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A Putative APSES Transcription Factor Is Necessary for Normal Growth and Development of *Aspergillus nidulans*

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The *nsdD* gene encoding a GATA type transcription factor positively controls sexual development in *Aspergillus nidulans*. According to microarray data, 20 genes that were upregulated by deleting *nsdD* during various life cycle stages were randomly selected and deleted for functional analysis. None of the mutants showed apparent changes in growth or development compared with those of the wild-type except the AN3154 gene that encodes a putative APSES transcription factor and is an ortholog of *Saccharomyces cerevisiae swi4*. Deleting AN3154 resulted in retarded growth and development, and the gene was named *rgdA* (*r*etared *g*rowth and *d*evelopment). The *rgdA* deletion mutant developed a reduced number of conidia even under favorable conditions for asexual development. The retarded growth and development was partially suppressed by the *veA1* mutation. The conidial heads of the mutant aborted, showing reduced and irregular shaped phialides. Fruiting body development was delayed compared with that in the wild-type. The mutant did not respond to various nutritional or environmental factors that affected the development patterns. The *rgdA* gene was expressed at low levels throughout the life cycle and was not significantly affected by several regulators of sexual and asexual development such as *nsdD*, *veA*, *stuA*, or *brlA*. However, the *rgdA* gene affected *brlA* and *abaA* expression, which function as key regulators of asexual sporulation, suggesting that *rgdA* functions upstream of those genes.

Keywords: *Aspergillus nidulans*, APSES transcription factor, *rgdA*, asexual development, VeA

Introduction

Aspergillus nidulans has both sexual and asexual reproductive life cycles during which complicated morphogenesis takes place. After acquiring competence (Axelrod *et al*., 1973) the aerial hyphae form foot cells from which conidiophores develop, followed by sequential development of phialides and conidia (Smith, 1977). It has been predicted that more than 1,300 genes might be specifically involved in asexual sporulation (Timberlake, 1980). A number of regulators that control the decision for asexual development, stage progression, and spore maturation and stability have been identified. Activation of *brlA*, which encodes a C_2H_2 zinc finger transcription factor, is essential for asexual development (Adams *et al*., 1988, 1990; Prade *et al*., 1993). Additionally, six regulators (FluG and FlbA-E) act upstream of BrlA and are required for *brlA* expression (Wieser *et al*., 1994). VosA, one of the velvet proteins similar to VeA, VelB, and VelC, functions as a key negative feedback regulator of conidiation and directly represses the *brlA* gene (Ni and Yu, 2007).

As the sexual cycle is more complicated and responds widely to various environmental conditions, many more genes may be necessary for sexual reproduction. Many genes that control sexual development have been identified through forward and reverse genetic methods (Han *et al*., 1990; Jeong *et al*., 2000; Lee *et al*., 2001; Min *et al*., 2007). Among them, VeA, NsdC, and NsdD are key regulators of sexual development (Han *et al*., 2001; Kim *et al*., 2002, 2009), and their loss of function mutants show a common phenotype of no fruiting bodies. VeA is a key regulator of sexual development and also makes complexes with FphA, VelB, and LaeA to control the light-dependent preference of asexual development, conidial maturation, and secondary metabolism, respectively (Bok and Keller, 2004; Ni and Yu, 2007; Bayram *et al*., 2008; Purschwitz *et al*., 2008). The *nsdC* gene encodes a putative transcription factor carrying a zinc finger DNA binding domain consisting of two C_2H_2 and a C_2HC motif (Kim and Han, 2006). NsdC not only plays a positive role as a regulator of sexual development but also as a negative regulator of asexual development (Kim *et al*., 2009). NsdD, a GATA type transcription factor, is also necessary for sexual development and repression of asexual development (Han *et al*., 2001). There is no evidence for dependency among the three positive regulators and no reliance of gene expression on the others (Kim, *et al*., 2009). Several genes, which are components of G protein-coupled signaling, transcription factors, or involved in metabolic regulation, have been identified as members required for forming fruiting bodies. Although the regulators are supposed to control a number of genes that may be involved in sexual development, few genes have been identified as actual targets.

Suppressor mutants, which develop fruiting bodies in the absence of *nsdD*, were isolated to search for the genes that might interact with or be up or downregulated by nsdD (Lee *et al*., 2011). Several loci scattered on various linkage

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Table 1. **Strains used in this study**

Strains	Genotype	Source
A4	*veA*$^{+}$	FGSC[a]
KOD2	Δ*argB*::*trpC*; Δ*nsdD*::*argB*; *choA2*; *veA*$^{+}$	Kim *et al.* (2009)
veA28	*pabaA1*; *argB2*; *pyroA4*; *choA1*, Δ*veA*::*argB*	Kim *et al.* (2009)
AMBP3	*pabaA1*, *yA2*; Δ*brlA*::*argB*, *veA*(?)	From T.H. Adams
STU10	Δ*stuA*::*argB*, *pabaA1*; *argB2*; *pyroA4*; *chaA1*	From S.K. Chae
HSY2	*anA1*; Δ*argB*::*trpC*; *veA*$^{+}$	Kim *et al.* (2009)
JSGP	*pyrG89*; *argB2*(?); Δ*nkuA*::*argB*, *pyroA4*; *veA*$^{+}$	Han *et al.* (2010)
JSGP-PG	*pyrG89*; *argB2*(?); Δ*nkuA*::*argB*, *pyroA4*; *veA*$^{+}$; *pAfpyrG*	In this study
JEL1	*pyrG89*; Δ*nkuA*::*argB*, *pyroA4*; *veA*$^{+}$; Δ*rgdA*::*AfpyrG*	In this study
JEL2	*pyrG89*; Δ*nkuA*::*argB*, *pyroA4*; *veA1*; Δ*rgdA*::*AfpyrG*	In this study

[a] Fungal Genetics Stock Center

groups have a common function as negative regulators of sexual development. However, it had not been clarified whether these genes are under the control of the *nsdD* gene. Genes that are up or downregulated by NsdD have been massively screened using microarray (our unpublished data). The transcription profile of the *nsdD* deletion mutant was compared to that of the wild type (WT), and the transcription of hundreds of genes was affected in the *nsdD* mutant. In this study, 20 of these genes were randomly selected and deleted to identify their functions during development. Unexpectedly, only one gene encoding an APSES transcription factor showed an obvious developmental phenotype and was analyzed to determine the details of its function.

Materials and Methods

Strains and culture conditions

FGSC A4 was used as the WT strain, and JSGP, a *veA*$^{+}$ strain carrying Δ*nkuA* (Han *et al.*, 2010), was used as the recipient strain for transformation with Af*pyrG*$^{+}$ vectors. FGSC A26 was the *veA1* mutant strain used to cross with JEL1 to isolate Δ*rgdA, veA1* recombinants (JEL2). JSGP was transformed with pAf*pyrG*, which carried the *pyrG*$^{+}$ gene of *Aspergillus fumigatus,* and was used as a positive control. The genotypes and sources of the *A. nidulans* strains used in this study are listed in Table 1. Minimal medium (MM) and complete medium (CM) were prepared as described previously (Han *et al.*, 2001). One M KCl or 1.2 M sorbitol was added to the MM to observe salt or osmotic stress. Light was illuminated in a growth chamber equipped with white fluorescent and metal SP lamps (maximum 20,000 Lux) and a temperature control system. For submerged culture, 1–2×10^{5}/ml conidia were inoculated and incubated at 37°C with agitation at 200 rpm. Mycelia cultured for 16 h in broth were harvested, washed, and overlaid on plates and cultured further to induce asexual or sexual development. Sexual development was induced by sealing the culture plates with Parafilm.

Table 2. **List of genes selected for mutagenesis and their brief characteristics**

Gene No.	Domain	Up/Down Regulation[a]	Phenotype
AN6810	ThiJ/PfpI	Dn; V 16 h	No
AN8369	Glycosyl Transferse	Up; V, A 3 h	No
AN11016	Malate Transport	Dn; V 16 h	No
AN11530	Unknown	Dn; A 12 h	No
AN3480	Unknown	Dn; S 6 h	No
AN8638	HHE	Dn; S 6 h	No
AN10678	Unknown	Dn; S 48 h	No
AN8466	LysM superfamily	Up; V 9 h	No
AN0635	Unknown	Dn; S 6 h	No
AN5015	Conidiation specific	Dn; A 3 h, Up; S 1 h	No
AN9205	Integral memb.	Dn; S 3 h	No
AN0497	Major Facilitator	Up; V 16 h	No
AN3872	Unknbown	Dn; V 16 h	No
AN10499	NADP-glycerol DeH	Dn; S 6 h	No
AN3679	Reductase family pro.	Dn; S 6 h	No
AN9038	Mo-cofactor biosynthesis	Dn; S 6 h	No
AN1073	MFS peptide transporter	Dn; S 48 h	No
AN9443	TIM P-binding SF	Dn; S 24-48 h	No
AN1719	Unknown	Dn; Hy (?)	No
AN3154	APSES	Up; V 9-16 h	Growth & Development retardation

[a] Dn, downregulation; Up, upregulation; V, vegetative growth period; A, asexual developmental period; S, sexual developmental period

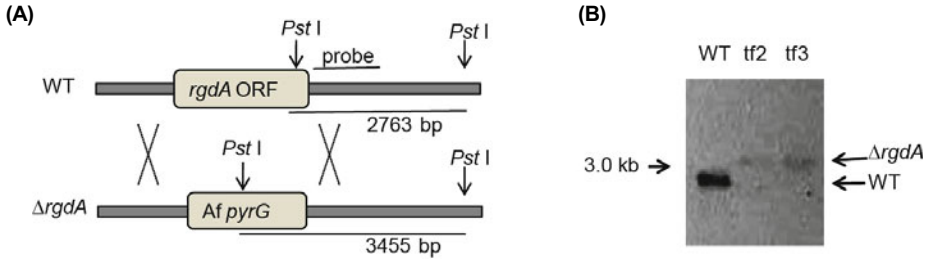


Fig. 1. Confirmation of the *rgdA* deletion mutant by Southern analysis. The *rgdA* gene was replaced by *pyrG* of *A. fumigatus*. If the *rgdA* open reading frame (ORF) is successfully replaced by *pyrG*, about 3.5 kb of the *Pst*I/*Pst*I fragment would be yielded instead of 2.7 kb (A). Southern hybridization revealed that the *rgdA* ORF was successfully deleted in two transformed strains (B).

When necessary, a loopful of conidia was overlaid on the broth prepared in 12-well plates and surface cultured. Standard fungal techniques were carried out for observation and genetic analyses, as described previously (Kafer, 1969; Han *et al*., 2001).

Measurement of growth rate and conidial and cleistothecial development

Radial growth was measured every 24 h after inoculation at the center of the plate. To count the number of conidia, 5 mm diameter agar blocks were removed, suspended in 1 ml of 0.8% Tween 80 solution and scored by hemacytometer under a light microscope. The number of cleistothecia that produced ascospores was counted as mature cleistothecia.

Molecular techniques

Genomic DNA of *A. nidulans* was prepared as described previously (Kim *et al*., 2009). Total RNA was isolated from mycelia frozen with liquid nitrogen by grounding using a mortar with Trizol (Invitrogen, USA), according to the manufacturer's instructions. Southern and northern blots were carried out as described previously (Han *et al*., 2001). Probes were labeled with [γ-^{32}P]dCTP (Amersham Pharmacia, USA) using a random priming kit (TaKaRa Bio, Japan) and purified using a Prober kit (iNtRON Biotechnology, Korea). DNA mediated transformation of *A. nidulans* was performed as described by Yelton *et al.* (1984).

Microscopy and photography

Photomicrographs were taken using an Olympus C-5060 digital camera through an Olympus BX50 microscope. Photographs were taken using a Samsung STW-NV7 digital camera.

Results

Isolation of NsdD-regulated gene deletion mutants

Among the genes that were up or downregulated by NsdD according to the microarray data carried out with the *nsdD* deletion mutant (our unpublished data), 20 genes were randomly selected to be deleted for functional analyses (Table 2). Deletion mutants were isolated by transforming JSGP, a *veA*$^{+}$ recombinant carrying the *nkuA* deletion mutation (Table 1, Han *et al*., 2010), using deletion cassettes purchased from the Fungal Genetic Stock Center (Colot *et al*., 2006). Deletion of all mutants was confirmed by Southern hybridization (data not shown). Their growth and asexual and sexual development phenotypes under various conditions were examined. Mutants of all genes except the AN3154 gene showed no phenotypic change. Growth and sexual and asexual development of the AN3154 gene deletion mutants were retarded, and the gene was named *rgdA*. The deduced RgdA protein carried an APSES DNA binding domain at its N terminus and an ankyrin repeat in the midst of the polypeptide, which is highly conserved in most fungi. The gene was an ortholog of *S. cerevisiae swi4* and was designated previously as An*swi4* (Fujioka *et al*., 2007).

RgdA is necessary for normal growth and development

Among 40 *pyrG*$^{+}$ transformants isolated according to the *rgdA* deletion strategy (Fig. 1A), 32 strains with a common phenotype were selected and replacement of *rgdA* with Af*pyrG* was verified by Southern hybridization (Fig. 1B). All mutants showed pleiotropic growth and development phenotypes. Apical growth rate of JEL1, a representative mutant strain, was reduced to half that of the WT in MM (Fig. 2).

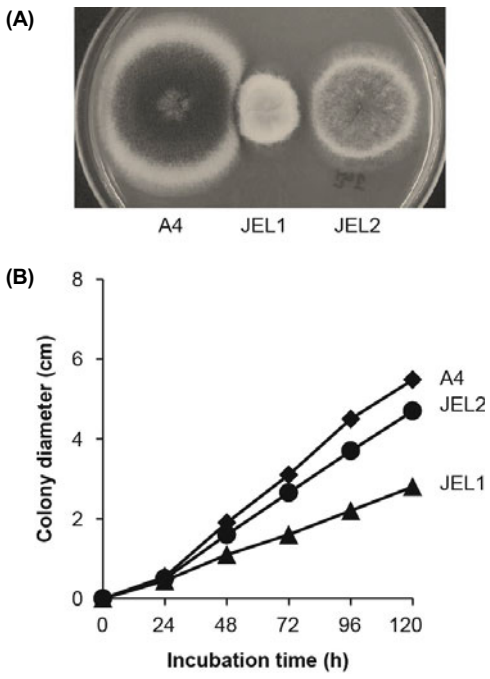
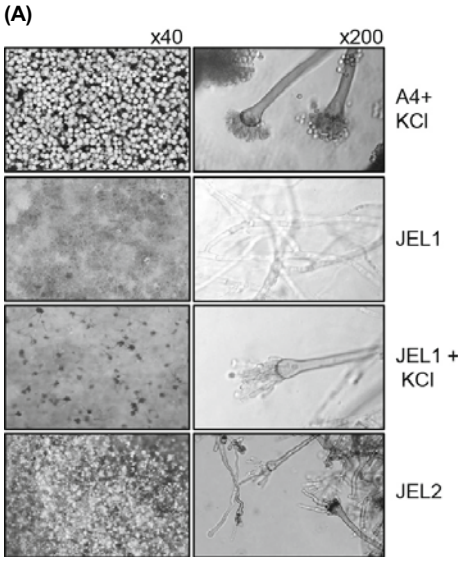


Fig. 2. Growth of the *rgdA* deletion mutant. (A) Wild type (A4), JEL1 (Δ*rgdA*, *veA*$^{+}$), and JEL2 (Δ*rgdA*, *veA1*) strains were point-inoculated on minimal medium (MM) containing 1% glucose and incubated at 37°C for 4 days. (B) Growth curves of the strains on MM are shown.



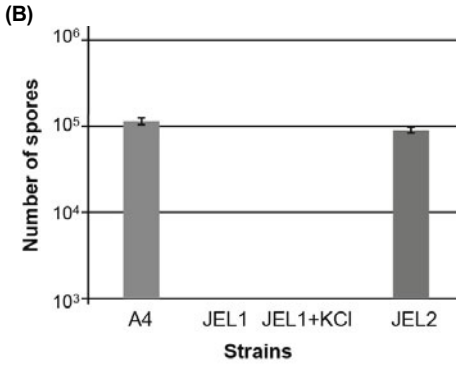


Fig. 3. Asexual development of the *rgdA* deletion mutant. Development of conidial heads (left lane) and their fine microscopic morphology (right lane) in wild-type and mutants on various media (A). No conidial heads and only a few were found on KCl supplemented medium when the *rgdA* deletion mutant (JEL1) was cultured; thus, the number of conidia per 0.25 πcm^2 did not exceed 10^3 (B). Development of conidia recovered almost to the wild type level in the Δ*rgdA* mutant with a *veA1* background (JEL2).

This retarded growth was also observed in CM and did not recover under any of the cultural conditions, such as changing C or N sources, shifting culture temperature (25, 30, or 42°C), or adding osmotic stabilizers (0.6 M KCl or 1.2 M sorbitol), suggesting that the growth defect was not conditional (data not shown). Asexual sporulation in the mutant was seriously affected. As shown in Fig. 3, no conidial heads were detected on CM and only a few were found on 1 M KCl supplemented medium, which preferentially induced asexual sporulation (Fig. 3A). The shapes of the phialides of the mutant grown on a KCl plate were abnormal and bore few conidia (Fig. 3A), resulting in the maintenance of conidia at <10^3/cm^2. The mutant showed similar phenotypes under other environmental conditions that promote asexual development such as adding 1.2 M sorbitol or supplying acetate as the sole C source (Fig. 5). These results suggest that normal function of RgdA is necessary not only for determining asexual development but also for morphogenic differentiation. Sexual development in the *rgdA* deletion mutant was delayed almost 3 days compared to that in the WT (Fig. 4A). The WT (A4) formed mature ascospores within most cleistothecia in 4 days, whereas the mutant developed only Hülle cells and young cleistothecia (Fig. 4B). Mature ascospores of the mutant were detectable after 6 days. It took 2–3 days to produce ascospores from the young fruiting bodies, which was slighlty longer than that of the WT, indicating that the maturation process was slightly affected. Development was far more retarded on media supplemented with lactose, glycerol or maltose, which promoted sexual development of the WT (Fig. 5). Although development of cleistothecia and ascospores was delayed, their shape and size was normal, indicating that the morphogenic process of sexual sporulation was not affected by deleting *rgdA* (Fig. 4B). The phenotypes of the *rgdA* deletion mutant suggested that the *rgdA* gene is necessary for normal apical growth and development and normal morphogenesis of conidia. When the *rgdA* gene was forcedly over-expressed under the *niiA* promoter, no significant phenotypic change was observed, suggesting that the excess RgdA did not affect growth or development (data not shown).

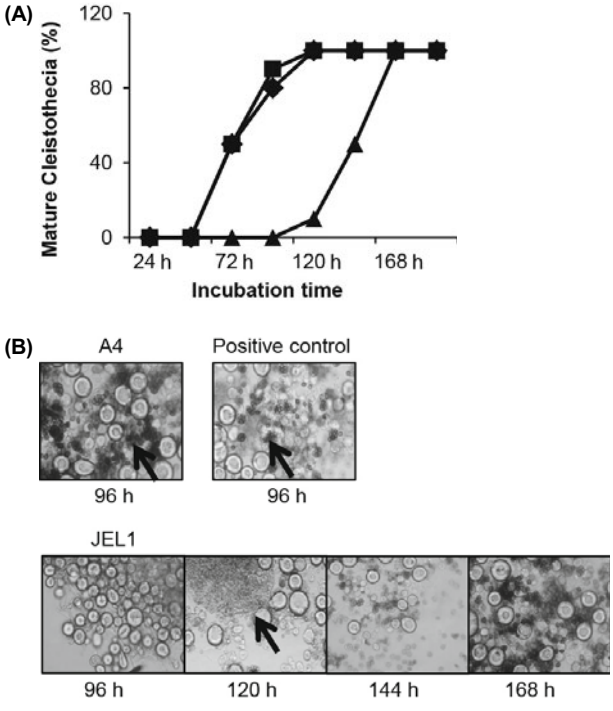


Fig. 4. Cleistothecia maturation rate of the *rgdA* deletion mutant. The rate of cleistothecia maturation was measured by scoring ascospore production among 20 randomly selected cleistothecia. Numbers represent time after induction of sexual development. Squares, A4; diamonds, positive control; triangles, JEL1 (A). A micrograph of crushed cleistothecia bearing ascospores. Arrows indicate ascospores (B).

RgdA positively controls *brlA* and *abaA* expression

The steady-state mRNA levels of the *rgdA* gene throughout the life cycle were examined by northern hybridization using

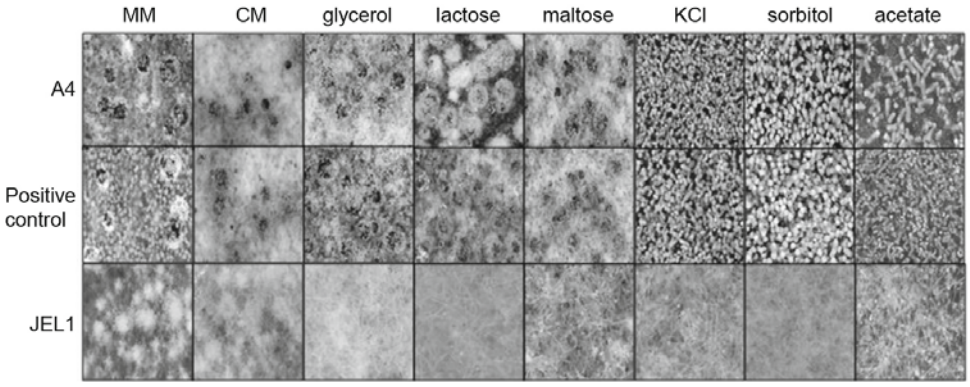


Fig. 5. Effect of various environmental conditions on development of the *rgdA* deletion mutant. No significant phenotypic changes were found under various conditions that favor sexual or asexual development. Minimal medium supplied with glycerol, lactose or maltose as a sole carbon source was used as favorite condition for sexual development and acetate as a sole carbon source or addition of 1 M KCl or 1.2 M sorbitol for asexual development.

a polymerase chain reaction-amplified 1.1 kb genomic DNA fragment within ORF as a probe. The *rgdA* transcript level was kept low not only during vegetative growth but also during developmental stages (Fig. 6). A slight decrease was observed during late asexual and early sexual development stages. The change of transcription levels in the deletion mutants of two sexual (NsdD and VeA) and two asexual (BrlA and StuA) regulators were examined to understand whether *rgdA* was under the control of known developmental regulators. The *rgdA* mRNA level increased slightly during vegetative growth in the *nsdD* deletion mutant but not in the *veA* or *brlA* deletion mutants (Fig. 6). No reduction in mRNA level was observed at the late stage of asexual sporulation in the *stuA* deletion mutant encoding other APSES transcription factors necessary for asexual sporulation. The change in *rgdA* transcription level caused by other mutations was not so significant, making it difficult to conclude that *rgdA* expression was under the regulation of any of those genes. The expression of those genes was examined to determine if RgdA controlled any of the sexual or asexual regulators. As shown in Fig. 7, *nsdD*, *veA*, and *stuA* mRNA levels were not significantly affected by deleting *rgdA*. However, *brlA* and *abaA* transcription was completely eliminated during asexual sporulation, suggesting that RgdA controls asexual development upstream of BrlA and AbaA.

Discussion

According to the transcription profile of the *nsdD* deletion mutant, >200 were up or downregulated compared with that of the WT (unpublished data). Those genes were expected to function in development as the *nsdD* deletion affects both sexual and asexual development (Han *et al.*, 2001; Kim *et al.*, 2009). About 20 genes were randomly selected and mutated by replacing the gene. Unexpectedly, most of the gene mutations did not result in any phenotypic changes in growth or development (Table 2). Northern blot analysis of randomly selected genes implicated few significant errors in the microarray experiment (data not shown). The possible explanations for the result are that NsdD controls genes that are not directly involved in development or the expression of

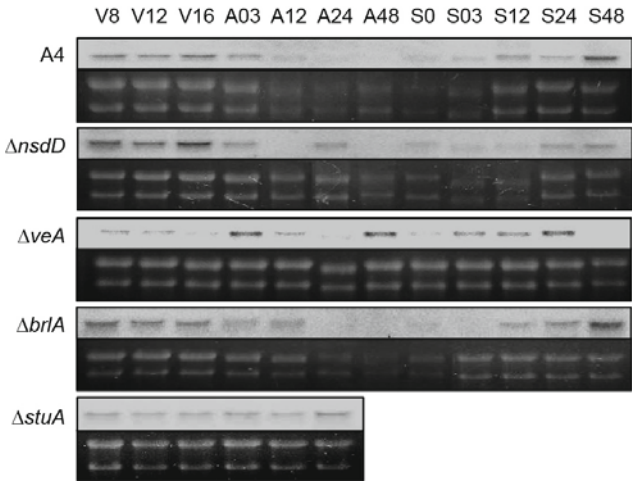


Fig. 6. Effect of various developmental regulators on *rgdA* expression. Steady-state level of *rgdA* mRNA in the mutants of sexual or asexual development regulatory genes were analyzed by northern hybridization. V, vegetative stage; A, asexual development; S, sexual development. Numbers represent time after inoculation or induction. No significant changes in mRNA levels were found in developmental regulator mutants.

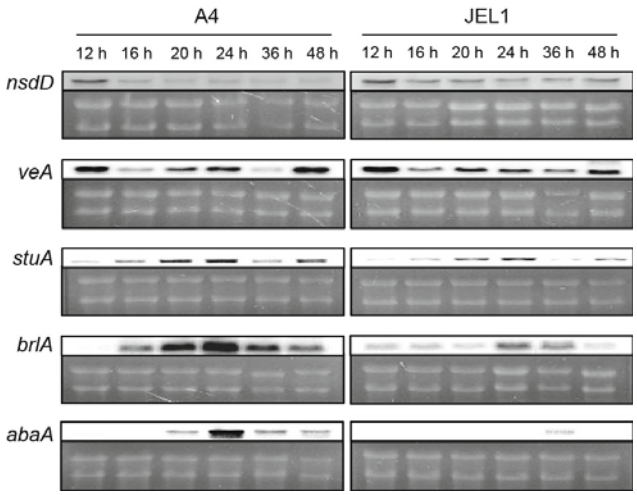


Fig. 7. Effect of *rgdA* on various developmental regulatory genes. The mRNA levels of sexual or asexual developmental regulatory genes in the wild type and the *rgdA* deletion mutant were analyzed by northern hybridization. V, vegetative stage; A, asexual development; S, sexual development. Numbers represent time after inoculation or induction.

those genes that are not directly related to *nsdD* is affected because of changes in the cellular system caused by the lack of NsdD.

Among 20 genes selected for deletion in this study, only one gene, AN3154, showed a notable phenotype. AN3154 has been identified previously as an ortholog of *swi4* in *S. cerevisiae*, which is highly conserved in most fungi (Fujioka *et al*., 2007). Swi4p is an APSES transcription factor that binds to the Swi4/6 cell-cycle box (SCB) in the promoters of a broad range of target genes (Bean *et al*., 2005). The Mpk1p-associated Swi4p forms a SCB binding factor complex with Swi6 and regulates genes involved in cell wall biogenesis during the G1/S stage (Kim *et al*., 2008, 2010). However, no evidence for similar regulatory function of the orthologs (AnSwi4-AnSwi6) and a relationship with *mpkA* is found in *A. nidulans* (Fujioka *et al*., 2007). The An*swi4* deletion mutant was isolated but showed no obvious phenotype change except a lighter conidial color. In contrast, the deletion mutant phenotype of the same gene in our study was quite different, as it showed delayed growth and development. Additionally, the mutant developed a very small number of conidiophores on KCl supplemented media that bore elongated phialides with a few conidia. The different phenotypes of the two mutants may be due to a difference in the *veA* genotype of the host strains used for gene targeting in the two experiments. The strain used in our study (JSGP) was the *veA*$^{+}$ WT, whereas the FGSC A89 used by Fujioka *et al*. (2007) had the *veA1* mutation. The double mutant carrying Δ*rgdA* and *veA1* was isolated from the meiotic cross between A26 and JEL1 to clarify the role of the *veA1* mutation on phenotypic expression of the *rgdA* deletion. The *veA1* mutation in JEL2 has been verified by Han *et al*. (2010). As shown in Fig. 2, growth in the double mutant recovered to 70% of the WT level, and the conidia with the lighter color developed into almost the normal level of color (Fig. 3). This result suggests that the reason why Fujioka *et al*. (2007) could not find any obvious phenotypic change in the An*swi4* deletion mutant was that *veA1* acted as a suppressor. VeA, a key regulator of sexual development, complexes with FphA, VelB, and LaeA to control the light-dependent preference of asexual development, conidia maturation, and secondary metabolism, respectively (Bok and Keller, 2004; Bayram *et al*., 2008; Purschwitz *et al*., 2008). As the *veA1* mutation causes a lack of N terminus harboring NLS, the VeA1 protein cannot enter nuclei, which results in preferential development of conidia even under unfavorable conditions (Kim *et al*., 2002; Han *et al*., 2003). The partial recovery of asexual development due to the *veA1* mutation has already been observed in some mutants such as *argB2*, *trpC801*, and *npgA1* (Han and Han, 1993; Han *et al*., 1994). Similarly, the defect in the Δ*rgdA* mutant during asexual sporulation might be suppressed by *veA1*. The difference in the Δ*rgdA* growth phenotype in different *veA* genotypes raises the argument of an additional role for VeA in the control of apical growth. Faster growth has often been observed in the *veA1* mutant caompred to that in the WT under certain circumstances unfavorable for asexual sporulation such as hyper nutrition, which supports the role of VeA in growth control (data not shown). However, evidence for the role of VeA in vegetative growth is insufficient. *rgdA* expression was not affected by *veA* and *vice versa* (Fig. 6), indicating that either gene is not directly regulated by the other. However, the abnormal shape of the phialides was not suppressed by *veA1*, indicating that the phenotype was entirely dependent upon the *rgdA* gene.

APSES proteins represent a conserved class of transcription factors that regulate cellular differentiation in ascomycetes. Phd1 and Sok2 in *S. cerevisiae* and Efg1p in *Candida albicans* control the yeast-to-hypha transition (Gimeno and Fink, 1994; Ward *et al*., 1995; Lo *et al*., 1997; Stoldt *et al*., 1997). In filamentous ascomycetes, the Asm1 in *Neurospora crassa* (Aramayo *et al*., 1996) and the StuA in *A. nidulans* (Dutton *et al*., 1997) control the maturation of ascospores and conidiospores, respectively. The loss of StuA function results in the stunted sterigmata which do not bud out conidia (Clutterbuck, 1969; Clutterbuck *et al*., 1977). Another APSES protein in *A. nidulans*, RgdA, controls differentiation of sterigmata. In contrast to Δ*stuA*, the *rgdA* mutant showed elongated sterigmata from which a few conidia were produced (Fig. 3). Both APSES transcription factors control phialide differentiation but during different stages. Thus, it is likely that StuA is necessary for sterigmata elongation and that RgdA is necessary for terminating elongation.

The *rgdA* transcript was maintained at a relatively low level throughout the life cycle except during late asexual sporulation when it decreased slightly, suggesting that the gene is elaborately regulated. Although the *rgdA* gene is important for growth and development, expression of the gene is not under the control of any known sexual and asexual development regulators. Furthermore, RgdA does not control the expression of sexual development regulators; however, it affects *brlA* and *abaA* transcription which was expected according to the mutant phenotype. Further study with more development regulators will be necessary to understand the role of *rgdA* in the regulatory network of development.

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