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csmA, a gene encoding a class V chitin synthase with a myosin motor-like domain of *Aspergillus nidulans*, is translated as a single polypeptide and regulated in response to osmotic conditions

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Abstract

The *csmA* gene of *Aspergillus nidulans* encodes a polypeptide that consists of an N-terminal myosin motor-like domain and a C-terminal chitin synthase domain. *csmA* null mutants showed marked abnormalities in polarized growth, hyphal wall integrity, and conidiophore development. Furthermore, the growth of the *csmA* null mutants was sensitive to low osmotic conditions. In an effort to investigate the intracellular behavior of the *csmA* product (CsmA) and the regulation of its production, we constructed strains that produced CsmA tagged with nine repeats of the hemagglutinin A (HA) epitope at its COOH terminus (CsmA–HA) instead of CsmA. Western blot analysis with anti-HA antibody showed that the entire coding region of *csmA* was translated as a single polypeptide with an approximate molecular mass of 210 kDa. CsmA–HA was produced during vegetative growth; however, its yield was significantly reduced under high osmotic conditions, suggesting that the role of CsmA in growth and morphogenesis is particularly important under low osmotic conditions.

Keywords: *Aspergillus nidulans*; Chitin synthase; Myosin; CsmA; Filamentous fungi; Cell wall; Osmotic conditions; Post-transcriptional regulation

The filamentous fungus *Aspergillus nidulans* grows by generating ordered networks of filaments or hyphae that form a mycelium. The temporal and spatial regulation of cell wall polymer synthesis plays an important role in fungal morphogenesis. Chitin, a β-1,4-linked homopolymer of *N*-acetylglucosamine (GlcNAc), is one of the major structural components of the fungal cell wall. Chitin metabolism, including synthesis, degradation, assembly, and cross-linking to other cell wall components, plays a critical role in hyphal growth and morphogenesis of many filamentous fungi [1–3].

Chitin synthases are membrane-bound proteins that catalyze the polymerization of GlcNAc using UDP-GlcNAc as a substrate. These synthases have been classified into five groups (classes I–V) on the basis of their conserved region amino acid sequence [4–6]. We have isolated five chitin synthase genes from *A. nidulans* and designated them *chsA*, *chsB*, *chsC*, *chsD*, and *csmA*, which correspond to classes II, III, I, IV, and V, respectively [7–10]. *csmA* encodes a unique protein (1852 amino acids) consisting of an N-terminal myosin motor-like domain (approx. 800 amino acids) and a C-terminal chitin synthase domain (approx. 750 amino acids) [10].

Myosins are mechanoenzymes that convert chemical energy, liberated through ATP hydrolysis, into mechanical force along actin filaments. In *A. nidulans*, only one gene (*myoA*) encoding a class I myosin has been cloned and characterized thus far. *MyoA* is essential for hyphal growth and plays a role in the secretion and endocytosis processes [11,12]. In *Saccharomyces cerevisiae*, the class I myosins (Myo3p and Myo5p) are involved in endocytosis and polarization of the actin cytoskeleton [13–16]. Additionally, it has been shown that Myo2p, a class V myosin, is essential for the proper localization of Chs3p [17]. The N-terminal myosin motor-like domain of CsmA contains typical myosin

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consensus motifs such as a P-loop, Switch I and Switch II sequences [10], and belongs to myosin family class XVII [18]. In filamentous fungi, actin is concentrated at the hyphal tips, the septa, and the branching sites, where cell wall or septal synthesis is active [19,20]. It is possible that the N-terminal myosin motor-like domain is involved in the transport of CsmA and/or the polarization of the actin cytoskeleton.

Orthologues of the entire coding region of *csmA*, *csm1* of *Magnaporthe grisea* [21], *chs2* of *Blumeria graminis* [22], *chsY* of *Aspergillus oryzae* (Accession No. AB066447), and *chs5* of *Exophiala dermatidis* (Accession No. AF469116) have been isolated. A hypothetical gene encoding a Csm-type protein is also present in the genome of *Neurospora crassa*, whose genome sequence has almost been determined. Since no orthologue exists in the genome of the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*, it is possible that Csm-type proteins have unique functions that are peculiar to filamentous growth.

In a previous investigation, we constructed and characterized *csmA* null mutants [23]. The growth of the *csmA* null mutants was sensitive to low osmotic conditions. Depolarized swollen tubes, balloons, or intrahyphal hyphae appeared, predominantly in old regions. Abnormal conidiophore morphologies, such as short stalks and a small population of metulae on the vesicles, were occasionally observed. These phenotypes in growth and conidiophore formation were suppressed to some extent with osmotic stabilizers. However, they were not suppressed when only the chitin synthase domain-coding region of *csmA* was expressed under control of the *alcA* promoter. Thus, we concluded that CsmA plays an important role in polarized cell wall synthesis and the maintenance of cell wall integrity, with the myosin motor-like domain being indispensable for these functions. Little is known about the transcriptional and translational regulation of the *csmA* gene. *csmA* mRNA contains three short ORFs upstream of the main ORF [10], suggesting that CsmA production could be regulated at the translational level.

The purpose of this study was to investigate the regulation of *csmA* expression and CsmA production. We constructed strains that produced CsmA tagged with hemagglutinin A (CsmA–HA) and monitored the temporal changes in the amount of CsmA–HA protein using Western blot analysis. *csmA* transcript levels were analyzed by Northern blot analysis. CsmA–HA was detected throughout the 5 days of cultivation and its amount was significantly lower under high osmotic conditions. The results of these experiments suggest that CsmA plays a crucial role in growth and morphogenesis, particularly under low osmotic conditions. In an effort to investigate the transcriptional control of *csmA*, we cloned and sequenced the *csmA* promoter region. An *abaA* response element, two HAP complex binding sites, osmotic response consensus sequences, and stress response elements (STRE) were all found in the promoter region.

Materials and methods

Strains and media. The *A. nidulans* strains used in this study were FGSC89 (*biA1 argB2*), ABPU1 (*biA1 pyrG89 argB2 pyroA4 wA3*) [8], ABPU/U (*biA1 pyrG89 argB2 pyroA4 wA3* [pP1] [8]), as well as CA2 and CA3 (*biA1 pyrG89 argB2 pyroA4 wA3 csmA*::9HA::*pyrG*). YG medium (0.5% yeast extract, 1% glucose, and 0.1% trace elements) and minimal medium (MM) for *A. nidulans* were generally used [24]. YG and MM plates were YG and MM containing 1.5% agar, respectively. MM was supplemented with arginine at 0.2 mg/ml, biotin at 0.02 μg/ml, pyridoxine at 0.5 μg/ml, and uridine at 2.44 mg/ml, when necessary.

Bacterial and fungal transformations. Transformation of *A nidulans* was performed as described previously [25]. Transformants were grown in MM with appropriate supplements. *Escherichia coli* MV1190 was used as a host strain for plasmid amplification and grown in Luria broth [26]. *E. coli* transformation and plasmid extraction were performed by standard methods [26].

Plasmid construction. Plasmids for the introduction of the coding sequence of nine repeats of HA epitope into the 3′ terminus of the *csmA* coding region were constructed as follows. The 0.9-kb *Bgl*II–*Nsp*I fragment from pM-ALC-CHS5 [23] was ligated with *Sal*I-digested pUC18, to yield p18MAC. A *Not*I site was introduced into the 3′ end of the *csmA* coding sequence of p18MAC by using TAKARA LA PCR in vitro Mutagenesis Kit (TAKARA) with the primer 5′-GCAACCTGTGCGGCCGCTTCCCCCCC-3′ (the underlined sequence represents the *Not*I recognition site), to yield pUCNOT1. A primer set of 5′-GGGCGGCCGCTTCGAGCTCATCTTTTAC-3′ (the underlined sequence represents the *Not*I recognition site) and 5′-GGTCTAGATCACTATAGGGCGAATTG-3′ (the underlined sequence represents the *Xba*I recognition site and the wavy line represents two tandem stop codons of 3xHA epitope) was used to amplify a 3xHA-encoding epitope sequence of pMPY-3xHA [27]. The 0.15-kb PCR-amplified product digested with *Not*I and *Xba*I was ligated with *Not*I and *Xba*I-digested pBluescript II, to yield pBSHA. The 0.15-kb *Not*I–*Pst*I fragment of pBSHA was ligated with *Not*I and *Pst*I-digested pUCNOT1, to yield pUCNH1. The 1.6-kb *Nde*I–*Xho*I fragment containing *pyrG* of *A. nidulans* from pJR15 [28] was ligated with *Sma*I-digested pUC119, to yield pUCPYR1. The 1.6-kb *Eco*RI–*Pst*I fragment of pUCPYR1 was ligated with *Eco*RI and *Pst*I-digested pUCNH1, to yield pUCNHP1. The 0.9-kb *Nae*I–*Stu*I fragment of pMK10 [10] was ligated with *Eco*RI-digested pUCNHP1, to yield pUCNHP10. Primer sets of A-5′-HA (5′-CGGCGGCCGCTACC CATACGATGTTCCTGAC-3′ in which the *Not*I recognition site is underlined) and A-3′-HA (5′-CCACTAGTAGCGTAATCTGGAAC GTCATAT-3′ in which the *Spe*I recognition site is underlined), as well as B-5′-HA (5′-CGACTAGTTACCCATACGATGTTCCTGAC-3′ in which the *Spe*I recognition site is underlined) and B-3′-HA (5′-CGGAATTCGCGGCCGCTAGCGTAATCTGGAACGTCATA T-3′ in which the *Eco*RI and *Not*I recognition sites are underlined) were used to amplify a 3xHA-encoding epitope sequence of pMPY-3xHA. The 0.1-kb PCR-amplified product using A-5′-HA and A-3′-HA as primers was digested with *Not*I and *Spe*I, and ligated with *Not*I and *Spe*I-digested pBluescript II, to yield pHA3. The 0.1-kb PCR-amplified product using B-5′-HA and B-3′-HA as primers was digested with *Eco*RI and *Spe*I, and ligated with *Eco*RI and *Spe*I-digested pHA3, to yield pHA6. The 0.2-kb *Not*I fragment of the 6xHA-encoding epitope sequence from pHA6 was ligated with *Not*I-digested pUCNHP10, to yield pHA9.

Subcellular fractionation and Western blot analysis. Total cellular extracts were prepared by grinding mycelia (approximately 200 mg) with Metal-corn (YASUIKIKAI) and then with glass beads using

Multi-beads shocker (YASUIKIKAI) in 200 μl extraction buffer (0.8 M sucrose, 10 mM Tris–HCl, pH 8.2) in the presence of protease inhibitor cocktail (Sigma). This was followed by centrifugation at 1000*g* for 5 min at 4 °C to remove cellular debris. The cellular extracts were centrifuged at 10,000*g* for 15 min at 4 °C to prepare low-speed pellets (LSP). The supernatants were centrifuged at 100,000*g* for 1 h at 4 °C to obtain high-speed supernatant (HSS) and high-speed pellets (HSP). The protein concentrations in the samples were measured using a BCA protein assay kit (PIERCE). Samples containing approx. 10 μg protein were separated by electrophoresis on 6% polyacrylamide gel containing sodium dodecyl sulfate and electroblotted to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were incubated with a mouse anti-HA primary antibody (BAbCO) at a 1:1000 dilution. Then, horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (cell signaling) at a 1:5000 dilution was added. The HRP was visualized with the ECL Western Blotting Detection System (Amersham Pharmacia Biotech) as recommended by manufacturer's instructions.

Northern blot analysis. Total RNA was isolated using an RNeasy Total RNA Kit (Qiagen) according to manufacturer's instructions. Northern blot analysis was perfomed as described previously [23]. A 1.0-kb *Spe*I and *Xho*I-digested fragment of pM-ALC-CHS5 was used as a probe for the detection of transcripts of *csmA* [23].

PCR amplification of the DNA fragment of the csmA promoter region. Total DNA was extracted from FGSC89 as previously described [28], digested with *Hin*dIII, and self-ligated. Inverse-PCR amplification was done with Taq DNA polymerase (TaKaRa Ex Taq) and the primer sets of Re-69 (5′-ACCGACTACGTTTTCATTCTAT-3′) and C-578 (5′-GATTCAACCCTTACTCGATC-3′) using the self-ligated DNA as a template. DNA sequencing was done as previously described [8]. PCR amplification was done with KOD DNA polymerase (TOYOBO) and a primer set of Re-69 and C-800 (5′-ATATGAC AGTTCCTCGCCAG-3′) using the total DNA from FGSC89 as a template. DNA fragments were ligated with pGEM-T Vector (Promega). The sequences of inserted DNA from three discrete clones were determined.

Nucleotide sequence accession number. The genomic DNA sequence of *csmA* was deposited in the DDBJ database (AB000125).

Results

Construction of CsmA–HA producing strains

To determine the intracellular amount of CsmA, we employed homologous recombination to introduce the coding sequence of nine repeats of the HA epitope into the 3′ terminus of the *csmA* coding region using the *Bgl*II–*Eco*RI fragment of pHA9. Successful integration of the DNA fragment that encoded the HA epitopes at the 3′ terminus of *csmA* was confirmed by Southern blot analysis. Two transformants, CA2 and CA3, were found amongst the ninety-six tested (Figs. 1A and B). Translation into CsmA–HA would be terminated at the end of the 9xHA coding region in these strains (see Materials and methods). Since no phenotypic difference was found between the two transformants under the various conditions tested, we used CA2 for further analysis as described below. It was supposed that CsmA–HA was functional since CA2 did not display any growth or

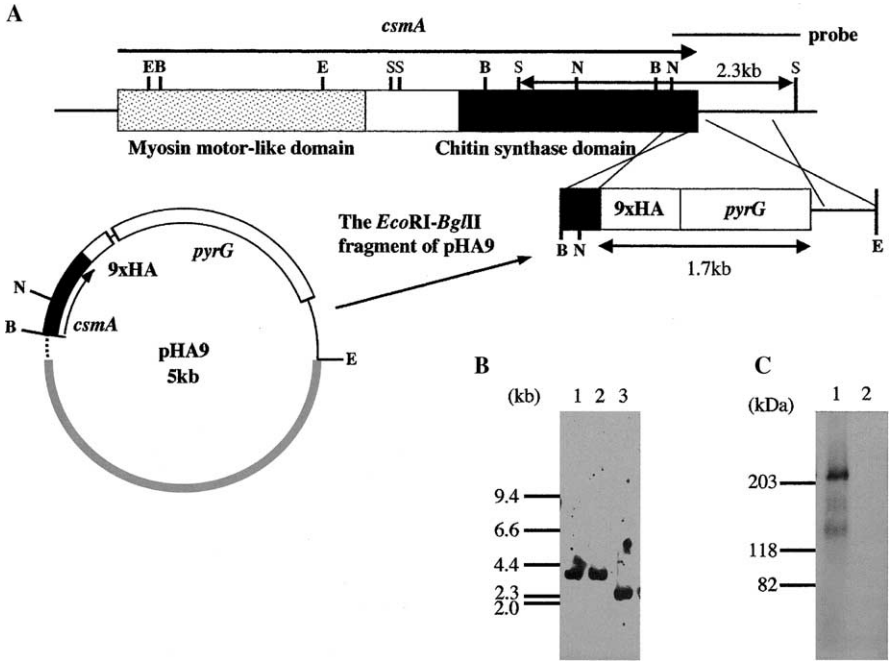

Fig. 1. Construction of CsmA-HA-producing strains. (A) Scheme of construction. The direction of the predicted open reading frame of *csmA* is indicated by an arrow. The *csmA* coding region is shown by the box. The myosin motor-like domain and the chitin synthase domain are indicated by a stippled box and a black box, respectively. The region used as a probe for Southern blot analysis is shown. Abbreviations: B, *Bgl*II; E, *Eco*RI; N, *Nde*I; S, *Sph*I. (B) Southern blot analysis of *Sph*I-digested total DNA of strain CA2 (lane 1), strain CA3 (lane 2), and strain ABPU1 (lane 3) probed with the 1.2-kb *Nde*I–*Sph*I fragment from pMK10. (C) Western blot analysis of cell extracts of strain CA2 (lane 1) and strain ABPU/U (lane 2) using anti-HA antibody.

morphological defects in comparison with the wild-type strain. The intracellular amount of CsmA–HA was monitored by Western blot analysis using anti-HA antibody. After 24 h of cultivation, a major band was visualized in an extract of CA2 at an approximate molecular mass of 210 kDa; however, the band was not seen in cell extracts of the wild-type strain (ABPU/U). This indicates that the entire coding region of *csmA* was translated as a single polypeptide (Fig. 1C).

When total cell extracts of CA2 were fractionated by centrifugation, CsmA–HA was found predominantly in the LSP and was not detected in the HSS (data not shown). This is consistent with CsmA presumably being an integral membrane protein due to the presence of the predicted transmembrane regions in the chitin synthase domain of CsmA.

Temporal change of CsmA–HA protein and csmA transcript levels

Temporal changes in CsmA–HA levels were investigated. Western blot analysis showed that the 210 kDa band of CsmA–HA displayed maximum signal intensity at day 1 and then gradually diminished, but was still detected even after 5 days of cultivation (Fig. 2A). Another band of 140 kDa was detected and its intensity reached a maximum at day 2. The intensity of this band was higher than that of the 210 kDa band at day 3 (Figs. 2A and C). Examination of the change in band intensities might suggest that the protein represented by the 140 kDa band is likely to be a degradation product of CsmA–HA lacking the myosin motor-like domain. The 210 and 140 kDa proteins appeared to reach a maximum at days 1 and 2, respectively, and gradually decreased after 3 days of cultivation. On the other hand, the amount of *csmA* transcript gradually increased over the first 4 days of cultivation, at which point it reached a level of 130% (relative to day 1) and then decreased to 70% after 5 days of cultivation (Figs. 2B and D). RNA was extracted from the wild type strain (ABPU/U) and the CA2 transformant grown under the same conditions. Since no difference was found in *csmA* transcript levels between the two under the various conditions tested, the data derived from the ABPU/U strain are shown. The amount of *csmA* transcript was maintained at a high level on days 3 and 4, when CsmA–HA levels were being reduced. Therefore, CsmA levels may be regulated at the post-transcriptional or the post-translational level.

Change in the amount of CsmA–HA and csmA transcript in response to external osmolarity

The growth of the *csmA* null mutants was sensitive to low osmotic conditions (YG plate and 1/2YG plate). This growth defect was remedied to some extent under moderate osmotic conditions (YG + 0.6 M KCl) [23,29].

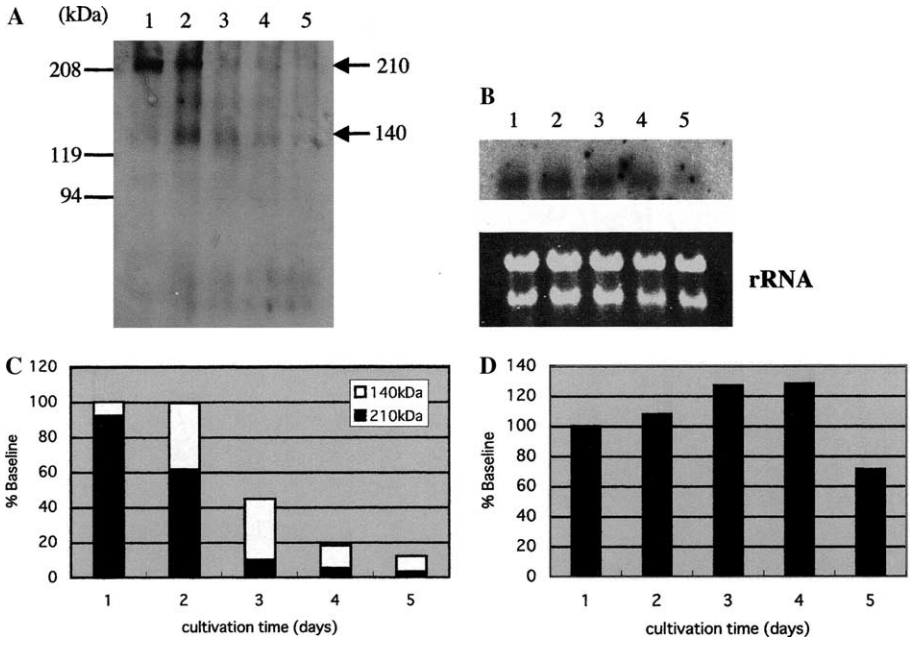

Fig. 2. Temporal change of CsmA–HA protein and *csmA* transcript levels. Each strain was grown for 1 day (lane 1), 2 days (lane 2), 3 days (lane 3), 4 days (lane 4), and 5 days (lane 5) in YG liquid culture. (A) Western blot analysis of LSP of cell extracts of strain CA2 grown in YG liquid culture. (B) Northern blot analysis of the total RNAs of ABPU/U. Approximately 10 μg RNA was loaded on each lane. The 1.0-kb *Spe*I–*Xho*I fragment of pMALK-CHS5 was used as a probe. (C) Scanning densitometry analysis of the amount of CsmA–HA as detected by Western blot analysis. Signals were displayed as percentage of the baseline values (1 day). Symbols: ■, 210 kDa; □, 140 kDa. (D) Scanning densitometry analysis of *csmA* mRNA levels as detected by Northern blot analysis. Signals were displayed as a percentage of the baseline values (1 day). Intensities of rRNA bands were used as internal standards.

We therefore investigated whether intracellular CsmA–HA levels changed in response to alterations in external osmolarity. The CA2 strain was cultivated on YG, YG + 0.6 M KCl, or YG + 1.2 M KCl. When cultivated in the respective solid or liquid media, the growth rates differed significantly. To obtain only young hyphae from these cultures, hyphae at the edge of 5-day-old colonies were collected and extracts were prepared. CsmA–HA was more abundant in hyphae grown under low osmotic conditions (YG plate) than in those grown under moderate osmotic conditions (YG + 0.6 M KCl plate). CsmA–HA was scarcely detected in hyphae grown under high osmotic conditions (YG + 1.2 M KCl plate) (Fig. 3A). The band that is present between 210 and 140 kDa may represent an intermediate degradation product of CsmA–HA. The level of *csmA* transcript was higher in hyphae grown on the YG plate than those grown on the YG + 0.6 M KCl or YG + 1.2 M KCl plate (Fig. 3B). The growth of the *csmA* null mutants was sensitive to low osmotic conditions and the defects in growth and conidiophore formation of the *csmA* null mutants were suppressed with osmotic stabilizers to some extent. The results obtained in this investigation suggest that the level of *csmA* transcript is regulated in response to an alteration in external osmolarity and that the role of CsmA in growth and morphogenesis is particularly important under low osmotic conditions.

Nucleotide sequence of the promoter region of csmA

To analyze the promoter structure that controls the transcriptional regulation of *csmA*, a 1.4 kb DNA fragment of the promoter region of *csmA* was amplified by inverse-PCR using the Re-69 and C-578 primer set. The nucleotide sequence was determined and a new PCR primer (C-800) was designed. The promoter region of *csmA* was amplified (using the Re-69 and C-800 primers) and the nucleotide sequence of three independently amplified clones was determined (see Materials and methods). An *abaA* response element (ARE) [30] and two CCAAT sequences, to which the HAP complex binds [31,32], were found in the promoter region of *csmA*. In *S. cerevisiae*, the Rlm1p and Msn2p/Msn4p transcription factors are known to be involved in the response to osmotic stress. Consensus sequences that are recognized by Rlm1p and Msn2p/Msn4p are present in the promoter region of *csmA* (Fig. 4).

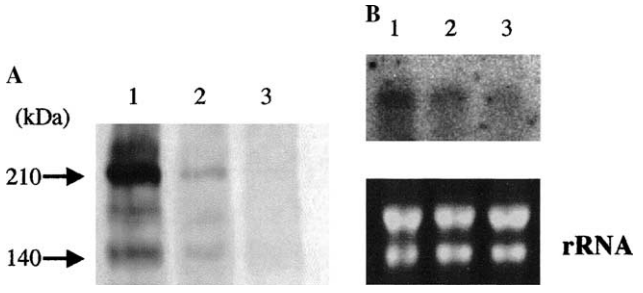

Fig. 3. Change in the amounts of CsmA–HA and *csmA* transcript in response to external osmolarity. Each strain was grown on YG (lane 1), YG + 0.6 M KCl (lane 2), or YG + 1.2 M KCl (lane 3) plate. (A) Western blot analysis of LSP of cell extracts of strain CA2, (B) Northern blot analysis of the total RNAs of ABPU/U. Approx. 10 μg RNA was loaded on each lane.

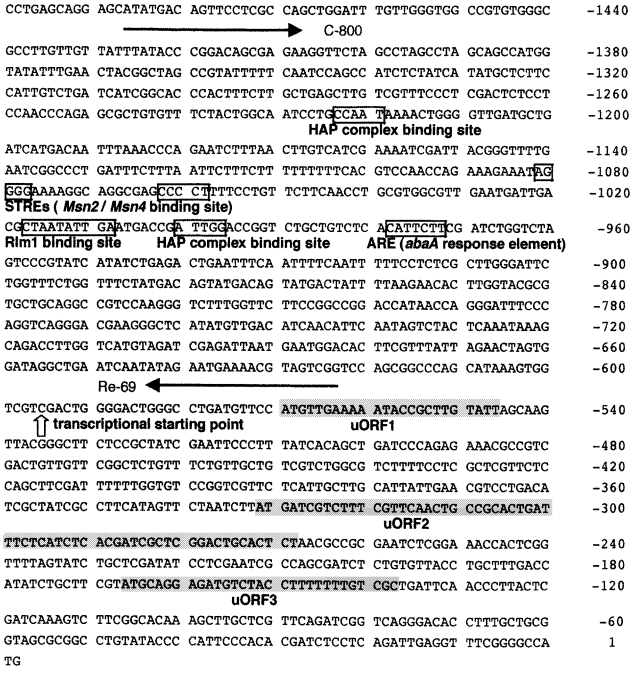

Fig. 4. Nucleotide sequence of the promoter region of *csmA*. Negative numbers on the right refer to nucleotides upstream from the *csmA* ATG translation start codon. Arrows indicate PCR primers (C-800 and Re-69). An open arrow indicates transcriptional starting point. Three uORFs are shaded. HAP complex binding sites, *abaA* response element (ARE), Rlm1p binding site, and stress response element (STRE) are boxed.

Discussion

In this paper, we showed that the entire coding region of *csmA* was translated as a single polypeptide with an approximate molecular mass of 210 kDa. This is the first demonstration that the *csmA*-type gene is translated as a single polypeptide containing both the myosin motor-like domain and the chitin synthase domain. CsmA–HA, a 210 kDa protein, was detected throughout the 5 days of cultivation and its levels were significantly reduced under high osmotic conditions.

csmA transcript levels were regulated in response to an alteration in external osmolarity. In *S. cerevisiae*, the protein kinase C-mediated MAP kinase pathway responds to hypo-osmotic stresses and regulates the maintenance of cell wall integrity [33]. Rlm1p transcription factor is the downstream target of this MAP kinase cascade. Most of the genes known, or suspected,

to encode cell wall proteins or proteins that are involved in cell wall biogenesis, are regulated by Rlm1p in response to activation of the PKC pathway [34]. The predicted DNA-binding site consensus sequence for Rlm1p is CTA(A/T)$_4$ TAG [34,35]. A similar sequence was found in the promoter region of *csmA*, suggesting that an orthologous gene of *RLM1* may be present in *A. nidulans* and that its product may regulate *csmA* expression in response to alterations in external osmolarity.

Exposure of cells to hypo-osmotic stresses results in an increase in turgor pressure. The remodeling or reinforcement of the cell wall must be achieved in order to maintain cell shape under these conditions. The growth of the *csmA* null mutants was sensitive to low osmotic conditions [23]. Furthermore, the levels of CsmA–HA protein and *csmA* transcript were significantly reduced under high osmotic conditions. Taken together, these results suggest that CsmA plays an important role in the maintenance of cell wall integrity under low osmotic conditions.

The *csmA* promoter contains potentially functional promoter elements. STREs, well-conserved elements amongst fungi, are also present in the promoter region of *csmA*. In *S. cerevisiae*, the expression of genes that contain STREs in their promoter regions is induced under high osmotic, heat shock, or oxidative stress conditions [36]. Thus, the transcription of *csmA* may similarly be induced under these stress conditions. The *abaA* response element (ARE) was found in the promoter region of *csmA*. AbaA is a known transcription factor necessary for conidiation. *abaA* is thought to activate the genes required for the differentiation of metulae and phialides [37,38]. Consistent with the presence of an ARE in the *csmA* promoter, abnormal morphologies of conidiophores were occasionally observed in *csmA* null mutants [23]. It is possible that *csmA* is induced by AbaA under conditions that induce conidiation and is in some way involved in conidiation.

The levels of CsmA–HA protein and *csmA* transcript displayed different temporal patterns. This suggests that CsmA protein levels are regulated at the post-transcriptional or post-translational level. A well-known form of post-transcriptional regulation is that in which short ORFs in the 5′ untranslated region of the mRNA are involved. The translational regulation of *GCN4* in *S. cerevisiae* is mediated by four short open reading frames (uORFs) upstream of the main ORF. These uORFs inhibit *GCN4* translation under non-starvation conditions by restricting the progression of scanning ribosomes through the leader to the *GCN4* start codon [39,40]. Elimination of the start codons of all four uORFs resulted in high levels of Gcn4p production under both amino acid starvation and non-starvation conditions. Similar post-transcriptional control has been reported with *GCN4* orthologues of other fungi. The *csmA* transcript has three uORFs upstream of the main ORF. The function of these uORFs in the regulation of *csmA* is an interesting subject for future research.

The temporal change in CsmA–HA levels and the appearance of a 140 kDa protein suggest that CsmA is cleaved between the myosin motor-like domain and the chitin synthase domain after 2 days of cultivation. The band at 140 kDa corresponds to the size of CsmA–HA without the myosin motor-like domain. Although the function of the myosin motor-like domain remains unclear, it might be involved in the transport or proper localization of CsmA. Post-translational processing of CsmA might occur as a means of activating the chitin synthase domain. This could conceivably be done at the sites where actin filaments are concentrated and where cell wall or septal synthesis is active, such as the hyphal tips, the septa, and the branching sites. The function of the myosin motor-like domain is currently being investigated.

Acknowledgments

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