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, the LACS in fungi is not well known. 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 retardant hyphal growth and a sharp reduction of conidial production. Most importantly, FigA is essential for the homothallic mating (self-fertilization) process; further, FigA is required for heterothallic mating (outcrossing) in the absence of HACS midA. Interestingly, in a figA deletion mutant, adding extracellular Ca^{2+} rescued 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 mature hyphal cells, to the location between vesicles and metulae, and between the junctions of metulae and phialides in conidiophores. Thus, our findings suggest that FigA, apart from being a member of a calcium uptake system in *A. nidulans*, may play multiple unexplored roles during hyphal growth and asexual and sexual development.
of fertile fruiting body development in the mating type a strain. Besides being involved in sexual development, figA has been associated with thigmotropism in C. albicans (23) and with vegetative growth and macronoucin production in F. graminearum (19). Although some advances have been achieved in studies of fungal fig1, its functions have been characterized for only a few species. *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 the HACS components CchA and MidA play unique and complex roles in regulating conidiation, hyphal polarity, and cell wall components in low-calcium environments (14). Compared to the function of 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 and asexual and sexual development, were investigated by studying calcium homeostasis, gene expression, and protein localization. Furthermore, to better understand the relationship between FigA and the HACS components, different double mutants were generated and analyzed. Our results indicate that FigA may function either synergistically or separately with the MidA/CchA complex during the different developmental stages in *A. nidulans*.

### MATERIALS AND METHODS

#### Strains, media, and culture conditions.

All *A. nidulans* strains used in this study are listed in Table 1. Growth conditions, genetic crosses, and the rich medium YUU, minimal medium MM, and MMPDR (MM plus 0.5 mg/liter pyridoxine, 2.5 mg/liter riboflavin, 5 mM uridine, 10 mM uracil) have been described previously (14, 24). Expression of genes under the control of the alcohol dehydrogenase (*alcl*) promoter was regulated by different carbon sources: MMPDR (on which genes were repressed), MMPGR (same as MMPDR but replacing glucose with 1% glycerol [vol/vol]), and MPPGRT (MMPGR with 6.25 mM threonine) (25). For gene expression analysis, vegetative growth and synchronized developmental induction were carried out as described previously (26, 27), with some modifications. Briefly, 1 × 10^7 conidia of control strain TNO2A7 and appropriate mutants were inoculated into 100 ml liquid MMPDR with 0.1% yeast extract in 250-ml flasks and incubated at 37°C and 250 rpm. For assexual- and sexual-development induction, mycelia after 24 h of vegetative growth were transferred to solid MMPDR and the plates were air exposed for assexual-development induction or tightly sealed and blocked from light for sexual-development induction.

**Construction of gene deletion mutants and alcAp-driven strains.** The figA gene was replaced with the selectable nutritional marker pyrG from *Aspergillus fumigatus* (ApypG). The deletion cassettes were created by double-joint PCR (28). In brief, ~1.0-kb sections of the flanking regions of the figA gene of *A. nidulans* were amplified using primers P1/P3 and P4/P6. The pyrG gene was previously amplified from plasmid pXDRFP4 using primers PyrgF/PyrG. 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 transformed into *A. nidulans* strain TNO2A7 to create a figA knockout strain. The transformation was performed as previously described (29, 30). A diagnostic PCR assay was performed to detect figA replaced by ApypG at the original figA locus using primers P1/Cpyrg. Furthermore, reverse transcription-PCR (RT-PCR) was performed to confirm the deletion of the figA gene using primers Chga/CfigaR. To construct figA and midA double-deletion mutants, the midA gene was replaced by a pyroA insertion as a selectable nutritional marker in the figA deletion background. The transformants were selected on minimal media without pyridoxine. To construct figA and cchA double-deletion mutants, the ΔfigA strain was crossed with the ΔcchA-1 strain, and the progeny were screened according to a standard protocol (31). To obtain an alcAp-driven figA conditional-expression strain, the intact figA gene was cloned into the pQA-pyroA vector. The final cassette of alcAp-figA-pyroA was transformed into a figA deletion strain and the TNO2A7 background strain.

**Complementation assay for figA in S. cerevisiae.** All *S. cerevisiae* strains used (see Table S2 in the supplemental material) are w303 derivatives. A PCR-generated DNA fragment including *S. cerevisiae*’s FGI1 open reading frame (ORF) plus 500 bp upstream of ATG and 200 bp downstream of the stop codon was obtained using primers 464 and 465 and then cloned into the BamHI/PacI sites of the integrative pRS306H vector (32). The Nael-linearized pRS306H-FIG1 vector was integrated into the URA3 locus, and integration was verified by colony PCR as previously described (33). The *A. nidulans* figA ORF was inserted to replace the FGI1 ORF of the pRS306H-FIG1 vector by restriction-free (RF) cloning using primers 471 and 472 and a figA cDNA as a template (34). The Nael-linearized pRS306H-FigA vector was integrated into the URA3 locus of the selected fig1::KAN strains. Cell-cell fusion assays were performed by monitoring cytoplasmic mixing as previously described (33). Briefly, cells of opposite mating types, with MATa strains expressing PGK1-mCherry, were grown to mid-log phase. Equal numbers of cells of all mating types were mixed and vacuumed to a nitrococellulos filter. The filter was placed cell side up on yeast extract-peptone-dextrose (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) and then washed and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) buffer before fluorescence microscopy analysis using an Olympus IX81 wide-field fluorescence microscope.

### TABLE 1 A. nidulans strains used in this study

| Strain    | Genotype                          | Reference or source |
|-----------|-----------------------------------|---------------------|
| TNO2A7    | pyrG89; pyroA4 nkuA::argB2; riboB2 veA1 | 35                  |
| GR5       | pyrG89; wA3; pyroA4; veA1          | FGSC                |
| R21       | pabaA1 ; yA2; veA1                | FGSC                |
| ΔmidA mutant | pyrG89; ΔmidA::pygG; pyroA4 | 14          |
| ΔcchA mutant | pyrG89; ΔcchA::pyroA; pyroA4 | 14          |
| ΔmidA ΔcchA mutant | pyrG89; ΔmidA::pyroA; pyroA4; nkuA::argB2 ΔcchA::pyG riboB2 veA1 | 14          |
| ΔcchA-1 mutant | pyrG89; pabaA1 yA2; nkuA::argB2; ΔcchA::pyG veA1 | This study |
| ΔfigA mutant | pyrG89; pyroA4 nkuA::argB2; ΔfigA::pyrgG; riboB2 veA1 | This study |
| ΔfigA ΔmidA mutant | pyrG89; ΔfigA::pyroA; pyroA4 nkuA::argB2; ΔfigA::pyrgG; riboB2 veA1 | This study |
| ΔfigA ΔcchA mutant | pyrG89; pyroA4 nkuA::argB2; ΔfigA::pyrgG; ΔcchA::pyrgG riboB2 veA1 | This study |
| Cfl       | pyrG89; pyroA4 nkuA::argB2; ΔfigA::pyrgG alcA(p)::figA::pyroA; riboB2 veA1 | This study |
| Ot2       | pyrG89; pyroA4 nkuA::argB2; alcA(p)::figA::pyroA; riboB2 veA1 | This study |
| FigA-GFP  | pyrG89; pyroA4 nkuA::argB2; figA:: GFP::pyrg; riboB2 veA1 | This study |
| ΔpmaA mutant | pyrG89; pyroA4 nkuA::argB2 ΔpmaA::pyroA; riboB2 veA1 | This study |
Plate assays. To assess the role of osmotic stress on conidiation, 0.8 M NaCl and 1.2 M sorbitol were separately added to MMPDR. For the calcium-related chemical sensitivity tests, 100 mM calcium and 2 mM EGTA were added to MMPDR. For the cell wall integrity test, 50 μg ml⁻¹ calcfluor white and 300 μg ml⁻¹ Congo red were added to MMPDR. Two microliters of 1 × 10⁶ conidia ml⁻¹ from mutants and the control strain TN02A7 was spotted onto relevant media and cultured for 2 or 3 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 the relevant strains were spread on YUU and minimal agar media. Next, sterile coverslips were inserted into the agar at an angle of 45 degrees. The plates were cultured while exposed to air at 37°C to process asexual development. After incubation for 24 h or 48 h, the coverslips were taken out of the medium and then mounted on the slides for microscopic observation. This approach was also used to localize the FigA-green fluorescent protein (GFP) fusion in developing conidiophores. Examination of self-fertilization development was carried out as previously described (27). In brief, conidia of the appropriate strains were point inoculated at the center of a solid medium and incubated at 37°C for 2 to 3 days, and then the plates were sealed and further incubated at 37°C for 7 days. The morphology of fruiting bodies was observed with a stereoscope. Ootropus and progeny analyses were performed according to standard protocols (31), except that a 20-fold amount of standard riboflavin was added into the medium to promote the 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 as that described previously (35) was used to construct the FigA-GFP fusion cassette. In brief, an ~1.0-kb fragment immediately upstream of the figA stop codon and an ~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 into strain TN02A7, and the transformants embedding homologous integration were verified by PCR.

Microscopic observation. For hyphal microscopic observations, conidia were inoculated onto precooled glass coverslips overlaid with liquid media. Strains were grown on coverslips at 37°C for the times indicated in the figures prior to observation under a microscope. Hyphal septa were stained using calcfluor white after the cells had been fixed with 4% paraformaldehyde (Polysciences, 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 the MetaMorph/MetaFluor combination software package (Universal Imaging, West Chester, PA).

Quantitative real-time PCR analysis. The samples were harvested at various time points, and the total RNA was extracted using TRIzol (Roche) by following the manufacturer’s instructions. The samples were treated with DNase I (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 determining the comparative change in cycle threshold (36) and normalized against the expression of the A. nidulans tubulin gene. Primers are listed in Table S1 in the supplemental material.

RESULTS
Identification of the yeast Fig1 homolog in A. nidulans. The amino acid sequences of Fig1 in S. cerevisiae were used to search for its homologs in the A. nidulans genome database. There are two A. nidulans FigA homologs, FigA (KEGG accession number AN3036.2) and FigB (AN7093.2, also referred to as FigA-like). Both of them have the conserved topology structure and the claudin motif shared by all Fig1 homologs. However, no detectable phenotypes were observed when we knocked out the full length of the figB open reading frame (data not shown). Therefore, figB was not 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. In Fig. 1A, the stars indicate the glycine and cysteine residues of a conserved Gly-Cys motif near the end of the first transmembrane domain. The diamonds show the conserved claudin motif, which is composed of the sequence in the box. The larger letters in the box display the conserved claudin motif [GφG]C, aa, amino acids. (B) Levels of figA mRNA expression in various developmental stages in A. nidulans. Numbers indicate the time (h) of incubation in liquid submerged culture (vegetation medium [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 deviations from three independent replicates. Different lowercase letters on the bars indicate significant differences in values among the stages (by Tukey’s honestly significant difference test, P < 0.05).

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

**FigA is involved in asexual development.** Hyphal growth defects were caused by the loss of figA, but colonies formed by the ΔfigA strain were notably devoid of conidia on minimal medium. The numbers of conidia produced by the ΔfigA mutant were approximately 100-fold lower than those of TN02A7 on MMPDR (Fig. 2C). However, there were no exacerbated conidiation defects of the ΔfigA ΔmidA and ΔfigA ΔcchA double mutants compared with the ΔfigA single mutant (Fig. 2A and C). Unexpectedly, a similar conidiation defect in the ΔfigA mutant was also observed on rich medium, YUU (Fig. 3A). To further analyze the details 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 develop into conidiophores, with visible phialides connected to numerous conidia, resulting in a distinct “aspergillum” appearance. Differently, in the ΔfigA mutant, only a few, if any, metulae and phialides were 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 hyphal morphogenesis. The results showed that there were no obvious differences in polarized growth and septum formation between the ΔfigA mutant and TN02A7 on both rich and minimal media (Fig. 3C).

In our previous study, loss of the putative high-affinity calcium transport protein CchA was also accompanied by the loss of conidiation defects associated with sporulation (14). These results are consistent with the observation that CchA is involved in conidiation and the formation of conidiophores in A. nidulans (33). Therefore, it seems likely that calcium is involved in the development of conidiophores and conidia in A. nidulans. In the future, we will explore the potential relationship between CchA and other known calcium transporters, such as MidA, to elucidate the calcium transport mechanism in A. nidulans. FIG 2 Plate assay. (A) The colony morphology of the control strain (TN02A7) and the ΔfigA, ΔfigA ΔmidA, ΔfigA ΔcchA, and ΔmidA ΔcchA mutants grown on minimal medium (MM in the figure indicates MMPDR), minimal medium with 1.2 M sorbitol, minimal medium with 0.8 M NaCl, and minimal medium with 100 mM CaCl₂ at 37°C for 2.5 days. (B) Quantitative data for the diameters of the colonies of the strains in panel A. (C) Quantitative numbers of conidia for the strains shown in panel A. Error bars represent standard deviations from three replicates. Letters represent significant differences among values for the strains (Tukey’s honestly significant difference test, P < 0.05). FIG 3 FigA is involved in asexual development. (A) Colonies of TN02A7 and the ΔfigA strain grown on solid YUU at 37°C for 2 days. (B) Conidiophores of TN02A7 and the ΔfigA mutant. (C) Hyphal morphology of the ΔfigA mutant stained with calciofluor white (septa) and DAPI (4',6-diamino-2-phenylindole) (nuclei). The ΔfigA strain was cultured on YUU broth at 37°C for 10 h. Arrows indicate the locations of septa. Bars, 10 μm.
channel CchA or MidA resulted in a reduction of conidial production, while the conidiation defects were rescued by either extracellular Ca\(^{2+}\) or osmotic stress (14). Unlike the ΔcchA, ΔmidA, and ΔcchA ΔmidA mutants, which showed conidiation defects only in MMPDR, the ΔfigA mutants showed much more severe conidiation defects on both rich and minimal media. We then investigated whether extracellular calcium or osmotic stress might rescue the conidial phenotype displayed by the ΔfigA, ΔfigA ΔmidA, and ΔfigA ΔcchA mutants. As shown in Fig. 2A and C, addition of 100 mM calcium onto MMPDR could not significantly restore the conidiation defects in the ΔfigA, ΔfigA ΔmidA, and ΔfigA ΔcchA mutants. In comparison, under salt stress (0.8 M NaCl) or cell wall stabilizer stress (1.2 M sorbitol) conditions, the ΔfigA, ΔfigA ΔmidA, and ΔfigA ΔcchA knockout mutants showed increased conidiation compared to conidiation under 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; extracellular calcium could not rescue the conidiation defects, but stress, such as salt and cell wall stabilizer stresses, can partially rescue these defects.

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

**FigA is required for sexual development.** In A. nidulans, sexual fruiting bodies (cleistothecia) can be formed under both homothallic (self-fertilization) and heterothallic (outcross) conditions. To test the self-fertilization body formation ability of the strains, the ΔfigA, ΔfigA ΔmidA, ΔfigA ΔcchA, and ΔmidA ΔcchA mutants and their parent control strain, TN02A7, were point inoculated onto minimal and rich media. After cultivation at 37°C for 2 days, agar plates of all the above-described strains were sealed to be induced for sexual development. As a result, compared to TN02A7, the ΔfigA, ΔfigA ΔmidA, and ΔfigA ΔcchA mutants did not produce either any visible cleistothecia or aggregated Hülle cells (Fig. 5A). In contrast, deletion of midA, cchA, or midA and cchA showed almost normal cleistothecium formation compared to that of the wild type under the same conditions, suggesting that figA but not midA and cchA might be essential for self-fertilization in A. nidulans. Interestingly, 50 mM additional extracellular calcium could not rescue the fruiting body formation defects in the ΔfigA, ΔfigA ΔmidA, and ΔfigA ΔcchA mutants (Fig. 5B).

In order to test if FigA was required for outcrossing, we carried
out the following sexual crosses according to the standard protocol described in Materials and Methods: ΔfigA mutant × GR5 (wild type), ΔfigA mutant × R21 (wild type), ΔfigA mutant × ΔcnaA mutant (calcineurin A is a catalytic subunit of the calmodulin-dependent protein phosphatase), ΔfigA mutant × ΔpmrA mutant (a putative calcium-transporting ATPase), and ΔfigA mutant × ΔcchA-J mutant. All the tested crosses resulted in the formation of normal cleistothecia containing ascospores with normal viability. Importantly, when ΔfigA cells were crossed with the ΔmidA or ΔmidA ΔcchA strain, we could not find any hybridized cleistothecia under the same conditions as described above. This result indicates that in the absence of midA or midA and cchA, figA is essential for outcrossing. All together, 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 which expressed soluble cytoplasmic mCherry, were incubated, allowed to mate, and then analyzed by fluorescence microscopy in order to score cell–cell fusion efficiency (33). As previously described (17), Δfig1 mutants have a mild but noticeable cell fusion defect (see Fig. S2 in the supplemental material). 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’s roles is to promote sexual development (Fig. S2).

figA deletion dramatically downregulates the expression of brlA and nsdD. Since the deletion of figA abolished both conidiation and cleistothecium formation, we asked whether figA could affect the expression of brlA, nsdA, and steA, which had been verified as key regulators of asexual and sexual development. Consistently 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 exposure to conditions for asexual development induction (37, 38). Differently, in ΔfigA cells, brlA expression was not great under the same conditions (Fig. 6A). Based on previous reports showing that nsdD and steA are transcription factors involved in sexual development in 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, nsdD mRNA levels started to accumulate in the early phase of vegetative growth, reaching higher levels as sexual development proceeded. However, in ΔfigA cells, nsdD mRNA levels remained almost unchanged after sexual induction. Interestingly, nsdD mRNA levels in vegetatively growing ΔfigA cells were higher than in TN02A7 cells. Since steA is a homolog of S. cerevisiae ste12, which positively regulates cleistothecium development in A. nidulans, the expression of steA was tested accordingly. Unexpectedly, loss of figA did not significantly decrease but instead slightly increased the expression of steA during both the vegetative growth and sexual-development stages (Fig. 6C). Collectively, those results suggest that loss of figA decreased the expression of brlA and nsdD in A. nidulans asexual and sexual differentiation, respectively.

FigA is located at 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 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 to D, FigA::GFP accumulated at the center of septum sites of mature hyphae but could not be detected in conidia or germlings. To gain insight into the exact location of FigA at the septum, a three-dimensional scanning image was obtained by using confocal microscopy. The result clearly showed that FigA::GFP is located just to the right of the center of the septum (Fig. 7E). In addition, as shown in Fig. 7F, in the architecture of conidiophores, FigA localized to the junctions between vesicles and metulae and between metulae and phialides but not in the junctions between phialides and spores or conidia. Considering these results, a model showing a FigA localization pattern in the architecture of conidiophores is presented in Fig. 7G, indicating that FigA is located at the vesicle-metula and metula-phialide, but not phialide-sparse, interfaces.

**DISCUSSION**

Fig1 is a member of a fungus-specific family of proteins that have topology characteristics similar to those 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 epi-
The specific location of FigA in *A. nidulans* indicates that the function of FigA may fit well with those of nonfungal 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 the hyphal septum (probably around the pore) indicates that *figA* may also have selective 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*, the 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 the pheromone during mating (3, 4, 17). Recently, it was reported that *fig1* is involved in sexual development in the 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 processes under both homothallic (self-fertilization) and heterothallic (outcross) conditions. Our results showed that *figA* is essential for self-fertilization but not required for outcrossing 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 phenotype is similar to that of the putative G protein-coupled receptors *gprA* and *gprB*, which are also required only for self-fertilization, perhaps because of reduced or no 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 evidenced 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 processes under either homothallic or heterothallic conditions. However, *ΔfigA* mutant × *ΔmidA* mutant and *ΔfigA* mutant × *ΔcchA ΔmidA* mutant crosses were unsuccessful, indicating that *figA* in combination with *midA* is involved in sexual development in *A. nidulans*. In other words, *figA* is essential for outcrossing in the absence of *midA* and *vice versa*.

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 the sexual development of *A. nidulans*, while *SteA* is a homolog of *S. cerevisiae* Ste12p, which positively regulates cleistothecium development in *A. nidulans* (39, 40). Moreover, in *S. cerevisiae*,...
Ste12p plays a key role in coupling mitogen-activated protein kinase (MAPK) signal transduction to the 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 ΔfigA 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 having an essential function in sexual development, FigA is involved in asexual differentiation in A. nidulans. The ΔfigA mutant showed an ~100-fold-decreased conidial production compared to TN02A7. Unlike the Δmida, ΔchhA, and Δmida ΔchhA strains, which had an asexual phenotype that was evident only on minimal media, the ΔfigA mutant showed identical asexual phenotypes on both rich and minimal media. Recently, it was shown that in the plant-pathogenic fungus Fusarium graminearum, fig1 is involved in asexual development and Δfig1 mutants present a 70-fold reduction in macroconidium production compared to that of the wild type (19). However, the function of fig1 in asexual development is divergent. For example, the deletion of fig1 in the filamentous fungus N. crassa did not affect conidiation. In Aspergillus, 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 the asexual stage, suggesting that figA affects conidiation possibly through the downregulation of brlA expression. Furthermore, we found that FigA is located at the vesicle-metula and metulaphialide junctions in conidiophores. The specific location of FigA in those junctions indicates that figA may play important roles in 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 yet unknown; 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 act 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 cells 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, phoromone-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 restores the hyphal growth defect but could not promote fruiting body formation or asexual development in the figA strain. 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 acts mainly as a calcium uptake system component, while in asexual and sexual development, it works as a regulator involved in the regulatory program for asexual and sexual differentiation. Moreover, the specific pattern of FigA localization at the center of separation sites indicates that it may play important roles in selective permeation or trafficking or that it may behave as a scaffolding protein during growth and 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.

ACKNOWLEDGMENTS

This work was financially supported by the National Natural Science Foundation of China (NSFC; grant 31200057 to S. Zhang and grant 81330035 to L. Lu), the Natural Science Foundation of the Jiangsu Province of China (grant BK2012451 to S. Zhang), the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (grant 11JK1A180005 to L. Lu), an ANII-Caldeyaro Barcia Fellowship (to N. Carbo), the International Centre for Genetic Engineering and Biotechnology (ICGEB; grant CRP/URU11-01 to P. S. Aguilar), the Agencia Nacional de Investigación e Innovación (ANII-INNOVA grant DGI-AL3A/2007/19.040 URU-UE to P. S. Aguilar), and FOCEM (MERCOSUR Structural Convergence Fund, grant COF 03/11 to P. S. Aguilar).

A. nidulans strain TN02A7 was a gift of B. R. Oakley (Ohio State University, Columbus, OH); strains GR5 and R21 and plasmids pXDRFP4 and pFNO3 were from the FGSC (http://www.fgsc.net). Plasmid pQA-pyroA was a gift from H. M. Park (Chungnam National University, South Korea).

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