

Conserved Roles of MonA in Fungal Growth and Development in *Aspergillus* Species

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ABSTRACT

MonA is a subunit of a guanine nucleotide exchange factor that is important for vacuole passing and autophagy processes in eukaryotes. In this study, we characterized the function of MonA, an orthologue of *Saccharomyces cerevisiae* Mon1, in the model fungus *Aspergillus nidulans* and a toxigenic fungus *A. flavus*. In *A. nidulans*, the absence of *AniMonA* led to decreased fungal growth, reduced asexual reproduction, and defective cleistothecia production. In addition, *AniMonA* deletion mutants exhibited decreased spore viability, had reduced trehalose contents in conidia, and were sensitive to thermal stress. In *A. flavus*, deletion of *AflmonA* caused decreased fungal growth and defective production of asexual spores and sclerotia structures. Moreover, the absence of *monA* affected vacuole morphology in both species. Taken together, these results indicate that MonA plays conserved roles in controlling fungal growth, development and vacuole morphology in *A. nidulans* and *A. flavus*.

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1. Introduction

Aspergillus species are widespread around humans through the air [1]. Some species in this genus have beneficial effects in medicine, the food industry, and the environment [2,3]. But others exert detrimental effects by producing toxic secondary metabolites called mycotoxins [1,4]. Among these species, *A. nidulans* has been a useful model system for investigating gene functions, not only of individual genes but also for elucidating networks of gene interactions [5,6]. *A. flavus* is an opportunistic pathogenic fungus that produces many toxic secondary metabolites, such as aflatoxin [7] and cyclopiazonic acid [8].


A vacuole is a membrane-bound organelle in eukaryotes that play important roles in the *cis*- or *trans*- transport of substances to their target organelles [9,10]. Through targeting proteins to specific membranes and fusing with other membrane-bound cellular structures, vacuoles help cells maintain their morphology and pH homeostasis and conduct functions such as autophagy or degradation of misfolded proteins [11]. These functions involve various proteins including SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor), Rab GTPase Ypt7p, and class C VPS/HOPS (class C

vacuole protein sorting/homotypic fusion and protein sorting) [12–15].

Among multiplex proteins, Mon1 is a member of the guanine nucleotide exchange factor (GEF) family and interacts with Ccz1 in *Saccharomyces cerevisiae*. This Mon1-Ccz1 complex converts GDP to GTP form to activate Ypt7p on late endosomes and regulate endosomal maturation [16–18]. The orthologs of Mon1 related to the Cvt (cytoplasm to vacuole targeting) and autophagy pathways have been studied in plant and in human pathogenic fungi, including *Magnaporthe oryzae*, *Fusarium graminearum*, and *Cryptococcus neoformans*. In these species, this protein is associated with fungal development and pathogenicity [19–21]. However, Mon1 has not yet been studied in *Aspergillus* species despite its important vacuolar functions, such as survival, morphology, and transport.

To investigate the roles of MonA, the Mon1 ortholog in *Aspergillus* spp. related to survival and morphology, we tested its functions. Mon1 orthologs in *A. nidulans* (*AniMonA*) and *A. flavus* (*AflMonA*) influence proper growth, asexual development, and the formation of normal sexual structures. In addition, MonA increases spore viability in both strains. However, only *AniMonA* significantly affects tolerance to thermal stress. Thus, MonA is essential for fungal growth and development.

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 Supplemental data for this article can be accessed [here](#).

2. Materials and methods

2.1. Strains, media, and culture conditions

The *Aspergillus* strains used in this study are listed in Table 1. For general purposes, all *A. nidulans* strains were grown on solid or liquid minimal media with 1% glucose (MMG) for 5–7 days [22]. For sexual development in *A. nidulans*, wild type (WT) and mutant strains were grown on solid sexual medium (SM) at 37 °C for 7 days. For *A. flavus* strains, MMG with 0.1% yeast extract (MMGY) was used for general purposes [23].

2.2. Generation of the *monA* mutant strains

Oligonucleotides used in the present study are listed in Table 2. To generate the *monA* deletion ($\Delta monA$) cassettes, double-joint PCR (DJ-PCR) was used as previously described [24]. The 5' and 3' flanking regions of the *AnimonA* gene were amplified using the primer pairs OHS091/OHS092 and OHS093/OHS094, respectively, from *A. nidulans* FGSC4 genomic DNA. The *pyrG* marker was amplified from *A. fumigatus* AF293 genomic DNA with the primer pair OHS089/OHS090. The final deletion cassette was amplified with the primer pair OHS095/OHS096 and introduced into RJMP1.59 protoplasts generated by the VinoTaste® Pro (Novozymes, Bagsvaerd, Denmark) [25,26]. Multiple transformants were selected on MMG medium without uracil and uridine and confirmed by PCR and restriction enzyme digestion.

For *A. flavus*, the 5' and 3' flanking regions of the *AflmonA* gene were amplified using the primer

pairs OHS435/OHS436 and OHS437/OHS438, respectively, from *A. flavus* NRRL3357 genomic DNA. The *pyrG* marker was amplified from *A. fumigatus* AF293 genomic DNA with the primer pair OHS089/OHS090. The *AflmonA* deletion cassette was amplified with the primer pair OHS439/OHS440 and introduced into the recipient strain AFL 3357.5 [24]. Multiple candidates were isolated on MMGY medium without uracil and uridine and confirmed by PCR and restriction enzyme digestion.

2.3. Spore viability test

Spore viability was tested as described previously [27]. Briefly, fresh conidia from 2-day-old or 10-day-old cultures of wild-type and mutant strains were collected and counted. Approximately 100 conidia were inoculated onto solid MMG or MMGY and incubated at 37 °C for 48 h in triplicate. Survival rates were calculated as the ratio of the number of viable colonies to the number of spores inoculated in triplicates.

2.4. Trehalose analysis

The amount of trehalose (α -d-glucopyranosyl α -d-glucopyranoside) in conidia was measured as previously described [28,29]. Briefly, 10^8 conidia were suspended in 200 μ l of ddH₂O and incubated at 95 °C for 20 min. The supernatant was separated by centrifugation. Fifty microliters of supernatant were transferred to a new tube, mixed with an equal volume of 0.2 M sodium citrate (pH 5.5), and incubated with or without (as a negative control) trehalose (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 8 h to generate glucose. After incubation, the amount of glucose generated from the trehalose was assayed with a Glucose Assay Kit (Sigma-Aldrich, St. Louis, MO, USA).

2.5. Heat stress response test

Heat stress response test was carried out as previously described [28,29]. Suspensions containing 10^3

Table 1. *Aspergillus* strains used in this study.

Strain name	Relevant genotype	References
FGSC4	<i>A. nidulans</i> wild type, <i>veA</i> ⁺	FGSC ^a
RJMP1.59	<i>pyrG89</i> ; <i>pyroA4</i> ; <i>veA</i> ⁺	[25]
TNJ36	<i>pyrG89</i> ; <i>Afupyrg</i> ⁺ ; <i>pyroA4</i> ; <i>veA</i> ⁺	[32]
TYE8.1 ~ 3	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>AnimonA</i> :: <i>Afupyrg</i> ⁺ ; <i>veA</i> ⁺	This study
NRRL 3357	<i>A. flavus</i> wild type	FGSC ^a
NRRL 3357.5	<i>A. flavus pyrG</i>	[33]
TYE9.1 ~ 3	<i>pyrG</i> ; Δ <i>AflmonA</i> :: <i>Afupyrg</i> ⁺	This study

^aFungal Genetic Stock Center.

Table 2. Oligonucleotides used in this study.

Name	Sequence (5' → 3') ^a	Purpose
OHS091	TAGCCAGACTCTCGCGTCC	5' flanking region of <i>AnimonA</i>
OHS094	CTGGCTTGTCTACCTGCAGG	3' flanking region of <i>AnimonA</i>
OHS093	TTGGTGACGACAATACCTCCCGAC CAACTGGTCAGGTCATCGC	5' <i>AnimonA</i> with <i>Afupyrg</i> tail
OHS092	GGCTTTGGCCTGTATCATGACTTCA TAGTTGGGGCGGAGAAGCTC	3' <i>AnimonA</i> with <i>Afupyrg</i> tail
OHS095	TAACGCTCCGCATGAACGGC	5' nested of <i>AnimonA</i>
OHS096	TGGAGCATCATCGGCCAGAG	3' nested of <i>AnimonA</i>
OHS089	GCTGAAGTCATGATACAGGCCAAA	5' <i>Afupyrg</i> marker
OHS090	ATCGTCGGGAGGTATTGTGCTCAC	3' <i>Afupyrg</i> marker
OHS435	CAGGTAAGCCAGCACTCC	5' flanking region of <i>AflmonA</i>
OHS438	GCTCCACGATCAGTGCA	3' flanking region of <i>AflmonA</i>
OHS437	TTGGTGACGACAATACCTCCCGAC GCTGCTCATAACCTCATCC	5' <i>AflmonA</i> with <i>Afupyrg</i> tail
OHS436	GGCTTTGGCCTGTATCATGACTTCA CCTAGTACAGTAGCGACGTGG	3' <i>AflmonA</i> with <i>Afupyrg</i> tail
OHS439	GTCACACCACCAACCTTGATC	5' nested of <i>AflmonA</i>
OHS440	CCAGTCTATGCACGGACATACG	3' nested of <i>AflmonA</i>

^aTail sequences are shown in italics.

conidia were incubated for 15 or 30 min at 37°C before adding 900 μ l of ddH₂O. Approximately 100 conidia were spread onto solid MMG or MMYG. After incubation at 37°C for 48 h, colony numbers were counted and survival was calculated as the ratio of colonies from treated versus untreated controls (in triplicate).

2.6. Microscopy

Colony photographs were taken with a Pentax MX-1 digital camera. Photomicrographs were taken with a Zeiss Lab.A1 microscope equipped with AxioCam 105c and AxioVision (Rel. 4.9) digital imaging software.

2.7. Staining of vacuoles

For vacuoles staining, WT and mutants conidia were inoculated in the YG (0.5% yeast extract, 1% glucose) media and culture for 24 h. The culture hyphae were stained with 10 μ M CMAC (7-amino-4-chloromethylcoumarin) (Invitrogen, Carlsbad, CA, USA) for 30 min. The stained hyphae were washed fresh YG media for 30 min. The fluorescence of CMAC was observed using Nikon eclipse 80i microscope with 353 nm as the excitation wavelength and 466 nm as the emission wavelength.

2.8. Statistical analysis

Statistical differences between WT and mutant strains were evaluated by Student's unpaired *t*-test. Mean \pm standard deviation (SD) are shown. A value of *P* < 0.05 was considered statistically significant.

3. Results

3.1. MonA in *Aspergillus* spp

The Mon1 gene is conserved in most Ascomycota and Basidiomycota [21]. To identify the *S. cerevisiae* Mon1 orthologs in *Aspergillus* spp., the *S. cerevisiae* Mon1 (ScMon1) protein sequence was blasted against genomes of 19 *Aspergillus* species in the AspGD database (www.aspgd.org). All *Aspergillus* species contain Mon1 orthologs that contain a trafficking protein MonA domain (Pfam03164) in their C-terminal regions (Figure 1(A)). The *AnimonA* gene (AN2093) encodes a 635-amino acid protein and *AflmonA* (AFLA_035140) gene encodes a 630-amino acid protein (418 a.a.) (Figure 1(B)). The MonA domain is highly conserved among *Aspergillus* species. To study the functions of AniMonA and AflMonA, three independent strains of *monA* deletion mutants in *A. nidulans* and *A. flavus* were generated (Supplementary Figure S1).

3.2. Role of AniMonA during asexual development in *Aspergillus nidulans*

To study the functions of AniMonA in *A. nidulans*, WT and *AnimonA* deletion mutant strains were inoculated onto MMG and fungal growth and development were compared (Figure 2(A)). Deletion of *AnimonA* leads to decreased colony diameters during growth in dark conditions (Figure 2(B)). The Δ *AnimonA* mutant strains exhibit decreased conidial production under both light and dark conditions (Figure 2(C)). In addition, the Δ *AnimonA* mutants produce light green conidia, a phenotype that differs from the conidia of WT strains (Figure 2(A)). To test whether *AnimonA* is also associated with sexual development, WT and Δ *AnimonA* mutants were inoculated on SM and sexual fruiting bodies were examined. As shown Figure 2(D,E), the cleistothecia of Δ *AnimonA* mutants are much smaller than those of WT. These results demonstrate that AniMonA is essential for proper growth, asexual and sexual development.

3.3. Role of AniMonA in *Aspergillus nidulans* conidia

To test the functions of AniMonA in asexual spores, we evaluated the viability of conidia. WT and Δ *AnimonA* mutant conidia from 2- and 10-day old culture plates were isolated and inoculated onto MMG media. The conidia of Δ *AnimonA* mutant strains exhibit defects in spore viability (Figure 3(A)). Previous studies demonstrated that trehalose in conidia is crucial for spore viability and stress tolerance in *Aspergillus* spp [28]. Therefore, we then examined the trehalose content in WT and mutant conidia. As shown in Figure 3(B), the amount of trehalose in Δ *AnimonA* mutant conidia is lower than in WT conidia. We then examined whether the absence of *AnimonA* could cause increased sensitivity of the spores to heat stress. The Δ *AnimonA* mutant conidia from 10-day old culture plates are more sensitive to thermal stress than those from equivalent WT cultures (Figure 3(C)). Taken together, these results demonstrate that AniMonA is required for conidia maturation.

3.4. Conserved role of AflMonA in *Aspergillus flavus*

Since AniMonA plays an important role in growth and development, we evaluated the role of MonA in another *Aspergillus* species, *A. flavus*. *AflmonA* deletion (Δ *AflmonA*) mutant strains were generated. WT and Δ *AflmonA* mutant strains were inoculated onto MMGY and cultured under both light and

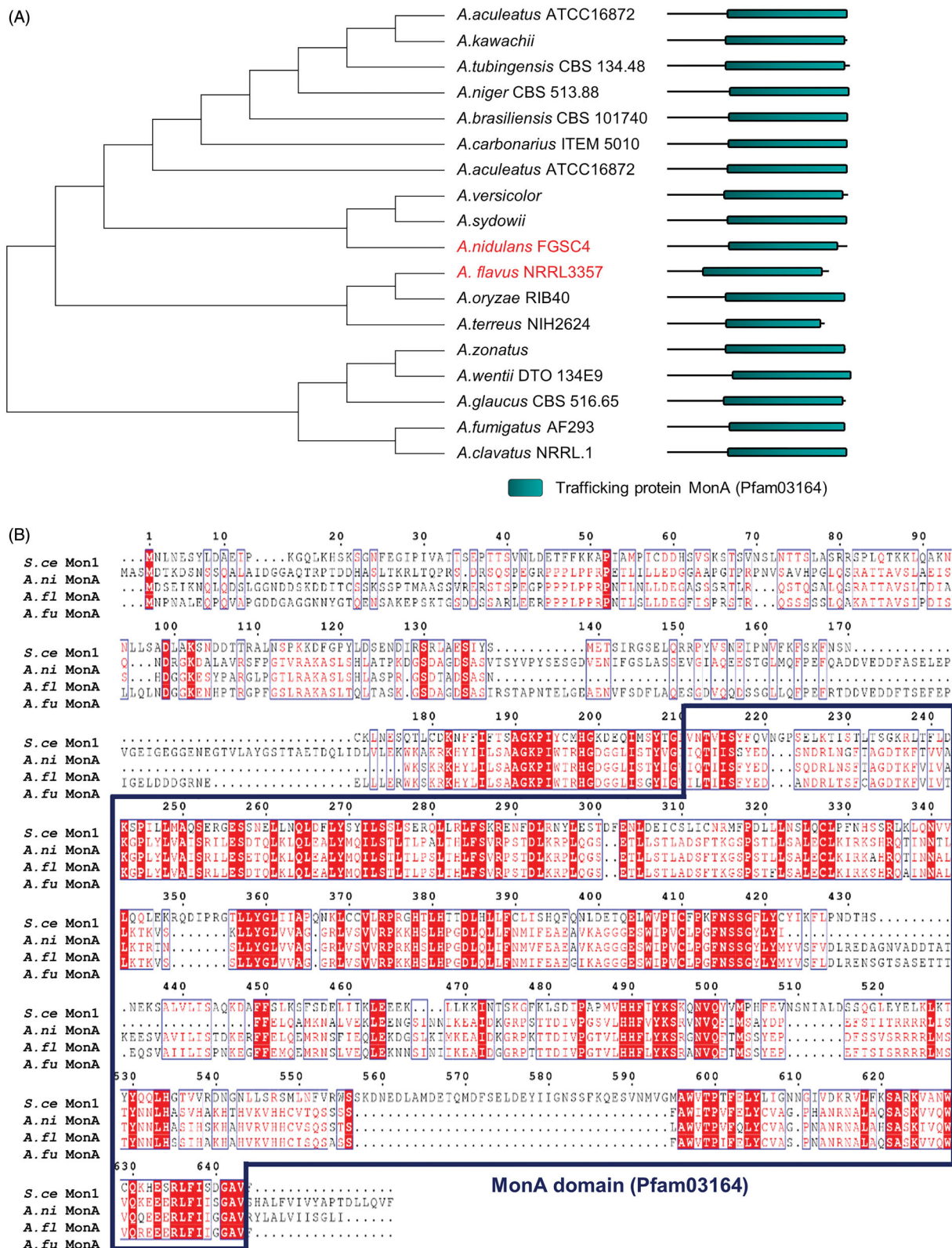


Figure 1. The MonA in *Aspergillus* spp. (A) A phylogenetic tree of MonA proteins from *A. oryzae* RIB40 (XP_023089908.1), *A. terreus* NIH2624 (XP_001215181.1), *A. clavatus* NRRL 1 (XP_001272699.1), *A. flavus* NRRL3357 (RMZ46160.1), *A. zonatus* (XP_022584673.1), *A. carbonarius* ITEM 5010 (OOF98920.1), *A. fumigatus* Af293 (Q4WHL1.2), *A. sydowii* (OJJ64521.1), *A. versicolor* (OJJ98619.1), *A. wentii* DTO 134E9 (OJJ30605.1), *A. aculeatus* ATCC 16872 (XP_020058629.1), *A. glaucus* CBS 516.65 (XP_022402549.1), *A. nidulans* FGSC4 (CBF86164.1), *A. niger* CBS 513.8 8 (XP_001394465.2), *A. brasiliensis* CBS 101740 (OJJ77853.1), *A. tubingensis* CBS 134.48 (OJJ82919.1), *A. luchuensis* (GAT22443.1), and *A. kawachii* IFO 4308 (GAA83795.1) was made with MEGA 7 software (<http://www.megasoftware.net/>) program neighbor-joining method with 1000 bootstrap replicates of phylogenetic relationships. The positions of the C-terminal trafficking domains of MonA in *Aspergillus* spp are shown (right); (B) Alignment of MonA in *S. cerevisiae* S228C (NP011391.2), *A. nidulans* FGSC4 (CBF86164.1), *A. flavus* AFL3357 (RMZ46160.1), and *A. fumigatus* AF293 (Q4WHL1.2). Bioedit and ESPript3 (<http://esprict.ibcp.fr/ESPript/ESPript/>) were used for the alignment.

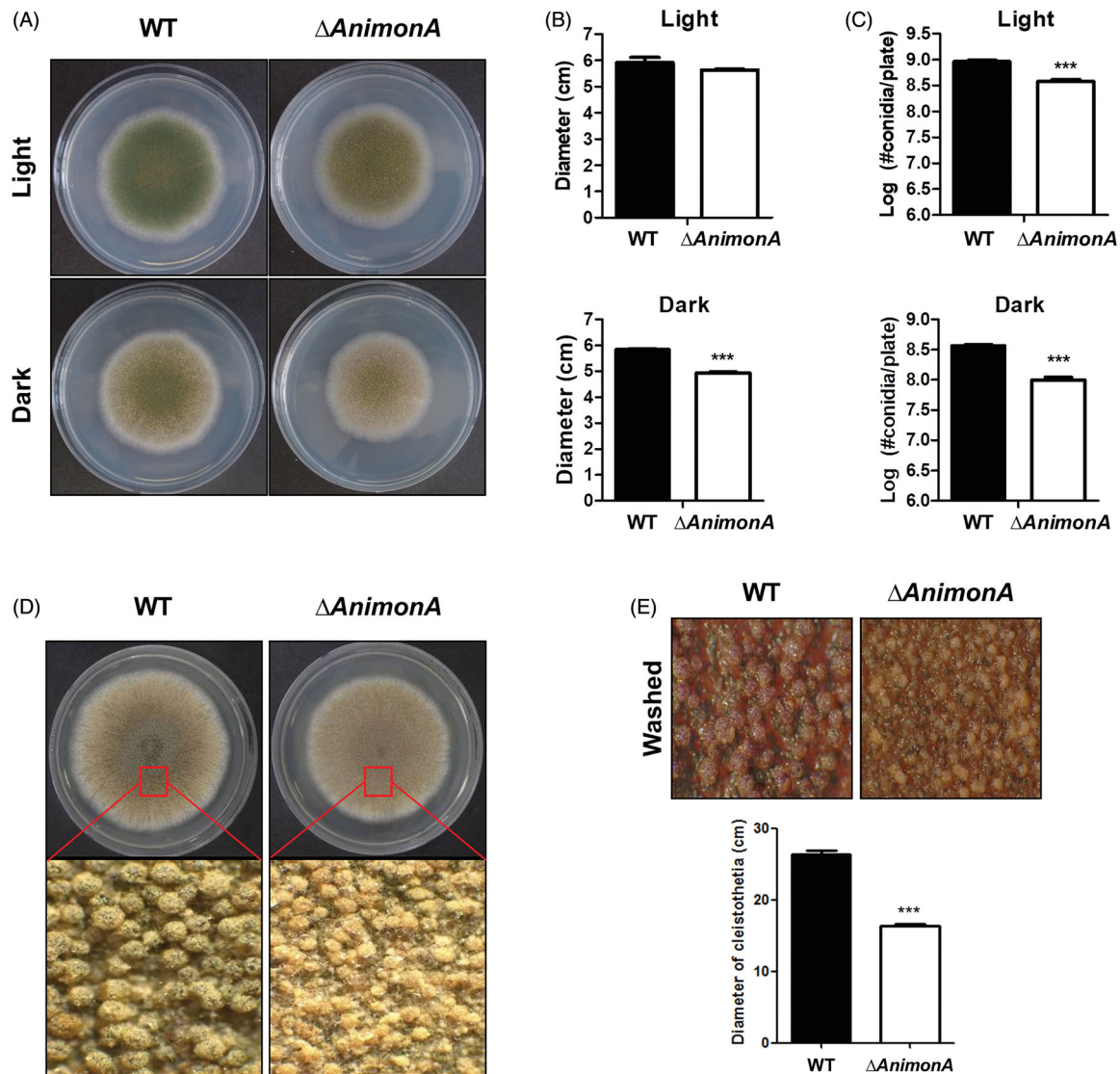


Figure 2. Phenotypes of $\Delta monA$ mutants in *A. nidulans*. (A) Colony photographs of WT (TNJ36) and $\Delta AnimonA$ (TYE8.1) strains. WT and mutants were point inoculated onto solid MMG and incubated at 37 °C for 5 days in light and dark conditions; (B) Conidial diameter of conidiospores in WT and $\Delta AnimonA$ (TYE8.1) mutants; (C) Conidia were measured 5 days after incubation on MMG plates under the light or dark conditions. The number of conidia per plate was counted in triplicate; (D) WT (TNJ36) and $\Delta AnimonA$ (TYE8.1) strains were point inoculated onto solid SM and incubated at 37 °C for 7 days in dark condition. Panels below represent magnified images from the middle of each plate; (E) Colonies of WT and $\Delta AnimonA$ (TYE8.1) mutants were washed with 100% ethanol to enable the visualization of cleistothelia. Bottom panel shows diameter of cleistothelia in WT and $\Delta AnimonA$ (TYE8.1) mutants grown in SM for 7 days. Error bars represent standard deviation (differences between the *AnimonA* mutants and WT strains. *** $p < .001$). Statistical analysis was performed using the Student's *t*-test.

dark conditions (Figure 4(A)). Reduced colony growth, similar to that seen in $\Delta AnimonA$ strains, is observed with $\Delta AflmonA$ mutant strains (Figure 4(B)) as well as decreased conidial production (Figure 4(C)) under the light and dark conditions. When WT and $\Delta AflmonA$ mutant strains were cultured under dark conditions for 7 days, WT strains produce sclerotia, whereas the $\Delta AflmonA$ mutant strains do not produce sclerotia (Figure 4(D,E)). Because *AniMonA* is required for spore viability and heat stress, the role of *AflMonA* in conidia was examined. Decreased spore viability is seen in $\Delta AflmonA$ mutant strains (Figure 4(F)). Overall, these results demonstrate that *MonA* has

conserved roles in fungal growth, differentiation, and spore viability in *Aspergillus* spp.

3.5. Role of *MonA* in *Aspergillus vacuole* formation

Because *MonA* is involved in vacuole formation in various fungi [20,30], we examined the vacuole formation in WT and *monA* deletion mutants in both *A. nidulans* and *A. flavus* by CMAC staining. As shown in Figure 5, the vacuoles of WT hyphae were stained by CMAC, whereas the CMAC fluorescence was dispersed in the $\Delta monA$ mutant hyphae in both *A. nidulans* and *A. flavus*, suggesting that *MonA*

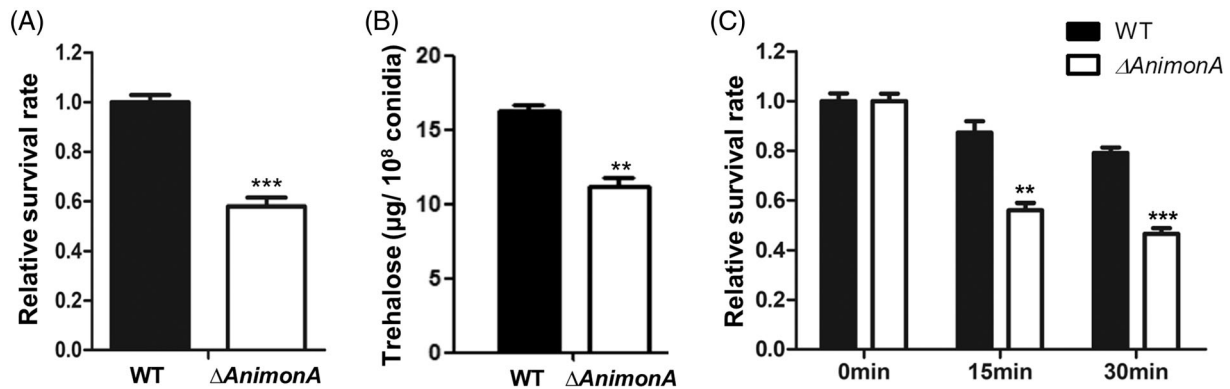


Figure 3. The roles of monA in *A. nidulans* conidia. (A) Viability of conidia collected from WT (TNJ36) and Δ AnimonA (TYE8.1) strains grown at 37°C for 10 days (triplicate measurements); (B) Trehalose in WT (TNJ36) and Δ AnimonA (TYE8.1) strain conidia; (C) Tolerance of WT (TNJ36) and Δ AnimonA (TYE8.1) conidia against thermal stress. All strains were grown for 2 days and 10² conidia per strain were heated at 50°C for 0, 15, and 30 min (triplicate measurements). Error bars represent standard deviation (differences between the AnimonA mutants and WT strains. *** P < 0.001; ** P < 0.01; * P < 0.05). Statistical analysis was performed using the Student's *t*-test.

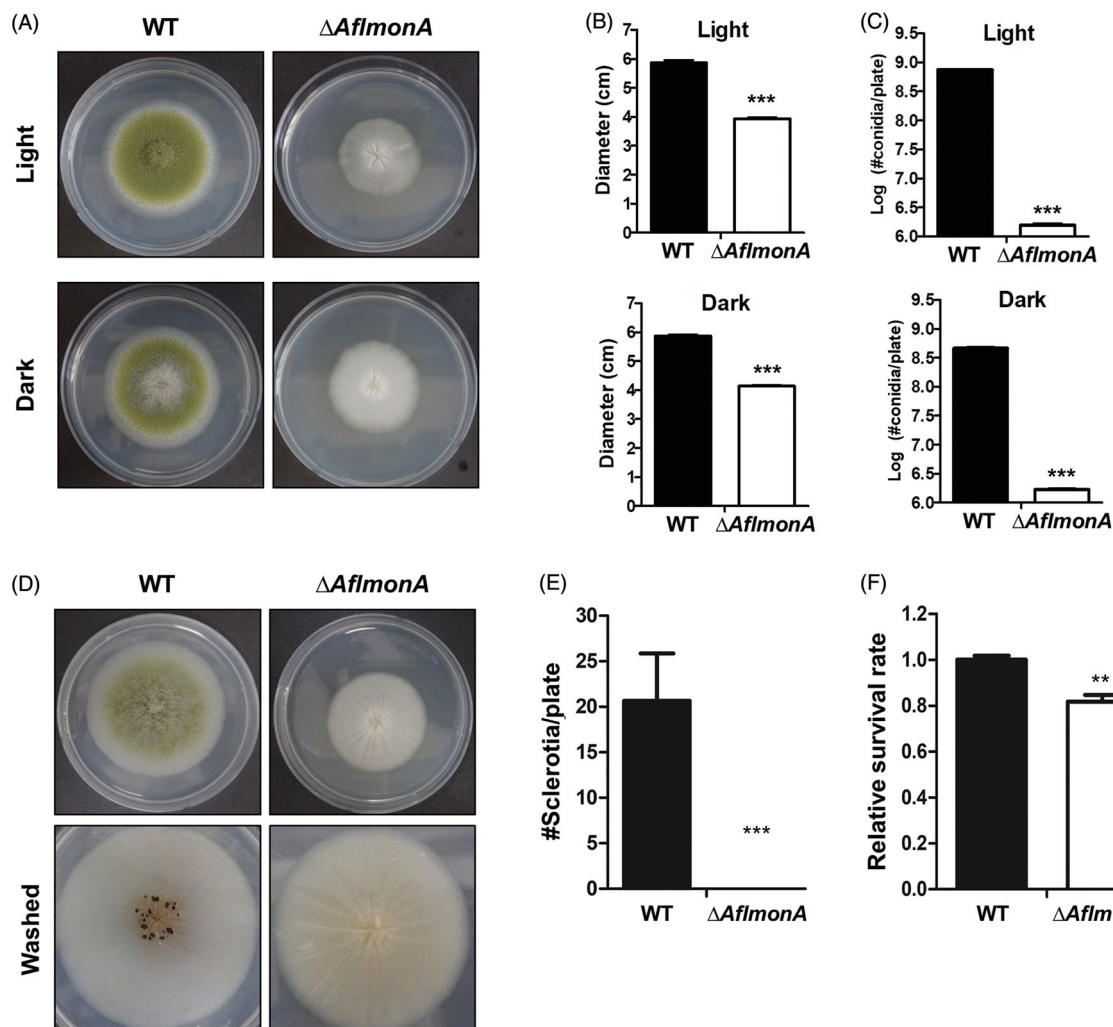


Figure 4. Phenotypes of Δ monA mutants in *A. flavus*. (A) Colony photographs of WT (NRRL3357) and Δ AflmonA (TYE9.1) strains. WT and Δ AflmonA (TYE9.1) mutant strains were point inoculated onto solid MMGY and incubated at 37°C for 5 days in light and dark conditions; (B) Conidial diameter of WT and Δ AflmonA (TYE9.1) mutants was measured 5 days after incubation on MMY plates under light or dark conditions; (C) The number of conidia per plate was counted for WT and Δ AflmonA (TYE9.1) mutant strains; (D) WT and Δ AflmonA (TYE9.1) strains were point inoculated onto solid MMYG, incubated at 37°C for 7 days in dark condition. Panels below represent colonies of WT and Δ AflmonA (TYE9.1) mutant after washing with 100% ethanol to enable the visualization of sclerotia; (E) Number of sclerotia in WT (NRRL3357) and Δ AflmonA (TYE9.1) strains; (F) Viability of conidia collected from WT (NRRL3357) and Δ AflmonA (TYE9.1) strains grown at 37°C for 10 days (triplicate measurements). Error bars represent standard deviation (differences between the AflmonA mutants and WT strains. *** P < 0.001; ** P < 0.01; * P < 0.05). Statistical analysis was performed using the Student's *t*-test.

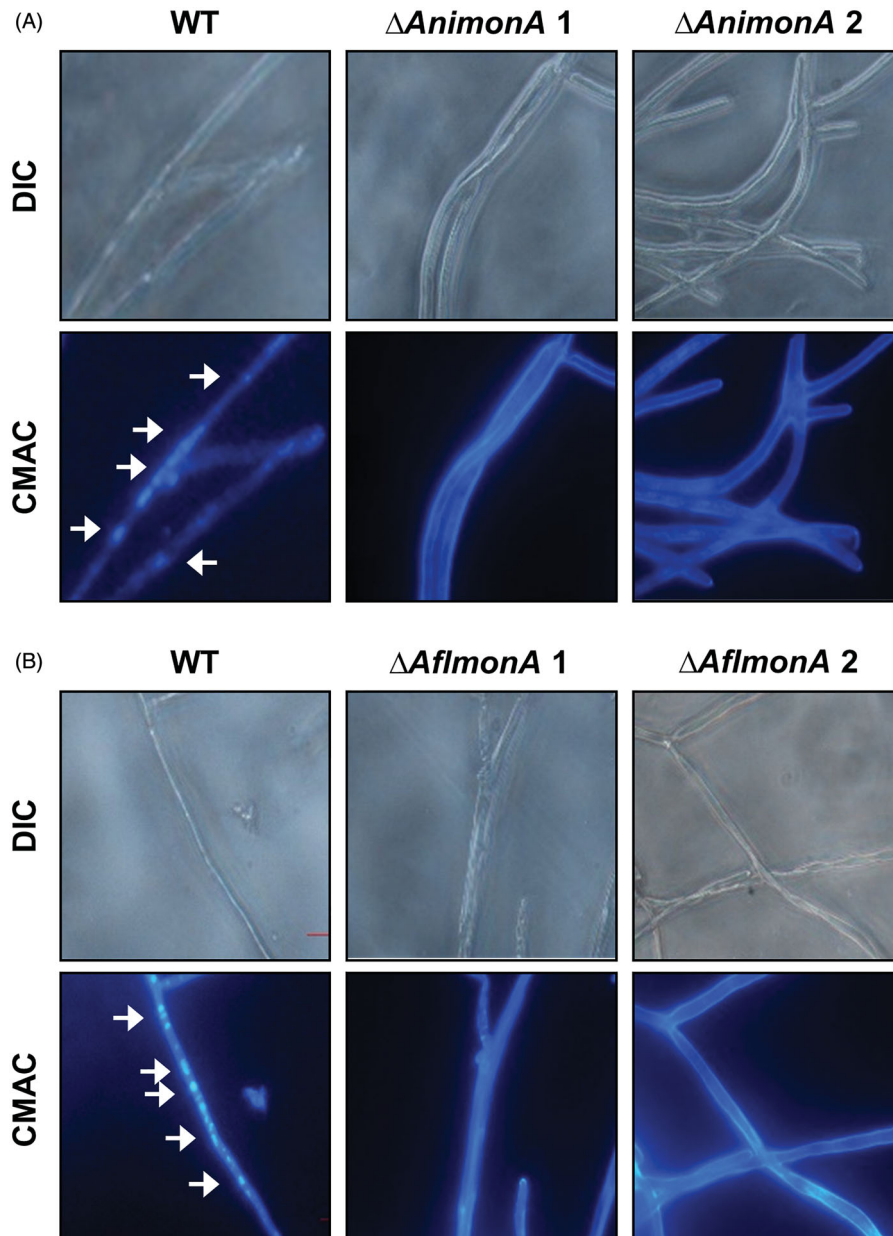


Figure 5. Vacuole morphology of Δ monA mutants in *Aspergillus* spp. Hyphae of WT and Δ monA mutant strains in *A. nidulans* (A) or *A. flavus* (B) were stained with CMAC and examined by DIC or fluorescence microscopy. Arrows indicate the vacuoles.

plays an important role in vacuole formation in *A.* both *Aspergillus* species.

4. Discussion

Mon1 is a conserved protein that plays a vital role in eukaryotes [31]. In *S. cerevisiae*, Mon1 interacts with Ccz1, forms a Mon1-Ccz1 complex that activates the formation of a *trans*-SNARE complex, and influences subsequent vacuole fusion and autophagy [16,31]. In filamentous fungi, Mon1 orthologs are also crucial for various processes involved in vacuole formation, fungal development, virulence, and mycotoxin production [20,30]. First, Mon1 is involved in fungal growth and development. In *F. graminearum*, *FgMON1* deletion mutants exhibit defects of conidial germination and sexual

development [20]. In *M. oryzae*, deletion of *MoMON1* causes reduced hyphal growth and decreased conidial production [30]. Second, Mon1 is required for pathogenicity in both plant and human pathogenic fungi. In human pathogenic fungi *C. neoformans*, deletion of *Camon1* causes defects in virulence in insect and murine inhalation models [21]. Third, the Mon1 ortholog affects production of detrimental secondary metabolites. *FgMon1* regulates expression of certain gene transcripts involved in trichothecene biosynthesis that control deoxynivalenol production [20].

Because Mon1 orthologs are important in development, pathogenicity, and the production of secondary metabolites in fungi, we hypothesized that MonA plays a crucial role in two *Aspergillus* species. *AnimonA* and *AflmonA* deletion mutant strains

showed defects in the production of asexual and sexual structures, suggesting that MonA is required for proper asexual and sexual development (Figures 2 and 4). We also examined the role of MonA in mycotoxin production. However, the Δ AnimonA and Δ AflamonA mutant strains still produce sterigmatocystin and aflatoxin, respectively (data not shown).

While the roles of MonA in *Aspergillus* spp. were studied through phenotypic analyses, detailed molecular mechanisms are unknown. In both yeast and filamentous fungi, Mon1 orthologs are required for vacuole formation, but the underlying regulatory mechanisms might be different. Because *S. cerevisiae* and *C. neoformans* produce the Ccz1 protein, a partner protein of Mon1, Mon1 can form the Mon1-Ccz1 complex that regulates vacuole formation and autophagy. However, the Ccz1 orthologs could not be found in the genome database of several filamentous fungi including *Fusarium* spp., *Aspergillus* spp., and *Penicillium* spp [20]. These observations imply that MonA could regulate vacuole formation in different ways.

In conclusion, we confirmed that MonA is essential for growth, asexual and sexual reproduction, vacuole formation, and spore maturation in *Aspergillus* species. Additional studies to elucidate the role of MonA in pathogenicity and mycotoxin production in *Aspergillus* will increase our understanding.

Disclosure statement

No potential conflict of interest was reported by the authors.

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