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Differential expression of the *chsE* gene encoding a chitin synthase of *Aspergillus nidulans* in response to developmental status and growth conditions

Jeong Im Lee, Yeong Man Yu, Yoo Mi Rho, Bum Chan Park, Jeong Hwan Choi, Hee-Moon Park, Pil Jae Maeng *

Department of Microbiology, School of Bioscience & Biotechnology, Chungnam National University, Daejeon 305-764, Republic of Korea

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Abstract

Expression of *chsE* encoding one of the five chitin synthases of *Aspergillus nidulans* was analyzed. Expression of *chsE* was moderate in conidiophores, but somewhat weaker in vegetative mycelia. During sexual development, *chsE* was expressed strongly in young cleistothecia and hülle cells, but little in mature sexual structures. Deletion of *chsE* caused a significant decrease in the chitin content of the cell wall during early sexual development. Expression of *chsE* was increased by substituting glucose with lactose or by addition of 0.6 M KCl or NaCl, but affected little by substituting glucose with sodium acetate. Consequently, *chsE* was shown to have a mode of expression distinct from those of the other chitin synthase genes, *chsA*, *chsB* and *chsC*.

Keywords: *Aspergillus nidulans*; *chsE*; Chitin synthase; sGFP; Differential expression

1. Introduction

Chitin, a β-1,4-linked polysaccharide of *N*-acetylglucosamine, is one of the major structural components of the fungal cell wall, and its biosynthesis is thought to be important for many fungi to maintain the physical strength of cell walls as well as to form their specific shape during cell growth and differentiation [1–5]. Chitin synthases (EC 2.4.1.16) are integral membrane proteins which catalyze polymerization of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine. The primary structures of many fungal chitin synthases are mainly deduced from their gene structures, and they have been divided into five groups, classes I–V, on the basis of their structures in the conserved region.

Five chitin synthase genes, *chsA* [6], *chsB* [2,6], *chsC* [7], *chsE* (identical to *chsD* reported by Motoyama et al.) [3,4], and *csmA*/*chsD* [3,8,9] corresponding to classes II, III, I, IV, and V, respectively, have been isolated and identified in the filamentous fungus *Aspergillus nidulans*. Disruption of *chsA* [6], *chsC* [7], or *chsE* (*CAL1* homolog) [3,4] does not cause any defect in cell growth or morphology during the asexual cycle, supporting the conclusion that none of the three genes is essential for hyphal growth. Double disruption of *chsA* and *chsE*,

* Corresponding author. Tel.: +82 42 821 6415; fax: +82 42 822 7367.
E-mail address: pjmaeng@cnu.ac.kr (P.J. Maeng).

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however, causes a remarkable decrease in the efficiency of conidia formation, indicating that *chsA* and *chsE* serve redundant functions in conidia formation [4,10]. Mutant strains in which both *chsA* and *chsE* are disrupted exhibit loss of integrity of hyphal wall and remarkable abnormalities during its asexual development [5]. It has also suggested that the functional importance of *chsE* increases when the expression of *chsB* is limited [11]. *chsA* is mainly expressed in the metulae, phialides, and conidia, whereas *chsC* is expressed in hyphae as well as conidiophores, which implies that ChsA and ChsC share critical functions in hyphal wall integrity and differentiation [5]. It has been thus revealed that *chsA*, *chsC*, and *chsE* perform redundant functions in the process of conidiation. The disruptant of *chsB* grows as minute colonies without conidia, and produces hyphae with enlarged tips, a high degree of branching, and disorganized lateral walls, which is not remedied by osmotic stabilizers. However, its mycelium is not deficient in chitin content and shows no evidence of lysis. It is thus probable that chitin synthesized by the ChsB enzyme does not substantially contribute to the rigidity of the cell wall but is necessary for normal hyphal growth and organization [2]. *csmA*, a part of which was cloned and designated as *chsD* by Specht et al. [3], encodes a novel protein (1852 amino acids, 206 kDa) consisting of a C-terminal class V chitin synthase domain and an extra N-terminal myosin motor-like domain [8]. The *csmA* null mutants show sensitivity to a chitin-binding reagent, Calcofluor white or Congo red, and morphological abnormalities in tip growth and septum formation [9]. The CsmA enzyme has important roles in polarized cell wall synthesis and maintenance of cell wall integrity, and its myosin motor-like domain is indispensable for these functions [12,13].

As an effort to understand the role of the chitin synthase genes in *A. nidulans*, we have analyzed the expressions of *chsA*, *chsB* and *chsC* [14]. While *chsB* is expressed ubiquitously throughout the fungal body and quite independently of the change in developmental status of the cells, *chsA* is expressed specifically during asexual differentiation. The expression of *chsC* is moderate in young vegetative mycelia and in sexual structures, i.e., such as young cleistothecia and mature ascospores, but weak in old vegetative mycelia and in asexual structures. In the present study, we analyzed the mode of expression of the chitin synthase gene, *chsE*, both by Northern blotting and by a vital reporter system with *sgfp* encoding a modified version of green fluorescent protein, sGFP [15]. Herein, we present some evidence indicating that *chsE* has a distinctive mode of expression from those of the other chitin synthase genes of *A. nidulans* previously reported, *chsA*, *chsB* and *chsC*, and also that the gene is subject to differential expression in response to developmental status and environmental factors.

2. Materials and methods

2.1. Strains, media, cultivation and transformation

Aspergillus nidulans FGSC A26 (*biA1*) (Fungal Genetics Stock Center; Kansas City, KS, USA) was used for RNA preparation, and *A. nidulans* W × 24 (*npgA1 biA1*; *sB3*; *chaA1 trpC801*) [16] was used as a recipient strain for transformation. *A. nidulans* D3-2 (*biA1 pyrG89*; *pyroA4*; *wA3*; *argB2*; *chsE::argB*) [17] and ABPU/A2 (*biA1 pyrG89*; *pyroA4*; *wA3*), which were kindly provided by Dr. Horiuchi, were used for analysis of chitin content. FGSC A26 and the transformants from W × 24 were maintained on *Aspergillus* complete medium (CM) [18], W × 24 on CMW (CM + 4 mM tryptophan) medium, and D3-2 and ABPU/A2 on CMU (CM + 10 mM uridine + 10 mM uracil) medium.

Transformation of *A. nidulans* W × 24 was performed as described by Lee et al. [14], and the protoplasts of transformants were regenerated on 1% glucose minimal medium (MM) [18] supplemented with 0.1 μM biotin, 4 mM methionine, and 0.6 M KCl (MBMK). When necessary, the transformants were grown on MM supplemented with 0.1 μM biotin and 4 mM methionine (MBM), and W × 24 on MBM supplemented with 4 mM tryptophan (MBMW).

Preparation of vegetative mycelia and induction of asexual and sexual differentiation was performed as described by Lee et al. [14].

2.2. Construction of plasmids

The vector pTchsE-p::sgfp used for analyzing the expression of *chsE* was constructed as follows. First, the presumptive promoter region of *chsE* (from nucleotide −1105 to −1) was amplified from the genomic DNA of *A. nidulans* by PCR using two primers, PE1 5′-GGTACCATGCGGTTGGTTGATCAG-3′ and PE2 5′-AGAATTCGATAACTATTCAATAGGCGAA-3′ [3,4] (Fig. 1A). The resulting 1.1-kb PCR product was cloned into pT7Blue(R) vector to yield pT7/chsE-11. Then the 1.1-kb *Kpn*I–*Eco*RI fragment was excised from pT7/chsE-11 and inserted into *Kpn*I–*Eco*RI-digested pTsgfp vector (Fig. 1B) [14] upstream of *sgfp* to yield pTchsE-p::sgfp (6.5 kb).

2.3. Molecular techniques

DNAs from *A. nidulans* strains were prepared by using miniprep procedures [19]. Southern blot analysis was performed according to the standard method [20] by using an ECL labeling and detection kit (Amersham; Buckinghamshire, UK). To confirm the correct integration of the vectors at the *trpC* loci of the transformants, a 0.4-kb *Sal*I-digested *trpC* fragment (from nucleotide −266 to 20) was used as a probe.

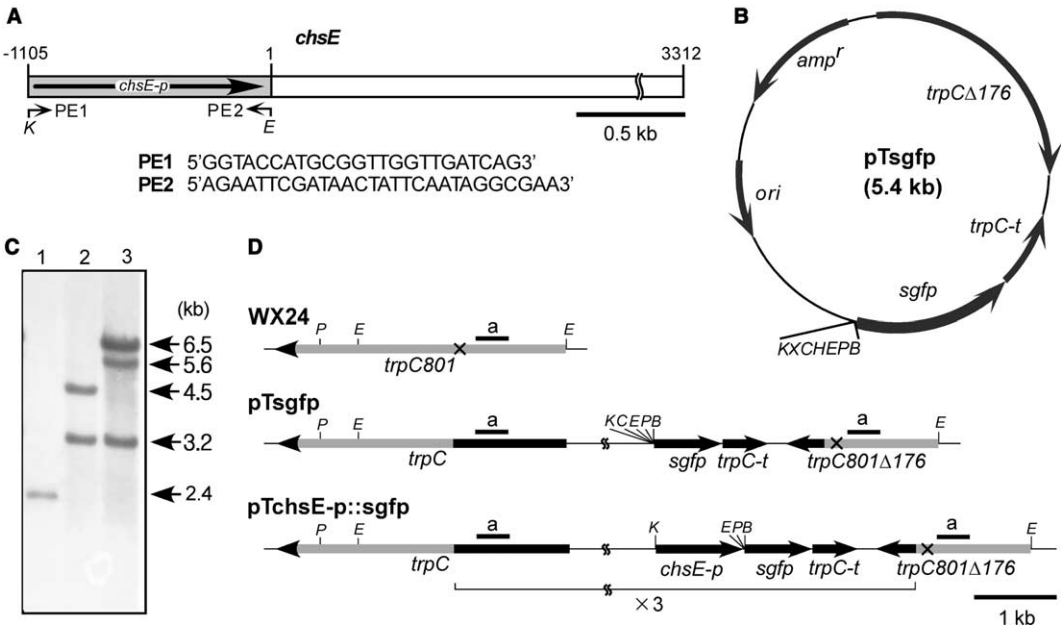

Fig. 1. Construction of the targeting vector pTchsE-p::sgfp and its integration into the *trpC* locus of *A. nidulans*. (A) Design of the primers for amplification of the promoter region of *chsE* (*chsE-p*) by PCR. Numerals above each bar refer to the distance from the translation start site of the gene. (B) Structure of the promoter analysis vector pTsgfp into which the *chsE-p* fragment was inserted. The 5′ 1.76-kb segment of *trpC* is designated as *trpC*Δ176, the 0.53-kb *trpC* terminator as *trpC-t*, and the cDNA encoding sGFP as *sgfp*. The *trpC*Δ176 fragment can function as a selectable marker for homologous integration of the transforming vectors derived from pTsgfp into *trpC801* (a mutant allele containing a point mutation in the 3′ portion of *trpC*) locus of the host genome. (C) Southern blot analysis of *A. nidulans* strains. Genomic DNAs of W × 24 (lane 1), pTsgfp transformant (lane 2), and pTchsE-p::sgfp transformant (lane 3) were digested with *Eco*RI, and hybridized with 0.4-kb *Sal*I-digested *trpC* fragment. (D) Restriction maps of the *trpC* loci of *A. nidulans* strains predicted on the basis of the results of Southern blot analysis. Dark arrows or bars represent the essential components from the recombinant vectors, and gray ones in the chromosomes of the host strain. The modes of integration at the *trpC* loci were estimated to be single in pTsgfp transformant and tandem triple (×3) in pTchsE-p::sgfp transformant. The regions homologous to the probe are shown as bars designated as 'a'. Abbreviations for restriction enzymes: *B*, *Bam*HI; *C*, *Cla*I; *E*, *Eco*RI; *K*, *Kpn*I; *P*, *Pst*I; X, *Xho*I.

Total RNAs were prepared from the liquid nitrogen-frozen and ground mycelia of *A. nidulans* at the vegetative growth, asexual development, or sexual development stages by modified guanidine thiocyanate/CsCl density gradient ultracentrifugation [20]. Poly(A)$^+$ RNAs were isolated by an Oligotex mRNA purification kit (Quiagen; Valencia, CA, USA) according to the manufacturer's instructions. Northern hybridization was performed according to standard procedures [20], in which the DNA probes were labeled with ^{32}P using a Random Primed DNA Labeling Kit (Boehringer Mannheim; Mannheim, Germany). A 1.1-kb *chsE*-specific probe for Northern blot analysis was prepared from gel-purified RT-PCR fragments amplified by using the primers, PEN1 (5′GACCTGCGATCCAGATGATT3′) and PEN2 (5′CCAACAGCACTCTTGACGAA3′). The nucleotide sequences of the cloned DNAs were determined by using an automatic DNA sequencer ABI 377 (Perkin–Elmer).

2.4. Confocal microscopy

Confocal microscopic observations of *A. nidulans* cells were performed using a confocal microscopy system (TCS SP; Leica; Heerbrugg, Switzerland). To observe sGFP fluorescence, fungal cells were excited with the wavelength of 488 nm, and the fluorescent emission with the wavelength of 500–520 nm was detected. All sGFP images were generated under standardized conditions in which the main parameters are adjusted as follows: PMT gain, 690–710; PMT offset, −10; Pinhole, 150; number of sections, 8; number of scans for each section, 8. The intensity of green fluorescence developed in the confocal micrographs was analyzed by using an image analysis system (GAIA Material V5.3.2.1; Mirero Inc., Seoul, Korea).

2.5. Determination of chitin content

One milligram of lyophilized powder of SDS-extracted cell walls was hydrolyzed in 0.2 ml of 6 N HCl (H-0636; Sigma; St. Louis, MO, USA) at 100 °C for 17 h, together with 0–200 °C of *N*-acetylglucosamine standards. After removing the HCl by using a speed vacuum concentrator (Savant; Ramsey, MN, USA) at 50 °C, dried samples were resuspended in 1 ml of water and centrifuged to remove insoluble material. To 0.1 ml of sample, 0.1 ml of solution A [1.5 M Na_2CO_3 in 4%

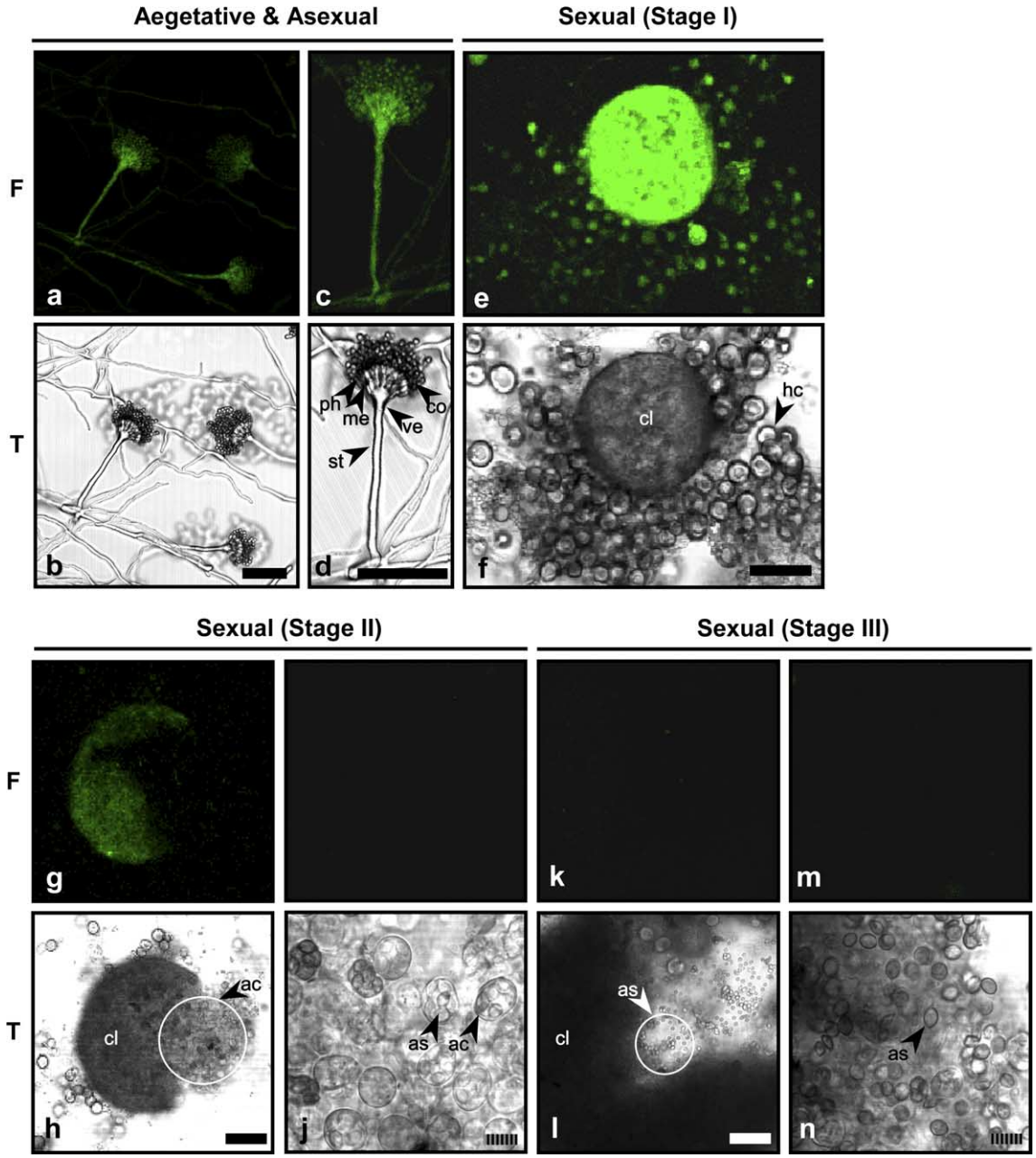

Fig. 2. Analysis of expression of *chsE* during vegetative growth, asexual differentiation, and sexual differentiation by using sGFP as a vital reporter in the transformant carrying tandem triple copy of pTchsE-p::sgfp at the *trpC* locus. For observation of vegetative mycelia and asexual structure, the transformant was slide-cultured for about 2 days on 1% glucose MBM agar plates (panels a–d). For induction of sexual differentiation, the mycelia of each transformant grown in CM broth were transferred onto 1% glucose MBM plates, incubated for another 24 h while the plates were sealed closely, and incubated thereafter under unsealed conditions. The expression of sGFP was observed by confocal microscopy. Stages of sexual differentiation: Stage I, 0–2 days after induction (panels e and f); Stage II, 3–5 days after induction (panels g–j); Stage III, 6–8 days after induction (panels k–n). Circled areas in panels h and l were observed by higher magnification and the results are presented in panels j and n, respectively. Asexual and sexual structures: st, stalk; ve, vesicle; me, metula; ph, phialide; co, conidium; cl, cleistothecium; hc, hülle cell; ac, ascus; as, ascospore. Images: F, sGFP images generated by excitation with 488 nm and detection of 500–520 nm emission; T, transmission images. Scale bars: black or white bars, 50 μm; striped bars, 10 μm.

(w/v) acetylacetone] was added and the mixture was incubated at 100 °C for 20 min. After cooling to room temperature, 0.7 ml of 96% ethanol and 0.1 ml of solution B (1.6 g *p*-dimethylaminobenzaldehyde in 30 ml concentrated HCl and 30 ml 96% ethanol) was added and incubated for 1 h at room temperature. Triplicate samples were incubated for 1 h at room temperature before the absorbance was read at 520 nm [21,22].

3. Results

3.1. Construction of A. nidulans transformants

To analyze the expression of *chsE* using *sgfp* cDNA as a vital reporter, pTsgfp and pTchsE-p::sgfp plasmids (Fig. 1B and D) were individually introduced into *A. nidulans* W × 24. Transformants which grew in the absence of tryptophan were expected to carry the wild-type *trpC* gene which was formed by homologous recombination of *trpC*Δ176 (5′ 1.8-kb segment of *trpC*) of the transforming vectors with the chromosomal *trpC801* allele. To confirm correct integration of the vectors at the *trpC* locus of the transformants isolated on MBM plates, chromosomal DNAs from one of the transformants and the host strain were digested with *Eco*RI and analyzed by Southern blotting using a 0.4-kb *Sal*I-digested *trpC* fragment (from nucleotide −366 to +19) as a probe (Fig. 1C). The DNA from *A. nidulans* W × 24 showed a single 2.4-kb fragment hybridized with the probe. On the other hand, the transformants exhibited differently sized DNA fragments hybridized with the *trpC* probe: specifically, the pTsgfp transformant showed 3.2- and 4.5-kb fragments; and the pTchsE-p::sgfp transformant 3.2-, 5.6-, and 6.5-kb fragments. While the two positive fragments from the pTsgfp transformant showed similar intensity, the 6.5-kb fragment of the pTchsE-p::sgfp transformant showed intensity about twice as high as the other two. Thus, the integration of the targeting vectors at *trpC* loci were estimated to be single in the pTsgfp transformant and tandem triple in the pTchsE-p::sgfp transformant (Fig. 1D).

3.2. Differential expression of the chsE gene in response to developmental status

To figure out the mode of differential expression of *chsE* gene during vegetative growth, asexual differentiation, and sexual differentiation, the level of sGFP in mycelia and differentiated structures of the pTchsE-p::sgfp transformant was monitored by confocal microscopy in comparison with the host strain W × 24 and the pTsgfp transformant.

When slide-cultured on minimal media which supported vegetative growth and asexual development, the host strain W × 24 and the pTsgfp transformant did not show any detectable level of sGFP fluorescence in any part of substrate mycelia or asexual structures (data not shown). Similarly, sexually differentiated structures, such as hülle cells and cleistothecia, of the two strains did not show any sGFP fluorescence (data not shown). These results suggest that autofluorescence of the fungal cells does not affect the reliability of the sGFP reporter system.

In the pTchsE-p::sgfp transformant slide-cultured on 1% glucose MBM, sGFP fluorescence was visible in most part of conidiophores and in conidia (Fig. 2, panels a–d). The intensity of sGFP fluorescence seemed to increase in accordance with the progress of asexual development, and to be stronger in vesicles, metulae and phialides than in other parts of conidiophores. However, a lower level of sGFP fluorescence was observed in vegetative hyphae and foot cells throughout the culture period.

sGFP fluorescence was also examined during sexual development, which was divided into three arbitrary stages: Stage I when hülle cells and young cleistothecia were formed (0–2 days after induction), Stage II when cleistothecia enlarged and ascospore formation occurred (3–5 days after induction), and Stage III when mature asci were liberated from the spontaneously rupturing cleistothecia (6–8 days after induction).

At the early stages of sexual development (Stage I), the pTchsE-p::sgfp transformant showed quite strong sGFP fluorescence both in young cleistothecia and hülle cells (Fig. 2, panels e and f). As the process of sexual development proceeded (Stage II; Fig. 2, panels g–j), the sGFP fluorescence in the cleistothecia declined strikingly, and thus only a faint fluorescent was visible in the cleistothecial shell. Furthermore, we could not find any sign of fluorescence in the cleistothecial contents, i.e., asci and ascospores. Accordingly, it is probable that the sGFP fluorescence was absent in the content of young cleistothecia at Stage I. At the late stages of sexual development (Stage III; Fig. 2, panels g–j), no sGFP fluorescence was observed in any part of the sexual structures.

The level of *chsE* transcript in the cells of three different developmental status was monitored by Northern blot analysis using the *chsE*-specific probe (Fig. 3). While only a little *chsE* transcript was detected in young vegetative mycelia grown for 9 or 12 h, quite a higher level of the transcript was observed in older ones grown for 15 h. During asexual development, the level of *chsE* transcript was roughly as high as that of later vegetative stage and remained almost constant. These results are considerably accordant with the result of confocal microscopic observation of the pTchsE-p::sgfp transformant.

During sexual development, Northern blot analysis was performed only for the cells of Stage I because we could not obtain adequate amounts of RNAs at the later stages. The expression of *actA*, a member of house-keeping genes and used as an internal control, was not constant but fluctuated during sexual development as reported previously [23]. The level of *chsE* transcript reached to the highest point at the very early stage of sexual differentiation (10 h after unsealing) and rapidly decreased thereafter. Thus, the amount of *chsE* transcript was almost negligible at the later moments of Stage I.

The results of confocal microscopy and Northern blot analysis in sum indicate that *chsE* is expressed

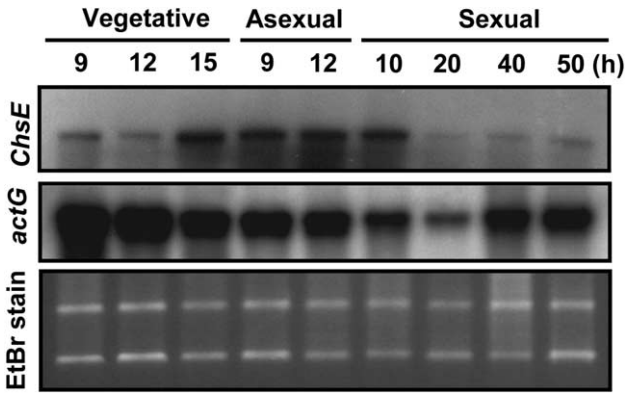

Fig. 3. Northern blot analysis of *chsE* expression during different developmental status. Total RNAs were prepared from the mycelia of *A. nidulans* FGSC A26 at the three different developmental status, vegetative growth, asexual differentiation, and early stage of sexual differentiation (Stage I). For preparation of vegetative mycelia, conidia of each strain were inoculated in CM broth and grown for ≈18 h at 37 °C . Induction of asexual differentiation was performed by spreading the vegetative mycelia onto agar plates and incubation at 37 °C. Short aerial hyphae were observed after 2–4 h and mature conidiophores including stalks, vesicles, metulae, phialides, and conidial chains are formed within 15 h. For induction of sexual differentiation, the vegetative mycelia were transferred onto agar plates, incubated for another 24 h while the plates were sealed closely, and incubated thereafter under unsealed conditions. hülle cells, cleistothecial primordia, and immature cleistothecia appeared gradually within 30 h. Each lane was loaded with 3 μg of mRNA based on reading of absorbance at 260 nm (A_{260}). The RNAs were hybridized with the *chsE*-specific probe labeled with ^{32}P. The blot of RNAs was rehybridized with a restriction fragment containing the whole ORF of the *A. nidulans actG* gene as an internal control.

considerably during the later stage of vegetative growth and throughout the process of asexual differentiation, and thus that it contributes to the synthesis of chitin contained in the cell walls of vegetative mycelia, conidiophores and conidia. They also show that the expression of *chsE* during sexual development is not only temporally specific to the early stage (Stage I) but also spatially differentiated, i.e., strong in young cleistothecial shells and in hülle cells, but negligible in other parts of the sexual structures, such as asci and ascospores.

Chitin contents in the cells of *chsE* deletion mutant (D3-2) of three different developmental stages, i.e., vegetative growth, asexual development, and sexual development, were analyzed in comparison with those of relevant wild-type strain (ABPU/A2) (Table 1). During vegetative growth and asexual development, the chitin contents in the cell walls of the mutant was about 90% of those of wild-type strain, respectively. On the other hand, the chitin content of the mutant cells decreased to ≈ 65% of that of wild-type strain at the early stage of sexual differentiation (Stage I). The effect of *chsE* deletion on the chitin synthesis thus seems to be much more significant during early sexual development than during either vegetative growth or asexual development.

3.3. *Effect of osmostress on the expression of* chsE *gene*

The effect of osmostress on the expression of *chsE* gene was analyzed by observing sGFP fluorescence in the cells of the pTchsE-p::sgfp transformant slide-cultured on 1% glucose MBM plates containing 1.2 M KCl (or NaCl). The transformant showed increased levels of sGFP fluorescence throughout the whole fungal body including substrate mycelia, conidiophores, and conida in the presence of KCl (Fig. 4A, panels c and d) or NaCl (data not shown). Image analysis of the confocal micrographs showed ≈54%, 66%, and 52% increment of green fluorescence in conidiophores, in substrate mycelia, and in the whole thallus, respectively (Fig. 4B). This result thus suggests that osmostress stimulates the expression of *chsE* in the whole fungal body.

3.4. *Effect of carbon source on the expression of* chsE *gene*

The effect of three different carbon sources, i.e., glucose, lactose, and sodium acetate, on the expression of *chsE* gene was examined by monitoring sGFP fluorescence in the cells of the pTchsE-p::sgfp transformant slide-cultured on MBM plates containing 1% of each carbon source.

Table 1
Chitin contents of the wild-type and *chsE* mutant

Strain	Relevant genotype	Chitin content [μg GlcNAc (mg cell wall dry weight)$^{-1}$][a]		
		Vegetative[b]	Asexual[c]	Sexual (Stage I)[d]
ABPU/A2	Wild-type	281 (100) ± 35	276 (100) ± 14	359 (100) ± 10
D3-2	*chsE::argB*	255 (91) ± 28	239 (87) ± 16	233 (65) ± 17

[a] Means ± SD were calculated from the results of triplicate samples. The ratio of chitin content of the mutant to that the wild-type strain is shown in parentheses.
[b] For preparation of vegetative mycelia, conidia were inoculated in CMU broth and grown at 37 °C for 15 h.
[c] Asexual differentiation was induced by spreading vegetative mycelia onto agar plates and incubation at 37 °C for 12 h.
[d] For induction of sexual differentiation, vegetative mycelia were transferred onto agar plates, incubated for another 24 h while the plates were sealed closely, and incubated thereafter under unsealed conditions for 24 h (Stage I).

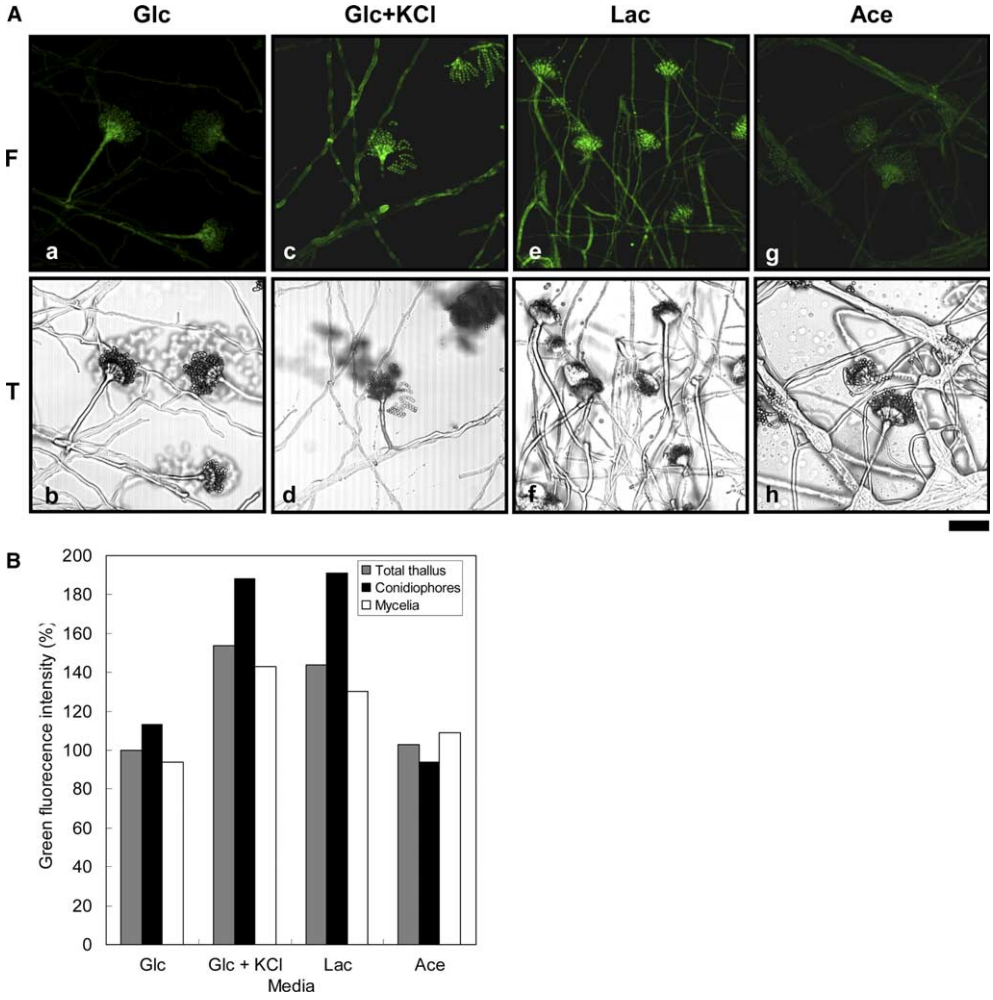

Fig. 4. Analysis of the effect of osmostress and carbon sources on the expression of *chsE* during vegetative growth and asexual differentiation using sGFP as a vital reporter. (A) Confocal micrographs of the transformant carrying tandem triple copy of pTchsE-p::sgfp at the *trpC* locus. The transformant was slide-cultured on MBM plates containing either 1% glucose (Glc; panels a and b), 1% glucose plus 1.2 M KCl (Glc + KCl; panels c and d), 1% lactose (Lac; panels e and f), or 1% sodium acetate (Ace; panels g and h), and the expression of sGFP was monitored by confocal microscopy. Images: F, sGFP images generated by excitation with 488 nm and detection of 500–520 nm emission; T, transmission images. Scale bar: 50 μm. (B) Analysis of the intensity of green fluorescence in confocal micrographs. The intensity of green fluorescence developed in the confocal micrographs was measured using an image analysis system (GAIA Material V5.3.2.1). The intensity of green fluorescence in total thallus, conidiophores, and mycelia was calculated by dividing the sum of green fluorescence detected in each part by the area of corresponding part, respectively, and the relative intensity of green fluorescence (%) was presented in comparison with that in total thallus grown on MBM plates containing 1% glucose.

The transformant showed higher levels of sGFP fluorescence on lactose than on glucose both in substrate mycelia and asexually differentiated structures, i.e., conidiophores and conidia (Fig. 4A, panels e and f). Image analysis of the confocal micrographs showed ≈44%, 69%, and 38% increment of green fluorescence in conidiophores, in substrate mycelia, and in the whole thallus, respectively. Quite differently from the case of lactose, the transformant showed similar sGFP intensity on sodium acetate as on glucose (Fig. 4A, panels g and h). These results suggest that the expression of *chsE* is increased to some extent by substituting lactose for glucose as a sole carbon source, however, affected little by sodium acetate.

4. Discussion

In the present study, we analyzed the expression mode of *chsE* in *A. nidulans* and found that this gene is subject to differential expression in response to developmental status and environmental stress in quite a distinctive manner from any other *chs* genes of this fungus previously reported [14].

As presented above, *chsE* was expressed at a considerable level during asexual differentiation and at a lower level during vegetative growth. During sexual development, the expression of *chsE* was quite strong in young cleistothecial shells and hülle cells, but was negligible in the asci and ascospores (Fig. 2).

The expression of *chsB* gene which is essential for cell growth [2,6] occurs strongly and ubiquitously throughout the whole fungal body, and is relatively independent of the change of developmental status of fungal cells [14]. Thus, the manner of *chsE* expression is similar to that of *chsB* during vegetative growth and asexual differentiation, however, is clearly distinguishable from that of *chsB* during sexual development. The functional relationship between *chsE* and *chsB* has been analyzed by using double mutant strains of *chsB* and *chsE*, in which *chsB* was placed under the control of *alcA* promoter [11]. The significance of *chsE* gene to the growth of mycelia under high-osmolarity conditions as well as to the process of conidiation increases when the expression of *chsB* is limited.

It has been well established by intensive mutation analysis that *chsA*, *chsC*, and *chsE* perform redundant functions in the process of conidiation [3–7,10]. However, differential expression of *chsA* and *chsC*, especially during sexual development, has been revealed only recently by Lee et al. [14]. *chsA* is specifically expressed in conidiophores and conidia during asexual differentiation, but is not essential for either sexual differentiation or vegetative growth. *chsE* is thus clearly distinct from *chsA* in that it was expressed during sexual differentiation as well as vegetative growth. *chsC* is expressed moderately during the early phase of vegetative growth, but weakly in old vegetative mycelia and in asexual structures. It is also expressed during sexual development, i.e., relatively strongly in mature ascospores and young cleistothecia, but negligibly in hülle cells. Therefore, *chsE* can be discriminated also from *chsC* in that it was expressed more in asexual structures than in vegetative mycelia, and furthermore in that it was not expressed in asci and ascospores during sexual development.

Deletion of *chsE* affected the chitin content of cell wall quite significantly during early sexual development (Table 1). Although *chsE* was expressed during asexual differentiation and vegetative growth as well as early sexual differentiation, its deletion caused only a little difference in chitin content of cell wall during either vegetative growth or asexual development. Thus, it is suggested that the role of *chsE* in chitin synthesis cannot be substituted by any other *chs* genes during early sexual development while it can be during vegetative growth and asexual development.

It is of interest that expression of *chsE* reached its highest level on media containing lactose, rather than glucose or acetate, as sole carbon source (Fig. 4). On the contrary, the expression of *chsA*, *chsB*, and *chsC*, are stimulated by acetate which was supplied as a sole carbon source but not by lactose [14]. It is thus suggested that the mode of regulation of *chsE* expression according to the carbon source is quite different from the other chitin synthase genes. In *A. nidulans*, genes encoding the enzymes committed to the catabolism of less preferred carbon sources [24], those involved in gluconeogenesis and glyoxylate cycle [25,26], and those involved in secondary metabolite synthesis [27] are subject to regulation by carbon sources. However, there have been there have been only limited reports of effect of carbon source on expression of chitin synthase genes [14].

We found in the present study that osmostress caused by high concentrations (up to 1.2 M) of KCl or NaCl stimulates the expression of *chsE* (Fig. 4). Similarly, osmostress stimulates expression of *chsA* mainly in conidiophores and neighboring substrate hyphae, and the expression *chsC* in the whole fungal body, while expression of *chsB* is not intimately responsive to osmostress [14]. It is thus suggested that *chsE*, together with *chsA* and *chsC*, can contribute to the resistance of fungal cells to osmostress causing dehydration and shrinkage of cells, while *chsB* does not necessarily participate to this process.

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