Journal of Microbiology (2014) Vol. 52, No. 11, pp. 940–947
DOI 10.1007/s12275-014-4517-y

Transcriptional Regulation of *fksA*, a β-1,3-Glucan Synthase Gene, by the APSES Protein StuA during *Aspergillus nidulans* Development

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(Received Sep 17, 2014 / Revised Oct 6, 2014 / Accepted Oct 6, 2014)

The temporal and spatial regulation of β-1,3-glucan synthesis plays an important role in morphogenesis during fungal growth and development. Northern blot analysis showed that the transcription of *fksA*, the gene encoding β-1,3-glucan synthase in *Aspergillus nidulans*, was cell-cycle-dependent and increased steadily over the duration of the vegetative period, but its overall expression during the asexual and sexual stages was fairly constant up until the time of transcription cessation. In an *A. nidulans* strain mutated in the eukaryotic bHLH-like APSES transcription factor *stuA1*, the transcriptional level of *fksA*, and consequently the content of alkali-insoluble cell wall β-glucan, significantly increased at the conidial chain formation and maturation stage. Electrophoretic mobility shift assays revealed that StuA was bound to StREs (StuA Response Elements) on the *fksA* promoter region. Promoter analysis with sGFP-fusion constructs also indicated the negative regulation of *fksA* expression by StuA, especially during asexual development. Taken together, these data suggest that StuA plays an important role in cell wall biogenesis during the development of *A. nidulans*, by controlling the transcription level of *fksA*.

***Keywords*:** *Aspergillus nidulans*, β-1,3-glucan synthase gene, transcription, StuA, EMSA, sGFP-fusion

Introduction

The fungal cell wall is essential for maintaining the shape of the cell and is a highly dynamic structure that changes in response to both external and internal cues (Klis, 1994). Thus, regulating changes in the structure and composition of the cell wall has to be the first step that a fungal cell undertakes for its normal growth and development (Cabib *et al.*, 1982).

Chitin and β-1,3-glucan are major structural components of the fungal cell wall (Klis *et al.*, 2002). Chitin, a β-1,4-linked polysaccharide of *N*-acetylglucosamine, is polymerized by chitin synthase. Ever since the first report of the chitin synthase gene from the yeast *Saccharomyces cerevisiae* was published (Bulawa *et al.*, 1986), the physiological roles and regulatory mechanism of eight chitin synthases in the filamentous fungus *Aspergillus nidulans* have been well characterized (Rogg *et al.*, 2012). β-1,3-Glucan, a β-1,3-linked polysaccharide of glucose, is polymerized by β-1,3-glucan synthase. Although 101 gene sequences for fungal β-1,3-glucan synthase are available from public databases and have been phylogenetically analyzed (Yang *et al.*, 2014), studies characterizing the genes and enzymes for β-1,3-glucan biosynthesis have been confined to only a few fungal species. In the *S. cerevisiae*, the enzyme complex containing Fks1 constitutes the β-1,3-glucan synthase activity found in vegetatively growing cells (Douglas *et al.*, 1994; Mazur *et al.*, 1995), and Fks2 is needed for β-1,3-glucan synthesis in sporulation (Mazur *et al.*, 1995). A third *FKS* homolog, *FKS3*, which was identified by homology searching, is required for normal spore wall formation (Ishihara *et al.*, 2007). Rho1p acts as a regulatory subunit, which specifically activates Fks1 in its GTP-bound form (Qadota *et al.*, 1996). In contrast to *S. cerevisiae*, only one *FKS1* homologous gene (*fksA*) has been isolated from *A. nidulans*, and the corresponding protein was purified by product entrapment in the fungus (Kelly *et al.*, 1996). However, neither has a regulatory partner been identified nor has the cellular localization of the β-1,3-glucan synthase complex been investigated in filamentous fungi.

In *A. nidulans*, fungal development and cell division are closely connected; for example, septum formation is dependent on the completion of third nuclear division (Harris *et al.*, 1994) and on a signal generated from mitotic nuclei in the germ tube during cell extension and growth (Wolkow *et al.*, 1996). Hyphal cells growing under optimal conditions spend a considerable amount of time in each stage of the nuclear division cycle, during which cells undergo constant morphogenetic changes, including cell wall biogenesis. Several reports have indicated that the interaction between developmental and cell cycle regulation is essential for normal morphogenesis in *A. nidulans* (Dutton *et al.*, 1997; Ye *et al.*, 1999; Park *et al.*, 2001).

In this paper, we report the expression level of the *fksA* gene during the cell cycle and various developmental stages of *A. nidulans*, and the analysis of the promoter region of the *fksA* gene by electrophoretic mobility shift assay (EMSA) and green fluorescent protein (GFP)-transcriptional fusion constructs. This study contributes to knowledge about the regulation of gene expression for β-1,3-glucan biosynthesis with respect to fungal growth and development.

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Materials and Methods

Strains, culture conditions, and transformation

A. nidulans strains FGSC A4 (wild type), FGSC A26 (*biA1*; *veA1*), FGSC A585 (*biA1*; *stuA1*; *veA1*), FGSC A781 (*wA2*; *nimA5*), and FGSC A237 (*yA2, pabaA1*; *trpC801*) were used in this study. *A. nidulans* complete medium (CM) was prepared as described previously (Käfer, 1977) and used for the cultures. Homogenous cell-cycle phase-specific cultures were prepared using *A. nidulans* A781 as described previously (Park *et al.*, 2001). The transformation, preparation of vegetative mycelia, and induction of asexual and sexual differentiation of *A. nidulans* were performed according to previously described methods (Lee *et al.*, 2004; Kang *et al.*, 2013). *Escherichia coli* DH5α was used for the propagation and preparation of recombinant plasmids, and *E. coli* strain BL21 was used for the expression of the DNA-binding domain of the StuA transcription factor StuAbd. *E. coli* transformation was performed according to a previously described method (Mandel and Higa, 1970). A standard LB medium was used for culturing the bacteria, and was supplemented with ampicillin (50 μg/ml) when necessary.

Nucleic acid manipulation

DNAs from the *A. nidulans* strains were prepared by using the miniprep procedure (Yelton *et al.*, 1984). DNA manipulations such as agarose gel electrophoresis and Southern blot analysis were performed according to standard methods (Sambrook *et al.*, 1989) by using an ECL labeling and detection kit (Amersham, UK). The correct integration of the vectors at the *trpC* loci of the transformants was confirmed by following the method of Lee *et al.* (2004). RNA preparation and northern hybridization were performed as described previously (Park *et al.*, 2003). The probes for northern hybridization were prepared from gel-purified RT-PCR fragments that had been amplified using specific primer sets for each gene, and then radiolabeled with [α-^{32}P]dCTP by using a random primed DNA labeling kit (Stratagene, USA) according to the manufacturer's instructions. For the EMSAs, two double-stranded DNA fragments containing StRE1 and 2 (210-bp-long SBS1·2) or StRE3 (215-bp-long SBS3) were PCR-amplified and used for the StuA binding and competition assays. Oligonucleotides pairs containing the StRE mutant sequence (mSBS1, 2, and 3) were also used for the competition assays. The nucleotide sequences of primers for PCR amplification and of the mutant oligonucleotides for the competition assay are listed in Table 1.

Preparation of GST-StuA fusion protein and EMSA

An expression vector for the glutathione *S*-transferase (GST)-fused form of StuA was constructed using pGEX-4T-1 (Amersham Biosciences, UK). A cDNA fragment of *stuA* corresponding to amino acids 1–304, which contains a sequence-specific DNA-binding APSES domain, was amplified by RT-PCR with specific primer pairs (Table 1) and cloned into the T-vector to generate pTStuAbd. The *Bam*HI/*Xho*I fragment of pTStuAbd was cloned into pGEX-4T-1, generating pGEX-StuAbd. The expression of GST-StuAbd in *E. coli* BL21 was induced by adding 1 mM IPTG into a culture of the bacteria that had reached an OD_{600} of 0.6–0.8 at 30°C. After 5 h of incubation, the cells were harvested and lysed by sonication, and the fusion protein was purified by affinity chromatography using glutathione-agarose beads. The fusion protein was eluted from the beads using reduced glutathione, dialyzed to remove excess glutathione, and stored at -80°C. EMSAs were performed as described previously (Park *et al.*, 2003).

Construction of plasmids to analyze the promoter region of *fksA*

A promoter analysis vector, pTsgfp, that had been constructed previously (Lee *et al.*, 2004), was used to analyze the expression of *fksA*, using *sgfp* cDNA as a vital reporter. Three different parts of the upstream region of the *fksA* gene were amplified by PCR as follows. First, the 688-bp upstream region of *fksA* (from nucleotides -688 to -1) was PCR-amplified from the genomic DNA of *A. nidulans* FGSC A4, using the primers fksA-CP1 and fksA-CP2. The resulting PCR product was cloned into the pGEM T-easy vector (Promega, USA) to yield pT-fksAp688. Then, the *Eco*RI fragment containing the PCR-amplified *fksA* promoter region was excised from pT-fksAp688 and inserted into *Eco*RI-digested pTsgfp, upstream of *sgfp*, to yield pT-fksAp688::sgfp. To clone the 985-bp upstream region of *fksA* (from nucleotides -985 to -1) by inverse PCR, the chromosomal DNA of *A. nidulans* FGSC A4 was digested with *Acc*I, *Sal*I, and *Pst*I, respectively, and then the presence of a 1.5 kb *Pst*I fragment carrying the 688 bp upstream region of *fksA* was confirmed by Southern blot

Table 1. Oligonucleotides used in this study

Name	Sequence (5′→3′)	Purpose
fksA-CP1	TGTTGGACAATCAGTTCCCTTT	Cloning of *fksA* promoter region
fksA-CP2	TATGACAAGCGACAATCACCG	Inverse PCR and cloning of *fksA* promoter region
fksA-CP3	TCCGTGTCCGTACTGGCTAT	Inverse PCR and cloning of *fksA* promoter region
fksA NP	CATGCCTACCTTCACCGTTT/CGCTGCATTGAAACACAGAT	Northern probe
stuA NP	GGATCCATGGCCAGCATGAATC/AAGCTTTCATTGCTGCAGCCC	Northern probe/ EMSA
SBS1·2	CAGCCACCAACGAAGCTTAAG/CCAGCTGCTTTCTGTGGCTT	EMSA
SBS3	ATCTTGTATTGGCAGTACGCC/GGGAGGGAGCAGACAAGTTAG	EMSA
mSBS1	CTACCAGTC**TA**GTTCAGCATA/TATGCTGAAC**TA**GACTGGTAG	EMSA and *in vitro* mutagenesis
mSBS2	CATAAAATTAC**TA**GGAATCTGCAA/TTGCAGATTCC**TA**GTAATTTTATG	EMSA and *in vitro* mutagenesis
mSBS3	AGCTGGTGTC**TA**GTACCTGA/TCATGGTAC**TA**GACACCAGCT	EMSA and *in vitro* mutagenesis

analysis, using the *Eco*RI-digested 688 bp from pT-fksAp688 as a probe. For inverse PCR, the 1.5 kb *Pst*I-digested genomic DNA fragment was self-ligated and used as a DNA template. From the circular DNA template, a 1,119 bp DNA fragment containing the 985 bp upstream region of *fksA* was PCR-amplified, using the primers fksA-CP2 and fksA-CP3, and then cloned into the pGEM T-easy vector to yield pT-fksAp985. Then, the 990 bp *Pst*I fragment was excised from pT-fksAp985 and inserted into *Pst*I-digested pTsgfp, upstream of *sgfp*, to yield pT-fksAp985::sgfp. To clone the 326 bp upstream region of *fksA* (from nucleotides -326 to -1), pT-fksAp985::sgfp was partially digested with *Xmn*I and then self-ligated to the plasmid fragment containing the 326 bp upstream region of *fksA* to yield pT-fksAp326::sgfp.

Other analytical methods

Confocal microscopy of *A. nidulans* cells and analysis of the intensity of green fluorescence developed in the confocal micrographs were performed according to previously described methods (Lee *et al*., 2005). Chemical analysis of the cell wall composition was determined by the method of Lee *et al*. (2002).

Results

Differential expression of the *fksA* gene in response to developmental stages

To determine whether the expression of *fksA* is cell-cycle-dependent, northern blot analysis was performed by using RNA preparations from homogenous cell-cycle phase-specific cultures of *A. nidulans*. As shown in Fig. 1A, a cell-cycle-regulated expression of *fksA* was revealed; the gene was expressed at a minimal level during the G2 phase and at a maximum level during the G1 phase, but high mRNA levels were also observed during the S and M phases. The level of *fksA* transcript in cells at three different developmental stages was monitored by northern blot analysis (Figs. 1B–1D). Whereas only a low level of *fksA* transcript was detected in young vegetative mycelia grown for 9 to 13 h, quite a higher level of the transcript was observed in older mycelia grown for 14 to 16 h. During asexual development, the level of *fksA* transcript was as high as that at the beginning of the later vegetative stage and remained almost constant thereafter, but then decreased sharply at 22 h post-induction. During sexual development, northern blot analysis was performed only for the cells of the early stage, in which Hülle cells and young cleistothecia are formed (0–2 days after induction), because adequate amounts of RNAs were not obtained at the later stages. The level of *fksA* transcript increased sharply at the early stage of sexual development (10 h after unsealing) and remained almost constant up to 50 h after induction.

Effect of StuA on the transcription level of *fksA*

Nucleotide sequence analysis of an approximately 1 kb 5′-upstream region of *fksA* revealed several sites for transcription regulation, including three StRE (StuA Response Element), four SRE (Stress Responsive Element), and one ARE (AbaA Response Element) sites. Among the transcription regulators, the APSES protein StuA acts as a morphological modifier and determines the differentiation of reproductive organs by dictating the spatial organization of conidiophores, the differentiation of sexual reproductive organs, and ascosporogenesis. A change in the *stuA* transcript level also influences temporal patterns of differentiation in reproductive organs (Wu and Miller, 1997).

To investigate the role of the three putative StREs in the *fksA* promoter *in vivo*, northern blot analysis was performed using the *stuA1* mutant strain. Mycelia from the wild-type and *stuA1* mutant strains cultured in liquid media at 37°C for 14 h (equivalent to 0 h post-induction) were transferred to solid agar media for the induction of conidiation by exposing differentiated hyphae to an air interface. The plates were

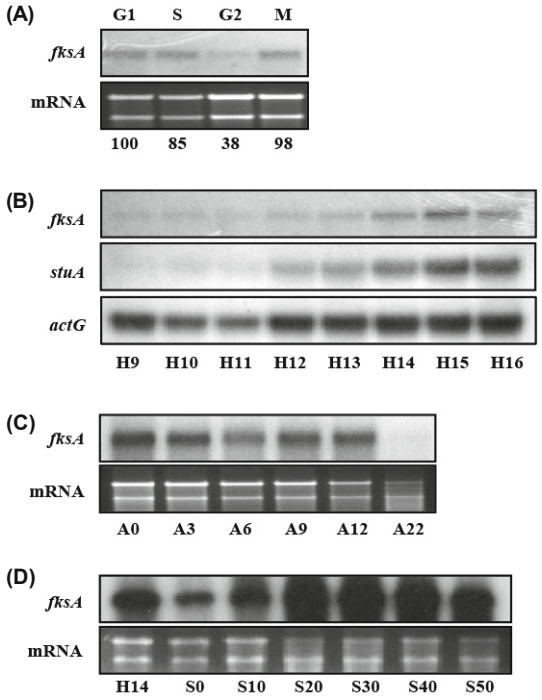

Fig. 1. Northern blot analysis of *fksA* expression during different developmental stages. For preparation of total RNAs, homogenous cell-cycle phase-specific cultures (A) of an *A. nidulans* cell cycle mutant (FGSC A781) and cultures of three different developmental stages (B, C, D) of the wild-type strain (FGSC A4) were used. (A) Expression level of *fksA* during four different cell cycles. The relative intensity of *fksA* mRNA normalized to total RNAs loaded on the gel was depicted at bottom of each lane by taking the normalized value for the G1 phase as 100%. (B) Expression levels of *fksA* and *stuA* during vegetative growth. Conidia were inoculated in complete liquid medium and cultured at 37°C, and mycelia were harvested at the time points indicated. The γ-actin gene (*actG*) was included as a control. H9 indicates the vegetative hyphae from a 9 h culture. (C) Expression level of *fksA* during asexual development. For induction of asexual differentiation, the vegetative mycelia from an 18 h culture were spread onto agar plates and incubated at 37°C. A0 indicates the time point at which induction of asexual differentiation was initiated. (D) Expression level of *fksA* during sexual development. For induction of sexual differentiation, the vegetative mycelia from an 18 h culture were transferred onto agar plates that were then sealed closed and incubated for another 24 h under dark condition, followed by incubation under unsealed condition. S0 indicates the time point at which the plates were unsealed. Ethidium-bromide-stained RNAs were included as an internal control for the amount of loaded RNA (A, C, and D).

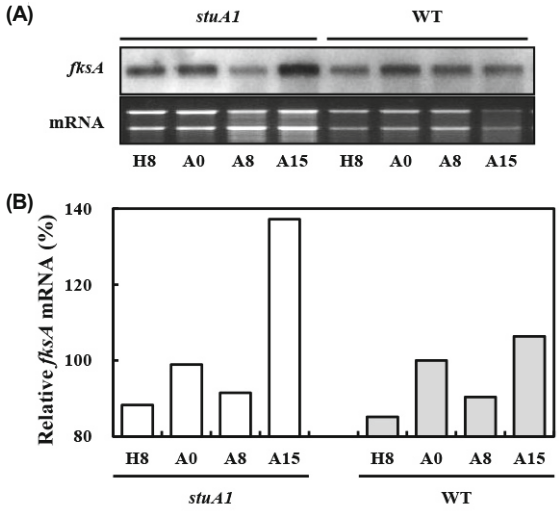

Fig. 2. Effect of *StuA1* mutation on *fksA* expression. Northern blot analysis was performed to determine whether the transcription of *fksA* was affected by *stuA1* mutation. RNAs were prepared from 8-h-old vegetative mycelia and from mycelia of asexual cultures at the time indicated post-induction. Representative photograph of northern blot analysis (A) and relative intensity of *fksA* mRNA normalized to total RNAs loaded on the gel (B). The normalized value for the wild-type strain at the time point of initiation of asexual differentiation (A0) was taken as 100%.

further incubated and samples were collected at desired time points. Under these culture conditions, short aerial hyphae were observed in about 2–4 h, aerial hyphae with mature vesicles after 4–6 h, metulae after 6 h, phialides after 9 h, immature conidia after 11.5 h, and conidial chains by 14 h post-induction. RNAs were extracted from developmental tissues taken at the indicated time of post-induction and probed with an *fksA*-specific cDNA fragment. As shown in Fig. 2, wild-type cells revealed low level of fluctuation (90.4% at A8 and 106.4% at A15) in the transcription of *fksA*, which may reflect a spatial regulation of *fksA* transcription during asexual development. Interestingly, the transcription level of *fksA* was increased significantly (137.2%) at 15 h post-induction in the *stuA1* mutant, suggesting the suppression of *fksA* transcription by StuA at the stage of conidial chain formation and maturation. These results indicate the spatiotemporal regulation of *fksA* gene expression by the APSES transcription factor StuA in *A. nidulans*.

Effect of *stuA1* mutation on cell wall composition

Fungal cell wall polysaccharides are differentially fractionated by sequential treatment with an alkali and an acid. In general, the alkali-soluble fraction contains α-glucans, and the alkali-insoluble fraction contains β-glucan as well as chitin. Subsequently, β-glucan in the alkali-insoluble fraction can be extracted by acid treatment.

To support the result from Fig. 2, the composition of the cell wall polysaccharides was analyzed by chemical fractionation of cell wall preparations from 9-h-old vegetative cultures in liquid media and from 15 h post-induction cells of asexual cultures on solid plates, respectively. In cell wall polysaccharides from the vegetative culture, the amounts of α-glucan, β-glucan, and chitin did not change significantly by *stuA1* mutation (P values of 0.235, 0.143, and 0.276, respectively). On the other hand, in the asexual cultures, the changes in the amount of α-glucan and chitin were insignificant (P values of 0.456 and 0.274, respectively), but the β-glucan fraction was significantly increased by 19% by the *stuA1* mutation (P value of 0.037) (Table 2). These results are in good agreement with those in Fig. 2, and thus indicate that cell wall β-1,3-glucan biosynthesis is affected by the StuA-dependent transcriptional regulation of *fksA* during asexual development in *A. nidulans*.

The GST-StuA fusion protein binds *in vitro* to StuA Response Element in the *fksA* promoter

As shown in Fig. 3A, sequence analysis of the promoter region of *fksA* revealed the presence of three consensus sequences ($^{A}/_{T}$CGCG$^{C}/_{A}$N$^{A}/_{C}$) for StRE, at positions -874 (StRE1), -855 (StRE2), and -777 (StRE3) relative to the ATG codon. A sequence (5′-CATTCG-3′) analogous to ARE was also found at position -363. To demonstrate the transcriptional regulation by StuA on a molecular level, a series of EMSAs were performed. A PCR-amplified cDNA fragment of *stuA* (corresponding to a sequence-specific DNA-binding APSES domain) was fused to GST, expressed, and purified from *E. coli*. For the EMSA, a 201 bp double-stranded oligonucleotide (SBS1·2) spanning both StRE1 and 2, which are separated only by 11 bp, and a 215 bp double-stranded oligonucleotide (SBS3) spanning StRE3 were PCR-amplified and used for the StuA binding assay. Figure 3B shows that the GST-StuAbd fusion protein was bound to SBS1·2 and SBS3 containing putative StREs, and that the intensity of retardation of the complexes was increased by an increase of the GST-StuAbd fusion protein. These complexes were specific, because all shifted bands were abolished when increasing molar excesses of unlabeled SBS1·2 and SBS3 were used as specific competitors (Fig. 3C). When unlabeled mutant oligonucleotides, mSBS1, 2, and 3 (of which the StRE

Table 2. Composition of cell wall polysaccharides

Developmental stage	Strain	Cell wall polysaccharides (μg/mg dry mycelia)[a]		
		α-Glucan	β-Glucan	Chitin
Vegetative	WT	82.4±11.9	150.5±15.4	149.9±6.5
	stuA1	89.9±11.3	164.6±12.1	153.3±6.2
Asexual	WT	75.7±10.7	330.2±33.7[b]	86.7±22.9
	stuA1	76.6±7.9	393.5±30.5[b]	97.9±18.2

[a] Chemical analyses of the cell wall preparations were performed three times with the wild type and mutant strains in both developmental stages. In each chemical analysis, samples for the carbohydrate assay were tripled.
[b] The P value for these two samples is 0.037.

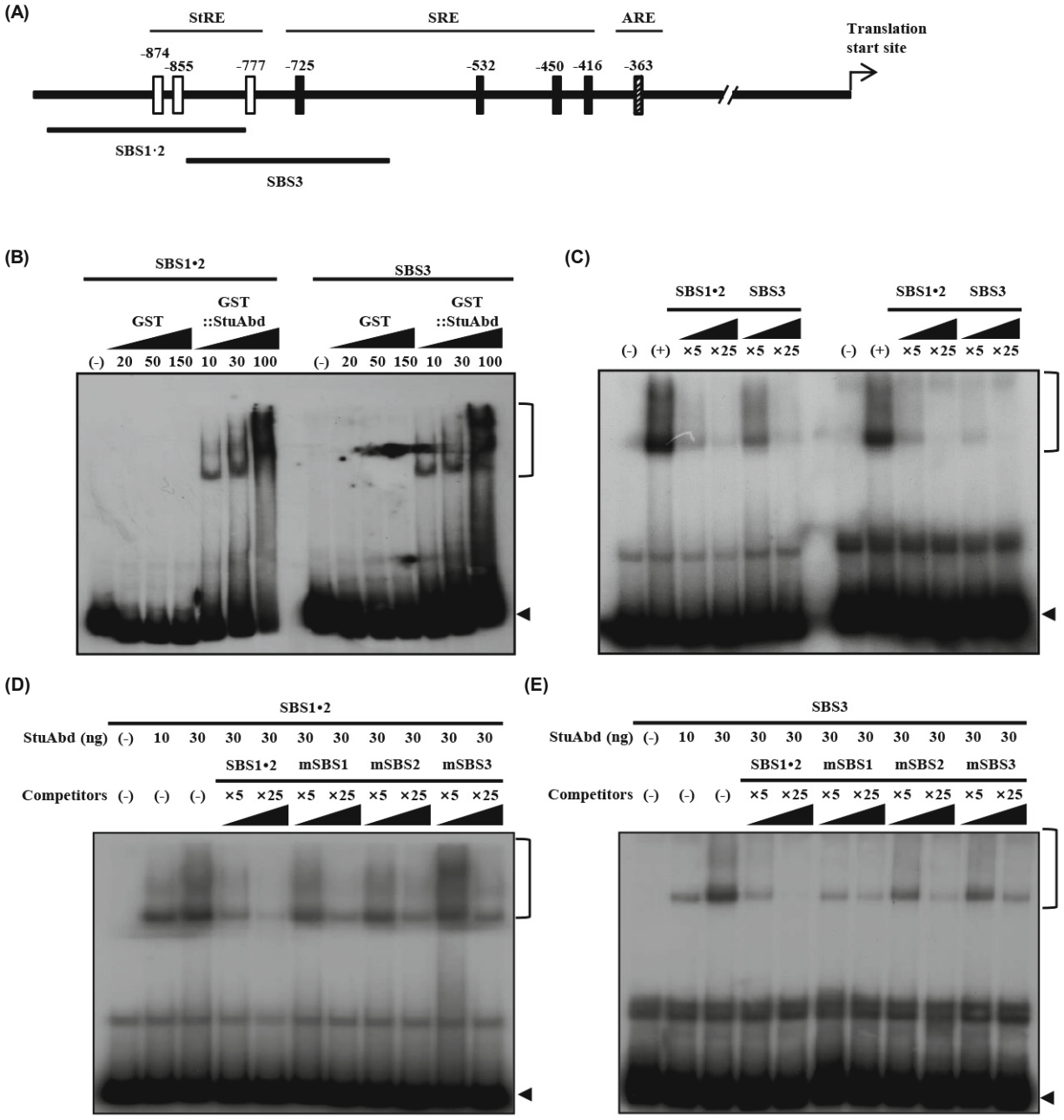

Fig. 3. Electrophoretic mobility shift assay of StuA binding to the *fksA* promoter. (A) Position of putative StREs (5'- $^{A}/_{T}$CGCG$^{C}/_{A}$N$^{A}/_{C}$-3') in the *fksA* promoter. Positions of PCR-amplified fragments spanning putative StRE motifs, SBS1·2 and SBS3, are marked with blocked bars below each element. StRE, SRE, and ARE stand for StuA Response Element, Stress Response Element, and AbaA Response Element, respectively. (B) Radiolabeled dsDNA probes were incubated with increasing amounts of glutathione *S*-transferase (GST)-StuAbd fusion protein or GST alone. Lanes marked with (–) indicate free probes incubated with no added protein. Bound probes (bracket) and unbound probes (arrowhead) are indicated. (C, D, E) Competition assays. Radiolabeled SBS1·2 and SBS3 probes and 30 ng of GST-StuAbd incubated with unlabeled specific competitors (C) and mutant competitors (D, E). All mutant competitors (mSBS1, 2, and 3) have altered sequences; that is, GC in the consensus sequence (5′-$^{A}/_{T}$CGCG$^{C}/_{A}$N$^{A}/_{C}$-3′) of StRE was substituted with TA to generate 5′-$^{A}/_{T}$CTAG$^{C}/_{A}$N$^{A}/_{C}$-3′. Bound probes (bracket) and unbound probe (arrowhead) are indicated.

motif 5′-CGCG-3′ was mutated to 5′-CTAG-3′) were used as the competitor, the capacity of these oligonucleotides to prevent complex formation was reduced considerably (Fig. 3D and 3E). These data demonstrate that StuA binds *in vitro* to putative StREs found in the 5′-upstream sequences of *fksA*.

Effect of StREs in the 5′-upstream region on *fksA* transcription

To test the effect of StRE on *fksA* transcription *in vivo*, a pT-fksAp::sGFP series of the reporter plasmids were constructed, in which a series of the 5′-upstream region of *fksA* for the promoters were fused to an open reading frame for

sGFP (Fig. 4A). These reporter plasmids were introduced into the *A. nidulans* wild-type strain, respectively, and the transformants, in which pT-fksAp::sGFPs were integrated into the chromosome by homologous recombination, were confirmed by Southern blot analysis (data not shown). Then, these strains were examined by confocal microscopy to determine the effect of the 5′-upstream region of *fksA* on the temporal and spatial expression of sGFP during vegetative growth and asexual development. The intensity of fluorescence emitted by the transformants was quantified and analyzed to compare the expression level according to the promoter construction and developmental stages. As summarized in Fig. 4B, the transformant with 326 bp of the 5′-upstream region of *fksA*, in which no known transcription factor binding site was included, showed weak expression of sGFP in the mycelia and asexual organs. However, the transformant with 688 bp of the 5′-upstream region of *fksA*, in which one ARE (-363) and three SRE (-416, -450, and -532) sites were included, showed a stronger expression of sGFP than did that with the 326 bp sequence in both the mycelia and asexual organs. Most interestingly, the transformant with 985 bp of the 5′-upstream region of *fksA*, in which one SRE (-725) and three StRE (-777, -855, and -874) sites were additionally added to that of the 688 bp sequence, showed a further increase of sGFP expression in the mycelia but a decrease in expression in the asexual organs. These observations support the possibility that StREs in the 5′-upstream region of *fksA* are important for the negative regulation of *fksA* expression at the stage of asexual organ development in *A. nidulans*.

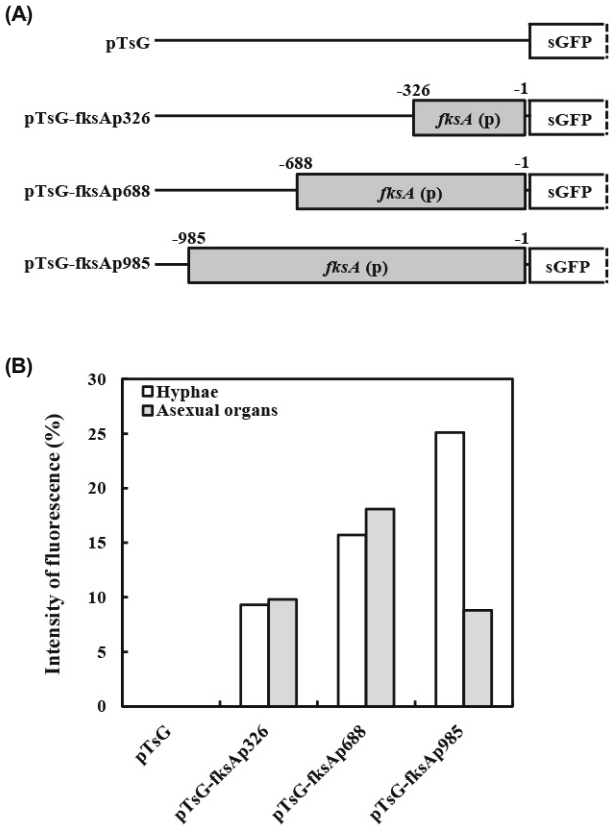

Fig. 4. Analysis of the effect of putative StREs on the expression of *fksA* during vegetative growth and asexual differentiation using modified green fluorescent protein (sGFP) as a vital reporter. (A) Schematic representation of the pT-fksAp::sGFP series of reporter plasmids utilized for the StuA transactivation tests. pT-fksAp326::sGFP contains 326 bp of the *fksA* promoter region without any site for known transcription regulators. pT-fksAp688::sGFP contains 688 bp of the *fksA* promoter region with one ARE and three SRE sites. pT-fksAp985::sGFP contains 985 bp of the *fksA* promoter region with one ARE, four SRE, and three StRE sites. (B) Analysis of the intensity of green fluorescence in the confocal micrographs. The fluorescence intensity developed in micrographs taken from the vegetative culture and asexual culture of each transformant was measured using an image analysis system (GAIA Material V5.3.2.1). The relative intensity of green fluorescence (%) was calculated in comparison with those from the corresponding cultures of the transformant with sGFP only. The increased amount (%) of relative intensity is depicted.

Discussion

As the results in this study reveal, transcription of *fksA* is cell-cycle-dependent and modulated by a morphological modifier, StuA, which determines the differentiation of asexual and sexual reproductive organs in *A. nidulans* (Wu and Miller, 1997).

In *A. nidulans*, interaction between developmental regulation and cell cycle regulation is essential for normal differentiation (Dutton *et al.*, 1997; Ye *et al.*, 1999; Park *et al.*, 2001; Lee *et al.*, 2013), during which cells undergo constant morphogenetic changes, including those of the cell wall structure. Previously, we reported the cell-cycle-regulated expression of genes for chitin biosynthesis, the pattern of which was classified into two groups (Park *et al.*, 2001). The first group includes *chsA* and *chsC*, which share critical functions in maintaining hyphal wall integrity and in developing asexual structures such as the conidiophores (Fujiwara *et al.*, 2000); the second group includes *csmA*, *chsB*, and *chsD*, which are functionally linked and overlapped with each other for hyphal growth and development (Borgia *et al.*, 1996; Specht *et al.*, 1996; Horiuchi *et al.*, 1999; Park *et al.*, 2001). However, no direct evidence for the cell-cycle-dependent regulation of β-1,3-glucan biosynthesis in *A. nidulans* has been presented. In this study, the cell-cycle-regulated expression of *fksA* in *A. nidulans* was revealed by northern blot analysis, using homogenous cell-cycle phase-specific cultures: a minimal level was expressed during the G2 phase, a maximum level during the G1 phase, and high levels during the S and M phases (Fig. 1A). The cell-cycle-dependent expression pattern of *fksA* is quite similar to that of the chitin synthase genes, *chsB*, *chsD*, and *cmsA* (Park *et al.*, 2001), and thus it can be postulated that the expression of *fksA* largely contributes to hyphal growth and development, in the same way that *chsB*, *chsD*, and *cmsA* do, but not to asexual development like *chsA* and *chsC*. It is also noteworthy that the APSES proteins, including StuA, control the critical G1/S cell cycle transition in yeasts, and that the DNA-binding motif of StREs is enriched with promoter sequences of *A. nidulans* cell cycle genes such as *nimE*, *nimO*, and *nimX* (Dutton *et al.*, 1997; Kang *et al.*, 2013).

In *A. nidulans*, StuA affects the morphogenesis of conidiophores by modulating the expression of the transcription factors *brlA* and *abaA*, which comprise the so-called cen-

tral regulatory pathway for asexual development. Sequence analysis of the 5′-upstream region revealed three putative StRE sites, and thus it is quite possible that the transcription of *fksA* is directly regulated by StuA. The results from northern blot analyses during the cellular life cycles (Figs. 1B–1D) and in the *stuA1* mutant (Fig. 2) support this possibility to some extent. The level of *stuA* expression is relatively low at the early stage of vegetative growth, starts to increase at 12 h after the initiation of vegetative growth (at which time the cells acquire competence for development), and reaches its maximum after 15 h of vegetative growth. It is noteworthy that the pattern of *fksA* expression during the vegetative growth stage did not show significant difference with that of *stuA* (Fig. 1B). In asexual development, an increase of *stuA* expression is observed even at 48 h post-induction, at which time the expression level is at least 15-fold higher than that at 12 h of vegetative growth (Kang *et al*., 2013). However, the *fksA* expression maintains the same level as that of 15 h of vegetative growth from 0 h (equivalent to H15) to 12 h post-induction, and then ceases almost completely at 22 h post-induction of asexual development, at which time conidial development and maturation prevail (Fig. 1C). In sexual development, *stuA* keeps its expression relatively constant level, even at 48 h post-induction of sexual development, the level of which is 5.5-fold higher than that of 12 h of vegetative growth (Kang *et al*., 2013). In Fig. 1D, *fksA* shows a rather constant expression until 50 h post-induction, at which time the maturation of cleistothecium prevail. Although *stuA* expression starts with the acquisition of competence in vegetative cells at around 12 h and is held above a certain level, the *fksA* expression seems to be influenced by *stuA* only at specific points of development for asexual spore production. These results suggest that the expression of *fksA* cannot be obligatorily dependent on *stuA* throughout the entire fungal life cycle; therefore, other regulators, such as AbaA and other stress response elements, may have roles in regulating *fksA* transcription, possibly in vegetative growth.

Northern blot analysis of *fksA* expression in the *stuA1* mutant and wild-type strain revealed no difference at the early stage of vegetative growth (H8) and asexual development (A0, 8), but a considerable increase in *fksA* expression in the *stuA1* mutant occurred at 15 h post-induction of asexual development (Fig. 2) when conidial chain formation and maturation prevail. These results suggest a role for StuA in the repression of *fksA* rather than in its activation at specific stages of asexual development. This postulation is supported by results from the chemical analyses of the polysaccharides (Table 2) in the cell walls of *A. nidulans* cultures, where liquid cultures of vegetative mycelia (V9) showed no significant difference between wild-type and *stuA1* mutant strains, but that from the asexual culture (A15) showed a statistically significant increase ($P = 0.037$) of the alkali-insoluble β-1,3-glucan fraction in the *stuA1* mutant (Table 2).

EMSA with the recombinant StuA-binding domain and DNA probes containing sequences of StREs in the 5′-upstream region of *fksA* also provided additional evidence at the molecular level to support the involvement of StuA in regulating *fksA* expression (Fig. 3). Results from the *fksA* promoter assay with sGFP-fusion constructs revealed that the longer the promoter region in the sGFP-fusion construct is, the stronger is the fluorescent intensity in vegetative hyphae. However, the longest fusion construct containing three StRE sites showed a specific decrease of sGFP intensity in the asexual organs, but not in the vegetative hyphae (Fig. 4B).

In summary, the results presented here indicate that expression of the *fksA* gene coding for β-1,3-glucan synthase is cell-cycle-regulated, and that binding of the developmental modulator StuA to StRE sites on the 5′-upstream region of *fksA* is important for the negative regulation of *fksA* expression, especially at the specific point of asexual development for conidial chain formation and maturation in *A. nidulans*. Although further studies are required, our results open up interesting avenues for further studies on the regulatory mechanism of the gene(s) for β-1,3-glucan synthase throughout the complicated life cycle of the filamentous fungi.

Acknowledgement

This work was supported by a grant from Chungnam National University [2014-0796-01].

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