FigA, a putative homolog of low-affinity calcium system Fig1 in yeast, is involved in growth, asexual and sexual development in *Aspergillus nidulans*

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Running title: The low-affinity calcium influx system FigA in *Aspergillus nidulans*

Key words: The low-affinity calcium influx system (LACS), *fig* (mating factor-induced gene), *Aspergillus nidulans*,

Abbreviations used:
Calcium-mediated signaling pathways are widely employed in eukaryotes and are implicated in the regulation of diverse biological processes. In *Saccharomyces cerevisiae*, at least two different calcium uptake systems have been identified—the high-affinity calcium influx system (HACS) and the low-affinity calcium influx system (LACS). Compared to the HACS, little is known about the LACS in fungi. In this study, FigA, a homolog of the LACS member Fig1 from *S. cerevisiae* was functionally characterized in the filamentous fungus *Aspergillus nidulans*. Loss of *figA* resulted in the retardant hyphal growth and the sharp reduction of conidia production. Most importantly, FigA is essential for the homothallic mating (self-fertilization) process, further, FigA is also required for heterothallic mating (outcross) in the absence of HACS-midA. Interestingly, in a *figA* deletion mutant, adding extracellular Ca$^{2+}$ was able to rescue the hyphal growth defects but could not restore asexual and sexual reproduction. Furthermore, quantitative PCR results revealed that *figA* deletion sharply decreased the expression of *brlA* and *nsdD*, which are known as key regulators during asexual and sexual development, respectively. In addition, green fluorescent protein (GFP) tagging at the C-terminus of FigA (FigA::GFP) showed that FigA localized to the center of the septum in the mature hyphal cells, to the location between vesicle-metulae, and between the
junctions of metulae-phialide in conidiophores. Thus, our findings suggest that FigA may play multiple unexplored roles during hyphal growth, asexual and sexual development apart from being a member of a calcium uptake system in *A. nidulans*.
INTRODUCTION

Calcium is a ubiquitous signaling molecule in eukaryotic cells and calcium-mediated signaling pathways are used by eukaryotic cells to regulate a wide variety of cellular processes through transient increases of cytosolic calcium ion (Ca^{2+}) (1, 2). In yeasts, at least two different calcium uptake systems, the high-affinity calcium influx system (HACS) and the low-affinity calcium influx system (LACS) have been identified during mating process (3-6). The HACS is primarily responsible for pheromone-induced calcium response in minimal medium, but in rich medium, the function of the HACS is strongly inhibited by calcineurin so that the LACS becomes essential for this response. The HACS consists of at least two known subunits, the voltage gated calcium channel (VGCC) homolog-Cch1 and the stretch activated calcium channel/regulatory protein Mid1, which usually form a complex as the major calcium entry route under low-calcium condition (5, 7-11). To date, the predicted homologs of Cch1 and Mid1 have been intensively studied in many types of fungi. Losses of mid1 and cch1 consistently cause one phenotype in common: inability to grow under the low-calcium condition, indicating that they are parts of the HACS. In addition, the nulls of mid1 and cch1 could result in the defects in vegetative growth, sexual or/and asexual development, and pathogenicity for some species (12-16).

Up to date, Fig1 is the only characterized member of the LACS in fungi. In S. cerevisiae, FIG1 is up-regulated in response to mating pheromone and loss of FIG1 results in incomplete fusion between the tips of mating shmoos (3, 4, 17). Therefore, the name FIG1 (mating factor-induced gene 1) is initially used to describe this gene.
Moreover, previous studies have verified that orthologs of FIG1 are also existed in the genomes of fission yeast, and filamentous fungi (5, 18, 19). As a member of PMP22/claudin superfamily, Fig1 shares several common structural characteristics with their mammalian orthologs such as the presence of four putative transmembrane domains and a conserved claudin motif [GφφGx(n)C, where “φ” is a hydrophobic amino acid and “n” is any number] in the large first extracellular loop (3, 19). In mammalian organisms, claudin superfamily members are involved in membrane-membrane interactions, cytoskeletal attachment, signaling, and vesicle trafficking (20-22). Based on previous published data, the known functions of fig1 in fungi fit with those of the mammalian claudin superfamily members to some extent, such as signal transduction and membrane-membrane interactions. Fig1 was involved in calcium influx and membrane fusion during the mating of S. cerevisiae and Candida albicans. However, unlike in S. cerevisiae, in which FIG1 deletion results in defective cell fusion during mating, such fusion defects are not observed in fig1 null mutant of C. albicans. In the plant pathogen Fusarium graminearum, fig1 also plays an important role in sexual development. Loss of fig1 fails to produce mature perithecia, and sexual development is halted prior to the formation of perithecium initials. In another filamentous fungus Neurospora crassa, deletion of fig1 leads to the failure of fertile fruiting bodies in the mating type a strain. Besides being involved in sexual development, fig1 has been associated with thigmotropism in C. albicans (23) and vegetative growth and macroconidium production in F. graminearum (19). Although some advances have been achieved on fungi fig1 studies, its functions have...
Aspergillus species are among the most abundant fungi worldwide. The model filamentous fungus *Aspergillus nidulans* develops both sexual and asexual spores through complicated regulated mechanisms. Our previous studies reported that HACS components CchA/MidA play unique and complex roles in regulating conidiation, hyphal polarity and cell wall components in low-calcium environment (14). Compared to the HACS, the function of the LACS during fungal development is barely known. In this study, the roles of figA during the life cycle of *A. nidulans*, especially for hyphal growth, asexual and sexual development were investigated by studying calcium homeostasis, gene expression, and protein localization. Furthermore, to better understand the relationship between FigA with the HACS components, different double mutants were generated and analyzed. Our results indicate that FigA may function either synergistically or separately with MidA/CchA complex during the different developmental stages in *A. nidulans*.

**MATERIALS AND METHODS**

**Strains, media and culture conditions.** All the *A. nidulans* strains used in this study are listed in Table 1. Growth conditions, genetic crosses and used media have been described previously (14, 24). Expression of genes under the control of alcA promoter was regulated by different carbon source: repression on MMPDR, de-repression on MMPGR, and induction on MMPGRT (25). For gene expression analysis, the vegetative growth and synchronized developmental induction was
carried out as described previously (26, 27) with some modifications. Briefly, $1 \times 10^7$
conidia of control strain TN02A7 and appropriate mutants were inoculated in 100 ml
liquid minimal medium MMPDR with 0.1% yeast extract in 250 ml flasks and
incubated at 37°C, 250 rpm. For asexual and sexual developmental induction, 24-h
vegetative growth mycelia were transferred to solid MMPDR and the plates were air
exposed for asexual developmental induction or tightly sealed and blocked from
light for sexual developmental induction.

Construction of gene deletion mutants and alcA (p)-driven strains. The figA gene
was replaced with the selectable nutritional marker pyrG from Aspergillus fumigatus.
The deletion cassettes were created by double joint PCR (28). In brief, about 1.0 kb
flanking regions of figA gene of A. nidulans were amplified using primers P1/P3 and
P4/P6, respectively. The pyrG gene was previously amplified from plasmid
pXDRFP4 using primers PyrgF/ PyrgR. The fusion PCR deletion construct was
amplified with primers P2 and P5. The primers for fusion PCR are listed in Table S1
in supplemental material. The final fusion PCR product was purified and used to
transform into A. nidulans strain TN02A7 to create figA knockout strain. The
transformation was performed as previously described (29, 30). A diagnostic
polymerase chain reaction (PCR) assay was performed to detect figA replaced by
AfpyrG at the original figA locus using primers P1/CpyrgR. Furthermore, RT-PCR
was performed to confirm the deletion of the figA gene using primers CfigF/CfigR.
To construct figA and midA double deletions, midA gene was replaced by pyroA
insertion as a selectable nutritional marker in figA deletion background. The
transformants were selected on minimal media without pyridoxine. To construct \( \text{figA} \) and \( \text{cchA} \) double deletions, the \( \Delta \text{figA} \) deletion was crossed with \( \Delta \text{cchA-1} \) and the progenies were screened according to standard protocol (31). To obtain \( \text{alcA} \)\( (p) \)-driven \( \text{figA} \) conditional expression strain (\( \text{alcA} = \) alcohol dehydrogenase), the intact \( \text{figA} \) gene was cloned to pQa-pyroA vector. The final cassette of \( \text{alcA} \)\( (p) : \text{figA} : \text{pyroA} \) was transformed into \( \text{figA} \) deletion and TN02A7 background strain, respectively.  

**Complementation assay for \( \text{figA} \) in \( \text{S. cerevisiae} \).** All used \( \text{S. cerevisiae} \) strains (Table S2) are w303 derivatives. A PCR-generated DNA fragment including \( \text{S. cerevisiae} \)’s \( \text{FIG1} \) ORF plus 500 bp upstream of ATG and 200 bp downstream of stop codon was obtained using primers 464 and 465 and then cloned in BamH1/PacI sites of the pRS306H integrative vector (32). Nael-linearized pRS306H-FIG1 vector was integrated into the URA3 locus and integration was verified by colony-PCR as previously described (33). \( \text{A. nidulans} \) \( \text{figA} \) ORF was inserted replacing \( \text{FIG1} \) ORF of the pRS306H-FIG1 vector by RFCloning using primers 471 and 472 and a \( \text{fig1A} \) cDNA as a template (34). Nael-linearized pRS306H-FigA vector was integrated into the URA3 locus of the selected \( \text{fig1}::\text{KAN} \) strains. Cell-cell fusion assays were performed by monitoring cytoplasmic mixing as previously described (33). Briefly, cells of opposite mating types, with \( \text{MATa} \) strains expressing PGK1-mCherry, were grown to mid-log phase. An equal number of cells of each mating type were mixed and vacuumed to a nitrocellulose filter. The filter was placed cell-side up on YPD plates and then incubated for 3 h at 30°C. Cells were scraped and stained with 0.4%
trypan blue for 10 min (to monitor cell lysis) then washed and fixed in 4% paraformaldehyde (PFA) in PBS buffer before fluorescence microscopy analysis using a Olympus IX81 wide-field fluorescence microscope.

**Plate assays.** To assess the role of osmotic stress on conidiation, 0.8 M NaCl and 1.2 M sorbitol were added into minimal medium MMPDR, respectively. For the calcium related chemical sensitivity tests, 100 mM calcium and 2 mM EGTA were added to MMPDR media, respectively. For cell wall integrity test, 50 μg ml⁻¹ Calcofluor White and 300 μg ml⁻¹ Congo Red were added to MMPDR media, respectively. 2 μl of 1×10⁶ conidia ml⁻¹ from the indicated strains were spotted onto relevant media and cultured for two or three days at 37°C, and then the colonies were observed and imaged. For each test at least three plates were prepared for each strain.

**Analysis of asexual and sexual development in *A. nidulans.*** To monitor conidiophore development, 1×10⁶ conidia of corresponding strains were spread on YUU and MM agar media. And then the sterile coverslips were inserted into the agar with an angle of 45 degrees. The plates were air-exposed cultured at 37°C to process asexual development. After incubation for 24 h or 48 h, the coverslips were taken out from the medium, and then mounted on the slides for microscopic observation. This approach was also used to localize FigA-GFP fusion protein in developing conidiophores. Examination of self-fertilization development was carried out as previous described (27). In brief, conidia of appropriated strains were pointed inoculated at the centre of solid medium and incubated at 37°C for 2-3 days, and then the plates were sealed and further incubated at 37°C for 7 days.
morphology of fruiting body was observed on stereoscope. Outcross and progeny analysis approaches were taken according to standard protocol (31), with an exception by using a twenty-fold amount of standard riboflavin was added into the medium to promote outcross between the riboB2 auxotroph strains.

Tagging of FigA with GFP under the native promoter. To localize FigA, a GFP-pyr4 fragment was amplified from plasmid pFNO3 using primer pairs Gfp-pyrGF/Gfp-pyrGR. The same approach described by (35) was used to construct the FigA-GFP fusion cassette. In brief, about a 1.0 kb fragment immediately upstream of the figA stop codon and about a 1.0 kb fragment immediately downstream of the figA stop codon were amplified from strain TN02A7 using primer pairs GfpfigP1/GfpfigP3 and GfpfigP4/GfpfigP6, respectively. The FigA-GFP fusion PCR cassette (using primer pairs GfpfigP1/P5) was transformed to strain TN02A7 and the transformants embedding homologous integration were verified by PCR.

Microscopic observation. For hyphal microscopic observations, conidia were inoculated onto pre-cleaned glass coverslips overlaid with liquid media. Strains were grown on coverslips at 37°C for the time as indicated prior to observation under microscope. Hyphal septa were stained using Calcofluor White after the cells had been fixed with 4% Para formaldehyde (Polyscience, Warrington, PA). Differential interference contrast (DIC) and fluorescent images of the cells were collected with a Zeiss Axio imager A1 microscope (Zeiss, Jena, Germany). These images were then collected and analyzed by a Sensicam QE cooled digital camera system (Cooke Corporation, Germany) with MetaMorph/MetaFluor combination software package.
Quantitative Real-time PCR analysis. The samples were harvested at various time points, and the total RNA was extracted using Trizol (Roche) following the manufacturer’s instructions. The samples were treated with DNaseI (TAKARA), and cDNA was generated using an iScript™ Select cDNA Synthesis Kit (Bio-rad). Real-time PCR was performed using an ABI one step fast thermocycler (Applied Biosystems), and the reaction products were detected with SYBR green (TAKARA). PCR was accomplished after a 10 min denaturation step at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Transcript levels were calculated by comparative Δcycle threshold (36) and normalized against the expression of *A. nidulans* tubulin gene. Primers are listed in Table S1.

RESULTS

Identification of yeast Fig1 homolog in *A. nidulans*. The amino acid sequences of Fig1 in *S. cerevisiae* were used to search its homologs in the *A. nidulans* genome database. There are two *A. nidulans* FigA homologs, FigA (AN3036.2) and FigB (AN7093.2, or referred as FigA-like). Both of them shared the conserved topology structure and the claudin motif shared by all Fig1 homologs. However, no any detectable phenotypes were observed when we knocked out the full length of *figB* open reading frame (data not shown). Therefore, *figB* had not been studied further in this study. The *figA* gene is 828 nucleotides long and contains three introns and four exons. It is estimated that FigA translates to a protein of 275 amino acids containing
four potential transmembrane domains, with two conserved motifs in the predicted extracellular loop region. As shown in Fig. 1A, the star indicates the glycine and cysteine residues of a conserved Gly-Cys motif near the end of the first transmembrane domain, and a second conserved motif that is characteristic of claudin motif \[G\Phi\Phi GXC(n)C\], where “\(\Phi\)” is a hydrophobic amino acid and “\(n\)” is any number \((3, 4)\). To investigate the expression profile of \(figA\) during \(A. nidulans\) life cycle, the expression levels of \(figA\) in vegetative growth (24 h), asexual development (24 h and 48 h after induction) and sexual development (72 h after induction) were analyzed by qRT-PCR. As shown in Fig. 1B, \(figA\) was expressed constitutively in all tested stages, but during some stages of the tested time-points in asexual development (Asex 48 h and Asex 24 h), and in sexual development (Sex 72 h), the expression levels were relatively increased, suggesting \(figA\) may have a relevant function during these stages.

**External calcium rescues the hyphal growth retardation in \(figA\).** To determine the possible function of \(figA\) in \(A. nidulans\), the \(figA\) deletion strain \(\Delta figA\) was generated by replacing the coding sequence with \(AfpyrG\) gene by homologous integration. Diagnostic PCR analysis showed that the fusion alleles located at the native gene loci. RT-PCR results showed that there were no \(figA\) transcripts in \(\Delta figA\) mutant (see Fig. S1 in supplemental material). To investigate the relationship between FigA with the HACS components-CchA and MidA, the \(\Delta figA\Delta midA\) and the \(\Delta figA\Delta cchA\) double mutants were generated either by homologous replacement or by genetic cross techniques described above. As shown in Fig. 2A and B, on minimal medium...
MMPDR, the colony size of \(\Delta figA\) is reduced compared to TN02A7 which indicated a significant loss of \(figA\) vegetative growth rate. Moreover, there was an exacerbated growth retardation phenotype in both of \(\Delta figA\Delta midA\) and \(\Delta figA\Delta cchA\) mutants compared to the \(\Delta figA\) strain, suggesting that FigA in combination with CchA/MidA, plays an important role on \(A.\ nidulans\) hyphal growth. As expected, the phenotypic growth retardation could be reversed by the addition of external 100 mM calcium on MMPDR. The colony diameter of \(\Delta figA\) was restored to almost the identical size of TN02A7, suggesting that exogenous calcium could completely rescue the hyphal growth defects caused by loss of \(figA\). In addition, calcium supplementation could also completely rescue the hyphal growth retardation in \(\Delta figA\Delta midA\) and \(\Delta midA\Delta cchA\) strains but partially in \(\Delta figA\Delta cchA\) mutants (Fig. 2A and B). These results suggest that FigA might be involved in calcium transport in hyphal growth of \(A.\ nidulans\). Salt stress (0.8 M NaCl) could not restore the hyphal growth retardation of \(\Delta figA\), \(\Delta figA\Delta midA\) and \(\Delta figA\Delta cchA\) strains but did so in \(\Delta midA\Delta cchA\) as previously showed (14). Furthermore, there were no obvious differences among the control strain TN02A7 and all the tested mutants in the sensitivity to the calcium chelating agent EGTA and cell wall disrupting agents Calcofluor White and Congo Red (data not shown).

**FigA is involved in asexual development.** Except the hyphal growth defects caused by loss of \(figA\), colonies formed by \(\Delta figA\) were notably devoided of conidia on minimal medium. The numbers of conidia produced by the \(\Delta figA\) mutant were approximately 100-fold lower than that of TN02A7 on MMPDR (Fig. 2C). However,
there were no exacerbated conidiation defects on ΔfigAΔmidA and ΔfigAΔcchA double mutants when compared with the single mutant ΔfigA (Fig. 2A and C). Unexpectedly, a similar conidiation defect in ΔfigA was also observed on rich medium YUU (Fig. 3A). To further analyze the detail of these conidiation defects, the morphogenesis of ΔfigA and TN02A7 conidiophores was observed. As shown in Fig. 3B, the vegetative mycelium of the control strain TN02A7 could be developed into conidiophores with visible phialides connected with numerous conidia resulting in a distinct ‘aspergillum’ appearance. Differently, in ΔfigA only a few, if any, metulaeas and phialides had been observed. Most significantly, the ΔfigA mutants were completely unable to form chains of conidia on phialides even after prolonged incubation times. Coverslip cultures were used to examine if loss of figA could affect the hyphal morphogenesis. The results showed there were no obvious differences in polarized growth and septum formation between ΔfigA and TN02A7 on both of rich and minimal medium (Fig. 3C).

In our previous study, loss of the putative high affinity calcium channel CchA or MidA resulted in a reduction of conidia production, while the conidiation defects were rescued by either extra-cellular Ca$^{2+}$ or osmotic stress (14). Different with ΔcchA, ΔmidA and ΔcchAΔmidA, which showed conidiation defects only in minimal medium MMPDR, ΔfigA showed much severe conidiation defects on both of rich and minimal medium. We then investigated whether extra-cellular calcium or osmotic stress might rescue the aconidial phenotype displayed by the ΔfigA and ΔfigAΔmidA and ΔfigAΔcchA mutants. As shown in Fig. 2A and C, addition of 100
mM calcium on MMPDR could not significantly restore the conidiation defects in ΔfigA, ΔfigAΔmidA and ΔfigAΔcchA. In comparison, under the salt stress (0.8 M NaCl) or cell-wall-stabilizer stress (1.2 M sorbitol) conditions, ΔfigA, ΔfigAΔmidA and ΔfigAΔcchA double knockout mutants showed increased conidiation when compared to normal growth conditions but all mutants were still defective in comparison to the control strain TN02A7 (Fig. 2A and C). Collectively, these results indicate that figA is involved in asexual development in A. nidulans, and extra-cellular calcium could not rescue the conidiation defects, but stress such as salt and cell wall stabilizer can partially rescue these defects.

To further confirm the function of figA on conidiation, a conditional strain Cf1 (alcA(p)::figA in ΔfigA background) was constructed. As shown in Fig. 4, When grown on repression medium MMPDR, the conditional strain displayed an identical phenotype to the ΔfigA strain. When grown on non-repression medium MMPGR, the conditional strain increased the conidia production (about 6-fold increased compared to that of on repression medium). In comparison, when grown on the induction medium MMPGRT, Cf1 produced conidia for 20-fold increased compared to that of on repression medium. Those results clearly indicate that with the increasing of the figA expression, conidiation could be enhanced accordingly, suggesting figA indeed plays important roles in asexual development. Additionally, another conditional strain Of1 (alcA(p)::figA in TN02A7) was constructed in a wild type context. No detectable different phenotypes were found between Of1 and TN02A7 under the both of repression and induction conditions. This result suggests that over-expression
of figA may not affect the asexual development in *A. nidulans* (data not shown).

**FigA is required for sexual development.** In *A. nidulans*, sexual fruiting bodies (cleistothecia) can be formed under both of homothallic (self-fertilization) and heterothallic (outcross) conditions. To test the ability of corresponding strains on self-fertilization body formation, the mutants, ΔfigA, ΔfigAΔmidA, ΔfigAΔcchA, ΔmidAΔcchA and their parent control strain TN02A7, were point-inoculated on minimal and rich medium. After cultivation at 37°C for two days, all above agar plates were sealed to be induced for sexual development. As a result, compared to TN02A7, ΔfigA, ΔfigAΔmidA and ΔfigAΔcchA mutants did not produce neither any visible cleistothecia nor aggregated Hülle cells (Fig. 5A). In contrast, deletion of *midA, cchA* or *midA/cchA* showed almost normal cleistothecia formation compared to wild type under the same conditions suggesting that *figA* but not *midA/cchA* might be essential for the self-fertilization in *A. nidulans*. Interestingly, 50 mM of additional extracellular calcium could not rescue the fruiting body formation defects in ΔfigA, ΔfigAΔmidA and ΔfigAΔcchA mutants (Fig. 5B).

In order to test if FigA was required for outcross, we carried out the following sexual crosses according to the standard protocol described in materials and methods: ΔfigA×GR5 (wild type), ΔfigA×R21 (wild type), ΔfigA×ΔcnaA (calcineurin A, a catalytic subunit of the calmodulin-dependent protein phosphatase), ΔfigA×ΔpmrA (a putative calcium-transporting ATPase), ΔfigA×ΔcchA-1. All the tested crosses resulted in the formation of normal cleistothecia containing ascospores with normal viability. Importantly, when ΔfigA cells were crossed with ΔmidA or with
ΔmidAΔcchA strains, we could not find any hybridized cleistothecia under the same conditions as above described. This result indicates that in the absence of midA or midA/cchA, figA is essential for outcross. Taking altogether, these results suggest a role for FigA in sexual development. To further test this hypothesis we asked if A. nidulans FigA was able to mimic S. cerevisiae Fig1 function during sexual mating.

For this purpose, S. cerevisiae strains of opposing mating types, one of them expressing soluble cytoplasmic mCherry were incubated, allowed to mate and then analyzed by fluorescent microscopy in order to score cell-cell fusion efficiency (33). As previously described (17), Δfig1 mutants have a mild but noticeable cell fusion defect (Figure S2). As expected, a single copy of FIG1 driven by its own promoter was able to complement the Δfig1 mutant defect. Remarkably, a single copy of the figA ORF driven by the FIG1 promoter is sufficient to suppress the Δfig1 mutant defect further supporting the idea that one of FigA roles is to promote sexual development (Figure S2).

figA deletion dramatically down-regulates the expression of brlA and nsdD.

Since the deletion of figA abolished both conidiation and cleistothecia formation, we asked whether figA could affect the expression of brlA, nsdA and steA, which had been verified as key regulators for asexual and sexual development, respectively. Consistent with previous reports, in wild-type TN02A7, the mRNA levels of brlA were very low during the vegetative growth stage, but the mRNA levels were quickly increased after exposition to conditions for asexual development induction (37, 38). Differently, in ΔfigA cells, brlA expression was not be greatly accumulated
under the same conditions (Fig. 6A). Based on previous reports showing that \textit{nsdD} and \textit{steA} are transcription factors involved in sexual development in \textit{A. nidulans} (39, 40) we decided to test the expression levels of these genes during sexual development. As shown in Fig. 6B, in wild-type cells, \textit{nsdD} mRNA levels started to accumulate in the early phase of vegetative growth reaching higher levels increased as sexual development proceeded. However, in \textit{ΔfigA} cells \textit{nsdD} mRNA levels remained almost unchanged after sexual induction. Interestingly, \textit{nsdD} mRNA levels in vegetative growing \textit{ΔfigA} cells were higher than in TN02A7 cells. Since \textit{steA} is a homolog of \textit{S. cerevisiae ste12} which positively regulates cleistothecia development in \textit{A. nidulans}, the expression of \textit{steA} was tested accordingly. Unexpectedly, loss of \textit{figA} could not significantly decrease but instead slightly increase the expression of \textit{steA} during both of vegetative growth and sexual development stages (Fig. 6C). Collectively, those results suggest that loss of \textit{figA} decreased the expression of \textit{brlA} and \textit{nsdD} in \textit{A. nidulans} asexual and sexual differentiation, respectively.

**FigA located on the septation sites.** So far, the subcellular location of FigA has not been verified in any filamentous fungus. In order to study the cellular location of FigA, a strain expressing a green fluorescent protein (GFP) tag at the C-terminus of FigA (FigA-GFP) under the control of its native promoter was generated. FigA::GFP transformants showed a wild-type phenotype indicating that the FigA-GFP fusion is fully functional. As shown in Fig. 7A-D, FigA::GFP accumulated at the center of septum sites of mature hyphae but could not be detected in conidia or germlings. To gain an insight into the exact location of FigA at the septum, a three-dimensional
scanning image was obtained using confocal microscopy. The result clearly showed that FigA::GFP located just right on the center of the septum (Fig. 7E). In addition, as shown in Fig. 7F, in the architecture of conidiophores, FigA also localized to the junction between vesicle-metulae and between metulae-phialide but not in the connection between phialide-spores or conidia. Considering these results, a model showing a FigA localization pattern on the architecture of conidiophore is presented on Fig. 7G, indicating that FigA is located at the interfaces of vesicle-metulae, metulae-phialide but not at phialide-spores interface.

DISCUSSION

Fig1 is a member of a fungus-specific family of proteins that contains similar topology characteristics found in the large mammalian claudin superfamily (3, 4, 19). The functions of mammalian claudin superfamily members are involved in membrane-membrane interactions such as epithelial tight junction formation which displays selectively permeation properties that allow ions, and other solutes to pass between cells (20-22). The specific location of FigA in A. nidulans indicates that the function of FigA may fit well with those of non-fungal proteins. In higher fungi, multicellular hyphae are compartmentalized by the formation of septa. However, a small pore is retained to enable communication between adjacent hyphal compartments (41, 42). The location of FigA at the center of hyphal septum (probably around the pore) indicates figA may also play selectively permeation properties that allow proper solutes to pass between cells.
Cell-to-cell communications are central to sexual development in fungi, and fig1 is involved in sexual development in all reported species. In *S. cerevisiae* and *C. albicans*, mating pheromone is able to induce *FIG1* expression. Moreover, it has been verified that the deletion of *fig1* results in the reduced calcium accumulation induced by pheromone during the mating (3, 4, 17). Recently, it was reported that *fig1* is involved in sexual development in filamentous fungi *F. graminearum* and *N. crassa*. However, the precise role for *fig1* in sexual development is still not known. The homothallic ascomycete *A. nidulans* can undergo sexual process under both of homothallic (self-fertilization) and heterothallic (outcross) conditions. Our results showed that *figA* is essential for self-fertilization but not required for outcross in most cases. Since *figA* is able to complement the bilateral absence of *S. cerevisiae FIG1* during mating, it can be speculated that *figA* is required for the specialized cell fusion event that leads to dikaryotic hypha formation during homothallic self-fertilization. However, forced hyphal fusion and heterokaryon formation between two different strains may bypass the requirement of *figA* in cell fusion. This is phenotypic similar with the putative G protein coupled receptors *gprA* and *gprB* which are also only required for self-fertilization maybe by reduced or lack of cell fusion or differential recognition of nuclei (27). In addition to the LACS, the HACS, which consists of both Cch1 and Mid1 can be stimulated by mating pheromones in *S. cerevisiae*. Thus, the HACS plays an essential role in the sexual life cycle in *S. cerevisiae*, as evidence by the fact that *mid1* deficient cells die during prolonged pheromone treatment. Interestingly, our results showed that the HACS is not essential in sexual development
in *A. nidulans*. Loss of *midA*, *cchA* and *midA/cchA* did not affect sexual process in both homothallic and heterothallic conditions. However, Δ*figA*×Δ*midA* and Δ*figA*×Δ*cchA*Δ*midA* crosses were unsuccessful indicating that *figA* in combination with *midA* is involved in sexual development in *A. nidulans*. In another word, *figA* is essential for outcross in absence of *midA* and *vice versa*. Additionally, Δ*figA* could mate with Δ*cchA* but not with Δ*midA*, suggesting the function of MidA may not share completely with that of CchA.

We further tested the gene expression of the key regulators *nsdD* and *steA* during sexual development. *nsdD* encodes a putative GATA-type transcription factor, which functions in activating sexual development of *A. nidulans* while SteA is a homolog of *S. cerevisiae* Ste12p which positively regulates cleistothecia development in *A. nidulans* (39, 40). Moreover, in *S. cerevisiae*, Ste12p plays a key role in coupling Mitogen Activated Protein Kinases (MAPK) signal transduction to cell-specific or morphogenesis-specific gene expression required for mating and pseudohyphal filamentous growth (43, 44). Interestingly, our results suggest that loss of *figA* can decrease the accumulation of *nsdD* but not *steA* during sexual development. However, in Δ*figA* cells both *nsdD* and *steA* showed higher expression levels during vegetative growth. These results suggest that *figA* is somehow involved in the transcriptional regulation of *nsdD* and *steA* during sexual differentiation in *A. nidulans*.

Besides the essential function of FigA on sexual development, it is also involved in asexual differentiation in *A. nidulans*. The Δ*figA* mutant showed approximately a 100-fold decreased conidial production when compared to TN02A7. Different with
the aconidial phenotype of ΔmidA, ΔcchA and ΔmidAΔcchA which was only evident
on minimal media, the ΔfigA mutant showed an identical aconidial phenotype on both
rich and minimal media. Recently, it was shown that in the plant pathogen fungus *F. graminearum*, fig1 is involved in asexual development and ΔfigI mutants present a
70-fold reduction in macroconidium production, when compared to wild type (19).
However, the function of fig1 on asexual development is divergent. Such as, the
deletion of fig1 in the filamentous fungus *N. crassa* did not affect conidiation. In
*Aspergillus*, the asexual reproduction is regulated by complicated regulatory pathways
(45). During conidiation, *brlA* is a well-known central regulatory factor, which
controls the temporal and spatial expression of conidiation specific genes (38, 46). As
expected, our results indicate that loss of figA greatly reduces the accumulation of
*brlA* in asexual stage, suggesting that figA affects conidiation possibly through the
down-regulation of *brlA* expression. Furthermore, we found that FigA is located on
the vesicle-metulae, metulae-phialide junctions in conidophores. The specific location
of FigA in those junctions indicates that figA may play important roles on trafficking
and/or act as a scaffolding protein, which is vital for asexual development in *A.
nidulans*. However, whether FigA interacts with BrlA directly or indirectly is
unknown yet; further protein interaction studies will address this question.

Various lines of evidence obtained from this study and others clearly indicate that
FigA and its homologs have different functions that are both calcium-dependent and
calcium-independent. Although Fig1 is involved in calcium uptake during cell fusion
in yeasts, its lack of homology to any known ion influx channel suggests that it may
be acting as an indirect facilitator of calcium influx (3). In *C. albicans*, deletion of *fig1* results in attenuation of the reorientation response but there is no measurable effect on calcium ion accumulation either in yeast or in hyphal cells (23). Additionally, exogenous calcium addition did not restore the vegetative growth rate defect observed in *F. graminearum* *fig1* mutants (19). Furthermore, pheromone-induced cell death is dependent on *fig1* but independent of its calcium uptake activity in yeasts (47). Thus, this information indicates that Fig1 homologs in fungi have multiple unexplored functions that operate beyond calcium uptake. Our results showed that adding extracellular calcium do restore the hyphal growth defect but could not promote fruiting body formation or asexual development in *figA*. Thus, although *figA* is undoubtedly involved in calcium uptake in hyphal growth, its roles in *A. nidulans* asexual and sexual development are still obscure.

The results presented here showed the functional flexibility of *figA* in the life cycle of *A. nidulans*. During vegetative growth *figA* mainly acts as a calcium-uptake-system component, while in asexual and sexual development it works as a regulator involved in the regulatory program for the asexual and sexual differentiation. Moreover, the specific pattern of FigA localization at the center of septation sites indicates that it may play important roles on selectively permeation, trafficking or behave as a scaffolding protein during growth, asexual and sexual development in *A. nidulans*. Finally, the end targets of FigA during developmental stages in *A. nidulans* are still not known, and investigations to identify them are in progress.
ACKNOWLEDGEMENTS

This work was financially supported by National Natural Science Foundation of China (NSFC) No. 31200057 to S. Zhang and No. 81330035 to L. Lu, and Natural Science Foundation of Jiangsu Province of China (No. BK2012451) to S. Zhang. Natural Science Foundation of the Jiangsu Higher Education Institutions of China (Grant No. 11KJA180005) to L. Lu. ANII-Caldeyro Barcia Fellowship, to N. Carbó, International Centre for Genetic Engineering and Biotechnology (ICGEB grant CRP/URU11-01) to P.S. Aguilar, Agencia Nacional de Investigación e Innovación (ANII-INNOVA grant DCI-ALA/2007/19.040 URU-UE) to P.S. Aguilar and FOCEM (MERCOSUR Structural Convergence Fund, COF 03/11) to P.S. Aguilar. *A. nidulans* strain TN02A7 was a gift of Oakley, B. R. (Ohio State University, Columbus, Ohio); strains GR5, R21 and Plasmid pXDRFP4, pFNO3 were from FGSC (http://www.fgsc.net); Plasmid pQa-pyro was a gift from Park, H. M. (Chungnam National University, Korea).
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Figure Legends

FIG 1 Bioinformatic identification and the expression of figA. (A) The schematic diagram of conserved motifs of FigA homologs in A. nidulans and S. cerevisiae. TM, transmembrane domain. The star icons indicate the glycine and cysteine residues in conserved Gly-Cys motif near the end of the first transmembrane domain. The diamond icons show the conserved claudin motif which was composed of the sequences in the box. The highlight letters in the box display the conserved claudin
motif [GφφGxC(n)C]. (B) Levels of figA mRNA expression in various developmental stages in A. nidulans. Numbers indicate the time (h) of incubation in liquid submerged culture (Veg), after asexual induction (Asex) and sexual induction (Sex). All values were normalized to the expression of the A. nidulans benA tubulin gene. The error bars indicate the standard deviation for three independent replicates. Different lowercase letters on the bars indicate significant differences among the stages (Tukey’s HSD, P < 0.05).

FIG 2 Plate assay. (A) The colony morphology of control strain (TN02A7), ΔfigA, ΔfigAΔmidA, ΔfigAΔcchA and ΔmidAΔcchA grown on minimal medium MM, MM with 1.2 M sorbitol, MM with 0.8 M NaCl and MM with 100 mM CaCl₂ at 37 °C for 2.5 d. (B) The quantitative data of colony diameter for (A). (C) The quantitative number of conidia for (A). Error bars represent standard deviation of three replicates. Letters represent significant differences among the strains (Tukey’s HSD, P < 0.05).

FIG 3 FigA is involved in asexual development. (A) Colonies of TN02A7 and ΔfigA strain grown on solid YUU at 37 °C for 2 d. (B) Conidiophores of TN02A7 and ΔfigA. (C) Hyphal morphology of ΔfigA stained by Calcofluor White (septa) and DAPI (nuclei). ΔfigA was cultured on YUU broth at 37 °C for 10 h. Arrows indicate the location of septa. Bars, 10 μm.

FIG 4 The phenotypic characterization of figA under the conditional promoter. The
full length figA gene was inserted into pQa-pyro vector under the regulation of alcA promoter. And then the cassette alcA(p)::figA: Pyro was transformed into ΔfigA (alc(p)::figA). alc(p), pQa-pyro blank vector was transformed into ΔfigA. The colony photographs of corresponding strains grown on repression medium MMPDR, derepression medium MMPGR and induction medium MMPGRT at 37 °C for 2.5 d are shown.

**Fig 5** FigA is essential in self-fertilized fruiting body formation. (A) Conidia of appropriate strains were point inoculated at the centre of solid MM and incubated at 37 °C for 2-3 d and then the plates were sealed and further incubated at 37 °C for 7 d to induce fruiting body production. Arrows indicate sexual fruiting bodies on plates. TN02A7 (WT), ΔmidA and ΔmidAΔcchA produced dark cleistothecia around by white Hüle cell but there were no detectable cleistothecia exist in ΔfigA, ΔfigAΔmidA, ΔfigAΔcchA under the normal sexually induced condition. (B) Extracellular calcium can not rescue the defects of fruiting body formation in ΔfigA, ΔfigAΔmidA, ΔfigAΔcchA under the same condition as (A) except for adding 50 mM of calcium to above described media.

**Fig 6** Expression analysis by quantitative PCR. The mRNA expression levels of (A) brlA, (B) nsdD and (C) steA were analyzed in TN02A7 and ΔfigA, respectively. Numbers in X axis indicate the incubated time (h) in liquid submerged culture (Veg), after post-asexual induction (Asex), sexual induction (Sex). All values were
normalized to the expression of the *A. nidulans* tubulin gene. The error bars indicate the standard deviation for three independent replicates. Asterisks indicated a significant difference between TN02A7 and ΔfigA at *p* < 0.05.

**FIG 7** The localization pattern of FigA-GFP. (A) The shape of hyphae was shown by DIC (Differential Interference Contrast). (B) The location of FigA-GFP was indicated by arrows. (C) The septa were stained by Calcofluor White. (D) The merged photo of septa and FigA-GFP. (E) A three-dimensional scanning image of Calcofluor White stained septa and FigA-GFP were obtained by using the confocal microscope. The images in the white rectangle appeared in (B), (D) and (E) are partial enlarged views. (F) The merged photo of DIC and FigA-GFP in conidiphores. (G) A model image showing the FigA localization in conidiphores in *A. nidulans*. Bars, 10 μm.
TABLE 1  *A. nidulans* strains used in this study

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tr>
<td>GR5</td>
<td><em>pyrG89, wA3; pyroA4, veA1</em></td>
<td>FGSC</td>
</tr>
<tr>
<td>R21</td>
<td><em>pabaA1, yA2; veA1</em></td>
<td>FGSC</td>
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<tr>
<td>ΔmidA</td>
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<tr>
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<td><em>pyrG89; ΔmidA::pyroA; pyroA4, nkuA::argB2, ΔcchA::pyrG, riboB2, veA1</em></td>
<td>This study</td>
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<tr>
<td>ΔcchA-1</td>
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<tr>
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