Role of LAMMER Kinase in Cell Wall Biogenesis during Vegetative Growth of *Aspergillus nidulans*

Yu Kyung Choi, Eun-Hye Kang and Hee-Moon Park*

Department of Microbiology and Molecular Biology, College of Bioscience and Biotechnology, Chungnam National University, Daejeon 035-764, Korea

Abstract Depending on the acquisition of developmental competence, the expression of genes for β -1,3-glucan synthase and chitin synthase was affected in different ways by *Aspergillus nidulans* LAMMER kinase. LAMMER kinase deletion, $\Delta lkhA$, led to decrease in β -1,3-glucan, but increase in chitin content. The $\Delta lkhA$ strain was also resistant to nikkomycin Z.

Keywords *Aspergillus nidulans*, Cell wall polysaccharides, Cell wall-related genes, LAMMER kinase, qRT-PCR

Fungal cells constantly remodel their rigid structure by reflecting differences in types of cell wall-related genes (CWGs) and their transcriptional regulation through cell signaling in respond to external stimuli and the cell cycle [1, 2]. In fungi, the cell wall integrity signaling (CWIS) pathway is responsible for cell wall remodeling and reinforcement following cell wall stress. The CWIS pathway in *Saccharomyces cerevisiae* is sequentially stimulated through the stress sensors Wsc1p and Mid2p; the GTP-binding protein, Rho1p; protein kinase C; the mitogen-activated protein kinase, mitogen-activated protein kinase (MAPK), cascade; and two types of transcription factors, e.g. MADS-box transcription factor Rlm1 and SBF (cycle box-binding factor) proteins Swi40 and Swip6 [3]. Although several homologous CWIS elements have been identified in *Aspergillus nidulans* [4], studies of cell wall biogenesis and regulation of the transcription of cell wall-related genes have been limited [2, 5]. Unlike in *S. cerevisiae*, the transcription of most CWGs in *A. nidulans* seems to be regulated by non-MpkA, a MAPK, signaling, with the exception of the α -1,3-glucan synthase genes (*agsA* and

agsB) [2].

The dual-specificity LAMMER kinases are involved in many cellular processes, including differentiation, the cell cycle, stress response, and reproduction. In *A. nidulans*, one of the prominent features revealed by deletion of the LAMMER kinase gene (*lkhA*) was the morphological changes during vegetative growth and various developmental stages. The patterns of germ tube emergence and hyphal polarity were altered and septation was increased by the deficiency of LAMMER kinase, which led to a detrimental effect on asexual and sexual differentiation [6]. In fission yeast, LAMMER kinase, Lkh1, regulates G1/S progression of the cell cycle by modulating the activity of cyclin-dependent kinase inhibitor, Rum1 [7]. Our previous reports also revealed that expression of the genes for the synthesis of chitin and β -1,3-glucan is cell cycle-dependent and developmental stage-specific [8-12]. From these results, it was postulated that LAMMER kinase may be involved in the non-MpkA signaling of CWGs in *A. nidulans*. In order to test this possibility, we analyzed the expression of CWGs, the composition of the major cell wall polysaccharides chitin and β -1,3-glucan, and the susceptibility to cell wall-damaging agents using a LAMMER kinase deficient strain ($\Delta lkhA$) of *A. nidulans* [6].

A. nidulans strain DLA1 (*yA2; argB2; pyroA4; veA⁺; $\Delta lkhA::argB$*), the LAMMER kinase deletion strain [6], and MCBA001 (*yA2; pyroA4; veA⁺*), the meiotic progeny produced by the cross between A4 and TJ1 (*yA2; argB2; pyroA4; veA⁺*), were used in this study. *A. nidulans* complete medium was prepared and used for culture and preparation of vegetative mycelia by the previously described method [6].

RNA preparation was performed as described previously [6]. Briefly, the cultured cells were harvested and ground to a powder with a mortar and pestle in liquid nitrogen. GT buffer (4 M guanidine thiocyanate, 25 mM sodium acetate [pH 6.0], 0.5% *N*-lauroylsarkosine, 0.84% β -mercaptoethanol)

Mycobiology 2014 December, 42(4): 422-426
<http://dx.doi.org/10.5941/MYCO.2014.42.4.422>
pISSN 1229-8093 • eISSN 2092-9323
© The Korean Society of Mycology

***Corresponding author**

E-mail: hmpark@cnu.ac.kr

Received October 2, 2014

Revised October 8, 2014

Accepted November 4, 2014

© This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Table 1. Oligonucleotides used for qRT-PCR analysis

Name	Sequence (5'-3')	Gene
actA-861-for	accacacattctacaacgag	Actin
actA-941-rev	gtcatcttctcacgggtgga	
agsA-5464-for	acagaatcatggctcgggta	β -1,6-glucan synthase A
agsA-5546-rev	cgtagtcgaactggctgga	
agsB-2392-for	agatgcgtccgagacgggt	β -1,6-glucan synthase B
agsB-2474-rev	cggttccgtggaagagtt	
chsA-1240-for	acgcaaaaggggtataaac	Chitin synthase A
chsA-1321-rev	agtgggtttctcctcgtt	
chsB-1283-for	aatcccagaggtctgcttc	Chitin synthase B
chsB-1364-rev	ctccgagatcttctgctt	
chsC-1560-rev	ctgtgtcctactcgatgct	Chitin synthase C
chsC-1641-rev	accacaagcacctccacac	
chsD-1408-for	gcctcccaggtgtttgt	Chitin synthase D
chsD-1489-rev	ttggagcaagggaacctg	
csmA-1839-for	tggtcaagaagccttcag	Chitin synthase CmsA
csmA-1920-rev	agccatgcaggcattcgt	
csmB-1212-for	aaagaggacggctccag	Chitin synthase CmsB
csmB-1293-rev	ctgtcgtaatcccaaggtc	
fksA-307-for	gtcaaggcagaggacgaca	β -1,3-glucan synthase
fksA-391-rev	tagtccatgtcggcagcac	

qRT-PCR, quantitative real-time polymerase chain reaction.

was added to the cell powder. After vortexing, the debris was removed by centrifugation. The supernatant was then overlaid with 2 mL of 5.7 M CsCl solution (5.7 M CsCl, 25 mM sodium acetate, pH 6.0). The RNA pellet was isolated by centrifugation at 10°C, 125,000 $\times$ g for 16 hr using an SW 55Ti rotor (Beckman Coulter, Brea, CA, USA). The pellet was resuspended in 400 μ L TES buffer (10 mM Tris-Cl, pH 7.4, 5 mM EDTA, 1% sodium dodecyl sulfate) and extracted with phenol/chloroform (pH 4.5). The RNA was concentrated by ethanol precipitation, dissolved in DEPC-treated water, and stored at -70°C.

Relative mRNA levels were determined by quantitative real-time (qRT) PCR using the specific primer sets

designed for each gene (Table 1). Briefly, total RNA was reverse transcribed into cDNA using the M-MLV Reverse Transcriptase (Elpis Biotech, Inc., Daejeon, Korea) according to the manufacturer's instructions. Each gene-specific primer was optimized for expression analysis through RT-PCR on a CFX96 Real-Time PCR (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using TOPreal qPCR 2 $\times$ PreMIX (Enzynomics, Daejeon, Korea). The PCR cycling parameters were 5 min at 95°C, followed by 45 cycles of 20 sec at 95°C, 20 sec at 57°C, and 15 sec at 72°C. A melting curve analysis to confirm specificity was performed for each primer set. Gamma-actin (*gAct*) was used for normalization, and the relative expression was calculated according to the method previously reported [13].

Chemical analysis of the composition of the cell wall was performed using the method described previously [14]. Sensitivity toward cell wall-perturbing agents was investigated via the method described by Kovács *et al.* [5] using media supplemented with Calcofluor white (CFW) or Congo red (CR). Disk diffusion method [15] was used to evaluate the effect of antifungal drugs, nikkomycin Z and terbinafine.

A previous study revealed that the genes for chitin synthase and β -1,3-glucan synthase are expressed in a cell cycle-dependent manner [8-10]. Similar to LAMMER kinase in fission yeast [7, 16, 17], the involvement of LAMMER kinase in cell division of *A. nidulans* was also indicated with fluorescent staining to visualize the septation and nuclear division in vegetative hyphae [6]. Therefore, we tested the effect of LAMMER kinase on the expression of genes for cell wall polysaccharides in vegetative hyphae via qRT-PCR. When the expression levels of the genes for chitin synthase (*chsA*, *B*, *C*, *D*, *cmsA*, and *cmsB*), α -1,3-glucan synthase (*agsA* and *B*), and β -1,3-glucan synthase (*fksA*) were quantified from the RNA of a 9-hr-old vegetative mycelia of DLA1, only *fksA* was discovered to be at a statistically significant diminished level ($p = 0.012$) (Fig.

Fig. 1. Characterization of gene expression changes of cell wall-related genes. RNA was purified from vegetative cultures from 9-hr (A) and 15-hr (B) samples for quantitative real-time polymerase chain reaction (qRT-PCR). Three biological samples were taken for the first cDNA synthesis, and subsequent real-time PCR analysis was performed using specific primer sets for cell wall genes. Conditions for qRT-PCR are described in the text. The asterisk indicates statistically significant change due to the deletion of LAMMER kinase ($\Delta lkhA$) as calculated with the Student's t-test for $p \leq 0.05$.

1A). On the other hand, qRT-PCR of RNA from a 15-hr-old vegetative mycelia of DLA1, which had already acquired developmental competence, the *chsC* and *chsD* genes for chitin synthase revealed statistically significant increase ($p = 0.006$) and decrease ($p = 0.021$), respectively (Fig. 1B). These results indicate that, in *A. nidulans*, LAMMER kinase positively regulates the expression of *fksA* before the acquisition of developmental competence, and LAMMER kinase affects the expression of only two chitin synthase genes, *chsC* and *chsD*, in different ways after the acquisition of developmental competence during vegetative growth; *chsC* was negatively but *chsD* was positively regulated. It is also noteworthy that *chsC* and *chsD* have different functions; *chsC* is required for maintenance of hyphal wall integrity and development of asexual structures such as the conidiophores [18], whereas *chsD* plays a role in hyphal growth and development [19].

Since changes in the expression of the genes for cell wall polysaccharide synthesis were apparent in the *lkhA*-deletion strain, the content of cell wall chitin and β -1,3-glucan were determined using a 15-hr-old mycelia from both the wild-type and DLA1. In good agreement with the results from Fig. 1, the amount of β -1,3-glucan was decreased by more than 20% in the DLA1 strain versus wild-type, but the

Table 2. Composition of cell wall polysaccharides

Strain	Cell wall polysaccharides ($\mu\text{g}/\text{mg}$ dry mycelia) ^a	
	Chitin	β -Glucan
WT	112 $\pm$ 6	225 $\pm$ 17
$\Delta lkhA$	145 $\pm$ 10	179 $\pm$ 8

^aChemical analyses of the cell wall preparations were performed three times with wild type (WT) and LAMMER kinase-deletion strain ($\Delta lkhA$) cultured on complete solid medium at 37°C for 15 hr. In each chemical analysis, samples for the carbohydrate assay were tripled.

amount of chitin was increased about 30% (Table 2). The increase of chitin contents in DLA1 can also be explained by a compensatory mechanism, which may be a back-up for the cell wall defects caused by a low level of *fksA* expression and, thus, of β -1,3-glucan content.

In yeast, the binding of cell wall-perturbing agents to stress sensors activates signaling pathways like CWIS and high-osmolarity glycerol [20], and, therefore, modulates β -1,3-glucan [21] and chitin synthesis at a transcriptional level [22]. Sensitivity to cell wall-perturbing agents is indicative of the composition of the cell wall, depending on which component the agent reacts with. CFW interacts

Fig. 2. Sensitivity to cell wall-damaging agents. A, Identical volumes of 10-fold serial dilutions of exponentially growing wild-type cells and cells lacking LAMMER kinase ($\Delta lkhA$) were spotted onto minimal medium (MM) containing 160 $\mu\text{g}/\text{mL}$ of Congo red (CR) or Calcofluor white (CFW) and incubated at 37°C. Colony growth was inspected after 48-hr incubation at 37°C; B, The equal volume of conidia suspension (1×10^7 cells/mL) was spread uniformly onto MM agar plate. The 6 mm-diameter paper disk was saturated with 2 μg of terbinafine or nikkomycin Z and placed onto inoculated plate. Plates were incubated at 37°C for 72 hr to observe the formation of inhibition zone.

specifically with β -1,4-linkage of polysaccharides such as chitin. CR forms a complex with chitin and β -glucan and inhibits chitin synthases [5, 23]. Thus, we tested the sensitivity of our strains toward cell wall-perturbing agents such as CFW and CR. As shown in Fig. 2A, DLA1 revealed no significant difference in sensitivity to the β -glucan-specific CR as well as the chitin-specific CFW. Antifungal drug test, however, revealed that deletion of *lkhA* made the cells resistant to the nikkomycin Z, which is a specific inhibitor for type I chitin synthase such as ChsC [24], but not to the terbinafine, which inhibits ergosterol synthesis and thus affects cell wall synthesis (Fig. 2B).

In summary, the results presented here indicate that the *A. nidulans* LAMMER kinase, LkhA, affects the cell wall polysaccharide composition of vegetative hyphae by modulating the expression of the cell wall genes *fksA*, *chsC* and *chsD*. Although further studies are required, our results unlock interesting avenues for further investigation into the regulatory mechanism of the gene(s) for cell wall biogenesis in filamentous fungi.

ACKNOWLEDGEMENTS

This work was supported by the Basic Research Infrastructure Supporting Program (to EHK, Grant no. 2013R1A6A3A01027651) and the NRF-NSFC Cooperative Program (to HMP, Grant no. 201200144) of the National Research Foundation of Korea.

REFERENCES

- Smits GJ, Kapteyn JC, van den Ende H, Klis FM. Cell wall dynamics in yeast. *Curr Opin Microbiol* 1999;2:348-52.
- Fujioka T, Mizutani O, Furukawa K, Sato N, Yoshimi A, Yamagata Y, Nakajima T, Abe K. MpkA-dependent and -independent cell wall integrity signaling in *Aspergillus nidulans*. *Eukaryot Cell* 2007;6:1497-510.
- Baetz K, Moffat J, Haynes J, Chang M, Andrews B. Transcriptional coregulation by the cell integrity mitogen-activated protein kinase Slr2 and the cell cycle regulator Swi4. *Mol Cell Biol* 2001;21:6515-28.
- de Groot PW, Brandt BW, Horiuchi H, Ram AF, de Koster CG, Klis FM. Comprehensive genomic analysis of cell wall genes in *Aspergillus nidulans*. *Fungal Genet Biol* 2009;46(Suppl 1):S72-81.
- Kovács Z, Szarka M, Kovács S, Boczonádi I, Emri T, Abe K, Pócsi I, Pusztahelyi T. Effect of cell wall integrity stress and RlmA transcription factor on asexual development and autolysis in *Aspergillus nidulans*. *Fungal Genet Biol* 2013;54:1-14.
- Kang EH, Kim JA, Oh HW, Park HM. LAMMER kinase LkhA plays multiple roles in the vegetative growth and asexual and sexual development of *Aspergillus nidulans*. *PLoS One* 2013;8:e58762.
- Yu EY, Lee JH, Kang WH, Park YH, Kim L, Park HM. Fission yeast LAMMER kinase Lkh1 regulates the cell cycle by phosphorylating the CDK-inhibitor Rum1. *Biochem Biophys Res Commun* 2013;432:80-5.
- Park BC, Maeng PJ, Park HM. Cell cycle-dependent expression of chitin synthase genes in *Aspergillus nidulans*. *J Microbiol* 2001;39:74-8.
- Park BC, Park YH, Park HM. Activation of *chsC* transcription by AbaA during asexual development of *Aspergillus nidulans*. *FEMS Microbiol Lett* 2003;220:241-6.
- Park BC, Park YH, Yi S, Choi YK, Kang EH, Park HM. Transcriptional regulation of *fksA*, a β -1,3-glucan synthase gene, by the APSES protein StuA during *Aspergillus nidulans* development. *J Microbiol* 2014;52:940-7.
- Lee JI, Choi JH, Park BC, Park YH, Lee MY, Park HM, Maeng PJ. Differential expression of the chitin synthase genes of *Aspergillus nidulans*, *chsA*, *chsB*, and *chsC*, in response to developmental status and environmental factors. *Fungal Genet Biol* 2004;41:635-46.
- Lee JI, Yu YM, Rho YM, Park BC, Choi JH, Park HM, Maeng PJ. Differential expression of the *chsE* gene encoding a chitin synthase of *Aspergillus nidulans* in response to developmental status and growth conditions. *FEMS Microbiol Lett* 2005;249:121-9.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 2001;25:402-8.
- Lee HH, Park JS, Chae SK, Maeng PJ, Park HM. *Aspergillus nidulans* *sod^{VI}C1* mutation causes defects in cell wall biogenesis and protein secretion. *FEMS Microbiol Lett* 2002;208:253-7.
- Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility by standardized single disc method. *Am J Clin Pathol* 1966;45:493-6.
- Kim KH, Cho YM, Kang WH, Kim JH, Byun KH, Park YD, Bae KS, Park HM. Negative regulation of filamentous growth and flocculation by Lkh1, a fission yeast LAMMER kinase homolog. *Biochem Biophys Res Commun* 2001;289:1237-42.
- Tang Z, Mandel LL, Yean SL, Lin CX, Chen T, Yanagida M, Lin RJ. The *kic1* kinase of *Schizosaccharomyces pombe* is a CLK/STY orthologue that regulates cell-cell separation. *Exp Cell Res* 2003;283:101-15.
- Fujiwara M, Ichinomiya M, Motoyama T, Horiuchi H, Ohta A, Takagi M. Evidence that the *Aspergillus nidulans* class I and class II chitin synthase genes, *chsC* and *chsA*, share critical roles in hyphal wall integrity and conidiophore development. *J Biochem* 2000;127: 359-66.
- Specht CA, Liu Y, Robbins PW, Bulawa CE, Iartchouk N, Winter KR, Riggle PJ, Rhodes JC, Dodge CL, Culp DW, Borgia PT. The *chsD* and *chsE* genes of *Aspergillus nidulans* and their roles in chitin synthesis. *Fungal Genet Biol* 1996;20:153-67.
- García-Rodríguez LJ, Durán A, Roncero C. Calcofluor antifungal action depends on chitin and a functional high-osmolarity glycerol response (HOG) pathway: evidence for a physiological role of the *Saccharomyces cerevisiae* HOG pathway under noninducing conditions. *J Bacteriol* 2000;182:2428-37.
- Roemer T, Paravicini G, Payton MA, Bussey H. Characterization of the yeast (1 $\rightarrow$ 6)- β -glucan biosynthetic components, Kre6p and Skn1p, and genetic interactions between the PKC1 pathway and extracellular matrix assembly. *J Cell Biol* 1994;127:567-79.
- Igual JC, Johnson AL, Johnston LH. Coordinated regulation

- of gene expression by the cell cycle transcription factor Swi4 and the protein kinase C MAP kinase pathway for yeast cell integrity. *EMBO J* 1996;15:5001-13.
23. Roncero C, Durán A. Effect of Calcofluor white and Congo red on fungal cell wall morphogenesis: *in vivo* activation of chitin polymerization. *J Bacteriol* 1985;163:1180-5.
24. Park BC, Park YH, Park HM. Detection of zymogenic ChsC activity in vegetative hyphae of *Aspergillus nidulans*. *Korean J Microbiol* 2004;40:178-82.