

SCIENTIFIC REPORTS

OPEN

Velvet-mediated repression of β -glucan synthesis in *Aspergillus nidulans* spores

Received: 22 December 2014

Accepted: 02 April 2015

Published: 11 May 2015

Hee-Soo Park¹, Yeong Man Yu², Mi-Kyung Lee¹, Pil Jae Maeng², Sun Chang Kim³ & Jae-Hyuk Yu¹

Beta-glucans are a heterologous group of fibrous glucose polymers that are a major constituent of cell walls in Ascomycetes and Basidiomycetes fungi. Synthesis of β (1,3)- and (1,6)-glucans is coordinated with fungal cell growth and development, thus, is under tight genetic regulation. Here, we report that β -glucan synthesis in both asexual and sexual spores is turned off by the NF- κ B like fungal regulators VosA and VelB in *Aspergillus nidulans*. Our genetic and genomic analyses have revealed that both VosA and VelB are necessary for proper down-regulation of cell wall biosynthetic genes including those associated with β -glucan synthesis in both types of spores. The deletion of *vosA* or *velB* results in elevated accumulation of β -glucan in asexual spores. Double mutant analyses indicate that VosA and VelB play an inter-dependent role in repressing β -glucan synthesis in asexual spores. *In vivo* chromatin immuno-precipitation analysis shows that both VelB and VosA bind to the promoter region of the β -glucan synthase gene *fksA* in asexual spores. Similarly, VosA is required for proper repression of β -glucan synthesis in sexual spores. In summary, the VosA-VelB hetero-complex is a key regulatory unit tightly controlling proper levels of β -glucan synthesis in asexual and sexual spores.

The cell wall is crucial for many aspects of fungal biology including growth, development, morphogenesis and pathogenicity^{1–4}. It maintains the shape of the fungal cells and provides a protection against internal turgor pressure and various environmental factors⁵. The major components of the fungal cell wall are glycoproteins, chitin, α -glucans, β -glucans, mannans and melanin^{6,7}. These components are cross-linked and form the fungal specific polysaccharide-based network^{4,5}. Since the structure and components of cell wall are fungi-specific, the cell wall has been considered an attractive target for anti-fungal drug development^{3,8,9}. In the model filamentous fungus *Aspergillus nidulans*, α -1,3-glucan, β -1,3-glucan and chitin are the main polysaccharides in the cell wall and associated genes include *agsA*, *agsB* (encoding α -1,3-glucan synthases), *fksA* (encoding β -1,3-glucan synthase) and *chsA* (encoding a chitin synthase)^{10–14}.

Filamentous fungi produce asexual spores (conidia in higher fungi) as the main propagules and infection particles^{15,16}. Conidia of *A. nidulans* are formed on the multicellular asexual reproductive structure conidiophore^{15,17}. After conidia are formed from the phialide apex, it subsequently undergoes the maturation process consisting of three stages^{18–20}. Stage I conidia contain walls consisting of C1 (outer) and C2 (inner) layers. At stage II, C1 becomes crenulated and C2 condenses. During stage III, two new wall layers, C3 (between C1 and C2) and C4 (the innermost wall) form and these two layers are required for conidial dormancy¹⁹. A key gene for conidia maturation in *A. nidulans* is *wetA*¹⁹. The *wetA6* mutant conidia are defective in the formation of the C3 and C4 wall layers and condensation of the C2 wall layer, resulting in the lack of spore pigmentation and lysis of conidia. Our studies further revealed that two *velvet* genes *vosA* and *velB* are also important for conidia maturation^{21–23}.

¹Department of Bacteriology, University of Wisconsin, Madison, WI, USA. ²Department of Microbiology and Molecular Biology, Chungnam National University, Daejeon, Republic of Korea. ³Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Dae-Jon, Republic of Korea. Correspondence and requests for materials should be addressed to J.H.Y. (email: jyu1@wisc.edu)

The *velvet* proteins (VeA, VelB, VelC and VosA) are fungi-specific transcription factors and contain the *velvet* domain with the NF- κ B-like DNA binding motif^{21,24–26}. These proteins form different complexes, which play differential roles in regulating developmental processes and synthesis of secondary metabolites in many filamentous fungi^{24,27}. Among them, the VosA-VelB complex acts as a functional unit controlling maturation (trehalose biogenesis), dormancy and germination of conidia^{21–23}. In addition, the *vosA* deletion mutant conidia are defective in the formation of the electron-light layer of the conidial cell wall²¹, suggesting that the VosA protein may regulate proper spore wall formation.

To gain further insights into their role in the formation of spore wall, we have performed a transcriptome analysis using the wild type (WT) and Δ *vosA* mutant conidia. We have found that several genes involved in the synthesis of major cell wall polysaccharides are up-regulated by the lack of VosA in conidia. Further targeted studies have revealed that the absence of *vosA* or *velB* results in elevated mRNA accumulation of *fksA*, *gelA* and *gelB* involved in the biosynthesis of β -1,3-glucan. Indeed, levels of β -glucan in the mutant conidia were much higher than those in WT. In addition, *in vivo* protein-DNA interaction studies employing chromatin-immuno-precipitation (ChIP) using the VosA and VelB proteins tagged with the epitope FLAG specifically enriched an *fksA* promoter region which contains the consensus DNA sequence predicted to be recognized by VosA²⁵. Moreover, the deletion of *vosA* causes increased levels of *fksA* mRNA and total β -glucan amount in sexual spores (ascospores), and VosA-FLAG-ChIP and VelB-FLAG-ChIP occupy an *fksA* promoter region. Taken together, we propose that the VosA-VelB complex represses β -glucan synthesis in fungal spores by directly binding to the promoter regions of *fksA* and other cell wall biosynthetic genes, thereby conferring proper spore wall formation during sporogenesis in *A. nidulans*.

RESULTS

Genome-wide expression studies reveal that VosA is necessary for proper repression of cell wall-related genes in conidia. To gain further insights into the complex regulatory roles of VosA during asexual sporogenesis, we carried out comparative transcriptome analysis of the WT and Δ *vosA* conidia. A total of 3,483 genes showed statistically significant differential expression between the Δ *vosA* and WT conidia (Fig. 1A, $P < 0.05$, T-test). A total of 2,117 genes exhibited more than two-fold reduction (1,104 genes), or induction (1,013 genes) in the Δ *vosA* conidia (Table S1). KOG (eukaryotic orthologous groups) analysis of genes up-regulated by VosA showed that the following categories of genes were over-represented: cell wall/membrane/envelope biogenesis, cytoskeleton, lipid transport/metabolism, and secondary metabolites biogenesis/transport/catabolism (Fig. 1B). Further GO analyses demonstrated that a large number of the cell wall-related genes (the MIPS PEDANT 3 database, <http://pedant.gsf.de/genomes.jsp?category=fungal>) is differentially up-regulated in the Δ *vosA* conidia (Fig. 1C). Several genes involved in β -1,3-glucan synthesis including *fksA*, *gelA* and *gelB* (β -1,3-glucan-transglucosylase genes), and chitin synthesis including *chsA* (class II chitin synthase gene)²⁸, *chsB* (class III chitin synthase gene)^{29,30}, *csmA* (class V chitin synthase gene)³¹ and *gfaA* (glutamine-fructose 6-phosphate transaminase gene) were shown to be highly induced in the Δ *vosA* conidia compared to the WT conidia (Table 1). These indicate that VosA is involved in repression of cell wall-related genes in spores, and may govern the spore wall polysaccharides composition and structure.

Both VelB and VosA are required for repression of spore wall-related genes. Previously we showed that VosA interacts with VelB, and forms the VosA-VelB complex which is a functional unit for spore maturation including trehalose biogenesis^{22,23}. To test whether VelB plays a role in proper spore wall formation, we first examined 8 days old conidia of WT, Δ *velB* and Δ *vosA* strains using transmission electron microscopy (TEM) (Fig. 2A). We found that, similar to Δ *vosA*, the Δ *velB* conidia appear to lack cytoplasm and clear organelle structures. In addition, the Δ *velB* conidia electron light layer was darker than that of WT. These results indicated that VelB, similar to VosA, plays a pivotal role in conidial wall formation and structure.

We then examined whether the absence of *velB* or *vosA* affected mRNA levels of key genes associated with synthesis and processing of β -1,3-glucan in conidia. We found that, while mRNA of 1,3- β -glucan synthetic genes including *fksA*, *gelA* and *gelB* was not detectable in WT conidia, mRNA levels of those genes were very high in the Δ *velB* and Δ *vosA* conidia (Fig. 2B). In accordance with the increased mRNA levels of β -1,3-glucan biosynthetic genes, β -glucan levels in the Δ *velB* and Δ *vosA* conidia were significantly higher than those of WT conidia (Fig. 2C). Moreover, the Δ *vosA* Δ *velB* double mutant conidia exhibited the essentially identical enhancement of mRNA accumulation patterns and increased β -glucan content in conidia (Fig. 2B,C). Taken together, these results suggest that both VelB and VosA are needed for proper repression of β -glucan synthesis in conidia, and they play an inter-dependent role.

VosA and VelB bind to the *fksA* promoter. As mentioned, a key and common property of these multi-functional *velvet* regulators is that they are DNA-binding proteins and function as transcription factors. Their DNA-binding *velvet* domain is similar to NF- κ B DNA binding motif²⁵. Previous chromatin immune-precipitation followed by microarray (ChIP-chip) studies have revealed promoter regions of a high number of genes were enriched by VosA, including several cell-wall related genes (Table 1)²⁵. One consensus DNA sequence predicted to be recognized by VosA is located at the promoter region of *fksA* (Fig. 3A). To test our hypothesis that the VelB and VosA hetero-complex is a functional unit

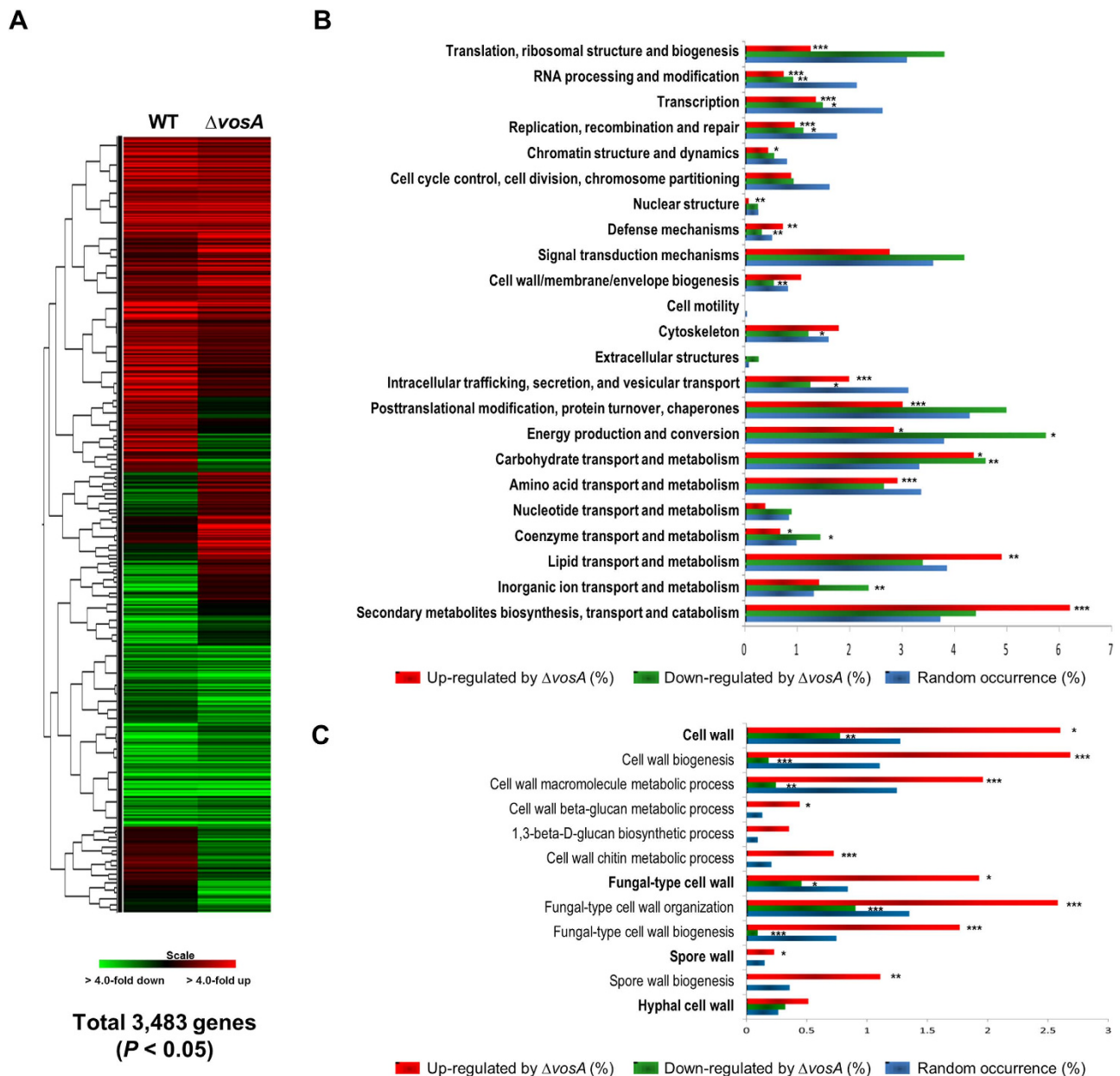


Figure 1. Genome wide analyses of *A. nidulans* genes whose expression is influenced by VosA in conidia. (A) Hierarchical clustering analysis of 3,483 genes which showed significant differential expression between the WT and $\Delta vosA$ conidia ($P < 0.05$, T-test) is presented. (B) Functional categories of up-, or down-regulated genes in the $\Delta vosA$ conidia compared to WT conidia are shown. Each group of genes was functionally categorized by KOG description (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). (C) Functional categories and expression responses of cell wall-, or sporulation-related genes in the $\Delta vosA$ conidia compared to WT conidia are presented. Each group of genes was functionally categorized by GO description (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

for repression of β -glucan synthesis in conidia, we checked whether both VosA-ChIP and VelB-ChIP enriched the promoter regions of *fksA* *in vivo*. As shown Fig. 3B, PCR-amplification of the *fksA* promoter region using DNA fragments derived from VosA-FLAG-ChIP and VelB-FLAG-ChIP gave rise clear amplicons, whereas DNA from ChIP of THS30 lacking FLAG-tagged VosA or VelB failed to do so. Collectively, these results indicate that the VosA-VelB hetero-complex binds to the promoter region of *fksA* and negatively regulates expression of *fksA*, and many other conidial wall biosynthetic genes.

VosA is required for β -glucan synthesis in ascospores. While asexual sporulation is the primary reproductive mode, *A. nidulans* can form sexual spores called ascospores. Our previous studies showed

Systematic name	Gene name	Description ^a	Expression ratio ($\Delta vosA$ /WT)	Occupancy of VosA-ChIP ^b
Genes involved in 1,3-β-glucan synthesis and processing				
AN3729.3	<i>fksA</i>	1,3-beta-glucan synthase	5.01	O
AN7657.3	<i>gelA</i>	Putative 1,3-beta-transglycosidase	2.34	
AN0558.3	<i>gelB</i>	Putative 1,3-beta-transglycosidase	2.20	
AN3730.3	<i>gelC</i>	Putative 1,3-beta-transglycosidase	2.73	O
AN10150.3	<i>btgA</i>	Putative beta-transglucosylase	4.17	
AN1551.3	<i>btgE</i>	Putative beta-glucosidase	3.17	
AN3053.3	<i>crhD</i>	Putative transglycosidase	2.91	O
AN0550.3		Putative glucan 1,3-beta-glucosidase	0.22	
AN0779.3		Putative glucan 1,3-beta-glucosidase	5.97	
AN4825.3		Putative glucan 1,3-beta-glucosidase	2.43	
AN4852.3		Putative glucan 1,3-beta-glucosidase	2.67	
AN6697.3	<i>sunA</i>	Putative Sun-family protein	4.59	O
Genes involved in 1,3-α-glucan synthesis and processing				
AN5885.3	<i>agsA</i>	alpha-1,3 glucan synthase	6.11	
AN1604.3	<i>agnE</i>	Putative alpha-1,3-glucanase	2.22	
AN6324.3	<i>amyE</i>	Putative alpha-amylase	2.76	
Genes involved in chitin synthesis and processing				
AN7032.3	<i>chsA</i>	Class II chitin synthase	2.13	
AN2523.3	<i>chsB</i>	Class III chitin synthase	2.16	
AN6318.3	<i>csmA</i>	Class V chitin synthase	3.37	O
AN10709.3	<i>gfaA</i>	Glutamine-fructose-6-phosphate transaminase	2.43	O
AN4871.3	<i>chiB</i>	Class V chitinase	3.23	
AN9390.3	<i>chiC</i>	Putative endochitinase	11.33	
AN11059.3		Putative chitinase	2.30	
AN0221.3		Putative chitinase	0.43	
AN8481.3		Putative chitinase	0.31	
AN1502.3	<i>nagA</i>	Extracellular N-acetyl-beta-glucosaminidase	2.26	O
Genes involved in other enzymatic function in cell wall biosynthesis				
AN8444.3	<i>celA</i>	Protein with similarity to cellulose synthase	2.02	
AN3883.3		Putative glucanase	2.36	O
AN6472.3	<i>dfgF</i>	Putative endo-mannanase	4.07	
AN10345.3	<i>dfgA</i>	Putative endo-mannanase	2.00	
AN4390.3	<i>ecmA</i>	GPI-anchored cell wall organization protein	2.50	O

Table 1. Relative expression of cell wall-related genes in $\Delta vosA$ and WT conidia. ^aASPGD and de Groot *et al.*, 2009. ^bO represent genes bound by VosA and having VosA-responsive element (Ahmed *et al.*, 2013).

that, similar to conidia, the deletion of *vosA* caused the formation of defective ascospores with dis-jointed cell wall lacking long-term viability²¹. We envisioned that if VosA is generally required for proper conidial wall formation and structure, it would function similarly during sexual sporulation. The $\Delta velB$ mutant could not produce sexual fruiting bodies, and was excluded in ascospore analyses. To further test the roles of VosA in sexual fruiting, we examined mRNA levels of *fksA* and total content of β -1,3-glucan in WT and $\Delta vosA$ ascospores. As shown Fig. 4A, mRNA of *fksA* accumulated at high level in $\Delta vosA$, but not in WT, ascospores. In addition, amount of β -glucan in $\Delta vosA$ ascospores was significantly higher than that of WT (Fig. 4B). Likewise in conidia, VosA-ChIP-PCR results show that VosA-ChIP specifically enriches the promoter regions of *fksA* in ascospores (Fig. 4C). Collectively, VosA represses expression of *fksA* and controls levels of β -1,3-glucan synthesis in ascospores.

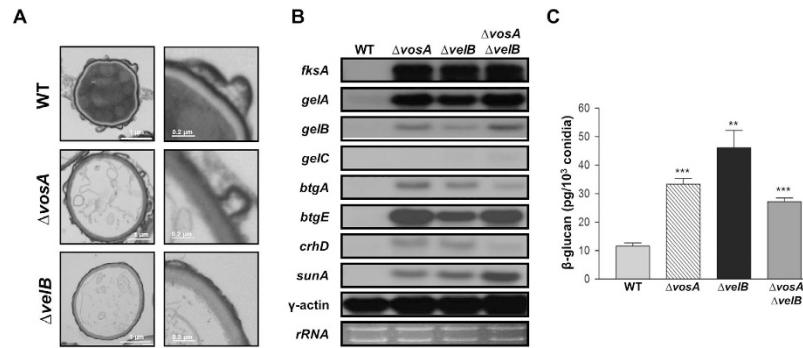


Figure 2. Requirement of *vosA* and *velB* in proper formation of conidial wall. (A) Transmission electron micrographs of WT (FGSC4), $\Delta vosA$ (THS15.1), $\Delta velB$ (THS16.1) mutant conidia are shown. All photomicrograph images were cropped (indicated by the red lines). (B) Levels of *fksA*, *gelA*, *gelB*, *gelC*, *btgA*, *btgE*, *crhD* and *sunA* mRNAs in conidia of WT (FGSC4), $\Delta vosA$ (THS15.1), $\Delta velB$ (THS16.1) and $\Delta velB \Delta vosA$ (THS14.1) strains. Equal loading of total RNA was confirmed by ethidium bromide staining of rRNA and hybridization with the γ -actin gene. All Northern blot and rRNA gel images were cropped (indicated by the red lines) to show the relevant data only. For each probe hybridization, equal amounts of total conidial RNA of four strains were separated in the same agarose gel, transferred to the one nylon membrane, and hybridized in the same bag. Thus, mRNA levels of each indicated gene can be directly compared among the four strains. (C) Amount of β -glucan (pg) per 10^3 conidia in 2 day old conidia of WT (FGSC4), $\Delta vosA$ (THS15.1), $\Delta velB$ (THS16.1) and $\Delta velB \Delta vosA$ (THS14.1) strains (measured in triplicates) (** $P < 0.01$; *** $P < 0.001$).

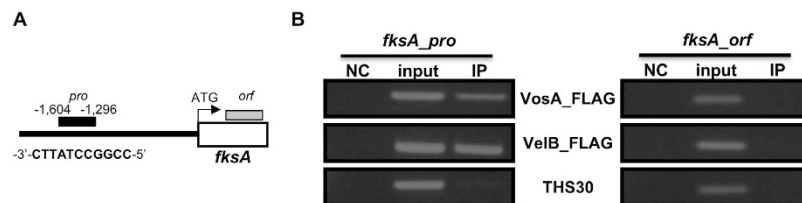


Figure 3. Both VosA and VelB bind to the *fksA* promoter in conidia. (A) Schematic presentation of the promoter region of *fksA*. The black box (the $-1,296 \sim -1,604$ region of the *fksA* promoter) and the gray box (a region of the *fksA* ORF) represents the regions subject to PCR (shown in B). The 11-nucleotide sequence predicted to be recognized by VosA is shown. (B) Results of VosA-ChIP-PCR: the PCR amplicons separated on a 2% agarose gel are shown in the bottom panel. The input DNA before immuno-precipitation (IP) was used as a positive control (input). The chromatin extract being incubated with bead only (without anti-FLAG antibody) was used as a negative control (NC). The samples of THS30 lacking FLAG-tagged VosA or VelB were used as negative controls, too. Representative results are shown (measured in duplicates). All gel images were cropped (indicated by the red lines) to show the relevant data only. The ChIP-PCR amplified samples were separated in the same agarose gel. The full-length gels are presented in Supplementary Fig. S1A online.

DISCUSSION

The VosA and VelB regulators are fungi-specific transcription factors that play multifaceted roles in various biological processes including spore maturation, trehalose biogenesis, conidial germination and conidial wall integrity^{21,22,25}. In this study, we have newly revealed following additional key roles of the VosA and VelB regulators during fungal sporogenesis. First, VosA negatively regulates expression of various genes including cell wall-related genes in conidia (Fig. 1, Table 1). Second, VosA and VelB directly bind to a promoter region of *fksA*, and thereby negatively control *fksA* expression and β -glucan synthesis in conidia (Figs. 2 and 3). Third, VosA plays a similar repressive role during sexual sporulation (Fig. 4). These results support that the VosA-VelB complex represses β -glucan synthesis in conidia and ascospores.

TEM results showed that 8 day old conidial wall of the $\Delta vosA$ and $\Delta velB$ mutant is different from that of WT. Our Northern blot analysis showed that *fksA* mRNA is detectable at relatively constant levels throughout the lifecycle of *A. nidulans* except in conidia (data not shown), suggesting that FksA may play a crucial role during fungal growth and development, but not in sporogenesis. While β -1,3-glucan is important for *A. nidulans* growth and development, the regulatory mechanism of β -1,3-glucan synthesis is largely unknown. Unlike *Saccharomyces cerevisiae*, transcription of *fksA* is regulated by non MpkA-RlmA pathway in *A. nidulans*^{32,33}. We have revealed a new regulatory mechanism of *fksA* in *A.*

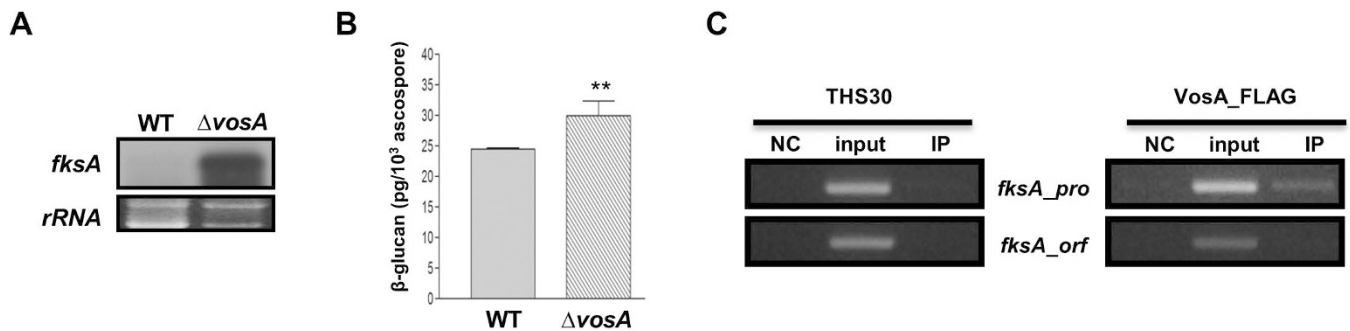


Figure 4. VosA represses β -glucan biogenesis in ascospores. (A) Levels of *fksA* transcript in ascospores of WT (FGSC4) and Δ *vosA* (THS15.1) strains. Equal loading of total RNA was confirmed by ethidium bromide staining of rRNA. (B) Amount of β -glucan (pg per 10³ ascospores) in 8 day old ascospores of WT (FGSC4) and Δ *vosA* (THS15.1) strains (measured in triplicates) (** P < 0.01). The Northern blot and rRNA gel images were cropped (indicated by the red lines). For *fksA* probe hybridization, equal amounts of total RNA of two strains' ascospores were separated in the same agarose gel, transferred to the one nylon membrane, and hybridized in the same bag. (C) Results of VosA-ChIP-PCR: the PCR amplicons separated on a 2% agarose gel are shown in the bottom panel. The input DNA before immuno-precipitation (IP) was used as a positive control (input). The chromatin extract being incubated with bead only (without anti-FLAG antibody) was used as a negative control (NC). The samples of THS30 lacking FLAG-tagged VosA were also used as negative controls. Representative results are shown (measured in duplicates). All four gel images were cropped (indicated by the red lines) to show the relevant data only. The ChIP-PCR amplified samples were separated in the same agarose gel. The full-length gels are presented in Supplementary Fig. S1B online.

nidulans that the VosA-VelB complex directly represses *fksA* expression in spores, thereby β -1,3-glucan synthesis.

Our discovery demonstrates that VosA plays a crucial role in both asexual and sexual spores. However, the role of VelB in *fksA* transcription and β -1,3-glucan synthesis could not be examined in sexual spores, because the Δ *velB* mutant fails to form any sexual fruiting bodies and sexual spores^{23,34}. To predict the VelB's role in β -1,3-glucan synthesis in sexual fruiting, we tested whether VelB-ChIP also enriches the promoter regions of *fksA* in ascospores. Our ChIP-PCR result showed that VelB-FLAG-ChIP enriched the *fksA* promoter region in ascospores (see Supplementary Fig. S1B online). Based on these results, we propose that the VosA-VelB hetero-complex directly regulates expression of *fksA* in both conidia and ascospores.

Beta-glucan is a fungal pathogen associated molecular pattern (PAMP) recognized by the pattern recognition receptors (PRRs) including Dectin-1 and Toll-like receptors (TLRs)^{4,35–37}. This cell wall component triggers innate immune responses to fungal pathogens including *Aspergillus fumigatus*^{38–40}. In *A. fumigatus*, the glucan synthase complex forms the catalytic and regulatory subunits⁴¹, where the *fks1* gene encodes the catalytic subunits. Previously, we have reported the conserved and distinct roles of the *velvet* genes in *A. fumigatus*⁴². To test whether VosA and VelB are involved in β -glucan synthesis in *A. fumigatus*, we briefly examined mRNA level of *fks1* *A. fumigatus* conidia. Our preliminary Northern blot data indicated that deletion of *vosA* or *velB* caused increased *fks1* expression in *A. fumigatus* conidia (data not shown), suggesting that VosA and VelB may play a conserved repressive role in β -glucan synthesis in *A. fumigatus* spores.

Our microarray data also showed that expression levels genes associated with chitin synthesis, including *chsA*, *chsB*, *csmA* and *gfaA*, are up-regulated in the Δ *vosA* conidia compared to WT (Table 1). These genes are required for normal hyphal tip growth, septum formation, cell wall integrity and conidiophore development^{31,43–46}. Previously we found that VosA-ChIP-chip enriched the promoter regions of *csmA* and *gfaA*²⁵. In addition, expression of *csmA*, *gfaA*, and other chitin biosynthetic genes was ~2-3 fold elevated in the Δ *vosA* conidia compared to the WT (Table 1; see Supplementary Fig. S2 online). Based on these results, one can speculate that the VosA-VelB complex may also directly represses chitin biosynthetic genes and chitin levels in conidia. However, as some chitinase encoding genes (*chiB* and *chiC*) were also up-regulated in the Δ *vosA* conidia (Table 1), additional studies of the cell wall polysaccharides composition and structure in the Δ *vosA* and Δ *velB* conidia need to be carried out.

An additional discovery of our transcriptome analysis is that the deletion of *vosA* causes increased expression of genes associated with secondary metabolism in conidia. Particularly, the members of the sterigmatocystin (ST) biosynthesis gene cluster, including *stcW*, *stcV*, *stcT*, *stcQ*, *stcI*, *stcF*, *stcE*, *stcA*, and *stcL*, were up-regulated by mutation of *vosA*. It has been proposed that the dynamic formation of the *velvet* complexes may govern the determination of fungal cellular processes⁴⁷. We speculate that

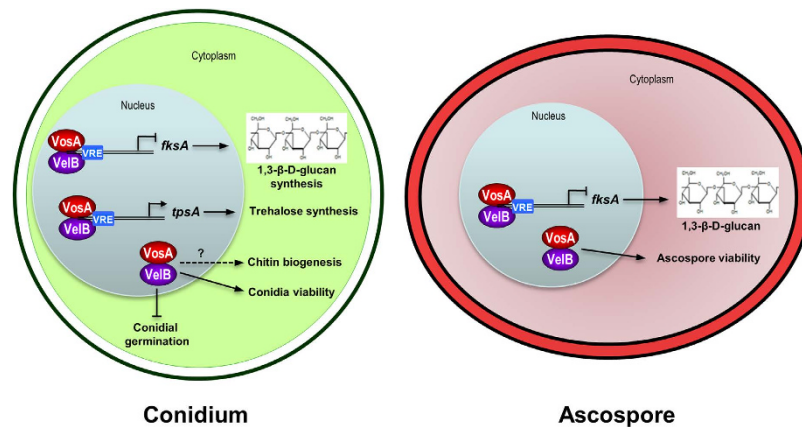


Figure 5. The roles of VosA and VelB in conidia and ascospores. A model depicting the regulatory roles of VosA and VelB in integrating morphological and chemical development during conidiogenesis and ascosporegenesis is shown (see Discussion).

Strain name	Relevant genotype	References
FGSC4	<i>A. nidulans</i> wild type, <i>veA</i> ⁺	FGSC ^a
RNI14.1	<i>biA1</i> ; Δ <i>vosA::argB</i> ⁺ ; <i>veA</i> ⁺	Ni and Yu, 2007
THS14.1	<i>pyroA4</i> ; Δ <i>vosA::pyroA</i> ⁺ ; Δ <i>velB::AfupyrG</i> ⁺ ; <i>veA</i> ⁺	Park et al., 2012a
THS15.1	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>vosA::AfupyrG</i> ⁺ ; <i>veA</i> ⁺	Park et al., 2012a
THS16.1	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>velB::AfupyrG</i> ⁺ ; <i>veA</i>	Park et al., 2012a
THS20.1	<i>pyrG89</i> ; <i>pyroA::velB(p)::velB::FLAG_{3x}::pyroA</i> ^b ; Δ <i>velB::AfupyrG</i> ⁺ ; <i>veA</i> ⁺	Park et al., 2012a
THS28.1	<i>pyrG89</i> ; <i>pyroA::vosA(p)::vosA::FLAG_{3x}::pyroA</i> ^b ; Δ <i>vosA::AfupyrG</i> ⁺ ; <i>veA</i> ⁺	Park et al., 2012a
THS30.1	<i>pyrG89</i> ; <i>AfupyrG</i> ⁺ ; <i>veA</i> ⁺	This study

Table 2. *Aspergillus* strains used in this study. ^aFungal Genetic Stock Center. ^bThe 3/4 *pyroA* marker causes targeted integration at the *pyroA* locus.

the deletion of *vosA* results in increased formation of the VelB-VeA-LaeA trimeric complex, which is required for ST production.

Taken together, we propose a model for the proposed roles of the VosA-VelB complex in both asexual and sexual spores (Fig. 5). Previous studies demonstrated that the VosA-VelB complex directly and/or indirectly controls developmental regulatory genes or sporogenesis-related genes, thereby regulating spore maturation, spore-wall integrity, and trehalose biogenesis in fungal spores. In addition, this hetero-complex directly binds to the *fksA* promoter region and negatively regulates *fksA* expression in conidia and ascospores. Overall, our data further corroborate the idea that the VosA-VelB complex is a master regulatory unit for structure, metabolism and physiology in both asexual and sexual spores in *A. nidulans*.

METHODS

Strains, media and culture conditions. *A. nidulans* strains used in this study are listed in Table 2. Strains were grown on solid minimal medium (MM), or sexual media (SM) with supplements as described previously^{47,48} at 37 °C.

Nucleic acid manipulation. The oligonucleotides used in this study are listed in Table 3. Total RNA isolation and Northern blot analyses were carried out as previously described²³. The DNA probes were prepared by PCR-amplification of the coding regions of individual genes with appropriate oligonucleotide pairs using FGSC4 genomic DNA as a template.

Name	Sequence (5' → 3')	Purpose
OMN143	ACTTATGCCAACGTTCTGCG	5' <i>fksA</i> probe
OMN144	AAAGAGCGGGCAGCATAATG	3' <i>fksA</i> probe
OHS518	GCTGCAAGCGTGACATTGCGAAG	5' <i>gelA</i> probe
OHS519	GTACCGCGTTCTGGCTACCAG	3' <i>gelA</i> probe
OHS520	GTAAGTTCACCGAGGTTCCAGGCC	5' <i>gelB</i> probe
OHS521	AGGCAGGTAACCAGCACCGTTTG	3' <i>gelB</i> probe
OHS972	ATGAAGTACTCTTTCGCCCTCACC	5' <i>sunA</i> probe
OHS973	CTAGTAGAAGACGAAGGTAGCGTC	3' <i>sunA</i> probe
OHS974	ATGACCCTTTTTCGAAACCTGGC	5' <i>crhD</i> probe
OHS975	TTAGCGCACAGGCTGGTAGCC	3' <i>crhD</i> probe
OHS976	ATGAGGGGAGCTATCCTGGCC	5' <i>btgE</i> probe
OHS977	GTGCTGCAAGCAGCTCCAAC	3' <i>btgE</i> probe
OHS978	ATGCGCGTCGCCGGGATTAT	5' <i>btgA</i> probe
OHS979	CTAGTTCGGGCACGACAAATCG	3' <i>btgA</i> probe
OHS980	ATGAAGTCTCCAGCATCCTTGC	5' <i>gelC</i> probe
OHS981	GATGCAGATGTGCCAGAGGGAC	3' <i>gelC</i> probe
OJA174	CATTGAGCACGGTGTGT	5' γ -actin probe
OJA175	ATCCCTGATCTCGTTTG	3' γ -actin probe
OHS622	AGCCGAGCGATCAACTCTTG	5' <i>fksA_pro</i> _ChIP-PCR
OHS623	CGATGTCTGGAAGTGGTATGCAG	3' <i>fksA_pro</i> _ChIP-PCR
OHS624	CTCGGTTGTGGATTCTTAATCTC	5' <i>fksA_orf</i> _ChIP-PCR
OHS625	AATAGGCTCCATGCCCTTGG	3' <i>fksA_orf</i> _ChIP-PCR

Table 3. Oligonucleotides used in this study.

Microarray and data analysis. Two-day old conidia of $\Delta vosA$ (RNI14.1) and WT (FGSC4) strains were collected and ground in liquid N_2 with a mortar and pestle. Total RNA was extracted by the acid-phenol method and harvested using CsCl density gradient ultracentrifugation. The fluorescent-labeled target cDNA probes were synthesized using the EZ start RNA linear amplification kit following manufacturer's instructions (GenomicTree, Korea). Briefly, total RNA (1 μ g) was reverse transcribed in the presence of 2 μ g of 5'-CTACGCTGGGCCGACCGGGCGCGGGAC-3'-dT24 primer (Bioneer, Korea) at 42 °C for 2 h to generate single stranded cDNA. The reaction mixtures were further treated with RNase H (Invitrogen), and double stranded cDNAs were synthesized in the presence of *Escherichia coli* DNA ligase and DNA polymerase I (Invitrogen). Double stranded cDNAs were then purified by the Qiaquick PCR purification kit (Qiagen, USA), and further labeled with Aminoallyl-dUTP (Sigma, USA) in the presence of 5'-CTACGCTGGGCCGACCGGGCGCGGGAC-3' primer (Bioneer) and Taq polymerase (Solgent). The amplified products were purified using a Qiaquick PCR purification kit (Qiagen), and further coupled with monofunctional cyanine dye as recommended by manufacturer (Amersham; Buckinghamshire, UK). The cDNAs synthesized from reference RNA were labeled with Cy3-dUTP, and cDNAs synthesized from total RNA taken from indicated time points were labeled with Cy5-dUTP. The labeled cDNA targets were purified using a Qiaquick purification kit (Qiagen), and dissolved in 100 μ l of hybridization solution containing 5 \times SSC, 0.1% SDS, 30% formamide, and 20 μ g of salmon sperm DNA (Invitrogen). The hybridization mixtures were heated at 100 °C for 3 min and directly applied onto the 70-mer glass slide DNA microarrays (Tiger-PFGRC, USA). Two biological replicates were performed using cultures grown in parallel.

After hybridization, microarray slides were imaged using the laser scanner (Axon 4000B; Axon, USA). The signal and background fluorescence intensities were calculated for each probe spot by averaging the intensities of every pixel inside the target region using GenePix Pro 4.0 software (Axon). The signal intensity for each spot is the difference between the median pixel signal intensity and the median local background intensity. Spots exhibiting obvious abnormalities were excluded from analysis. All data normalization, statistical analyses and cluster analyses were performed using the GeneSpring 7.3.1 (Agilent, USA). Genes were further filtered according to their intensity in both channels, and thus the spots with average intensity of both channels ≥ 10 after normalization were considered as reliable ones.

The information of each *A. nidulans* gene that was downloaded from AspGD (<http://www.aspergillusgenome.org/>), the chromosomal feature of *A. nidulans* FGSC A4 was updated on 2 March, 2014. The functional categories of *A. nidulans* FGSC A4 were assigned using the MIPS PEDANT 3 database (<http://pedant.gsf.de/genomes.jsp?category=fungal>)⁴⁹, KOGs database⁵⁰ was from the version p3_p130_Asp_nidul (calculation date 11 August, 2007), GO database was from the version p3_r40559_Asp_nidul (calculation date 19 September, 2013).

Transmission electron microscopy (TEM). Samples were prepared following the methods as described²¹. Briefly, conidia were collected from the 8 day cultures on solid media. Samples were fixed in Karnovsky's fixative, post-fixed in 2% osmium tetroxide, dehydrated in graded ethanol series. Polymerized samples were sectioned on a Leica UC6 ultramicrotome (80 nm) and stained with uranyl acetate and lead citrate. The stained sections were viewed on a JEOL 100CX transmission electron microscope, and documented with a SIS (Soft Imaging Systems, Lakewood, CO) MegaView III digital side mount camera.

β -glucan analysis. The β -1,3-glucan amounts in spores were determined by the Glucatell[®] assay (Associates of Cape Cod, USA) following the manufacturer's instructions⁵¹. Briefly, two-day old conidia, or eight-day old ascospores of WT and mutants were collected in ddH₂O. Samples of spores suspension ($10^2 \sim 10^5$) were prepared and resuspended in 25 μ l of ddH₂O. Each spores sample was mixed with 25 μ l of Glucatell[®] reagent and incubated at 37 °C for 30 minutes. After incubation, diazo-reagents were added to stop the reaction, and optical density at 540 nm was determined. This test was performed in triplicate.

Chromatin Immuno-precipitation followed by PCR (ChIP-PCR). Samples were prepared following the methods as described²⁵. Briefly, ChIP-PCR analysis was performed according to the manufacturer's instructions with a minor modification using MAGnify Chromatin Immuno-precipitation System (Invitrogen). Two-day-old conidia or eight-day-old ascospores (1×10^9) of each strain were cross-linked, washed and homogenized by a mini-bead beater. The cell lysates were sonicated for four cycles (30 s on, 60 s off) with a sonifier. The sonicated cell lysates were cleared by centrifugation. The diluted chromatin extracts were incubated with 2 μ g of anti-FLAG antibody-Dynabeads complex and washed with the IP buffer. The input control and chromatin samples were eluted from the beads at 55 °C for 15 min with a reverse crosslinking buffer with Proteinase K. Enriched DNA was purified by DNA purification Magnetic Beads (Invitrogen). For amplification by PCR, the GO Taq DNA polymerase (Promega) was used. As negative controls, the chromatin extract being incubated with bead only (without anti-FLAG antibody) and samples of the fungal strain THS30 without FLAG-tagged VosA or VelB were used. Individual input DNA samples before immune-precipitation (IP) were used as positive controls. Two biological replicates have provided the essentially identical ChIP-PCR results. The primer sets used for PCR are shown in Table 3.

References

- Klis, F. M., de Jong, M., Brul, S. & de Groot, P. W. Extraction of cell surface-associated proteins from living yeast cells. *Yeast* **24**, 253–8 (2007).
- Oshero, N. & Yarden, O. [The Cell Wall of Filamentous Fungi]. *Cellular and Molecular Biology of Filamentous Fungi* [224–237] (ASM Press, Washington, 2010).
- Latge, J. P. The cell wall: a carbohydrate armour for the fungal cell. *Mol Microbiol* **66**, 279–90 (2007).
- Latge, J. P. Tasting the fungal cell wall. *Cell Microbiol.* **12**, 863–72 (2010).
- Free, S. J. Fungal cell wall organization and biosynthesis. *Adv. Genet.* **81**, 33–82 (2013).
- Bowman, S. M. & Free, S. J. The structure and synthesis of the fungal cell wall. *Bioessays* **28**, 799–808 (2006).
- Klis, F. M., Mol, P., Hellingwerf, K. & Brul, S. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* **26**, 239–56 (2002).
- Butts, A. & Krysan, D. J. Antifungal drug discovery: something old and something new. *PLoS Pathog.* **8**, e1002870 (2012).
- Tada, R., Latge, J. P. & Aimanian, V. Undressing the fungal cell wall/cell membrane—the antifungal drug targets. *Curr. Pharm. Des.* **19**, 3738–47 (2013).
- Yoshimi, A. *et al.* Functional analysis of the alpha-1,3-glucan synthase genes *agsA* and *agsB* in *Aspergillus nidulans*: *agsB* is the major alpha-1,3-glucan synthase in this fungus. *PLoS One* **8**, e54893 (2013).
- He, X., Li, S. & Kaminsky, S. G. Characterization of *Aspergillus nidulans* alpha-glucan synthesis: roles for two synthases and two amylases. *Mol. Microbiol.* **91**, 579–95 (2013).
- Kelly, R., Register, E., Hsu, M. J., Kurtz, M. & Nielsen, J. Isolation of a gene involved in 1,3-beta-glucan synthesis in *Aspergillus nidulans* and purification of the corresponding protein. *J. Bacteriol.* **178**, 4381–91 (1996).
- Horiuchi, H. Functional diversity of chitin synthases of *Aspergillus nidulans* in hyphal growth, conidiophore development and septum formation. *Med. Mycol.* **47** (Suppl 1), S47–52 (2009).
- de Groot, P. W. *et al.* Comprehensive genomic analysis of cell wall genes in *Aspergillus nidulans*. *Fungal Genet Biol.* **46** (Suppl 1), S72–81 (2009).
- Adams, T. H., Wieser, J. K. & Yu, J.-H. Asexual sporulation in *Aspergillus nidulans*. *Microbiol. Mol. Biol. Rev.* **62**, 35–54 (1998).
- Ebbole, D. J. [The Conidium]. *Cellular and Molecular Biology of Filamentous Fungi*. [577–590] (ASM Press, Washington, 2010).
- Timberlake, W. E. Molecular genetics of *Aspergillus* development. *Annu. Rev. Genet.* **24**, 5–36 (1990).
- Mims, C. W., Richardson, E. A. & Timberlake, W. E. Ultrastructural analysis of conidiophore development in the fungus *Aspergillus nidulans* using freeze-substitution. *Protoplasma* **144**, 132–141 (1988).
- Sewall, T. C., Mims, C. W. & Timberlake, W. E. Conidium differentiation in *Aspergillus nidulans* wild-type and wet-white (*wetA*) mutant strains. *Dev. Biol.* **138**, 499–508 (1990).
- Ni, M., Gao, N., Kwon, N.-J., Shin, K.-S. & Yu, J.-H. [Regulation of *Aspergillus* Conidiation]. *Cellular and Molecular Biology of Filamentous Fungi* [559–576] (ASM Press, Washington, 2010).

21. Ni, M. & Yu, J.-H. A novel regulator couples sporogenesis and trehalose biogenesis in *Aspergillus nidulans*. *PLoS One* **2**, e970 (2007).
22. Sarikaya Bayram, O. *et al.* LaeA control of velvet family regulatory proteins for light-dependent development and fungal cell-type specificity. *Plos Genetics* **6**, e1001226 (2010).
23. Park, H.-S., Ni, M., Jeong, K. C., Kim, Y. H. & Yu, J.-H. The role, interaction and regulation of the velvet regulator VelB in *Aspergillus nidulans*. *PLoS One* **7**, e45935 (2012).
24. Bayram, O. & Braus, G. H. Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. *FEMS Microbiol Rev.* **36**, 1–24 (2012).
25. Ahmed, Y. L. *et al.* The velvet family of fungal regulators contains a DNA-binding domain structurally similar to NF- κ B. *PLoS Biol* **11**, e1001750 (2013).
26. Beyhan, S., Gutierrez, M., Voorhies, M. & Sil, A. A temperature-responsive network links cell shape and virulence traits in a primary fungal pathogen. *PLoS Biol.* **11**, e1001614 (2013).
27. Park, H.-S. & Yu, J.-H. Genetic control of asexual sporulation in filamentous fungi. *Curr Opin Microbiol* **15**, 669–77 (2012).
28. Fujiwara, M. *et al.* 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.* **127**, 359–66 (2000).
29. Yanai, K. *et al.* Isolation and characterization of two chitin synthase genes from *Aspergillus nidulans*. *Biosci. Biotechnol. Biochem.* **58**, 1828–35 (1994).
30. Fukuda, K. *et al.* Class III chitin synthase ChsB of *Aspergillus nidulans* localizes at the sites of polarized cell wall synthesis and is required for conidial development. *Eukaryot Cell* **8**, 945–56 (2009).
31. Motoyama, T. *et al.* The *Aspergillus nidulans* genes *chsA* and *chsD* encode chitin synthases which have redundant functions in conidia formation. *Mol. Gen. Genet.* **253**, 520–8 (1997).
32. Jung, U. S. & Levin, D. E. Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. *Mol. Microbiol.* **34**, 1049–57 (1999).
33. Fujioka, T. *et al.* MpkA-Dependent and -independent cell wall integrity signaling in *Aspergillus nidulans*. *Eukaryot Cell* **6**, 1497–510 (2007).
34. Bayram, O. *et al.* VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. *Science* **320**, 1504–6 (2008).
35. Brown, G. D. & Gordon, S. Fungal beta-glucans and mammalian immunity. *Immunity* **19**, 311–5 (2003).
36. Tsoni, S. V. & Brown, G. D. beta-Glucans and dectin-1. *Ann. N. Y. Acad. Sci.* **1143**, 45–60 (2008).
37. Romani, L. Immunity to fungal infections. *Nat. Rev. Immunol.* **11**, 275–88 (2011).
38. Steele, C. *et al.* The beta-glucan receptor dectin-1 recognizes specific morphologies of *Aspergillus fumigatus*. *PLoS Pathog.* **1**, e42 (2005).
39. Hohl, T. M. *et al.* *Aspergillus fumigatus* triggers inflammatory responses by stage-specific beta-glucan display. *PLoS Pathog.* **1**, e30 (2005).
40. Dubey, L. K., Moeller, J. B., Schlosser, A., Sorensen, G. L. & Holmskov, U. Induction of innate immunity by *Aspergillus fumigatus* cell wall polysaccharides is enhanced by the composite presentation of chitin and beta-glucan. *Immunobiology* **219**, 179–88 (2014).
41. Beauvais, A. *et al.* Glucan synthase complex of *Aspergillus fumigatus*. *J. Bacteriol.* **183**, 2273–9 (2001).
42. Park, H.-S., Bayram, O., Braus, G. H., Kim, S. C. & Yu, J.-H. Characterization of the velvet regulators in *Aspergillus fumigatus*. *Mol. Microbiol.* **86**, 937–53 (2012).
43. Horiuchi, H., Fujiwara, M., Yamashita, S., Ohta, A. & Takagi, M. Proliferation of intrahyphal hyphae caused by disruption of *csmA*, which encodes a class V chitin synthase with a myosin motor-like domain in *Aspergillus nidulans*. *J. Bacteriol.* **181**, 3721–9 (1999).
44. Culp, D. W. *et al.* The *chsA* gene from *Aspergillus nidulans* is necessary for maximal conidiation. *FEMS Microbiol Lett* **182**, 349–53 (2000).
45. Ichinomiya, M., Yamada, E., Yamashita, S., Ohta, A. & Horiuchi, H. Class I and class II chitin synthases are involved in septum formation in the filamentous fungus *Aspergillus nidulans*. *Eukaryot Cell* **4**, 1125–36 (2005).
46. Takeshita, N., Yamashita, S., Ohta, A. & Horiuchi, H. *Aspergillus nidulans* class V and VI chitin synthases CsmA and CsmB, each with a myosin motor-like domain, perform compensatory functions that are essential for hyphal tip growth. *Mol. Microbiol.* **59**, 1380–94 (2006).
47. Park, H.-S., Nam, T. Y., Han, K.-H., Kim, S. C. & Yu, J.-H. VelC positively controls sexual development in *Aspergillus nidulans*. *PLoS One* **9**, e89883 (2014).
48. Kafer, E. Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. *Adv Genet* **19**, 33–131 (1977).
49. Walter, M. C. *et al.* PEDANT covers all complete RefSeq genomes. *Nucleic Acids Res* **37**, D408–11 (2009).
50. Tatusov, R. L. *et al.* The COG database: an updated version includes eukaryotes. *BMC Bioinformatics* **4**, 41 (2003).
51. Odabasi, Z. *et al.* Beta-D-glucan as a diagnostic adjunct for invasive fungal infections: validation, cutoff development, and performance in patients with acute myelogenous leukemia and myelodysplastic syndrome. *Clin. Infect. Dis.* **39**, 199–205 (2004).

Acknowledgements

We thank our lab members for helpful discussion. The work at UW-Madison was supported by USDA CSREES Hatch (WIS01195) and the Intelligent Synthetic Biology Center of Global Frontier Project (2011-0031955) funded by the Ministry of Education, Science and Technology grants to JHY. The work at CNU (PJM) was supported by Grant 2009-0070380 from the National Research Foundation (NRF) of Korea.

Author Contributions

H.S.P., Y.M.Y. and M.K.L. performed the experiments. H.S.P., Y.M.Y., P.J.M., S.C.K. and J.H.Y. designed the experiments and analyzed the data. H.S.P., Y.M.Y., M.K.L. and J.H.Y. write the main manuscript text. H.S.P. and Y.M.Y. prepared all Figures and Tables. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Park, H.-S. *et al.* Velvet-mediated repression of β -glucan synthesis in *Aspergillus nidulans* spores. *Sci. Rep.* **5**, 10199; doi: 10.1038/srep10199 (2015).



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>