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Evidence That the *Aspergillus nidulans* Class I and Class II Chitin Synthase Genes, *chsC* and *chsA*, Share Critical Roles in Hyphal Wall Integrity and Conidiophore Development[1]

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Although many chitin synthase genes have been identified in a broad range of fungal species, there have been only a few reports about their role in fungal morphogenesis. In most cases, single gene disruption or replacement did not reveal their function, possibly because of functional redundancy among them. We obtained null mutants of *Aspergillus nidulans chsA* and *chsC* genes encoding non-essential class II and class I chitin synthases, respectively. The *ΔchsA ΔchsC* mutant exhibited growth defects on media supplemented with sodium dodecyl sulfate (SDS), high concentration of salts, chitin-binding dyes, or chitin synthase competitive inhibitors, suggesting loss of integrity of hyphal wall. Moreover, remarkable abnormalities of the double mutant were observed microscopically during its asexual development. The conidiophore population was drastically reduced. Interestingly, secondary conidiophores were occasionally produced from vesicles of the primary ones. The morphology of these conidiophores was similar to those of the *A. nidulans* developmental mutants, *medusa* (*medA*), *abacus* (*abaA*), and some kinds of *bristle* (*brlA*). *In situ* staining patterns suggested that *chsA* was mainly expressed in the metulae, phialides, and conidia, whereas *chsC* was expressed in hyphae as well as conidiophores. These results suggest that ChsA and ChsC share critical functions in hyphal wall integrity and differentiation.

Key words: *Aspergillus nidulans*, chitin synthase, conidiophore development, hyphal wall integrity, multigene family.

Chitin, a β-1,4-linked homopolymer of *N*-acetylglucosamine, is one of the major structural components of the fungal cell wall. Its synthesis, degradation and assembly are important for fungal morphogenesis (*1–5*). Chitin synthases, encoded by *chs* genes, are membrane-bound proteins responsible for the catalytic polymerization of *N*-acetylglucosamine from UDP-sugar. The *chs* genes constitute a multigene family, products of which have been structurally subdivided into five groups: class I to V. Class V is constituted of the recently identified CsmA (chitin synthase with a myosin motor–like domain)-type enzymes (*6–10*). The distribution of a set of *chs* genes in a broad range of fungal species suggests that chitin synthases belonging to each of these classes play distinct roles in cell wall biogenesis. Five chitin synthase genes representing each class have been isolated by our group from the filamentous fungus *Aspergillus nidulans* and designated *chsA* (class II), *chsB* (class III), *chsC* (class I), *chsD* [class IV, called *chsE* by Specht *et al.* (*11*)] and *csmA* [class V, a part of *csmA* is called *chsD* by Specht *et al.* (*11*)], respectively (*9*, *12–14*).

Studies on chitin synthases in filamentous fungi are less advanced than those in the budding yeast *Saccharomyces cerevisiae*, in which chitin is a minor cell wall component (*4*, *5*). However, previous studies have clarified the importance of the class III–chitin synthases on hyphal growth in *Neurospora crassa*, *A. nidulans*, and *Aspergillus fumigatus* (*12*, *15–17*). In addition, the *Aspergillus* class V–chitin synthase and CsmA-type products have been shown to be critical for the maintenance of hyphal wall integrity and the polarized synthesis of cell wall (*11*, *18*, *19*). In contrast, single gene disruptions or replacements of class I, II, and IV enzymes of *A. nidulans*, *N. crassa*, or *Ustilago maydis* did not result in noticeable effects (*7*, *11–14*, *20–23*). One possible explanation for this is that other enzymes with similar or overlapping function masked defects otherwise resulted from a single gene disruption. The recent finding that the *A. nidulans* ChsA and ChsD share functions in conidial formation might support this idea (*14*). However, the physiological functions of these three classes have not yet been demonstrated.

Asexual reproduction in filamentous fungi usually causes conspicuous morphological changes accompanied by the remodelling of wall structures. In *A. nidulans*, the mechanisms of remodelling have been extensively analyzed at the

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genetic and molecular levels (*24–26*). The multicellular reproductive machinery, the conidiophore, is composed of a stalk, a vesicle, sterigmata (a metula and a phialide), and asexual spores, called conidia. A linear core pathway composed of three sequentially expressed development regulatory factors, BrlA, AbaA, and WetA, plays a central role in conidiophore development (*26–32*). Two genes, *stuA* and *medA*, are known as modifiers that are necessary for proper cell patterning and spatial organization of conidiophores. They are involved in the temporal and spatial coordination of expression of the transcription factors BrlA and AbaA (*26, 33, 34*).

In this paper, we report that double disruption of the *A. nidulans chsA* and *chsC* genes causes severe defects in growth and development. Interestingly, the abnormal structures of conidiophores of the double mutant resembled those of the *medA, abaA*, and certain *brlA* mutants.

MATERIALS AND METHODS

Strains and Transformation—The *A. nidulans* strains used in this study are listed in Table I. Complete (CM), YG (0.5% yeast extract, 1% glucose, and 0.1% trace elements), and minimal (MM) media for *A. nidulans* were as described (*35*). Transformation was performed by the method of May (*36*), and the transformants obtained were grown in MM with appropriate supplements (arginine, 0.25 mg/ml; biotin, 0.02 μg/ml; pyridoxine, 0.05 μg/ml; uridine, 2.44 mg/ml).

Plasmid Construction—The 1.8-kb *Bam*HI–*Sph*I fragment of pSS1, containing the *argB* gene, was inserted into the *Nru*I site of pchsA (*14*) to yield pAΔNr5-4. The 2.0-kb *Pst*I–*Xba*I fragment of pP1 (*13*) was blunted and ligated to bacterial alkaline phosphatase–treated and blunted *Nsp*I–*Xho*I digested pchsC to yield pCXNΔP4. The 4.3-kb *Sca*I–*Cla*I fragment of pchsA, containing the *chsA* promoter (4 kb) and a part of *chsA* open reading frame (0.3 kb), and the 2.0-kb *Bgl*II–*Spl*I fragment of pchsC (*14*), containing the *chsC* promoter (1.6 kb) and a part of *chsC* open reading frame (0.5 kb), were ligated to the *Sma*I site of pSS-TB-LZ (*37*) to yield pAL and pCL, respectively.

TABLE I. ***A.nidulans*** **strains used in this study.**

Strain	Genotype	Reference
ABPU1	*biA1 pyrG89 wA3 argB2 pyroA4*	*14*
AU1	*pyrG89 wA3 argB2*	*13*
ABPU/A1	*biA1 pyrG89 wA3 argB2 pyroA4* [pSS1]	This study
ABPU/AU	*biA1 pyrG89 wA3 argB2 pyroA4* [pSS1] [pP1]	*14*
A-4	*biA1 pyrG89 wA3 argB2 pyroA4 chsA::argB*	*14*
C2-11	*biA1 pyrG89 wA3 argB2 pyroA4 chsC::pyr4*	*14*
C2-11/A	*biA1 pyrG89 wA3 argB2 pyroA4 chsC::pyr4* [pSS1]	This study
AC-7,8	*biA1 pyrG89 wA3 argB2 pyroA4 chsA::argB chsC::pyr4*	This study
ACo-2	*biA1 pyrG89 wA3 argB2 pyroA4 chsA::argB chsC::pyr4*	This study
CA-2,4,10,11, 12,15	*biA1 pyrG89 wA3 argB2 pyroA4 chsC::pyr4 chsA::argB*	This study
AL1	*pyrG89 wA3 argB2* [pAL]	This study
CL5	*biA1 pyrG89 wA3 argB2 pyroA4* [pCL]	This study

Gene Disruption and Southern Analysis—All of the *chs* gene disruptants were derived from ABPU1 (*14*), which was auxotrophic to arginine, biotin, pyridoxine, and uridine. *A. nidulans argB* and *N. crassa pyr4* were used as selective markers to complement arginine and uridine auxotrophy, respectively. Total DNA was extracted as described (*38*). For Southern analysis, DNA labelling and detection were carried out with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, UK). The *chsC* of strain A-4 (*ΔchsA*) was disrupted by pCΔP1 as described previously (*14*). Southern blot analysis yielded two disruptants, named AC-7 and AC-8 (data not shown). The *chsC* of strain A-4 (*ΔchsA*) was also disrupted by transformation with the 4.3-kb *Pst*I fragment from pCXNΔP4. Southern analysis of the *Bgl*II- and *Eco*RI-digested total DNA of 10 transformants probed with the 5.1-kb *Apa*I–*Kpn*I fragment from pchsC yielded one disruptant, ACo-2 (data not shown). The *chsA* of strain C2-11 (*ΔchsC*) was disrupted by transformation with the 6.0-kb *Apa*I fragment from pAΔNr5-4. Southern analysis of the *Sac*I-digested total DNA of 18 transformants probed with the 6.7-kb *Sac*I fragment from pchsA yielded six disruptants, CA-2, -4, -10, -11, -12, and -15.

Northern Analysis—Total RNA was isolated using an RNeasy Total RNA kit (QIAGEN) according to the manufacturer's instructions. Northern analysis was done as described previously (*9*). A 1.2-kb *Cla*I-digested fragment of pchsA (*14*) and a 1.1-kb *Spl*I-digested fragment of pchsC (*13*) were used as probes for detection of transcripts of *chsA* and *chsC*, respectively.

Reagents—The effect of reagents on hyphal growth was tested at 37°C in media supplemented with Calcofluor white (fluorescent brightner 28, Sigma), Congo red (Wako), nikkomycin Z (Calbiochem, USA), polyoxin B, and polyoxin D (kind gifts from Dr. Yamaguchi) at the indicated concentrations.

Conidiation Efficiency—Approximately 5×10^8 fresh conidia were spread on a CM plate containing arginine, biotin, pyridoxine, and uridine. After 3 days of culture at 37°C, conidia were collected and counted with a hemocytometer (Erma Tokyo, Japan).

Light and Fluorescent Microscopy—Mycelia from plates were stained with 0.01% Calcofluor white or 0.0001% 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Wako) solutions, and observed with a fluorescent microscope (BHS-RFK, Olympus) equipped with an automatic camera (PM-10ADS, Olympus).

Scanning Electron Microscopy (SEM)—Mycelia from plates were fixed for 1 h at 4°C with 2% glutaraldehyde, washed with phosphate-buffered saline (pH 7.4), and dehydrated in a graded ethanol series (50, 70, 80, 90, and 99.5%). After transfer to isoamyl acetate, samples were critical-point dried, coated with gold, and observed under a scanning electron microscope (HCP-2, Hitachi, Tokyo).

Construction of Strains to Monitor Expression of chsA and chsC—Strains in which expression of β-galactosidase was under the control of the *chsA* or *chsC* promoter were constructed as follows. pAL was digested with *Kpn*I and transformed into AU1 (*13*). Southern analysis of the *Eco*RI-digested total DNA of the transformants probed with the 1.2-kb *Cla*I-fragment from pchsA confirmed the integration of pAL into the genomic *chsA* locus in one strain, which was designated AL1. pCL was digested with *Bgl*II and

transformed into ABPU1. Southern analysis of the *Xba*I-digested total DNA from the transformants probed with the 1.1-kb *Stu*I–*Hin*dIII fragment from pSS1 (*14*) confirmed the integration of pCL into the genomic *argB* locus in one strain, which was designated CL5. Integration of one copy of the DNA fragment in question in each strain was confirmed by Southern analysis of the *Eco*RI- or *Sma*I-digested total DNA from AL1, or the *Bam*HI-, *Hin*dIII-, or *Xba*I-digested total DNA from CL5, probed with the 3.0-kb *Bam*HI fragment from pSS-TB-LZ.

Chitin Contents—Chitin content was determined by the method of Specht *et al.* (*11*) with the following modifications. The strains were grown in 200 ml of MM medium for 20 h at 37°C. Mycelia were collected, washed with distilled water, and suspended in 1 ml of 1.0 M KOH. The suspension was sonicated, heated in a boiling-water bath for 20 min, and centrifuged. The supernatants were assayed for protein concentrations. The pellets were washed twice with distilled water, then treated with enzymes as described by Specht *et al.* (*11*). This suspension was then centrifuged, and the GlcNAc content of the supernatant was determined by the method of Reissig *et al.* (*39*). The protein concentration was measured with a BCA protein assay kit (Pierce).

In Situ Staining of the β-Galactosidase Activity—The methods previously reported (*40*, *41*) were modified as follows. A 20-μl portion of conidial suspension (10^8/ml) was spread on a MM plate and cultured for 13 or 36 h at 37°C. Mycelia were sampled, treated with chloroform for 20 min at room temperature, then stained with a staining solution [0.05 M sodium phosphate, pH 7.5, 0.02% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal, Wako)] for 2 h at 37°C, and observed by light microscopy.

RESULTS

Double Disruption of A. nidulans chsA and chsC—We constructed three kinds of *ΔchsA ΔchsC* double mutants by targeted disruption of *chsC* and *chsA* in strains already carrying a deletion of *chsA* (strain A-4) (Fig. 1A) and *chsC* (strain C2-11) (Fig. 1C), respectively (Table I). Strains AC-7 and AC-8 (AC type) were derived from strain A-4 by replacing *chsC* on the chromosome of strain A-4 with *pyr4* of *N. crassa* (Fig. 1C). Strain ACo-2 (ACo type) was derived from strain A-4 by replacing the 1.9-kb *Xho*I–*Nsp*I fragment of *chsC* with the *pyr4* DNA of *N. crassa* (Fig. 1D). Strains CA-2, 4, 10, 11, 12, and 15 (CA type) were derived from strain C2-11 by disrupting *chsA* with the *argB* DNA of *A. nidulans* (Fig. 1B). Since all strains of AC type, ACo type, and CA type showed similar phenotypes in terms of growth rate, hyphal morphology, and conidiophore structure, strain AC-8 was selected for further experiments. Northern analysis of ABPU/AU (wild type), C2-11 (*ΔchsC*), A-4 (*ΔchsA*), and AC-8 (*ΔchsA ΔchsC*) is shown in Fig. 2, A and B. The absence of RNA expression of *chsA* or *chsC* was confirmed in AC-8. The growth rate of AC-8 was almost the same as that of the wild-type strain, but hyphal density was reduced considerably and pigmentation was observed (Fig. 3). In this case, ABPU/AU and AC-8 formed white conidia due to *wA3* mutation. In addition, AC-8 produced few aerial hyphae (data not shown).

Involvement of ChsA and ChsC in Hyphal Wall Assem-

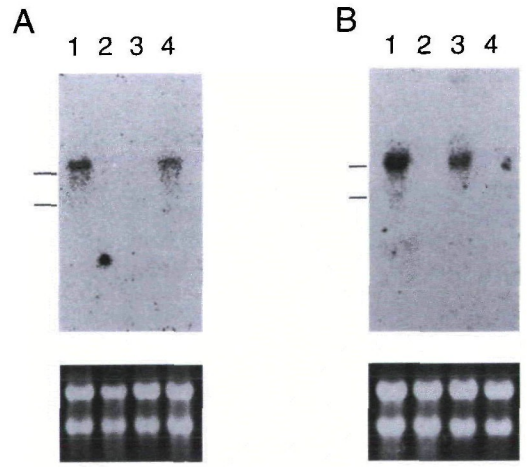

Fig. 2. **Northern analysis of expression of *chsA* (A) and *chsC* (B) in the wild-type strain (ABPU/AU) (lane 1), AC-8 (lane 2), A-4 (lane 3), and C2-11 (lane 4).** A 1.2-kb *Cla*I-digested fragment of pchsA (A) and a 1.1-kb *Spl*I-digested fragment of pchsC (B) were used as probes. Approximately 5 μg of total RNA was loaded on each lane. Positions of rRNAs are indicated by the bars on the left. EtBr staining of rRNA is shown in the lower panels.

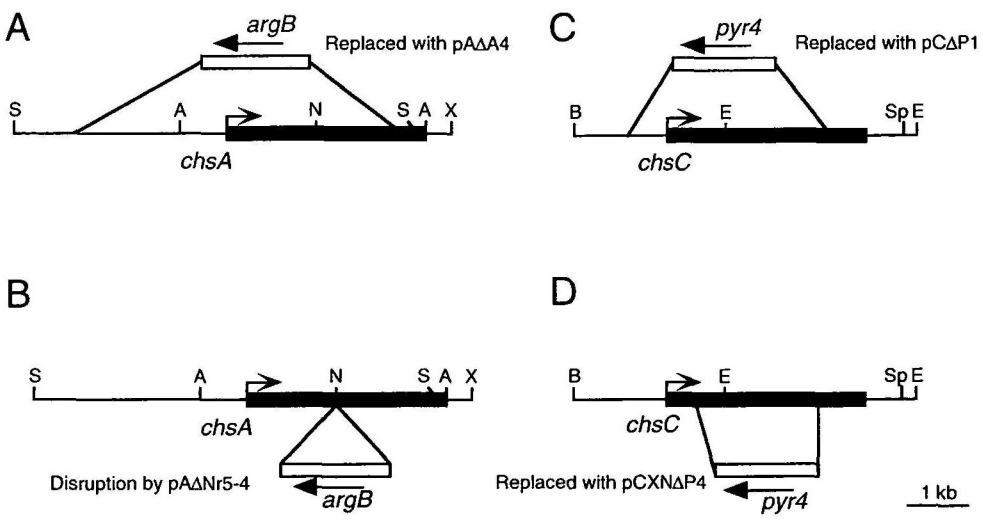

Fig. 1. **Construction of the *ΔchsA ΔchsC* mutants.** Schematic representation of the targeted disruption of *A. nidulans* chitin synthase genes. (A and B) *chsA*. (C and D) *chsC*. Black bold bars indicate open reading frames of *chsA* or *chsC*. Restriction sites are abbreviated as follows: A, *Apa*I; B, *Bgl*II; E, *Eco*RI; S, *Sac*I; Sp, *Sph*I; X, *Xba*I.

bly—We next characterized the defects of hyphal wall assembly of the double mutant in detail. Fluorescent microscopic analysis with Calcofluor white and SEM analysis revealed that aerial hyphae of the double mutant were morphologically similar to those of the wild-type strain (Fig. 4 and data not shown). Conidiophores frequently fell down (data not shown). We further tested the effect of various reagents on hyphal growth, including salts (NaCl [1.2 M], KCl [1.2 M]), SDS (10 μg/ml), chitin-binding re-agents (Calcofluor white [10 μg/ml], Congo red [10 μg/ml]), and chitin synthase inhibitors (nikkomycin Z [10 μg/ml], polyoxin B [1 μg/ml], polyoxin D [1 μg/ml]). All of these reagents severely inhibited the colony extension of AC-8 (*ΔchsA ΔchsC*) but did not affect that of the wild-type strain, A-4 (*ΔchsA*), or C2-11 (*ΔchsC*). No remarkable morphological differences were found microscopically between mycelia of strain AC-8 and the wild-type strain treated with these reagents (data not shown).

Defects of the Double Mutant in Conidiophore Development—To determine the effect of *chsA* and *chsC* null mutations on conidiation, we measured the conidia formation efficiency of *ΔchsA*, *ΔchsC*, and *ΔchsA ΔchsC* mutants. Conidiation of AC-8 (*ΔchsA ΔchsC*) was less than 0.01% of that of the wild-type strain (ABPU/AU) (Table II). Abnormal morphology of conidiophores was microscopically visible in AC-8 but not in ABPU/AU, A-4 (*ΔchsA*), or C2-11 (*ΔchsC*) (Figs. 4 and 5, data not shown). AC-8 formed branching chains of elongated sterigmata, and few conidia at the tips of sterigmata which were not separated completely (Figs. 4B and 5A). These sterigmata would be defined as metulae because they could branch. Each compartment was septated and contained one or two nuclei (Fig. 5, C and D), demonstrating that the abnormal sterigmata were not caused by defects of nuclear distribution, as they are in the *aps* mutants (*42*, *43*). Secondary conidiophores derived from a vesicle were occasionally found (Figs. 4C and 5B). These features are similar to those of the *medA*, *abaA*, and certain *brlA* mutants (*26*, *30*, *44*). In addition, sexual repro-

TABLE II. **Conidiation defect of the *ΔchsA ΔchsC* mutants.**

Strain	Relevant genotype	No. of conidia/mm² ×10⁴ (mean ± SE)
ABPU/AU	Wild type	22 ± 4
A-4	*ΔchsA*	17 ± 3
C2-11/A	*ΔchsC*	33 ± 3
AC-8	*ΔchsA*, *ΔchsC*	< 0.001

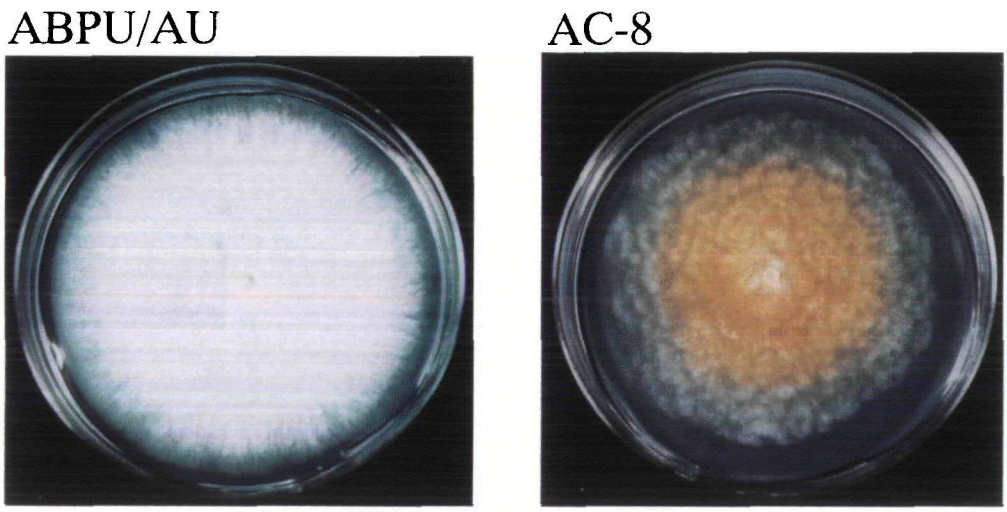

Fig. 3. **Colonies of the *A. nidulans* wild-type strain (ABPU/AU) and AC-8 (*ΔchsA ΔchsC*) on MM plates.**

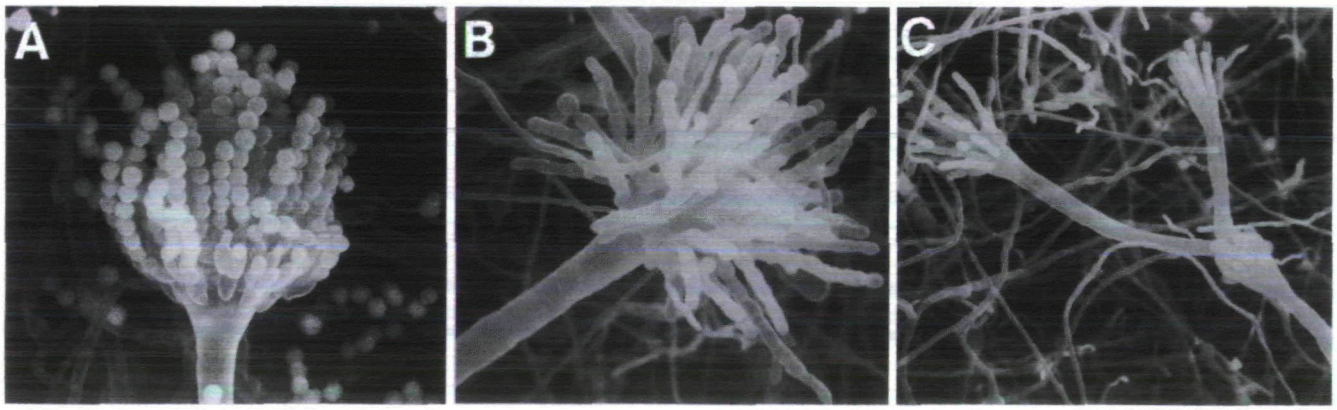

Fig. 4. **Scanning electron micrographs of conidiophores of the *ΔchsA ΔchsC* mutant.** (A) ABPU1. (B and C) AC-8. Bars represent 20 μm.

ductive cells, cleistothecia, were not observed in AC-8, whereas they were observed in A-4 and C2-11.

Chitin Contents of the Double Mutant—We next measured chitin contents of the mutants grown in a liquid medium. Chitin contents of AC-8, C2-11/A, A-4, and the wild-type strains ABPU/AU and ABPU/A1 are shown in Table III. To avoid the effect of the different genetic background of C2-11, we used C2-11/A instead. The chitin con-

TABLE III. **Chitin contents of various mutants.**

Strain	Relevant genotype	Chitin content (mg/mg protein)
ABPU/AU	Wild type	1.23 ± 0.06 (100%)
C2-11/A	*ΔchsC*	1.10 ± 0.11 (89%)
AC/8	*ΔchsA, ΔchsC*	1.28 ± 0.05 (104%)
ABPU/A1	*pyrG*	0.96 ± 0.02 (100%)
A-4	*pyrG, ΔchsA*	1.13 ± 0.06 (118%)

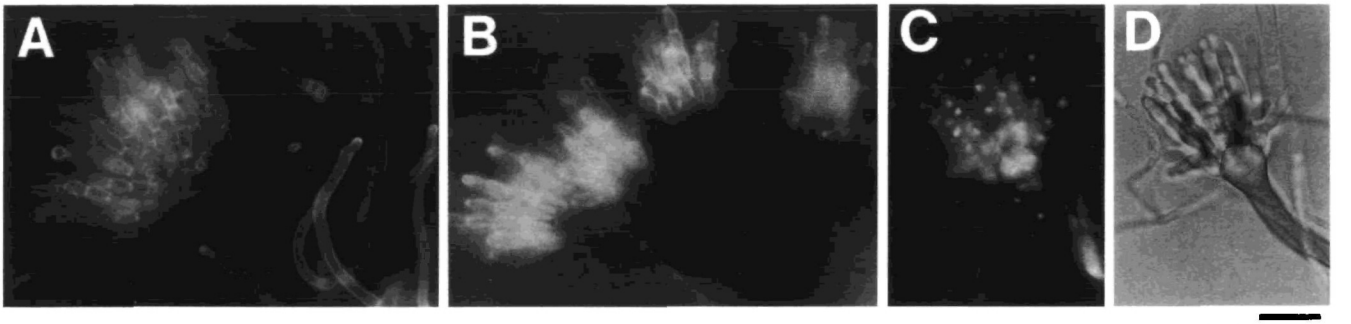

Fig. 5. **Morphology of the *ΔchsA ΔchsC* mutant.** (A) Calcofluor staining of aerial hyphae and a conidial head of strain AC-8. (B) Four secondary conidiophores produced from one vesicle. (C) Nuclear staining with DAPI. (D) Phase contrast nuclear staining reveals the presence of one or two nuclei in each compartment of sterigmata. A bar represents 10 μm.

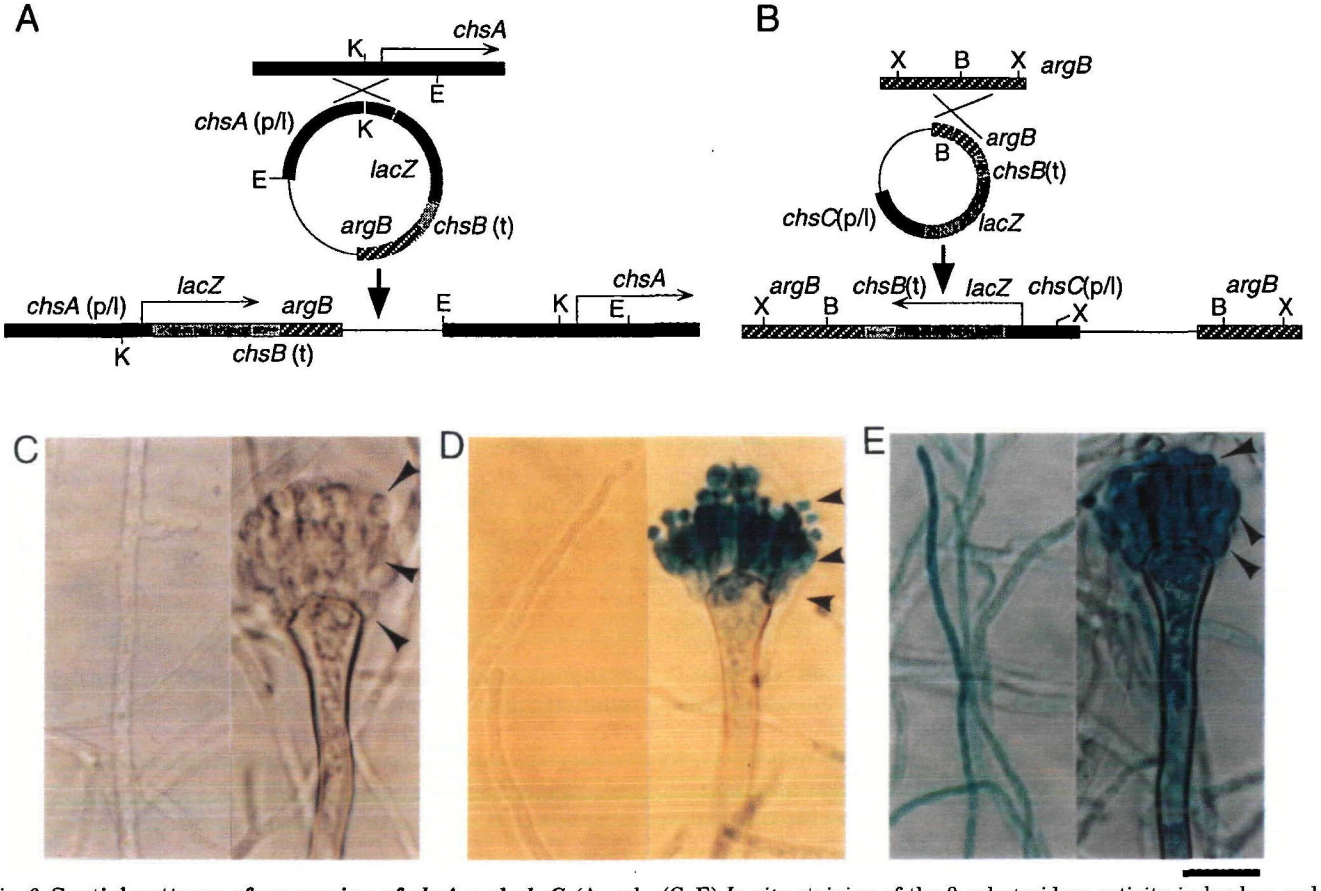

Fig. 6. **Spatial patterns of expression of *chsA* and *chsC*.** (A and B) Construction of AL and CL strains, respectively. Abbreviations are as follows: *chsA*(p/l), promoter and leader sequence of *chsA*; *chsB*(t), terminator of *chsB*; *chsC*(p/l), promoter and leader sequence of *chsC*. See "MATERIALS AND METHODS" for details of these strategies. (C–E) *In situ* staining of the β-galactosidase activity in hyphae and conidiophores. Left panels, hyphae; right panels, conidiophores. Three arrowheads in each panel indicate conidia (top), metulae (middle), and phialides (bottom). (C) ABPU/A1. (D) AL1. (E) CL5. The bar represents 10 μm.

tent of C2-11/A was approximately 89% of that of the wild-type strain ABPU/AU. The chitin content of strain A-4 was compared with that of ABPU/A1, because the *pyrG* mutation reduced chitin content (ABPU/AU *versus* ABPU/A1). The chitin content of A-4 was 118% of that of ABPU/A1. The chitin content of AC-8 was 104% of that of ABPU/AU. Taken together, these results suggest that the chitin content of AC-8 was the result of the combined effects of *ΔchsA* and *ΔchsC* mutations.

Intracellular Localization of chsA and chsC Expression—To examine the spatial control of expression of *chsA* and *chsC*, we constructed strains in which the *Escherichia coli* β-galactosidase was connected to the leader sequences of ChsA and ChsC, respectively, as translational fusions (Fig. 6, A and B). The resultant strains AL1 and CL5 were cultured on plates and stained *in situ* with X-Gal. The staining pattern of AL1 indicated that *chsA* was expressed in metulae and more strongly in phialides and conidia (Fig. 6D), whereas no signals were detected in the control strain ABPU/A1 (Fig. 6C). On the other hand, signals in CL5 were detected in both hyphae and entire conidiophores, but they were more intense in metulae, phialides, and conidia (Fig. 6E).

DISCUSSION

We analyzed the functions of the non-essential class I and class II chitin synthase genes, *chsA* and *chsC,* of *A. nidulans* by characterizing their null mutants. The *ΔchsA ΔchsC* double mutant (AC-8) showed defects in growth and morphology, whereas the single *ΔchsA* (A-4) and *ΔchsC* (C2-11) mutants did not. Hyphal growth of the double mutant was inhibited on media containing various reagents. The high sensitivity to SDS indicates the weakened structure of cell wall, as shown for *S. cerevisiae* (*45*, *46*). Sensitivity to chitin-binding dyes indicates alterations in cell wall assembly. The high sensitivity to chitin synthase inhibitors suggests that these inhibitors are targeted to chitin synthases other than ChsA and ChsC. Furthermore, aerial hyphae were developed poorly. These features apparently represent the loss of hyphal wall integrity in the double mutant. Furthermore, the efficiency of conidial formation of these strains was reduced to less than 0.01% of that of the wild-type strain. This was caused by infrequent production and the abnormal structure of conidiophores. Multiple chains of metulae septated into compartments, each containing one or two nuclei, were frequently seen. Scarce conidia at tips of sterigmata were incompletely separated from sterigmata. In addition, secondary conidiophores appeared on plates incubated over 3 days at 37°C. Thus, ChsA or ChsC is required for the normal asexual development. In agreement with these results, the expression of these two genes, as reported by β-galactosidase activity, was intense in the metulae, phialides, and conidia. The absence of cleistothecia in the *ΔchsA ΔchsC* double mutant is also indicative of involvement of two gene products in the sexual differentiation. Although 90% reduction of conidiation efficiency was observed with the *ΔchsA ΔchsD* double mutant (*14*), conidiophore morphology of the mutant was normal (data not shown). Colony extension of the *ΔchsA ΔchsD* double mutant was not affected when SDS (10 μg/ml), Calcofluor white (10 μg/ml), or Congo red (10 μg/ml) was added to the medium (data not shown). Together, these findings suggest that functional overlapping between *chsA* and *chsD* is small compared with that between *chsA* and *chsC*.

The chitin content of the *ΔchsA ΔchsC* mutant was a little higher than that of the wild-type strain, being intermediate between those of the *ΔchsA* and *ΔchsC* single mutants. These results suggests that the chitin contents of mutants do not reflect the malignancy of the defects of these strains. Elevated chitin content in the mutant of a chitin synthase (encoded by *chs2*) was also reported in *Candida albicans* (*47*). The activity of other chitin synthases may increase in *A. nidulans* and *C. albicans* when the activity of one chitin synthase is lost.

Developmental abnormalities of the *ΔchsA ΔchsC* double mutant raise questions regarding the interaction between cell wall chitin metabolism and asexual reproduction. ChsA and ChsC seem to play important roles in chitin synthesis in metulae, phialides, and/or conidia. Interestingly, one and three putative *cis*-acting elements for AbaA (CATTCC/T) (*32*) are present in *chsA* and *chsC* promoters, respectively. In addition, there are many sequences that partially match the consensus AbaA and BrlA (C/AG/AAGGGG/A) (*48*) in both promoters. This may imply the direct regulation of *chsA* and *chsC* gene expression by AbaA and BrlA at the transcriptional level. Recent genetic analysis suggests that the modifier MedA has a function in temporal and spatial control of the expressions of BrlA and AbaA (*34*). Thus, the expression of *chsA* and *chsC* may be regulated by MedA through BrlA and AbaA.

What are the mechanisms by which chained metulae and secondary conidiophores are formed in the *ΔchsA ΔchsC* double mutant? It is possible that some factors survey the integrity of cell wall of conidiophores or metulae and that, if abnormality is detected, they induce the formation of secondary conidiophores or another metulae on the vesicles or on the tip of metulae. It is also possible that the abnormal hyphal cell wall assembly in the *ΔchsA ΔchsC* double mutant directly or indirectly disturbs normal differentiation of conidiophores. This would explain the low population of conidiophores in the double disruptant. Poor development of aerial hyphae may be also caused by a similar mechanism. In this case, intracellular signals that induce a morphogenic change from vegetative growth to conidiophore formation would not be generated or properly transmitted in the *ΔchsA ΔchsC* double mutant.

The functions of the class I chitin synthases characterized in this study appear to be very similar to those of the class II chitin synthase. In fact, phylogenetic analyses on the highly conserved region of chitin synthases indicate a close relationship between these two classes (data not shown). In combination with the study of other chitin synthases in *A. nidulans*, it appears that the chitin synthases of different classes have some overlapping functions in building the complicated cell wall architecture of fungi.

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