalcsC and pNalcarg, respectively. The 2.5-kb MseI fragment of pBP4 was ligated into pNalcsC and pNalcarg digested with NotI, to yield pchiAalcsC and pchiAalcarg, respectively.

alc-ChiA–EGFP strain was constructed by transformation of the strain ChiA–EGFP with the 6.4-kb BamHI fragment of pchiAalcsC. Methionine-prototrophic transformants were selected, and their total DNAs were used for Southern blot analysis to obtain clones in which ChiA–EGFP fusion protein was expressed under the control of *alcA*(p) at *chiA* locus.

alc-ChiAn–EGFP strain was constructed by transformation of the strain ChiAn–EGFP with the 3.2-kb BamHI–BglI fragment of pchiAalcarg. Arginine-prototrophic transformants were selected, and their total DNAs were used for Southern blot analysis to obtain clones in which ChiAn–EGFP fusion protein was expressed under the control of *alcA*(p) at *chiA* locus.

alc-ChiA strain was constructed by transforming ABPUS14 with the 6.4-kb BamHI fragment of pchiAalcsC. Methionine-prototro-

Table 1
Aspergillus nidulans strains used in this study

Strain	Genotype	Reference
A26	*biA1*	FGSC*
ABPUS14	*biA1 pyrG89; wA3*; *argB2 sC114*; *pyroA4*	Yamada et al. (2005)
ABPUS/sC-2	*biA1 pyrG89; wA3*; *argB2 sC114*::*sC*; *pyroA4*	Yamada et al. (2005)
ABPUS/sC-2/argB8, 9	*biA1 pyrG89; wA3*; *argB2*::*argB sC114*::*sC*; *pyroA4*	This study
ABPUS/sC-2/argB9/pyroA17	*biA1 pyrG89; wA3*; *argB2*::*argB sC114*::*sC*; *pyroA4*::*pyroA*	This study
ABPUS/sC-2/argB9/pyroA17/pyrG20	*biA1 pyrG89*::*pyrG; wA3*; *argB2*::*argB sC114*::*sC*; *pyroA4*::*pyroA*	This study
alc-ChiA	*biA1 pyrG89; ΔchiA*::*sC-alcA*(p)-*chiA wA3*; *argB2 sC114*; *pyroA4*	This study
alc-ChiA/argB	*biA1 pyrG89; ΔchiA*::*sC-alcA*(p)-*chiA wA3*; *argB2*::*argB sC114*; *pyroA4*	This study
ChiA–EGFP	*biA1 pyrG89; ΔchiA*::*chiA-egfp-argB wA3*; *argB2 sC114*; *pyroA4*	This study
ChiAn–EGFP	*biA1 pyrG89; ΔchiA*::*chiAn-egfp-argB wA3*; *argB2 sC114*; *pyroA4*	This study
alc-ChiA–EGFP	*biA1 pyrG89; ΔchiA*::*sC-alcA*(p)-*chiA-egfp-argB wA3*; *argB2 sC114*; *pyroA4*	This study
alc-ChiAn–EGFP	*biA1 pyrG89; ΔchiA*::*argB-alcA*(p)-*chiAn-egfp-sC wA3*; *argB2 sC114*; *pyroA4*	This study

* Fungal Genetic Stock Center (University of Missouri, Kansas City).

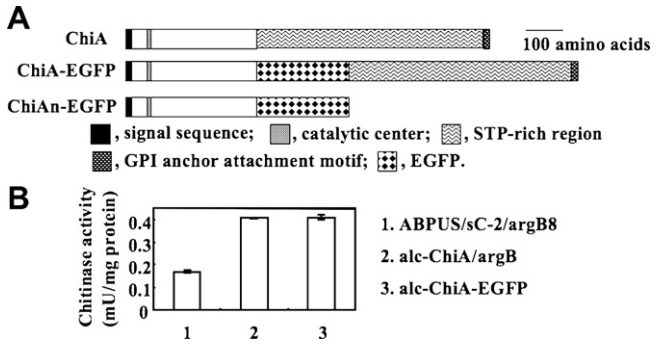

Fig. 1. Structure of ChiA. (A) Structures of ChiA, ChiA–EGFP and ChiAn–EGFP fusion proteins. (B) Chitinase activity of the cell extracts of the ABPUS/sC-2/argB8, alc-ChiA/argB and alc-ChiA–EGFP strains. Data are means ± standard error of mean (SEM) of the results of three independent experiments.

phic transformants were selected, and their total DNAs were used for Southern blot analysis to obtain clones in which *chiA* was expressed under the control of *alcA*(p) at *chiA* locus.

Strains alc-ChiA/argB and ABPUS/sc-2/argB8, 9 were constructed by transforming ABPUS/sC-2 with the pSS1 linearized by digestion with BglII containing *argB* of *A. nidulans*. Arginine-prototrophic transformants were selected, and their total DNAs were used for Southern blot analysis to obtain clones in which *argB2* was replaced by the wild-type *argB*.

Strain ABPUS/sC-2/argB9/pyroA17 was constructed by transforming ABPUS/sC-2/argB9 with the 2.7-kb BamHI–PstI fragment from pUCPYROA (Ichinomiya et al., 2005) containing *pyroA* of *A. nidulans*. Pyridoxin-prototrophic transformants were selected, and their total DNAs were used for Southern blot analysis to obtain clones in which the *pyroA4* mutation was replaced by the wild-type *pyroA*. Strain ABPUS/sC-2/argB9/pyroA17/pyrG20 was constructed by transforming strain ABPUS/sC-2/argB9/pyroA17 with the 1.4-kb NdeI–XhoI fragment from pJR15 (Oakley et al., 1987). Uridine and uracil-prototrophic transformants were selected, and their total DNAs were used for Southern blot analysis to obtain clones in which *pyrG89* was replaced by the wild-type *pyrG*.

2.3. Southern blot analysis

Southern blot analysis was performed with Gene Images AlkPhos Direct Labelling and Detection system (GE Healthcare) according to the manufacturer's instructions.

2.4. Gel electrophoresis and immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) under denaturing conditions was performed using the method of Laemmli (1970). For Western-blot analysis, proteins were transferred onto nitrocellulose membranes and probed with anti-GFP monoclonal antibody (invitrogen or Clontech) at a 1:1000 dilution, and then with horseradish peroxidase-conjugated goat anti-mouse IgG (Cell Signaling Technology) at a 1:5000 dilution. The antibody complexes were detected using the ECL system (GE Healthcare) following the manufacturer's instructions.

2.5. PI-PLC treatment

For preparation of membrane fraction, mycelia of the alc-ChiA–EGFP strain grown on 50 ml of YTFuu medium was suspended in 3 ml of LY buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) with 10 μl of protease inhibitor cocktail (Sigma) and broken using a Sonicator (Branson). The homogenates were centrifuged in a microcentrifuge for 5 min at 5000 rpm at 4 °C and the supernatants were collected (The pellet was used for laminarinase treatment, see below.). The supernatant was subsequently centrifuged for 15 min at 15,000 rpm at 4 °C and the pellet was used as a membrane fraction. The treatment of the membrane fractions with phosphatidylinositol specific phospholipase C (PI-PLC) was performed as described previously (Hamada et al., 1998) with some modifications. The membrane fraction was resolved in 100 μl of LY buffer without NaCl containing 0.2 U of PI-PLC from *Bacillus cereus* (Sigma P-5542). After incubation at 33 °C for 30 min, 1 ml of 10% Triton X-114 was added to the reaction mixture followed by the incubation on ice for 2 h. After the subsequent incubation at 33 °C for 3 min, the solution was centrifuged for 30 s at 10,000 rpm at room temperature and separated into an aqueous phase and a detergent phase. The aqueous phase was extracted three times by addition of a drop of 10% Triton X-114 followed by centrifugation as above and the detergent phase was extracted also three times by addition of 1 ml of LY buffer without NaCl. Proteins in both phases were separately precipitated by addition of trichloroacetic acid (10% final) with carrier bovine serum albumin (0.01% final). Precipitated proteins were washed three times with cold acetone, dried by a vacuum pump, and analyzed by SDS–PAGE and Western blotting.

2.6. Cell wall digestion

The pellet described in PI-PLC treatment was suspended into LY buffer containing 2% SDS, incubated for 10 min at 100 °C and centrifuged for 5 min at 5000 rpm at 4 °C. This step was repeated once again. The resulting pellet was washed five times with LM buffer (100 mM sodium acetate, pH 5.5, 1 mM EDTA) for the treatment with Laminarinase (Sigma) and Yatalase (TaKaRa) or with LP buffer (10 mM sodium phosphate, pH 7.5, 150 mM NaCl, 5 mM EDTA) for the treatment with Glucanex (gift from Novozyme Co.) and Lysing enzyme (Sigma). The washed cell walls were incubated with 500 μl of LM buffer containing 0.5 U of laminarinase or 10 mg of Yatalase or with 500 μl of LP buffer containing 50 μl of Glucanex or 5 mg of lysing enzyme for 90 min at 37 °C. The treated samples were centrifuged for 5 min at 15,000 rpm at 4 °C and resulting supernatant was analyzed by SDS–PAGE and Western blotting.

2.7. TFMS treatment

TFMS treatment was performed as described previously (Edge et al., 1981). Briefly, 0.2 g of wet mycelia grown on 50 ml of YTFuu medium were broken with metal-corn (YASUI KIKAI) and then glass beads (YASUI KIKAI) using Multi-beads shocker (YASUI KIKAI) in 200 μl of TNE buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing 5 μl of protease inhibitor cocktail (Sigma). After adding another 300 μl of TNE buffer and removal of cell debris by centrifugation for 5 min at 2600 rpm at 4 °C, the supernatant was centrifuged for 5 min at 15,000 rpm at 4 °C and the resulting supernatant was used as cell extract. The cell extract containing 1 mg of protein was freeze-dried and was dissolved in 1 ml of a mixture of anisole and TFMS, which was made by mixing 1 ml of anisole and 2 ml of TFMS in a glass tube and cooled to 0 °C. The reaction mixture was cooled to 0 °C and nitrogen was bubbled through the solution for 30 s followed by subsequent incubation on ice for 30 min. It was mixed with a 50-fold excess of diethylether containing 10% (v/v) of *n*-hexane and incubated at −40 °C for 1 h. If no precipitate formed within 20 min, a drop of pyridine was added to help to initiate protein precipitation. The precipitated protein was collected by centrifugation for 5 min at 2000 rpm at 4 °C, followed by the second ether wash and re-centrifugation. The resultant pellet was suspended with ice cold 95% ethanol, re-centrifuged, and then the pellet was dried by a vacuum pomp and analyzed by SDS-PAGE and Western-blot analysis.

2.8. Glycopeptidase F treatment

The alc-ChiA–EGFP strain grown on 50 ml of MMTFbpuu medium was broken after being frozen in liquid N_2. The resulting powder of 0.2 g was suspended in 500 μl of buffer (50 mM Tris–HCl, pH 8.3, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) with 5 μl of protease inhibitor cocktail (Sigma). The solution containing 14.5 μg of protein was subjected to the treatment of glycopeptidase F (TaKaRa) following the manufacturer's instructions and analyzed by SDS–PAGE and Western blotting.

2.9. Total RNA isolation and real-time RT-PCR analysis

Total RNA was isolated using RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer's instructions. Real-time RT-PCR was performed using ExScript™ RT reagent Kit (TaKaRa) according to the manufacturer's instructions. For the PCR, *chiA* specific primers, 5′-GATGAGACCGCCACGACAAG-3′ and 5′-GGGCAGATGCTGGTGTACGA-3′ were used. For making the standard, 1.0×10^1–10^7 copies of genomic DNA of *A. nidulans* A26 strain were used.

2.10. Chitinase activity assay

Conidia of 5.0×10^7 were grown on 50 ml of MMTFbpuu at 37 °C for 18 h. Wet mycelia of 0.2 g were used for preparation of cell extract. The cell extract was prepared as described in the TFMS treatment section. Chitinase activity of the cell extract of each strain was measured using colloidal chitin as a substrate as described by Yanai et al. (1992).

2.11. Fluorescent microscopic analysis

Mycelia on plates were observed under a fluorescent microscope (BX52, Olympus) equipped with an automatic camera (ORC-ER, Hamamatsu Photonics).

2.12. Sequencing of chiA gene

The published *chiA* sequence (Takaya et al., 1998a) contained some sequence errors. Correct sequence of the *chiA* gene was registered on DDBJ database (Accession No. D87895).

3. Results

3.1. Structure of the chiA gene product ChiA

The *chiA* gene of *A. nidulans* has an open reading frame (ORF) of 2883 bp that encodes a 961-amino acid protein. The deduced amino acid sequence of ChiA indicates two hydrophobic regions at the amino and carboxyl termini. A short C-terminal region is similar to the glycosylphosphatidylinositol (GPI) anchor attachment motif. The best position for the ω-site for GPI attachment predicted by the fungal big-II predictor is Gly^{936} and the second best position is Ala^{937} (Eisenhaber et al., 2004) (http://mendel.imp.univie.ac.at/gpi/fungi_server.html). Thus, ChiA apparently consists of an N-terminal signal sequence, a region including chitinase consensus motifs, a Ser/Thr/Pro-rich (STP-rich) region, and a C-terminal GPI-anchor attachment motif (Fig. 1A).

3.2. ChiA is an O-linked glycoprotein

In order to obtain an insight into the physiological function of ChiA, we attempted to express a ChiA–EGFP fusion protein using the endogenous *chiA* promoter. Unfortunately, we were unable to detect any specific signal when cell extracts of the ChiA–EGFP strain were subjected to Western-blot analysis using an anti-GFP antibody (data not shown). Therefore, we placed the ChiA–EGFP construct under the control of the *alcA* promoter (*alcA*(p)) at the *chiA* locus. Under the induced condition, the chitinase activity in the cell extract of this alc-ChiA–EGFP strain was almost the same as that of the alc-ChiA/argB strain in which wild-type *chiA* was expressed under the control of *alcA*(p). The observed activity was twofold higher than that of the wild-type strain ABPUS/sC-2/argB8. These results demonstrated that ChiA–EGFP was enzymatically active (Fig. 1B). It is to be noted, that the molecular mass of ChiA–EGFP was greater than 200 kDa (Fig. 2A) although the predicted molecular mass of ChiA–EGFP was 120 kDa, suggesting that ChiA–EGFP was subjected to posttranslational modifications.

ChiA has many potential *O*-linked glycosylation sites in its STP-rich region. To determine whether ChiA–EGFP was modified by *O*-linked glycosylation in its STP-rich region, we constructed the strain alc-ChiAn–EGFP that produced a ChiA–EGFP protein consisting of the N-terminal signal sequence and the subsequent region including chitinase consensus motifs tagged with EGFP (ChiAn–EGFP; Fig. 1A). Cell extracts of the alc-ChiA–EGFP and alc-ChiAn–EGFP strains were treated with trifluoromethanesulfonic acid (TFMS)—a reagent that removes O-linked glycans (Edge, 2003)—and ChiA–EGFP and ChiAn–EGFP were detected by Western-blot analysis. Two major bands of higher electrophoretic mobility than that of ChiA–EGFP were detected after the treatment of this protein with TFMS (arrows in Fig. 2B), whereas the size of ChiAn–EGFP was

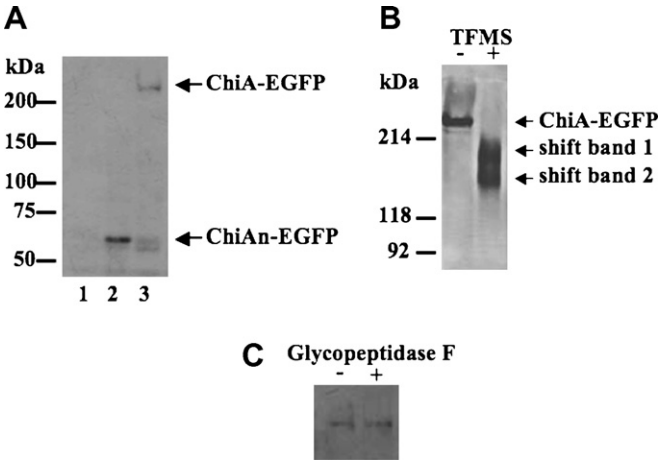

Fig. 2. ChiA is an O-linked but not N-linked protein. (A) The cell extracts of the ChiA–EGFP and ChiAn–EGFP strains were subjected to Western-blot analysis. Lane 1, alc-ChiA; lane 2, alc-ChiAn–EGFP; lane 3, alc-ChiA–EGFP. (B,C) The cell extract of the ChiA–EGFP strain was treated with TFMS (B) and glycopeptidase F (C) and subjected to Western-blot analysis.

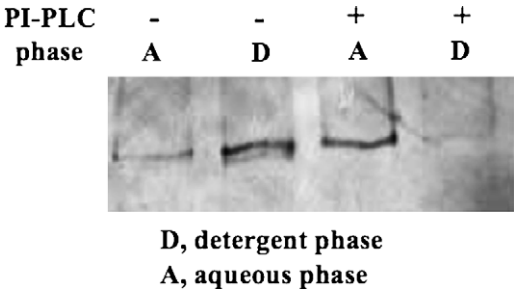

Fig. 3. ChiA is a GPI-anchored protein. The membrane extract of the alc-ChiA–EGFP strain was treated with or without PI-PLC followed by fractionation by Triton X-114 and subjected to Western-blot analysis.

not changed after the treatment (data not shown). Treatment with glycopeptidase F, that removes N-linked glycans, did not change the mobility of ChiA–EGFP in spite of the presence of a potential *N*-glycosylation site (^{650}NQT^{652}) (Fig. 2C). These results demonstrate that ChiA is modified by *O*-linked glycosylation at its STP-rich region.

3.3. ChiA is a GPI-anchored protein

We examined whether ChiA is GPI-anchored. When the membrane extract of the alc-ChiA–EGFP strain was fractionated with Triton X-114, ChiA–EGFP was recovered in the detergent phase (Fig. 3), suggesting that it is an integral membrane protein. Preincubation of the membrane extract with PI-PLC resulted in the recovery of ChiA–EGFP in the aqueous phase but not in the detergent phase, indicating that ChiA is a GPI-anchored protein.

It has been proposed that in yeast there are two types of GPI-anchored proteins: plasma membrane-resident and cell wall-targeted proteins (Ash et al., 1995; Chen et al., 1995; Conzelmann et al., 1988; van der Vaart et al., 1995). To investigate whether ChiA is a plasma membrane-resident or cell wall-targeted protein, the cell wall fraction of the alc-ChiA–EGFP strain was digested with cell wall-hydrolyzing enzymes (Laminarinase, Yatalase, Glucanex or Lysing enzyme) and the resulting supernatants were subjected

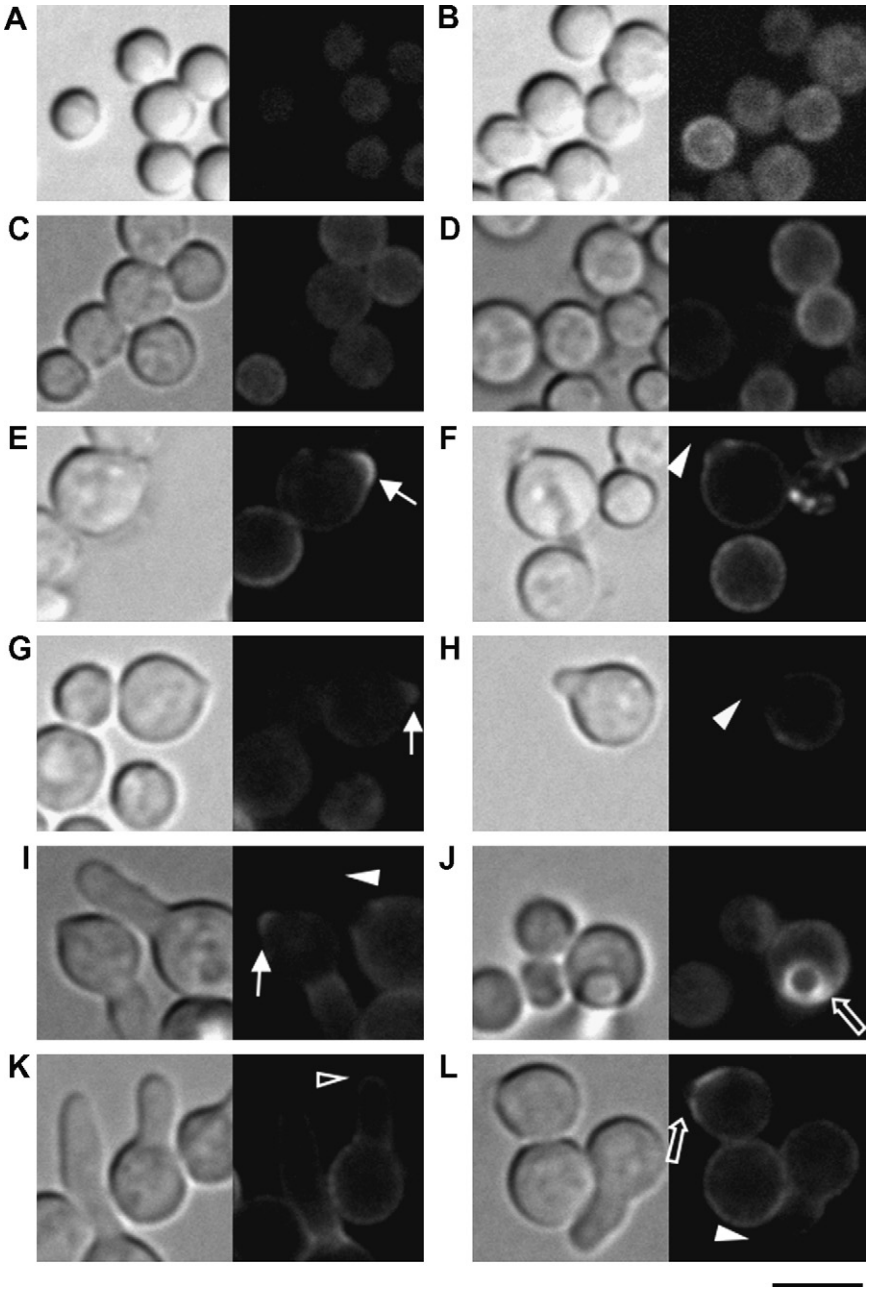

Fig. 4. Localization of ChiA–EGFP and ChiAn–EGFP fusion proteins during germination. Conidia of alc-ChiA–EGFP (A–L) and alc-ChiAn–EGFP (M–T) strains were incubated on MMTFbpuu for 1 h (A & M), 2 h (B & N), 3 h (C & O), 4 h (D&P), 5 h (E, F, G, H, Q & R) and 6 h (I, J, K, L, S & T). The left and right panels show DIC and EGFP fluorescent images, respectively, of the same field. Bars: 5 μm. Fluorescence at germination sites, that disappeared from the germinating tips, that of ring-like patterns, and that observed at the tip of the germ tube are indicated by arrows, arrowheads, open arrows, and open arrowheads, respectively. See text for details.

to Western-blot analysis. No signal was detected by anti-GFP antibody (data not shown), suggesting that ChiA is a plasma membrane-resident type GPI-anchored protein.

3.4. Localization of ChiA–EGFP

We examined the subcellular localization of ChiA–EGFP and ChiAn–EGFP. After 2 h of incubation in a germination medium, ChiA–EGFP fluorescence appeared as weak spots inside the conidia and at the conidial surface (Fig. 4B). After 3 to 4 h of incubation, the fluorescence was observed mainly at the surface of the cell (Figs. 4C and D). After 5–6 h of incubation, strong signals were observed at germination sites just beginning to bulge (arrows in Figs. 4E, G, and I). However, soon after this stage, the fluorescence disappeared from the germinating tips (arrowheads in Figs. 4F and H–J), but it appeared at the surface bordering the germination sites in a ring-like pattern (open arrows in Figs. 4J and L). At a later stage of incubation, weak signals were sometimes observed at the tip of the germ tube (open arrowheads in Fig. 4K). In contrast, no strong signals were observed at the germination sites and hyphal tips in the ChiAn–EGFP strain (Figs. 4M–T), although weak signals were observed inside the conidia and on the conidial surfaces after 1–3 h of incubation (Figs. 4M–T). These results suggest that the observed localization of ChiA–EGFP is dependent on its GPI-anchor and/or STP-rich region.

Next, we examined the localization of ChiA–EGFP during the hyphal growth phase. A strong ChiA–EGFP fluorescence was observed at hyphal branching sites (arrows in Figs. 5A and B). Strong fluorescence was detected around the branching sites in a ring-like pattern (open arrows in Figs. 5C and E); however, fluorescence at the branching tips was not observed (arrowheads in Figs. 5C and D). Strong fluorescence was sometimes observed at the hyphal tips (open arrowheads in Fig. 5A) and was also observed at almost all septa (stars in Figs. 5A–E). In contrast, strong ChiAn–EGFP fluorescence was only observed at the septa (stars in Figs. 5F–H), suggesting that the localization of ChiA–EGFP at septa could be dependent on its N-terminal signal sequence.

Using EGFP as a marker protein, our data demonstrate the subcellular localization of ChiA at germination sites, hyphal branching sites, and sometimes at hyphal tips and support the notion that the STP-rich region and the GPI-attachment motif are key elements for the specific subcellular distribution of this chitinase.

3.5. Expression pattern of chiA

The expression of *chiA* was investigated during conidia germination and hyphal growth by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). During conidia germination, the expression level of *chiA* dramatically increased until 2 h of incubation, and then gradually decreased (Fig. 6A). This suggests that *chiA* is involved in the isotropic growth and/or germ tube emergence of conidia. We dissected the colony of *A. nidulans* into three regions, corresponding to the growth and developmental stages of the mycelium (Masai et al., 2006) and measured the expression level of *chiA* in these regions by real-time RT-PCR (Fig. 6B). The outermost region comprises the tip cells of the hyphae growing actively along the surface of the agar medium (marginal growth region of the colony); this is followed by a white-colored region consisting of substrate hyphae and vertically growing aerial hyphae (middle region); the innermost region consists of

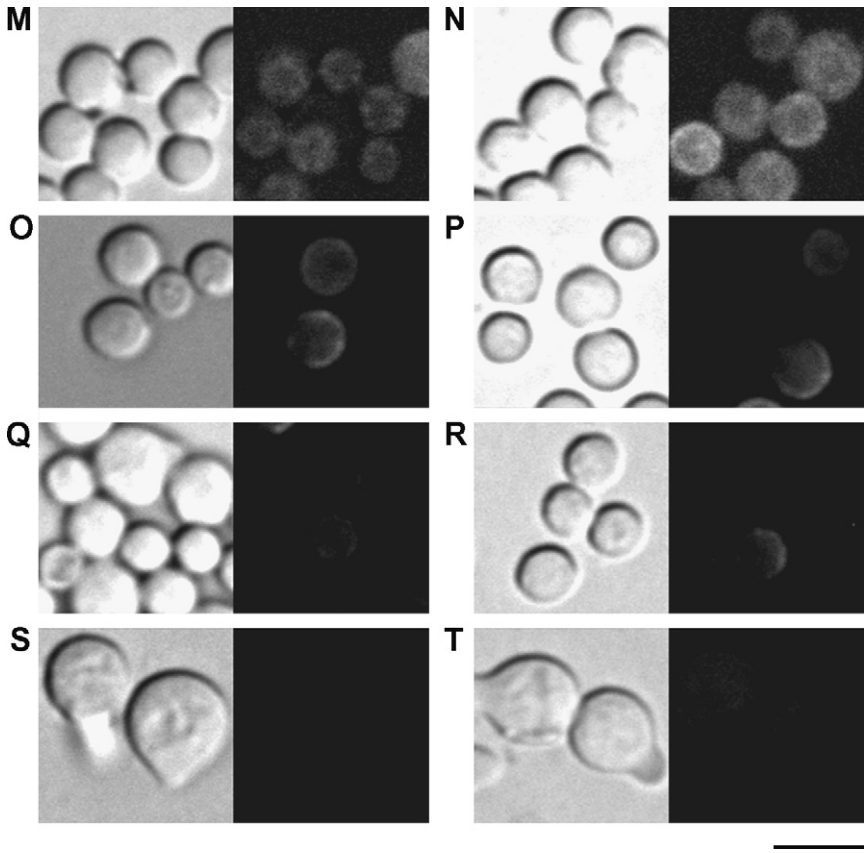

Fig. 4 (*continued*)

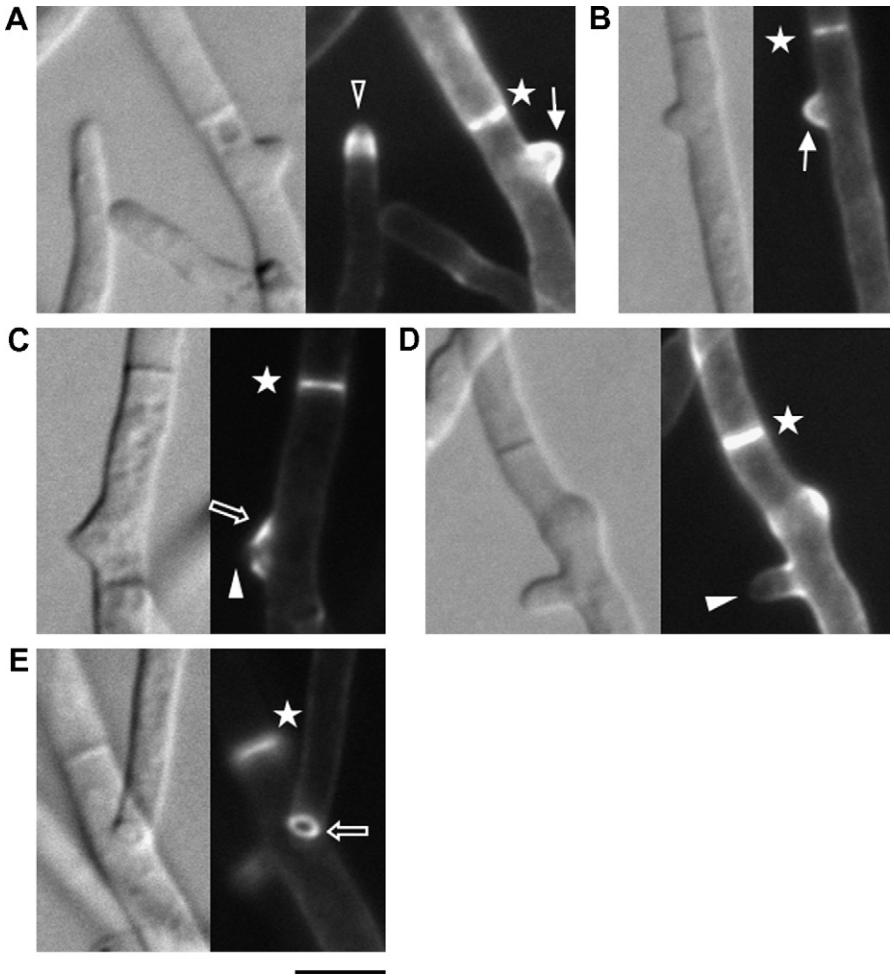

Fig. 5. Localization of ChiA–EGFP and ChiAn–EGFP fusion proteins during hyphal growth. Conidia of alc-ChiA–EGFP (A–E) and alc-ChiAn–EGFP (F–H) strains were incubated on MMTFbpuu for 44 h. The left and right panels show DIC and EGFP fluorescent images, respectively, of the same field. Bars: 5 μm. Fluorescence observed at hyphal branching sites, that of ring-like patterns, that not observed at the branching tips, that observed at the hyphal tips, and that observed at almost all septa are indicated by arrows, open arrows, arrowheads, open arrowheads, and stars, respectively. See text for details.

old substrate hyphae and fully differentiated conidiophores (basal region). The expression level of *chiA* was considerably higher in the marginal growth region of the colony than in the middle and basal regions (Fig. 6B). This result is consistent with the localization of ChiA–EGFP to the marginal growth regions and further supports our notion that *chiA* functions in the growing hyphal region.

4. Discussion

In the present study, we provide evidence that ChiA is an O-linked and GPI-anchored protein that is specifically localized at germination sites just before and after conidia germination, at hyphal branching sites, and sometimes at hyphal tips. We also demonstrated the specific expression of *chiA* during germ tube emergence and at hyphal tip regions. We hypothesize that ChiA remodels the rigid structure of cell wall chitin to a more loose structure at the germination sites of conidia and at the hyphal branching points and is involved in the maturation of the newly synthesized chitin at the hyphal tip regions.

The amino acid sequence of the C-terminus of ChiA is characteristic of GPI-anchored proteins; in fact, treatment of ChiA–EGFP with PI-PLC indicated that ChiA is a GPI-anchored protein (Fig. 3). In *S. cerevisiae* certain proteins initially anchored by a GPI moiety to the plasma membrane, and subsequently cross-linked to β-1,3-glucans through β-1,6-glucans, are thought to be major constituents of yeast cell wall organization (Klis et al., 2002). However, ChiA–EGFP was not recovered from the cell wall after cell wall-digesting enzyme treatment (data not shown), suggesting that ChiA is retained in the plasma membrane. The treatment of the ChiA–EGFP protein with TFMS demonstrated that ChiA–EGFP is an *O*-glycosylated protein (Fig. 2B). Two major bands of smaller derivatives of ChiA–EGFP were detected by Western-blot analysis after the treatment of ChiA–EGFP with TFMS. It is possible that there are two types of O-linked glycans on the surface of the ChiA protein—one type is easily cleaved by TFMS and the other type is hardly cleaved due to their locations in ChiA. It is also possible that the shift band 2 was derived from the shift band 1 by the cleavage of the acid-labile peptide bond in ChiA–EGFP during TFMS treatment. Since the sizes of ChiA–EGFP treated with TFMS were approximately 180 kDa and 150 kDa and were not coincident with the calculated size of ChiA–EGFP, it is likely that other modifications occurred in ChiA. Recently, it was reported that a *Neurospora crassa* ortholog of ChiA is possibly a GPI-anchored protein (Bowman et al., 2006).

Bartnicki-García presented a model showing that a "delicate balance between synthesis and lysis" exists in the growing hyphal apex (Bartnicki-García, 1999). In the case of conidia germination and hyphal branching, it is apparently considered that cell wall lytic enzymes are necessary for loosening and extending the cell wall structure before germ tube emergence and hyphal branching, but

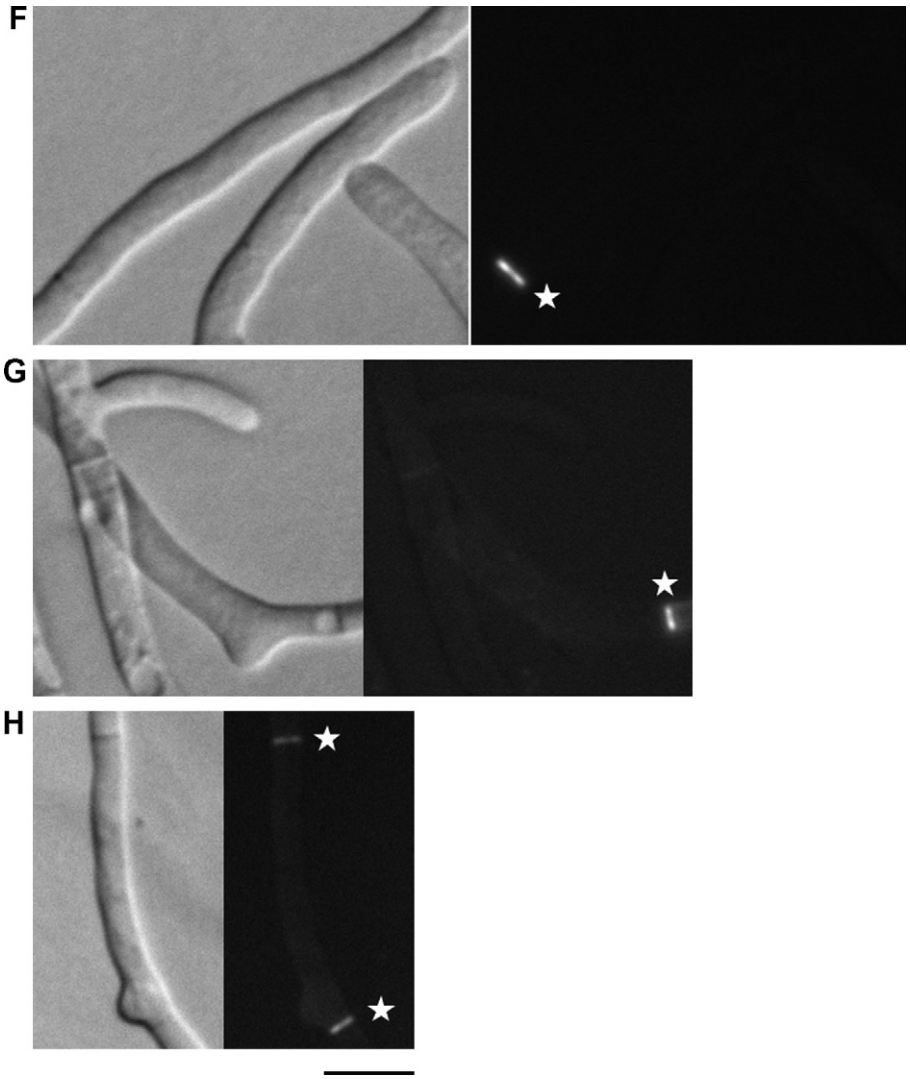

Fig. 5 (*continued*)

there is no report concerning the localization of cell wall lytic enzymes in filamentous fungi. In this study, we provide evidence that ChiA–EGFP is specifically localized at conidia germinating points and hyphal branching points when germination and branching just begin, respectively. The fluorescence of this fusion protein disappeared from the tip of the germ tube and the branching points but showed a ring-like pattern at the basal region (Figs. 4 and 5). It was reported that the tip growth of early germlings was slower and fluctuated compared with that of mature hyphae (Horio, 2007). Thus, the mechanism of tip growth of early germlings would be partly different from that of mature hyphae. These localization patterns were not observed for ChiAn–EGFP, suggesting that the distinctive localization of ChiA–EGFP was dependent on its GPI-anchor and/or the STP-rich region. Since some chitinases have transglycosidase activity as well as chitinase activity (Fukamizo et al., 2001; Jaques et al., 2003; Xia et al., 2001), it is likely that ChiA also has transglycosidase activity. If this is the case, ChiA–EGFP that remains at the basal region of the germ tube and branching hyphae, forming a ring-like fluorescence pattern, might function to maintain cell wall integrity in response to cell wall defects at newly developing regions that are susceptible to physiological stress from the outer environment. We speculate that GPI-anchor attachment is important for the proper localization of ChiA at germination sites, hyphal branching sites, and hyphal tips. Since proteins that have a signal sequence at their N-termini are known to localize at both hyphal tips and septa (Maruyama and Kitamoto, 2007), the localization of ChiA–EGFP at septa is likely to be dependent on its N-terminal signal sequence. The notion that ChiA functions in the morphogenesis of *A. nidulans* at polarized growth sites is compatible with the specific expression of *chiA* during germination and at the marginal growth region of colonies (Fig. 6). Our conclusion is further supported by two previous reports. First, in *A. nidulans chiA* was highly expressed in submerged cultures during the vegetative growth phase compared with autolytic phase (Pusztahelyi et al., 2006). Second, a chitinase gene-encoding ChiA1, the *A. fumigatus* ortholog of ChiA, was highly expressed in the early stages of growth (Taib et al., 2005). Since there are more than 15 putative genes-encoding chitinases in the *A. nidulans* genome (our unpublished result), other chitinases may play roles similar to those of ChiA when the function of ChiA is defective.

In conclusion, we provide first insight into the role of an *A. nidulans* chitinase in conidia germination and hyphal branching, a function that could involve loosening of the rigid chitin of the cell wall to permit the emergence of germ tubes and new hyphal tips. In addition, ChiA may function in the maturation of the cell wall architecture containing chitin at the hyphal tips.

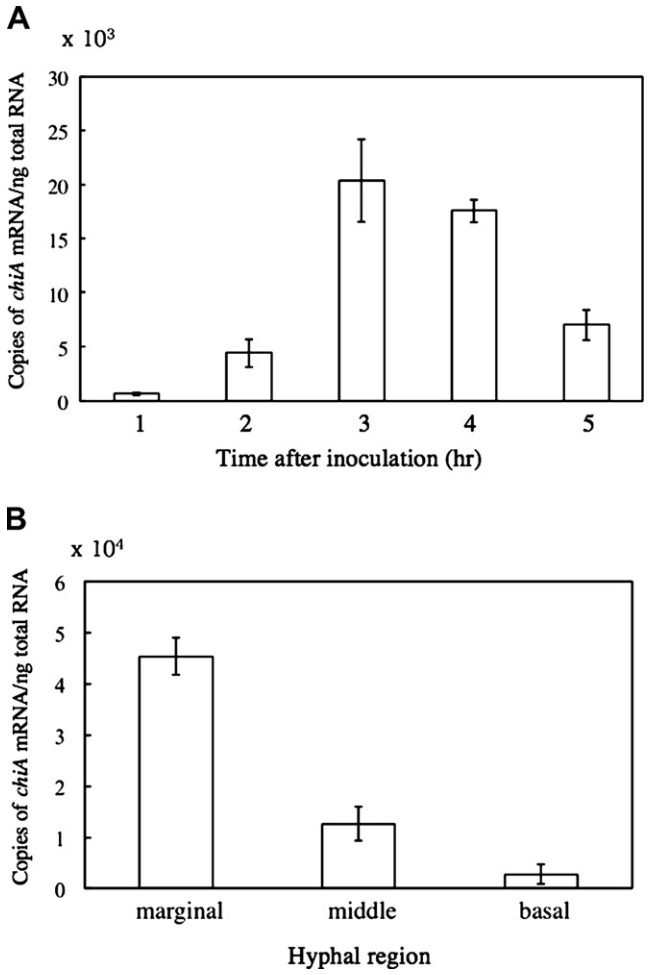

Fig. 6. The expression pattern of *chiA* during conidia germination and hyphal growth. (A) Conidia of 2.0×10^8 of ABPUS/sC-2/argB8/pyroA17/pyrG20 were incubated on 50 ml of YG liquid medium for indicated hours and the total RNA of growing conidia were subjected to real-time RT-PCR analysis. (B) Hyphal colony grown on YGuu medium for 3 days were divided in three regions according to square-plate culture method (Masai et al., 2006). The total RNAs of these samples were subjected to real-time RT-PCR analysis. Data are means ± S. E. M. of the results of three (A) or four (B) independent experiments.

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