Role of Fig1, a Component of the Low-Affinity Calcium Uptake System, in Growth and Sexual Development of Filamentous Fungi

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Calcium is a ubiquitous messenger in eukaryotic cells, acting both directly and indirectly to modulate protein activity, gene transcription, energy metabolism, endo- and exocytosis, and many other cellular processes (4, 13, 48). In fungi, two major calcium uptake pathways have been identified and characterized: the high-affinity calcium uptake system (HACS), active during low calcium availability, and the low-affinity calcium uptake system (LACS), active when calcium availability is high (24, 27, 38, 45, 50). A third calcium uptake pathway was recently described for *Saccharomyces cerevisiae* but has yet to have its genetic components identified (34). In the filamentous fungus *Fusarium graminearum* (sexual-stage Gibberella zeae), the causal agent of head blight of wheat and barley, calcium signaling has been shown to have a role in hyphal growth, sporulation, and fruiting body function, and the regulation of components of the HACS has been shown to be involved in these processes (17, 35, 62, 63).

The HACS is minimally composed of the voltage-gated Ca2+ channel (VGCC) Cch1 (27, 50), the stretch-activated calcium channel/regulatory protein Mid1 (38), and the PMP22_Claudin superfamily member regulatory protein Ecm7 (45). In yeasts, the HACS responds to environmental and endoplasmic reticulum stress and to exposure to mating pheromone (8, 9, 20, 36, 38, 42, 51, 65). In filamentous fungi, HACS mutants show both shared and variable phenotypes across species, including reduced vegetative growth and calcium homeostasis lesions for most species and effects on pathogenicity and sexual development for some species (6, 17, 35, 40).

The LACS is minimally composed of the Fig1 membrane protein, a PMP22_Claudin superfamily member, like Ecm7, and is involved in calcium influx and membrane fusion during the mating of *S. cerevisiae* and *Candida albicans* (49, 69), mating projection development and cell wall degradation at the fusion site of appressed shmoos in *S. cerevisiae* (24), and thigmotropism and the repression of hyphal growth in *C. albicans* (9). Mammalian PMP22_Claudin superfamily members are involved in membrane–membrane interactions such as epitheilal tight-junction formation and signal transduction such as ion flux (46, 64). The fungal members show little overall sequence identity with their mammalian orthologs, but most have similar secondary structures and topologies, including cytoplasmic N and C termini, four transmembrane domains, two extracellular loops and one intracellular loop, and a conserved GΦΦGXC(n)C motif (where Φ is F, L, M, or Y hydrophobic residues; 8 < n < 20 amino acids [aa]) in the first extracellular loop (9, 24, 73).

In *F. graminearum*, the deletion of *CCH1*, *MID1*, or both resulted in almost identical phenotypes, including a reduced vegetative growth rate, conidiation, ascospore development, and forcible ascospore discharge from asci. Chemical complementation with exogenous calcium rescued all phenotypes, at least partially, except for the abnormal ascospore development seen in strains lacking Mid1, including the Δcch1 Δmid1 double mutant (17, 35). Because the addition of calcium rescued most phenotypes, including those of the Δcch1 Δmid1 double mutant, and because the LACS is involved in calcium importation, is active in environments with a high calcium availability, and is involved in the sexual development of yeasts, we investigated the role of Fig1 in the growth and development of *F. graminearum*. We asked whether Fig1 is involved in the uptake of calcium, which is essential for the chemical complementation of the HACS mutant phenotype. We identified the putative *F. graminearum* *FIG1* ortholog and generated Δfig1, Δcch1 Δfig1, Δfig1 Δmid1, and Δcch1 Δfig1 Δmid1 strains to characterize the Fig1 function and interactions with HACS components. Additionally, we investigated the effect of the loss of Fig1 on growth, calcium homeostasis, and sexual develop-
Strains used in this study are described in Table 1. Strains of *F. graminearum* were obtained from the Fungal Genetics Stock Center (FGSC) and were maintained on synthetic crossing (SC) medium (22) slants at 20°C, as macroconidia (10⁶ to 10⁸ conidia/ml), and as colonized pieces of V8 agar at 80°C. Macroconidia were produced to the manufacturer’s instructions. Along with the universal primer in-
cluded in the kit, primers Fig1 RACE R1 and Fig1 RACE F1 for the 5’- and 3’-RACE reactions, respectively, and Phusion high-fidelity DNA polymerase (NEB) were used in the RACE reaction mixtures. Amplified fragments were then cloned into pCR2.1-TOPO by using the TOPO TA cloning kit (Life Technologies) and sequenced at Michigan State University’s Research Technology Support Facility by using an ABI Prism 3730 genetic analyzer (Life Technologies). Reverse transcription (RT)-PCR to detect FIG1 expression was performed by using primers Fig1 RT-R and Fig1 RT-R, which span the splice junctions between exons 1 and 2 and exons 3 and 4, with the 3’ ends consisting of 6 bp of exon 2 and exon 3, respectively. The expression of the gene for elongation factor 1A (EF1A) (FGSG_08811) was detected by using primers EF1A-F and EF1A-R, which do not span exon splice junctions.

Analysis of sexual crosses. All F. graminearum crosses were performed between NIT3 (FGSG_10250) (probable nitrate) nitrate-utilizing (nit’) and nit3 non-nitrate-utilizing mutant (nit’) strains. Cirrhi (spores oozing from perithecia) from single perithecia were collected individually, and the spores were suspended in water and distributed across the surface of MMTS medium, as previously described (7, 17). Recombinant perithecia can easily be identified by the presence of approximately equal numbers of nit’ and nit’ progeny, which have different growth phenotypes on this medium. For strains that did not form cirrhi (cch1 and mid1 mutants), 10 perithecia at the interface between strains were collected, crushed in bulk on a glass slide in sterile distilled H2O to release ascospores, and rinsed off the glass slides, and a portion of the suspension was spread onto the surface of MMTS agar. After 5 to 7 days of growth at room temperature, nit’ and nit’ colonies from recombinant perithecia or bulk mixtures were individually collected. To confirm the nit phenotype, cultures were subsequently transferred onto Czapek-Dox agar (60) and Czapek-Dox agar supplemented with 470 mM potassium chlorate and 0.05% glucose and fructose (68). PCR was used to confirm the presence or absence of CCH1, FIG1, and MID1.

To generate N. crassa fig1 mating type A mutants, a Δfig1 mating type A mutant strain (FGSG_17273) was crossed with a wt mating type A strain (FGSG_4200). To recover progeny, ascospores were then rinsed off the lids, subjected to heat shock to promote germination, and placed onto SC medium amended with 1% sorbose and 0.05% glucose and fructose, with nit3 mutants, 10 perithecia at the interface between strains were collected, crushed in bulk on a glass slide in sterile distilled H2O to release ascospores, and rinsed off the glass slides, and a portion of the suspension was spread onto the surface of MMTS agar. After 5 to 7 days of growth at room temperature, nit’ and nit’ colonies from recombinant perithecia or bulk mixtures were individually collected. To confirm the nit phenotype, cultures were subsequently transferred onto Czapek-Dox agar (60) and Czapek-Dox agar supplemented with 470 mM potassium chlorate and 0.05% glucose and fructose, with sucrose omitted for germination, as previously described (52, 58, 59).

Single-spore isolates were transferred into SC medium, and PCR was used to confirm the presence or absence of fig1 and mating type A-2 (present in mating type A and absent in mating type A). Characterization of Δfig1 phenotypes. To characterize the vegetative growth and sexual development of F. graminearum Δfig1 strains, individual strains were grown on carrot agar amended with 80 mM CaCl2 or 80 mM MgCl2 or un amended and incubated at room temperature under continuous light. Radial growth was measured at 24-h intervals for 3 days, with four biological replicates for each strain. The first day’s growth was discounted. The growth rate was calculated by subtracting the colony diameter at 24 h from the diameter at 48 h and was averaged between the replicates. Significance between the growth of Δfig1 strains compared to the growth of wt and among the treatments was assessed. Bilay’s medium (5) was supplemented to 1 mM with the extracellular calcium chelator 1,2-bis(2-aminophenoxy)ethene-N,N,N,N-tetraacetic acid tetrapotassium salt (BAPTA) to determine the ability of Δfig1 mutants to grow under conditions of limited calcium, as previously described for Δcch1 mutants (17). To determine the effect of increased intracellular calcium levels, strains were center inoculated onto V8 agar, and after 48 h of growth, a point treatment of 10 μL of either a 9.5 mM solution of the calcium ionophore A23187 (in ethyl alcohol [EtOH]) or 100% EtOH (control) was applied onto a point slightly ahead of the leading edge of the mycelial growth.

To quantify conidiation in F. graminearum mutants, strains were grown with shaking in 100 ml CMC for 4 days at room temperature. Macroconidia were harvested by filtration through sterilized Miracloth (Calbiochem). The macroconidia were pelleted by centrifugation, resuspended in 1 ml of sterile distilled H2O, and quantified.

PCR-identified Δfig1 mating type A progeny of N. crassa were reciprocally crossed to wt mating type a, wt mating type A, and Δfig1 mating type A strains both to test the mating type determination and to assess the effect of the loss of Fig1 on sexual development. Squash mounts of perithecia from the crosses were examined microscopically to observe ascus and ascospore development. Culture dish lids of the crosses were monitored for discharged ascospores. For the quantification of macroconidia, cultures were grown on synthetic crossing medium in plates sealed with Parafilm. After 3 days of growth, the Parafilm was removed, and the cultures were allowed to sporulate for 3 days. Spores were suspended in water with Tween 60 and quantified. Three trials were performed for each strain.

Pathogenicity assays were performed, as previously described (35), by the inoculation of a central flook of spring wheat cultivar Wheaton with 10 μl of the conidial stock of the appropriate strain. Ten wheat heads at anthesis were inoculated with each strain. Following 72 h in a misting chamber, symptoms were monitored for up to 2 weeks. The experiment was repeated twice.

Statistical analysis. To assess the significance of intragroup variations and intergroup interactions and to return associated P values for all pairwise comparisons, an analysis of variance (ANOVA) and Tukey’s honestly significant difference (HSD) test were performed using the R language and environment for Windows (33).

Illumina sequencing and bioinformatics. Genomic DNA from an insertional mutant generated from wt strain PH-1 was sequenced by using Illumina GAILI (San Diego, CA) with 76-bp paired-end reads and a 400-bp average library insert size. The 3’ ends of the reads were trimmed with a Biopython script (18) and a Phred scale quality cutoff score of 28. The reads were assembled by using the Velvet assembler (version 1.03), using k-mer values of 37 to 49 nucleotides (71, 72). No reads were manually discarded because of length, but reads shorter than the k-mer size used in an assembly are automatically ignored by Velvet. The FIG1 locus was located in the assembly by using standalone BLAST+ (11). A sequencing library was constructed from wt RNA collected from perithecia at 96 h after the induction of sexual development and sequenced by using Illumina GAILI 36-bp single-end reads and a 400-bp average cDNA library insert size (58a).

Burrows-Wheeler Aligner (BWA) (version 0.5.9) (41) was used to align the RNAseq reads to either the genomic FIG1 sequence from the Velvet assembly or the same sequence with the introns removed. The 5’- and 3’-RACE clone sequences were aligned manually. A multiple-sequence alignment of the sequence of the putative full-length F. graminearum Fig1 protein to Fig1 sequences from multiple fungi was performed with T-Coffee (23). The multiple-sequence alignment was viewed and edited with Jalview (66) and Seaview (32). The putative full-length F. graminearum Fig1 protein sequence was used to search the Conserved Domains Database (44).

Microscopy and imaging. For microscopic examination, samples were fixed in 2× phosphate-buffered saline (PBS) containing 4% (wt/vol) paraformaldehyde for 20 min on ice, washed twice with 2× PBS, and stained with 0.06% (wt/vol) toluidine blue in sterile H2O overnight. Stained samples were washed twice each in 50%, 75%, and 100% ethanol in sterile water before being stored in sterile H2O. To determine whether sexual development initiates at all or if it halts at a very early stage of development, the wt and the Δfig1 single mutants were inoculated onto carrot agar with several pieces of a cellulose membrane placed onto the surface of the medium. At 24 h and 48 h postinduction, the cellulose membranes were removed from the carrot agar, and the membranes were fixed, stained with toluidine blue, destained, and viewed by light microscopy.

A Zeiss (Göttingen, Germany) standard microscope was used to observe samples, and images were captured with a Zeiss AxioCam MRc color camera by using AxioVision 4.8.2. Nonmicroscopic images were captured with a Nikon (Tokyo, Japan) Coolpix 995 camera. Image processing and
RESULTS

Identification of FIG1 through RACE and Illumina sequencing.

The reference sequence of *F. graminearum* contained a contig gap in the region of FIG1 (assembly 3, contigs 204 and 205) (*Fusarium* Comparative Sequencing Project, Broad Institute of Harvard and MIT [http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/MultiHome.html]). Due to the gap, the FIG1 annotation (in both the MIPS *Fusarium graminearum* Genome Database and the Broad Institute *Fusarium* Comparative Database) contained two introns and three exons (Fig. 1A) ending 36 bp upstream of the contig gap, predicting a protein of 156 amino acids that is more than 100 aa shorter than most filamentous ascomycete homologs. To recover the missing sequence, 5' and 3' -RACE were performed, and two clones from each preparation were sequenced, revealing the presence of a third intron and a fourth exon (Fig. 1B). Subsequently, we performed Illumina sequencing on genomic DNA from a strain derived from the wt (data not shown). In addition, Illumina RNAseq was performed on developmental stages of the wt (58a). The FIG1 genomic sequence was identified from the Velvet assembly of the genomic DNA by BLAST. The sequence spanning the annotated end of the previous gene upstream to the start of the next gene downstream of FIG1 was used for the alignment. The RACE sequences and RNAseq reads were aligned to the Velvet FIG1 sequence, and both sets of data aligned well to the exons but not the introns (Fig. 1B). The introns were removed from the Velvet FIG1 genomic sequence, and the RNAseq reads were aligned to the putative spliced transcript, resulting in read coverage across the splice junctions instead of the loss of coverage seen with the introns included (Fig. 1C).

RNAseq data were also used to examine gene expression levels across vegetative growth and development. RPKM (reads aligned per kilobases mapped) values for MID1, CCH1, and MID1 are presented in Table S3 in the supplemental material. These values indicate that MID1 and CCH1 were expressed constitutively, but FIG1 showed the highest expression levels during vegetative growth, the lowest expression levels at 24 h after the induction of sexual development, and increased expression levels throughout the maturation of the asci (72 h to 144 h).

To assess whether the *F. graminearum* FIG1 sequence from the
Velvet assembly represented the full length of the gene, the translated protein sequence was aligned to 16 Fig1 homologs from ascomycetes (see Table S2 in the supplemental material). The Claudin motif is present in all Fig1 homologs (Fig. 2). The longer protein aligned well across the full length of the protein, especially to other filamentous species (data not shown), and a search of the Conserved Domains Database (44) found a full-length Fig1 domain (data not shown), indicating that the longer sequence included the complete F. graminearum FIG1 ortholog.

Generation and characterization of FIG1 mutants of F. graminearum. F. graminearum FIG1 mutants were generated by replacing the first 125 bp of the coding sequence with hph. From a single transformation experiment, 10 transformants resistant to hygromycin were obtained. PCR amplics of three transformants were consistent with the replacement of the targeted FIG1 sequence with hph, and the remaining 7 transformants were not tested (not shown). Two of these three transformants, designated Δfig1-1 and Δfig1-2, were used in subsequent experiments. RT-PCR detected FIG1 expression in wt cDNA but not wt genomic DNA or Δfig1-1 and Δfig1-2 CDAs (see Fig. S1 in the supplemental material). Δfig1-2 protoplasts were used to recover complemented transformants following selection with the calcium ionophore A23187. From equal numbers of protoplasts, two were recovered using 1.4 mM ionophore for selection, and none recovered from selection with 3.1 mM ionophore, more than 70

To generate Δchcl nit3 and Δchcl mid1 nit3 strains, crosses were initiated between Δchcl-T11 (Δchcl) and mn-11 (Δmid1 nit3). PCR screening of 12 progeny growing slowly and sparsely on MMTS agar (indicative of a nitatro phenotype) revealed two strains, cn-5 and cn-7, that produced the amplicons expected from a Δchcl genotype and two strains, cmm-1 and cmm-9, that produced the amplicons from a Δchcl Δmid1 nit3 genotype. Crosses of Δfig1-1 × mn-11, Δfig1-1 × cn-7 (Δchcl nit3), and Δfig1-1 × cmm-1 (Δchcl mid1 nit3) were performed to isolate Δfig1 Δmid1, Δchcl Δfig1, and Δchcl Δfig1 Δmid1 strains, respectively. Six progeny displaying slow and dense growth on MMTS agar (indicative of a nitatro phenotype) were recovered from each of the three crosses. Screening by PCR confirmed the presence of the appropriate amplicons for two Δfig1 Δmid1 strains (fm-1 and fm-5), two Δchcl Δfig1 strains (fc-1 and fc-6), and one Δchcl Δfig1 Δmid1 strain (fcm-2).

To examine the effects of the FIG1 deletion on vegetative growth, calcium uptake, and calcium homeostasis, all strains were grown on medium with or without the addition of calcium, magnesium, the calcium chelator BAPTA, and the calcium ionophore A23187 (which increases the cytosolic concentration of calcium). Differences in mycelial growth were tested under several conditions. Growth on unamended carrot agar revealed that all LACS and HACS mutants grew at a lower rate than the wt (Fig. 3A). Growth on Bilay’s medium containing 1 mM BAPTA was used to test growth in calcium-restrictive medium. As previously reported, strains lacking Mid1 or Cch1 grew slightly for 1 day and failed to colonize the BAPTA medium further (17, 35), but Δfig1 mutants of F. graminearum, F. chlamydosporium, F. verticillioides, F. poae, F. graminearum, and F. culmorum were recovered from each of the three crosses. Screening by PCR confirmed the presence of the appropriate amplicons for two Δfig1 Δmid1 strains (fm-1 and fm-5), two Δchcl Δfig1 strains (fc-1 and fc-6), and one Δchcl Δfig1 Δmid1 strain (fcm-2).

A quantification of growth rates and macroconidium production was performed to better understand the effect of the loss of Fig1 on vegetative growth and asexual reproduction. When grown on unamended carrot agar, Δfig1 mutants grew at a significantly lower rate than the wt, while mutants having both LACS and HACS defects grew at a significantly lower rate than the Δfig1
strains. The growth rates of complemented strains f12-C1 and f12-C2 were not significantly different from that of the wt (Fig. 4A). The Δfig1 Δmid1 and Δfig1 Δcch1 results were nearly the same as those previously reported for single Δmid1 and Δcch1 mutants (17, 35). The growth of strains on carrot agar amended with 80 mM calcium or 80 mM magnesium was not significantly different from the growth of the same strain on unamended carrot agar, except for the Δcch1 Δfig1 Δmid1 triple mutant, which grew significantly more slowly on carrot agar amended with magnesium than on both carrot agar and carrot agar amended with calcium. These results differed from previously obtained results for Δmid1, Δcch1, and Δmid1 Δcch1 mutants in that these mutants all showed significant increases in growth rates upon exogenous calcium treatment (17, 35). Additionally, the Δmid1 Δcch1 mutant did not have a decreased growth rate upon the addition of exogenous magnesium (17). All mutants produced significantly fewer macroconidia in CMC medium than the wt but were not significantly different from each other (Fig. 4B). The Δfig1 mutant conidiation phenotype was slightly more severe than that previously reported for Δmid1 mutants, and the numbers of conidia produced by the Δfig1 Δmid1, Δmid1 Δcch1, and Δfig1 Δmid1 Δcch1 mutants were an order of magnitude lower than those produced by the Δmid1 and Δmid1 Δcch1 mutants (17).

All strains lacking functional Fig1 failed to develop perithecia, including the double and triple mutants with the HACS components Mid1 and Cch1, although they produced the characteristic black pigment seen in the top 2 to 4 mm of the medium after induction (Fig. 5A). Previously reported results for Δmid1, Δcch1, and Δmid1 Δcch1 mutants showed slowed sexual development, reduced ascospore discharge, and some abnormally developed ascospores (17, 35). To determine whether sexual development initiates at all or if it halts at a very early stage of development, the wt and the Δfig1 single mutants were inoculated onto carrot agar with several pieces of cellulose membrane placed onto the surface of the medium. At 24 h, the wt developed perithecia initially, but the Δfig1 mutants had only small hyphae curving back onto themselves. By 48 h, the membranes with the wt contained larger immature perithecia with developing walls, and the curved hyphae of the Δfig1 mutant enlarged but failed to develop any further (Fig. 5B).

Pathogenicity assays showed that wheat inoculated with the Δfig1 and Δfig2 strains developed symptoms similar to those of the wt (all 10 heads developed symptoms), although full symptoms were delayed by 1 week in comparison to the wt. The Δfig1 Δmid1 and Δfig1 Δcch1 strains showed similar delays in symptom development and reduced pathogenicity, with fewer than 5 of the 10 inoculated heads developing symptoms with each strain in both experiments. The Δfig1 Δmid1 Δcch1 triple mutant failed to develop symptoms in any of the heads inoculated.

Characterization of N. crassa fig1 mutants. The N. crassa fig1 ortholog (NCU02219) was identified by BLAST using the S. cerevisiae Fig1 protein sequence. A Δfig1 mating type a strain, but not a Δfig1 mating type A strain, was generated previously by Colot et al. (19), so the Δfig1 mating type a strain was crossed to a wt mating type A strain to generate Δfig1 mating type A progeny. Twenty-four progeny were screened by PCR for the absence of fig1 and the presence of the mating type A-2 gene. PCR analysis revealed that three progeny, fig1A-18, fig1A-20, and fig1A-21, lacked fig1 but harbored the mating type A-2 gene. Strain fig1A-21 was used for
FIG 4 Characterization of *F. graminearum* growth and asexual development. (A) Growth rates on carrot agar with and without Ca\(^{2+}\) or Mg\(^{2+}\) supplementation (change of diameter in mm/day). Asterisks above brackets indicate a significant difference between all growth conditions of the bracketed strain and the wild type on carrot agar. Number signs above brackets indicate a significant difference between the bracketed strain on carrot agar and strain \(\Delta fig1-1\), but not \(\Delta fig1-2\), on carrot agar. (B) Macroconidium production. Error bars represent the standard deviations of the means from 3 biological replicates. *, \(P < 0.005\); #, \(P < 0.05\). Abbreviations for strains and genotypes are listed in the legend of Fig. 3.}

FIG 5 Sexual development of *F. graminearum* wt and \(\Delta fig1-1\). (A) Mature wt culture (right) with normal sexual development and \(\Delta fig1-1\) culture (left) with no perithecia. (B) Sexually induced cultures at 24 and 48 h stained with toluidine blue. Perithecia initials (arrowheads), which developed at 24 h in the wt, were barely initiated in \(\Delta fig1-1\) cells. By 48 h, there were immature walled perithecia (arrow) in wt cells; in \(\Delta fig1-1\) cells, perithecia initials have not matured and do not develop further. Scale bars, 20 \(\mu\)m. Abbreviations for strains and genotypes are listed in the legend of Fig. 3.

**DISCUSSION**

The results presented here demonstrate divergent roles for Fig1 in *F. graminearum* and *N. crassa*. In *F. graminearum*, Fig1 function affects all stages of the life cycle examined and is essential for sexual development. In *N. crassa*, the loss of Fig1 resulted in little to no effect on vegetative growth but reduced female fertility and arrested perithecia development in a mating type *a* background. Fig1 is required for both Ca\(^{2+}\) influx-dependent and -independent responses to exposure to mating pheromone in yeasts (24, 43, 69, 73); however, to date, there have been no reported functional studies of Fig1 in filamentous species. The apparent divergence of LACS function between *N. crassa* and *F. graminearum* is reminiscent of the previously reported divergence of HACS function between *F. graminearum* and *N. crassa* in which both similar (slowed vegetative growth and calcium homeostasis lesions) and divergent (reduced ascospore discharge and abnormal ascospore morphology in *F. graminearum* and altered hyphal electrophysiology in *N. crassa*) phenotypes were observed (17, 40). The finding that the roles of the HACS and LACS both show divergence points to the evolutionary malleability of calcium signaling.

Recent studies, including an analysis of genomes in the Origins of Multicellularity Database (http://www.broadinstitute.org/annotation/genome/multicellularity_project/MultiHome.html), have suggested that some components of calcium...
signaling once thought to be unique to either fungi or metazoans appeared before the divergence of these lineages approximately a billion years ago (10, 55, 57). The genome of *Thecamoenas trahens*, a member of the phylum Apusozoa, was reported to contain the only known ortholog of Mid1 outside the true fungi. The Apusozoa are a possible sister group of the opisthokonts, having diverged before the fungal-metazoan split (15, 16, 31). However, BLASTP, BLASTX, and TBLASTN searches of the *T. trahens* genome or predicted proteins using the full-length Mid1 protein sequences from *F. graminearum* and *S. cerevisiae* and default settings failed to find a match. In addition, *T. trahens* contains several homologs of the mammalian VGCC rather than a fungal Cch1 ortholog. Furthermore, *Allo myces macrognus*, a blastocladiomycete (39), possesses six Cch1 homologs (10). Although the presence of Fig1 and other PMP22_Claudin superfamily members was not examined by Cai and Clapman (10), multiple members are already known to exist in both metazoans and fungi, suggesting that at least one member was present in the common ancestor. Taken together, it appears that lineage-specific adaptation resulting in the loss, gain, or expansion of calcium signaling components in combination with the changes in specific roles may account for the differences seen in the extant species of unikont organisms.

Members of the PMP22_Claudin superfamily, of which Fig1 is a member, are involved in membrane-to-membrane interactions and also commonly form diffusion barriers that selectively allow ions, water, and other solutes to pass between cells (28, 33, 56, 68). The known functions of Fig1 in fungi fit well with those of non-fungal proteins in the PMP22_Claudin superfamily, involving cell-to-cell interactions and ion flux. Fig1 is involved in the sexual development of *C. albicans* and *S. cerevisiae*, and the results here show that it is also involved in the sexual development of *F. graminearum* and *N. crassa*. Fig1 expression and calcium uptake increase in response to mating pheromone, and Fig1 localizes to the mating projections and membranes destined for fusion during mating in both *S. cerevisiae* and *C. albicans*. The deletion of *FIG1*

**FIG 6** Reciprocal crosses of *N. crassa* wt and Δ*fig1* strains. Strains listed to the left served as females, developing protoperithecia that were fertilized by conidia of the strain listed in the upper right of each image. All crosses produced wt perithecia, except for crosses with Δ*fig1* mating type *a* strains as the female, which produced small infertile fruiting bodies. Abbreviations for strains are as follows: wt *a*, FGSC_4200; wt *A*, FGSC_2489; Δ*fig1* *a*, FGSC_17273; Δ*fig1* *A*, figA-18.

**FIG 7** *N. crassa* perithecium squash mounts from wt and Δ*fig1* crosses. (A) Perithecium from a wt *a* × Δ*fig1* *A* cross showing normal development, with asci and ascospores. (B) Infertile fruiting body from a Δ*fig1* *a* × wt*A* cross. (C) Infertile fruiting body from a Δ*fig1* *a* × Δ*fig1* *A* cross. Scale bars, 75 μm. Abbreviations for strains are described in the legend of Fig. 6.
resulted in lowered pheromone-induced calcium accumulation and decreased cell fusion during the mating of both yeasts. For S. cerevisiae, undigested cell wall remaining between the appressed shmoo was observed, which was rescued by the addition of exogenous calcium, and this phenotype was not seen for C. albicans (2, 24). Cell-to-cell interactions and membrane dynamics appear to be central to the function of Fig1 in yeast mating and point to a possible similar role in filamentous fungi that may explain the blocking of sexual development in F. graminearum and the reduced female fertility of N. crassa seen for Δfig1 mutants. Cellular fusion is critical to the formation of the coiled perithecium initials and the formation of parenchymatous tissue from hyphae during fruiting (61). Although the blocking of F. graminearum sexual development did not allow an investigation of the role of Fig1 in later stages of development in the present study, expression data suggest that Fig1 is involved at later stages of F. graminearum sexual development. Membrane dynamics and trafficking are essential for ascus development (3, 37, 54) and for the delimitation of ascospores by the formation of double membranes around the progeny nuclei (21, 67). As Fig1 is involved in membrane fusion, a role for Fig1 in ascus development and ascospore delimitation is possible.

The mating-type-specific defect of N. crassa Δfig1 mating type a strains, while different from the F. graminearum results, is reminiscent of the results seen for yeasts. For S. cerevisiae, Fig1 expression was triggered in MATa cells upon exposure to α-pheromone, and although expression in MATα cells was not reported, some mating defects were more severe in Δfig1 × Δfig1 crosses than in Δfig1 × wt (MATα) crosses (24), suggesting at least some Fig1 expression in MATα strains. In C. albicans, CaFIG1 expression occurs only in opaque-phase cells homoygous for mating type locus a (MTLa) upon exposure to α-pheromone and not in MTL heterozygotes or MTLα homoygotes (43). While the details are slightly different, the commonality is that Fig1 plays a greater role in one mating type, the α idiomorph, than in the other. In contrast to these systems, F. graminearum is homothallic and effectively always contains both mating types, which may have eliminated the mating type constraints on Fig1 expression and, possibly, function.

The role of Fig1 in vegetative growth is also variable among the fungi. The loss of Fig1 did not affect the growth rate or calcium accumulation of S. cerevisiae or C. albicans vegetative yeast cells (9, 49). During the hyphal growth of C. albicans, the CaFIG1 expression level was low, and fluorescence from CaFig1-green fluorescent protein (GFP) fusion proteins was undetectable but was increased upon exposure to mating pheromone (70). The deletion of CaFIG1 resulted in an increased conversion to hyphal growth of yeast cells grown on agar, indicating that CaFig1 plays a role in hyphal suppression in some environments (9). Additionally, the loss of CaFig1 did not decrease calcium uptake in C. albicans hyphae but did affect the thigmotropic response of hyphae to physical ridges, resulting in a reduced frequency of hyphal tip growth reorientation upon contact with a ridge compared to the wt (9). Our results show that the loss of fig1 had no detectable effect on N. crassa vegetative growth. However, in F. graminearum, the FIG1 deletion resulted in phenotypes similar to those for HACS mutants: reduced vegetative growth, reduced macroconidiation, and defective calcium homeostasis (17, 35). The mycelium of HACS mutants was fluffy compared to that of the wt, while mycelia of Δfig1 strains were appressed to the substrate surface, with few aerial hyphae.

The nutrient availability seemed to affect this phenotype, as fewer aerial hyphae were produced on V8 agar than on the more-nutrient-rich carrot agar. Another difference is that the Δfig1 mutants and the wt grew on calcium-limited medium supplemented with BAPTA, but Δmtd1 and Δcch1 strains failed to colonize the BAPTA-containing medium, suggesting the need for the HACS, but not the LACS, to support calcium influx in calcium-limited environments. It would be interesting to see if the LACS has a function in the hyphal electrophysiology of F. graminearum and N. crassa. A disruption of the HACS in N. crassa resulted in altered electrical properties, as measured by a voltage clamp (40). In contrast, a disruption of the HACS in F. graminearum did not alter the hyphal electrochemical properties (17).

The results presented here, taken together with the results of previous studies of the HACS and LACS in fungi and the analysis of calcium signaling components in the Origins of Multicellularity Database, show the evolutionary flexibility of calcium signaling in the different lineages of extant eukaryotes. Even within the Sordariomycetes, the roles of the HACS and LACS have diverged, as suggested by studies of C. chilensis, Mtd1, and Fig1 of F. graminearum and N. crassa. Although not examined here because of the block in sexual development, it is likely that Fig1 plays a role in sexual development after the transition from perithecium initials to the immature perithecium, and the application of sexual development stage specific or inducible RNA interference (RNAi) would allow the investigation of any such role. Additionally, the documented roles of calcium importation genes in sexual development suggest that other calcium signaling components will also be involved. Finally, the end targets of calcium signaling during sexual development are not known, and investigations to identify them are in progress.

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