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A non-pheromone GPCR is essential for meiosis and ascosporogenesis in the wheat scab fungus

Mingyu Ding[a,1], Shulin Cao[b,1], Daiying Xu[a], Aliang Xia[a], Zeyi Wang[c], Wanshan Wang[a], Kaili Duan[a], Chenyu Wu[a], Qinhu Wang[a], Jie Liang[a], Diwen Wang[c], Huiquan Liu[a], Jin-Rong Xu[c,2], and Cong Jiang[a,2]

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Meiosis is essential for generating genetic diversity and sexual spores, but the regulation of meiosis and ascosporogenesis is not clear in filamentous fungi, in which dikaryotic and diploid cells formed inside fruiting bodies are not free living and independent of pheromones or pheromone receptors. In this study, Gia1, a non-pheromone GPCR (G protein-coupled receptor) with sexual-specific expression in *Fusarium graminearum*, is found to be essential for ascosporogenesis. The *gia1* mutant was normal in perithecium development, crozier formation, and karyogamy but failed to undergo meiosis, which could be partially rescued by a dominant active mutation in *GPA1* and activation of the Gpmk1 pathway. *GIA1* orthologs have conserved functions in regulating meiosis and ascosporogenesis in Sordariomycetes. *GIA1* has a paralog, *GIP1*, in *F. graminearum* and other Hypocreales species which is essential for perithecium formation. *GIP1* differed from *GIA1* in expression profiles and downstream signaling during sexual reproduction. Whereas the C-terminal tail and IR3 were important for intracellular signaling, the N-terminal region and EL3 of Gia1 were responsible for recognizing its ligand, which is likely a protein enriched in developing perithecia, particularly in the *gia1* mutant. Taken together, these results showed that *GIA1* encodes a non-pheromone GPCR that regulates the entry into meiosis and ascosporogenesis via the downstream Gpmk1 MAP kinase pathway in *F. graminearum* and other filamentous ascomycetes.

ascosporogenesis | filamentous ascomycetes | G protein-coupled receptor | MAP kinase pathway | signal recognition

Significance

Meiosis is essential for generating genetic diversity and sexual spores, but the regulation of meiosis is not clear in filamentous fungi. Here, we functionally characterized GPCR (G protein-coupled receptor) genes with sexual-specific expression in *Fusarium graminearum*, a perithecium-forming ascomycete, and found that *GIA1* is essential for meiosis and ascospore formation. Upon recognition of the meiosis-specific ligand enriched in developing perithecia, Gia1 activated the downstream Gpmk1 mitogen-activated protein (MAP) kinase pathway to trigger meiosis and ascosporogenesis. Gia1 orthologs are functionally conserved in the model filamentous ascomycete *Neurospora crassa* and other Sordariomycetes. Taken together, our findings show a GPCR senses stage-specific non-pheromone signals for meiosis to activate a conserved MAP kinase (MAPK) pathway in *F. graminearum* and other filamentous fungi.

As in other eukaryotic organisms, sexual reproduction is important for increasing genetic variations and purging deleterious mutations in fungi (1). In the filamentous ascomycete *Fusarium graminearum*, a causal agent of the wheat head blight disease, sexual reproduction is a critical step in its infection cycle because ascospores (sexual spores) are the primary inoculum for infection of floral tissues (2). This fungal pathogen survives on plant debris and produces sexual fruiting bodies (ascocarps) known as perithecia. When environmental conditions are favorable in the spring, ascospores formed inside asci are ejected from perithecia and airborne ascospores are dispersed to wheat heads that are susceptible from anthesis to grain filling stages. Infectious growth then spreads from the initial infection site to other spikelets via the rachis (3, 4). *F. graminearum* also infects barley, oat, corn, and other grain crops and is a producer of harmful mycotoxins such as deoxynivalenol and zearalenone (3, 4).

Sexual reproduction in ascomycetes is controlled by the mating type (*MAT*) locus that contains transcription factor genes (TFs) (5). In the budding yeast *Saccharomyces cerevisiae*, a model organism forming naked asci (without fruiting bodies), *MAT*-encoded TFs regulate the expression of pheromone precursor and receptor genes. Upon binding with pheromones (small peptides), Ste2 and Ste3 G protein-coupled receptors (GPCRs) activate the downstream Ste11-Ste7-Fus3/Kss1 pheromone response pathway (6). Deletion of *STE2* or *STE3* or disruption of the downstream MAPK pathways leads to mating defects and sterility (6). After mating, *S. cerevisiae* can grow by budding as diploid cells. Under nitrogen starvation conditions, diploid yeast cells undergo meiosis and form four ascospores in each ascus, which is independent of MAT TFs or pheromones/pheromone receptors (2).

In filamentous ascomycetes that form perithecia or other forms of fruiting bodies, the roles of *MAT* TF genes are different from their yeast counterparts (7). In *F. graminearum*, MAT TFs are dispensable for the initial mating processes but important for the formation and growth of dikaryotic hyphae and late stages of sexual reproduction (8, 9). Furthermore, unlike in yeast, the fusion between compatible cells (plasmogamy) is not directly followed by karyogamy in filamentous ascomycetes. Dikaryotic cells formed by mating and plasmogamy can grow inside developing ascocarps, and karyogamy occurs only in croziers that are formed at the tip of dikaryotic hyphae. In the model filamentous fungus *Neurospora crassa*, meiosis occurs in developing asci, which is followed by one round of mitosis and

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[1]M.D. and S.C. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: jinrong@purdue.edu or cjiang@nwafu.edu.cn.

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the delimitation of eight uninucleate developing ascospores (10). In *F. graminearum*, developing ascospores undergo two additional rounds of mitosis and septation to form four-celled mature ascospores with one nucleus in each compartment (9).

Similar to many other ascomycetes, *F. graminearum* has two pheromone precursor genes, *PPG1* and *PPG2* that encode pheromones, and two pheromone receptor genes, *PRE1* and *PRE2* that encode GPCRs homologous to yeast Ste2 and Ste3, respectively. The *ppg1 ppg2* and *pre1 pre2* deletion mutants are reduced in fertility but still produce perithecia and ascospores (11). Therefore, pheromones and pheromone receptors are not essential for sexual reproduction, including perithecium development and ascospore formation in *F. graminearum*, a homothallic fungus. In two other homothallic fungi, mutants deleted of a pheromone receptor gene form fewer and smaller fruiting bodies in *Aspergillus nidulans* (12), but no obvious sexual development defect is observed when one pheromone or receptor gene is deleted in *Sordaria macrospora* (13). However, in *N. crassa*, a heterothallic fungus, the pheromone receptor gene *PRE-1* is essential for mating type-specific directional growth, fusion of trichogynes, and female fertility (14). Therefore, the roles of pheromones and pheromone receptors differ among different ascomycetes that vary in ascocarp formation and mating systems. In *S. cerevisiae* that lacks dikaryotic growth, nitrogen starvation triggers meiosis and ascosporogenesis in diploid cells (15). In filamentous ascomycetes, it is not clear what triggers the development of croziers, meiosis, and ascus development and whether regulation of these processes involves special signals or signal transduction pathways in developing ascocarps. In *N. crassa*, although its exact function is not clear, mutants deleted of *GPR-1*, a non-pheromone GPCR gene, produce fewer, smaller pale protoperithecia compared to the wild type (WT) when mated as the female (16). When the *gpr-1* mutant was mated as the male, fewer mature perithecia with reduced numbers of ascospores were formed and these perithecia often have morphological defects, including deformed beaks and the absence of ostioles (16).

In a previous study on systematical characterization of the 105 GPCR genes in *F. graminearum*, we found that a total of 18 non-pheromone GPCR genes were specifically expressed or highly up-regulated during sexual reproduction (17). To determine whether these GPCRs are involved in regulating ascus development and ascospore formation, in this study, we examined the defects of their mutants in postmating/fusion processes and found that one of them, FG4G26040 (=FGREES_17256) was essential for meiosis and ascosporogenesis (named *GIA1* for GPCR indispensable for ascosporogenesis 1). *GIA1* was specifically expressed during sexual reproduction and the *gia1* mutant had no other defects but was blocked in meiosis and ascosporogenesis after karyogamy, which could be partially suppressed by dominant active mutations in *GPA1*, *FST7*, or its orthologs from *Fusarium verticillioides* or *N. crassa*. *GIA1* has a paralog in *F. graminearum* that differs from *GIA1* in expression patterns, intracellular signaling, and functions during sexual reproduction. Domain swapping analysis with these two paralogs showed that the N-terminal (NT) region and extracellular loop 3 of Gia1 are responsible for binding with its stage-specific ligand, likely a peptide or protein enriched in developing perithecia. Taken together, these results showed that *F. graminearum* has two non-pheromone, paralogous GPCRs that are specifically required for perithecium formation and ascus/ascospore development, two different stages of sexual reproduction.

Results

***GIA1* Is Essential for Ascus and Ascospore Formation.** When mating cultures of mutants deleted of the 18 GPCR genes that had stage-specific expression or upregulation during sexual reproduction (17) were examined at 8 days postfertilization (dpf), all but two of them were normal in sexual development, with ascospore cirrhi released from the ostiole of mature perithecia, representing the formation of mature asci and release of ascospores (*SI Appendix*, Fig. S1 *A* and *B*). Whereas the FG3G08120 (named *GIP1*, for GPCR indispensable for perithecium formation 1) deletion mutant was blocked in perithecium formation as described in the earlier report (17), mutant strains deleted of FG4G26040 (named *GIA1* for GPCR indispensable for ascosporogenesis 1) formed morphologically normal perithecia but failed to produce asci or ascospores (Fig. 1*A* and *SI Appendix*, Fig. S1*B*). The *gia1* mutants were normal in vegetative growth, conidiation, conidium morphology, and plant infection (*SI Appendix*, Fig. S2 *A–D*). For complementation assays, we transformed the full-length *GIA1* gene into the *gia1* mutant. The resulting *gia1*/*GIA1* transformants produced perithecia with mature asci and were normal in ascospore discharge (Asc discharge) (Fig. 1*A*), indicating a full complementation. Based on published RNA-seq data (18), *GIA1* was not expressed in vegetative hyphae or infected plant tissues (Fig. 1*B*), which is consistent with its function during ascus development and ascospore formation in *F. graminearum*.

The *gia1* Mutant Is Blocked in Meiosis in Developing Asci. To further characterize its defect in sexual reproduction, perithecia formed by the *gia1* mutant were examined at 3-, 5-, and 7-dpf. In developing perithecia at 3-dpf, the *gia1* mutant and the WT PH-1 were similar in the initial growth of ascogenous tissues (Fig. 1*C*). Crozier formation was observed in the *gia1* mutant at 3.5-dpf (Fig. 1*D*). Perithecia formed by the *gia1* mutant also were normal in development and had typical ostioles (Fig. 1*C*). However, fertile asci with ascospores were observed only in perithecia formed by PH-1 at 5- and 7-dpf (Fig. 1*D*). The *gia1* mutant formed only a few developing ascus-like structures with limited elongation that lacked ascospores at 7-dpf (Fig. 1*D*). Even at 14- and 28-dpf, the *gia1* mutant failed to produce mature asci with ascospores (*SI Appendix*, Fig. S3).

When ascogenous tissues were stained with Hoechst 33258, dikaryotic croziers were observed in both the WT and *gia1* mutant at 3.5-dpf (Fig. 1*E*). At 4-dpf, a single nucleus with stronger fluorescence signals was observed in some croziers, indicating the occurrence of karyogamy in both strains. However, significant elongation of developing asci and meiosis in elongated asci were observed only in the WT at or after 5-dpf (Fig. 1*E*). In the *gia1* mutant, ascus mother cells with one diploid nucleus were only slightly elongated at 5-dpf. Although developing asci elongated over time, meiosis never occurred and only a single nucleus was observed in elongated developing asci or ascus-like structures, even at 28-dpf (Fig. 1*E* and *SI Appendix*, Fig. S3). When quantified, in comparison with the eight haploid nuclei in the WT asci, nuclei in elongated developing asci of the *gia1* mutant were approximately twofold larger in fluorescent areas stained with Hoechst 33258 (Fig. 1*F*), indicating the diploid status of these *gia1* nuclei. Therefore, although crozier formation and karyogamy were normal, meiosis was blocked in the *gia1* mutant. These results showed that signaling via *GIA1* inside perithecia is required for meiosis in developing asci in *F. graminearum*.

The *gia1* Mutant Is Normal in Male and Female Fertility. Whereas deletion of *PRE1* had no detectable phenotype, the *pre2* deletion mutant was reduced in fertility in selfing or female fertility in outcrossing (11). To determine whether deletion of *GIA1* affects male or female fertility, we crossed the *gia1* mutant as the female and male with H1-GFP transformants of PH-1 and Δ*mat1-1-1* mutant (Dataset S1), respectively. The *gia1* mutant had no obvious reduction

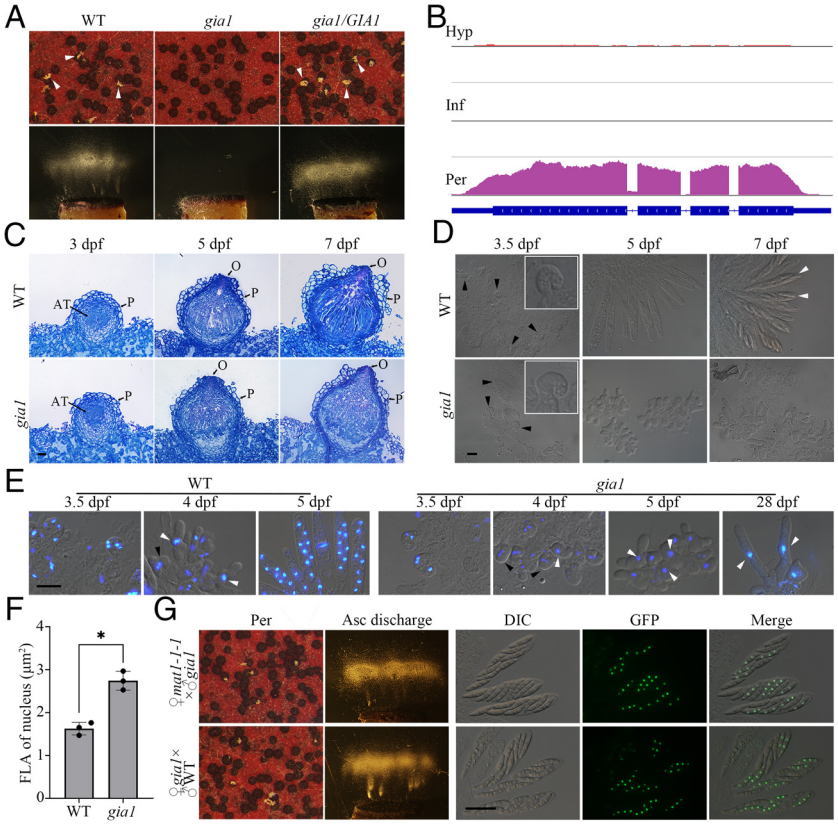

Fig. 1. Stage-specific function and expression of *GIA1* during sexual reproduction. (*A*) Mating cultures of the WT, *gia1* mutant, and *gia1/GIA1* complemented transformant were examined for ascospore cirrhi (white arrowheads) on perithecia (*Upper*) and assayed for Asc discharge (*Lower*) at 8 dpf. (*B*) IGV-Sashimi plots showing the read coverage of *GIA1* in published RNA-seq data of vegetative hyphae (Hyp), infected wheat heads (Inf), and perithecia (Per) with a diagram of its predicted ORF at the *Bottom*. (*C*) Semithin sections of perithecia harvested at marked time points were examined for the development of peridia (P), ostioles (O), and ascogenous tissues (AT) after staining with 0.5% toluidine blue. (*D*) Perithecia formed by the WT and *gia1* mutant were examined for croziers (black arrowheads) and ascus development. Mature asci with ascospores (white arrowheads) were not observed in the mutant. (*E*) Ascogenous tissues were stained with Hoechst 33258 and examined by DIC and epifluorescence microscopy. Croziers with two nuclei before karyogamy are marked with black arrowheads. White arrowheads point to ascus mother cells and elongated asci with a single nucleus. (*F*) Fluorescent Labeling Area (FLA) of nuclei inside developing asci of the WT and *gia1* mutant at 5-dpf were measured with the Fiji plugin 3D Object Counter. The average FLA was approximately twofold higher in the mutant. Mean and SD were estimated with data from three (n = 3) independent experiments (marked with black dots on the bars). The asterisk indicates significant differences based on Student's t test ($P < 0.05$). (*G*) Mating cultures of the *gia1* mutant crossed with *mat1-1-1* H1-GFP or WT H1-GFP were examined for perithecia (Per), Asc discharge, and GFP signals in ascospores at 8-dpf. H1-GFP signals were examined by epifluorescence microscopy and merged with DIC images. In both crosses, 1:1 segregation of H1-GFP signals in the nucleus was observed. Bar, 20 μm in all the panels.

in the number of perithecia formed in these crosses (Fig. 1*G*), further indicating that *GIA1* is dispensable for perithecium formation. Perithecia from these *gia1* × *GIA1* crosses formed mature asci with eight four-celled ascospores that segregated 1:1 for H1-GFP signals (Fig. 1*G*). Ascospore morphology and release (Fig. 1*G*) as well as ascospore germination were normal (*SI Appendix*, Fig. S2*E*). These results indicate that *GIA1* is dispensable for male and female fertility and gene dosage does not affect meiosis and ascosporogenesis in the *gia1/GIA1* dikaryotic or diploid cells. Furthermore, because all the ascospores from these outcrosses (1:1 segregation for *gia1*:*GIA1*) were normal in morphology and germination, *GIA1* must be dispensable for the postascospore delimitation events, confirming its specific function in meiosis and ascosporogenesis.

Gpmk1 MAP Kinase Functions Downstream from *GIA1*. Although Gia1 has the typical GPCR structural elements, to determine its functional relationship with trimeric G-proteins, we generated the $GPA1^{DA}$ allele with the R178C mutation (19) and transformed it into the *gia1* mutant because *GPA1* is the only Gα gene essential for sexual reproduction in *F. graminearum* (20). The resulting *gia1*/$GPA1^{DA}$ transformants had no obvious defects in growth, conidiation, or conidium morphology, and produced fertile perithecia with asci and ascospores (Fig. 2*A* and *SI Appendix*, Fig. S4 *A* and *B*). Although the number of asci and ascospores per perithecium was reduced, ascospore cirrhi were observed in the *gia1*/$GPA1^{DA}$ transformants (Fig. 2*A*). These results indicate that expression of $GPA1^{DA}$ in the *gia1* mutant partially rescued its defect in ascus development and ascospore formation.

G-protein signaling is known to function upstream MAP kinase pathways. In *F. graminearum*, all three MAP kinase pathways are important for sexual reproduction. For each MAP kinase cascade, mutants deleted of the MAPK, MEK, or MAP kinase kinase kinase (MEKK) genes have the same phenotype. It is a common practice to introduce dominant active mutations to the MEK genes in fungal studies (21). To determine which MAPK functions downstream from Gia1, we generated dominant active alleles of *FST7*, *FgMKK2*, and *FgPBS2* that are orthologous to yeast *STE7, MKK2,* and *PBS2* MEK genes, respectively (22–24), and transformed them into the *gia1* mutant. The resulting transformants expressing the $FST7^{DA}$, $FgMKK2^{DA}$, and $FgPBS2^{DA}$ alleles (Dataset S1) were increased in the phosphorylation of Gpmk1, Mgv1, and FgHog1, respectively, compared to the WT and *gia1* mutant (Fig. 2 *B–D*), confirming the effects of these dominant active mutations in the activation of MAPKs. All these transformants were normal in vegetative growth, conidiation, conidium morphology, and produced melanized perithecia (Fig. 2*A* and *SI Appendix*, Fig. S4 *A* and *B*). However, whereas perithecia formed by the $FgPBS2^{DA}$ and $FgMKK2^{DA}$ transformants were sterile and lacked asci or ascospores, the *gia1*/$FST7^{DA}$

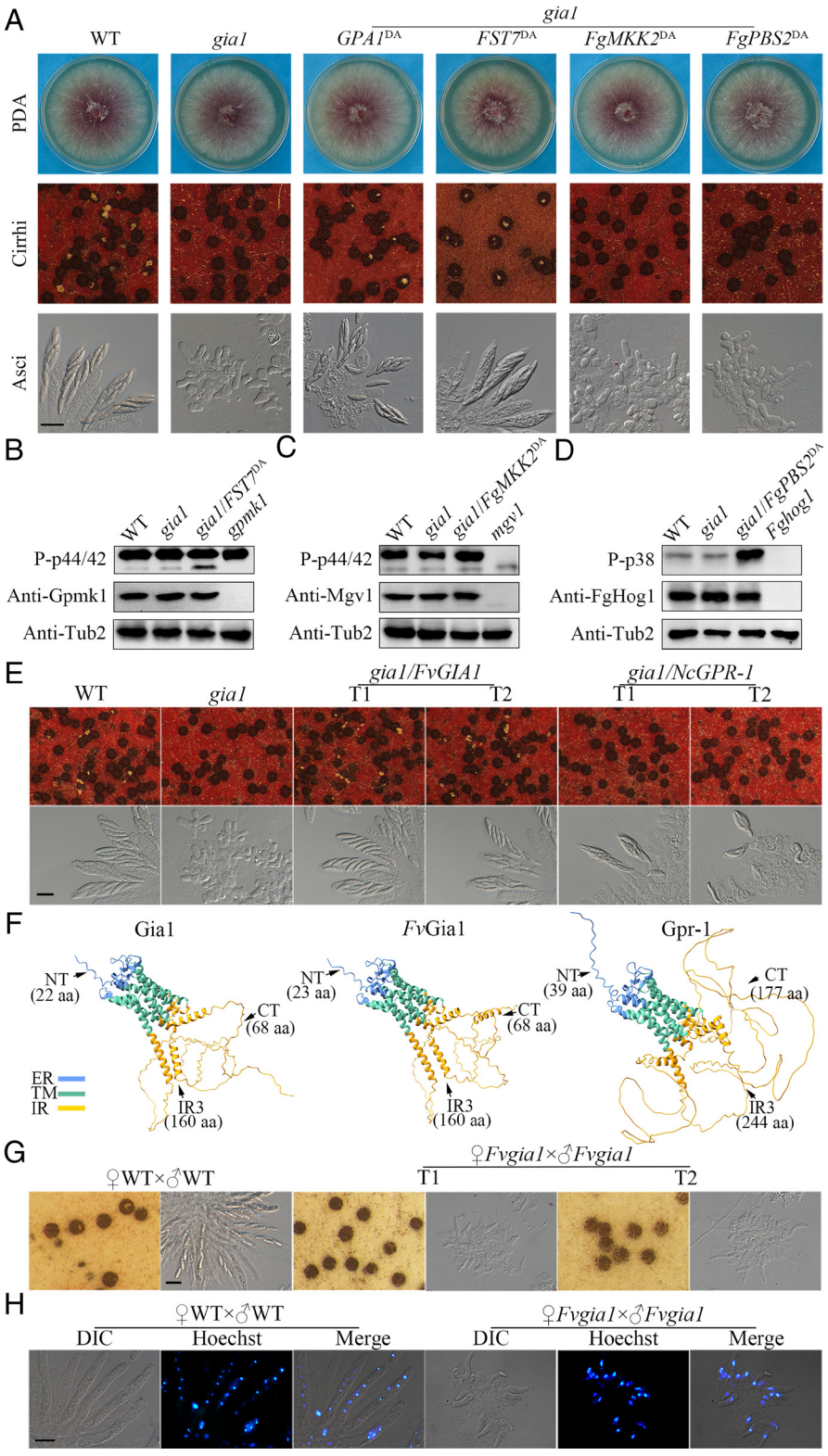

Fig. 2. Downstream signaling of Gia1 and conserved functions of its orthologs. (*A*) Cultures of the WT, *gia1* mutant, and transformants of *gia1* expressing the marked dominant active alleles of *GPA1*, *FST7*, *FgMKK2*, and *FgPBS2* were examined for colony growth on PDA, ascospore cirrhi, and ascus development. (*B*) Western blots of proteins isolated from vegetative hyphae of the WT, *gia1* mutant, *gia1/FST7*^{DA} transformant, and *gpmk1* mutant were detected with an anti-TpEY (*Top*), anti-Gpmk1 (*Middle*) or anti-Tubulin2 antibody (*Lower*). (*C*) Western blots of proteins isolated from vegetative hyphae of the WT, *gia1* mutant, *gia1/FgMKK2*^{DA} transformant, and *mgv1* mutant were detected with an anti-TpEY (*Top*), anti-Mgv1 (*Middle*) or anti-Tubulin2 antibody (*Lower*). (*D*) Western blots of proteins isolated from vegetative hyphae of the WT, *gia1* mutant, *gia1/FgPBS2*^{DA} transformant, and *Fghog1* mutant were detected with an anti-TpGY (*Top*), anti-FgHog1 (*Middle*) or anti-Tubulin2 antibody (*Lower*). (*E*) Mating cultures of the WT, *gia1* mutant of *F. graminearum*, *gia1/FvGIA1* transformants, and *gia1/NcGPR-1* transformants were examined for the production of ascospore cirrhi (*Upper*) and development of asci/ascospores (*Lower*). (*F*) Tertiary structures of Gia1, FvGia1 of *F. verticillioides*, and Gpr-1 of *N. crassa* predicted with AlphaFold v2 and visualized using the UCSF Chimera tool. Blue, green, and yellow areas indicate ERs, transmembrane regions (TM), and intracellular regions (IR), respectively. The NT and CT regions and third intracellular ring (IR3) are marked with arrows. (*G*) Mating cultures of the WT and *Fvgia1* mutant strains of *F. verticillioides*, a heterothallic fungus, were examined for perithecium formation (*Left*) and ascus development (*Right*) at 14-dpf. (*H*) Asci of the marked crosses of *F. verticillioides* stained with Hoechst 33258 were examined by DIC and epifluorescence microscopy. Bar, 20 μm in all the panels.

transformants produced asci with eight ascospores (Fig. 2*A*). Although the number of asci was reduced, ascospore cirrhi were observed in the *gia1*/*FST7*^{DA} transformants, indicating that expression of *FST7*^{DA}, but not *FgPBS2*^{DA} or *FgMKK2*^{DA}, partially rescued the *gia1* mutant. Therefore, the Gpmk1 MAPK pathway functions downstream from Gia1 in *F. graminearum*. The recognition of the meiosis-specific ligand (functioning as a meiogen inside developing perithecia) by Gia1 GPCR results in the activation of Gpa1, which then activates the Gpmk1 MAPK cascade to regulate ascus development and initiate meiosis in developing asci.

GIA1 Orthologs Have a Conserved Role in Meiosis and Ascus Development. To determine whether orthologs of *GIA1* have a conserved role in sexual reproduction, we first cloned its ortholog from *F. verticillioides*, a heterothallic fungus closely related to *F. graminearum*, behind the *GIA1* promoter and transformed the resulting P$_{GIA1}$-*FvGIA1* construct into the *gia1* mutant. The resulting *gia1*/*FvGIA1* transformants (Dataset S1), similar to the WT, produced ascospore cirrhi and formed asci with eight ascospores (Fig. 2*E*), indicating that *FvGIA1* fully complemented the *gia1* mutant. We also amplified *GPR-1* from *N. crassa* (16) and generated the P$_{GIA1}$-*GPR-1* construct. In the *gia1*/P$_{GIA1}$-*GPR-1* transformants, although many asci were aborted and no ascospore cirrhi was formed, approximately 10% asci with eight ascospores were observed (Fig. 2*E*), indicating partial complementation of *gia1* by *GPR-1*. Based on predicted tertiary structures, FvGia1 and Gia1 are highly similar but Gpr-1 differs significantly with them in the length of the N- and C-terminal (CT) regions as well as the third intracellular ring (Fig. 2*F*).

To further verify the conserved function of *GIA1* during sexual reproduction, we generated mutants deleted of *FvGIA1* in both mating types of *F. verticillioides* (Dataset S1). In the *Fvgia1 MAT1-1* × *Fvgia1 MAT1-2* crosses, darkly pigmented perithecia were formed but asci or ascospores were not observed (Fig. 2*G*). In perithecia examined at 8-dpf, whereas the WT had four or eight nuclei in elongated developing asci, only a single nucleus was observed in developing asci with limited elongation in the *Fvgia1* × *Fvgia1* cross (Fig. 2*H*). These results indicate that Gia1 orthologs have a conserved role in meiosis and ascosporogenesis in both homothallic and heterothallic Sordariomycetes.

The Paralog of *GIA1* Is Essential for Perithecium Formation in *F. graminearum*. Phylogenetic analysis with Gia1 homologs showed that *F. graminearum* and other Hypocreales species have a paralog of *GIA1*. However, almost all other non-Hypocreales species in Sordariomycetes (except two Coniochaetales species) have only one *GIA1* homolog (Fig. 3*A*). In *F. graminearum*, the paralog of *GIA1* is FG3G08120, the non-pheromone GPCR that has been reported to be essential for perithecium formation (17) and named *GIP1*. The *gip1* mutant was normal in vegetative growth, conidium morphology, and plant infection, indicating that the function of *GIP1* is sexual-specific (*SI Appendix*, Fig. S5 *A*–*D*). Among all the GPCRs in *F. graminearum*, Gip1 shares the highest amino acid sequence identity (41.1%) with Gia1. When the full-length *GIP1* gene was reintroduced into the *gip1* mutant, the *gip1*/*GIP1* complementation transformants were normal in perithecium formation and ascospore development (*SI Appendix*, Fig. S5*A*). Because the *mat1-1-1* deletion mutant formed small, melanized perithecia that lack asci or ascospores (8), Gip1 functions in earlier stages than *MAT1-1-1* during sexual reproduction in *F. graminearum*.

We also generated mutants deleted of *FvGIP1* in both mating types of *F. verticillioides* (Dataset S1). The *Fvgip1* × *Fvgip1* cross failed to form perithecia, confirming the conserved function of Gip1 orthologs in perithecium formation in heterothallic or homothallic species (*SI Appendix*, Fig. S5*E*). Examination of the 20-kb upstream and downstream sequences of *GIA1* or *GIP1* orthologs indicated that the flanking genes of the *GIP1* orthologs are highly conserved in *Fusarium* species but are completely different from flanking genes of *GIA1*, and vice versa. In comparison with *N. crassa* and *S. macrospora*, none of the flanking genes of *GPR-1* are present in the flanking sequences of *GIP1*. However, three of them are present in the flanking regions of *GIA1* in *F. graminearum* and *F. verticillioides* (*SI Appendix*, Fig. S6). Therefore, *GIP1* is a paralog of *GIA1* with diverged functions in *F. graminearum* and other Hypocreales species.

Divergence between *GIA1* and *GIP1* in Expression and Stage-Specific Functions during Sexual Reproduction. Although *GIA1* and *GIP1* were specifically expressed during sexual reproduction, *GIA1* differed from *GIP1* in expression patterns (Fig. 3*B* and *SI Appendix*, Fig. S7*A*). Expression of *GIP1* rapidly increased upon fertilization and became detectable at 1-dpf. Its transcript abundance peaked at 3-dpf and decreased rapidly afterward. In contrast, *GIA1* transcripts were rare at 1-dpf but rapidly increased after 3-dpf. Its transcript abundance peaked at 5-dpf and decreased rapidly afterward (Fig. 3*B*). To determine the effects of different expression patterns on their functions, we generated the P$_{GIA1}$-*GIP1* construct and transformed it into the *gia1* mutant. The *gia1*/P$_{GIA1}$-*GIP1* transformants formed perithecia but failed to form asci and ascospores (Fig. 3 *C* and *D*), indicating that expression of *GIP1* under the control of *GIA1* promoter failed to complement the *gia1* mutant. Similarly, we found that the expression of *GIA1* under the control of *GIP1* failed to complement the *gip1* mutant (Fig. 3 *C* and *E*). Therefore, the difference in their expression is not directly responsible for functional divergence between *GIA1* and *GIP1*.

We then generated the P$_{GIP1}$-*GPR-1* construct and transformed it into the *gip1* mutant. The resulting *gip1*/P$_{GIP1}$-*GPR-1* transformants formed mature perithecia with asci and ascospores (Fig. 3*E*), indicating that *GPR-1* of *N. crassa* could complement the *gip1* mutant. Because *GPR-1* also partially complemented the *gia1* mutant (Fig. 2*E*), it is likely that Gpr-1 has the functions of both Gia1 and Gip1 in *N. crassa*. Interestingly, although transcripts of *GPR-1* were almost undetectable in vegetative hyphae, its expression level rapidly increased upon fertilization at 1-dpf, decreased after that, but increased again after 4-dpf, and remained high even at 7-dpf in *N. crassa* (*SI Appendix*, Fig. S7*B*) based on published RNA-seq data (25). In *Chaetomium globosum*, another non-Hypocreales species in Sordariomycetes, the *GPR-1* ortholog had similar expression profiles with two peaks (26). Two peaks of *GPR-1* expression (1- and 7-dpf) may be related to its functions for recognizing stage-specific factors to regulate perithecium formation and ascus/ascospore development in *N. crassa*, which correspond to specific functions of *GIP1* and *GIA1* in *F. graminearum*.

***GIA1* and *GIP1* Differ in Downstream Signaling Pathways.** Because expression of *FST7*^{DA} partially rescued the *gia1* mutant, to determine its effect on the *gip1* mutant, we generated the *gip1*/*FST7*^{DA} transformants. Unlike the *gia1*/*FST7*^{DA} transformant, expression of *FST7*^{DA} failed to rescue the defects of the *gip1* mutant in perithecium formation (Fig. 3*F*) although the phosphorylation level of Gpmk1 was increased (Fig. 3*G*). We then transformed the *GPA1*^{DA} allele into the *gip1* mutant. The *gip1*/*GPA1*^{DA} transformants also increased the phosphorylation level of Gpmk1, but failed to form perithecia on mating plates (Fig. 3 *F* and *G*), indicating that the expression of *GPA1*^{DA} or activation of the Gpmk1 pathway is

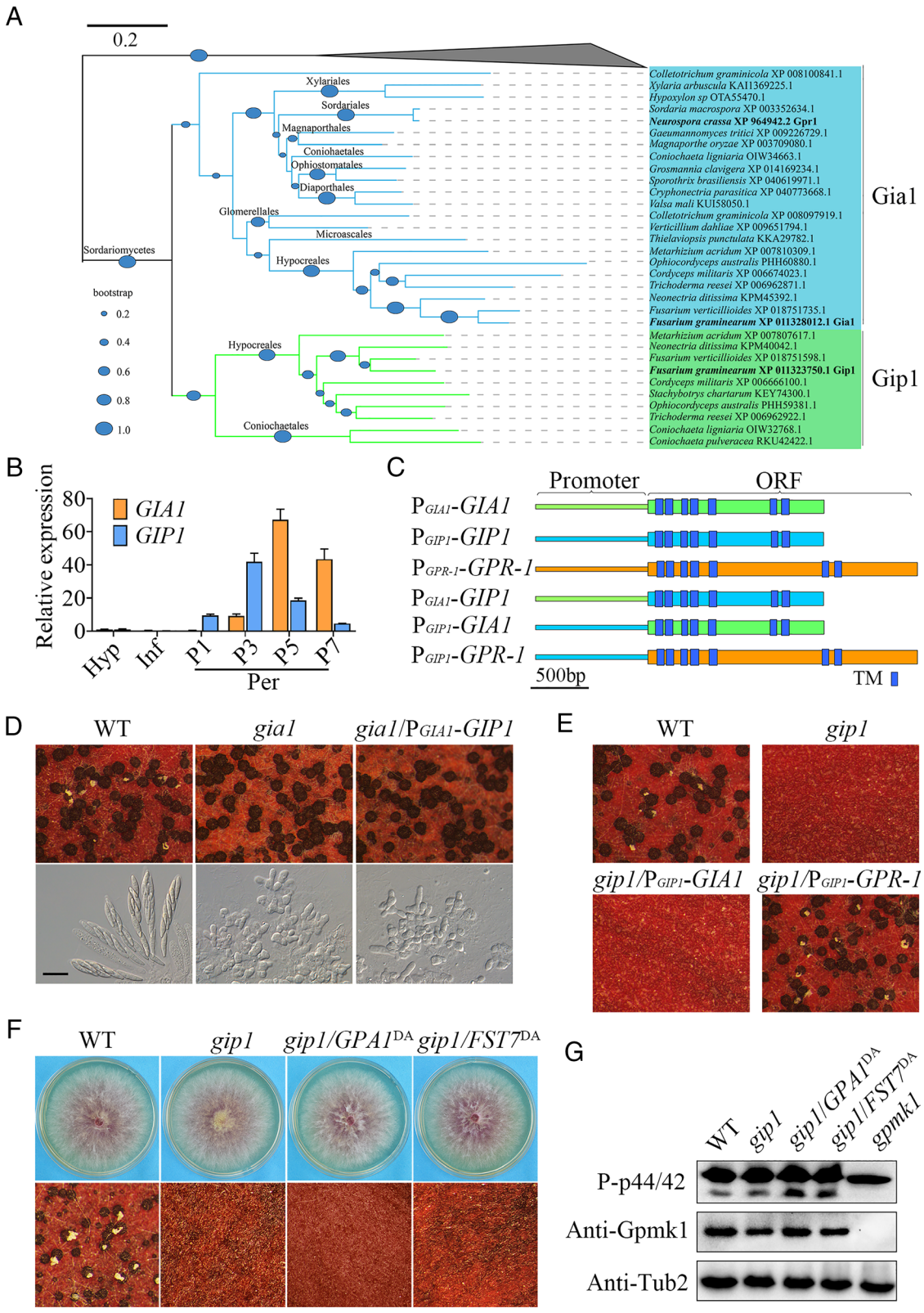

Fig. 3. Evolutionary relationship and functional divergence between Gia1 and its paralog. (*A*) A phylogenetic tree of the homologs of Gia1 and Gip1 from marked species was generated with the neighbor-joining method using MEGA version 6.0. The size of blue dots represents the percentage of their occurrence in 1,000 bootstrap replicates. The numbers behind the species names are GenBank accession numbers. (*B*) Relative expression levels of *GIA1* and *GIP1* were assayed by qRT-PCR with RNA isolated from vegetative hyphae (Hyp), infected wheat heads sampled at 3 dpi (Inf), and mating cultures sampled at 1-, 3-, 5-, and 7-dpf (P1, P3, P5, and P7). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as the internal control. Mean and SD were estimated with results from three ($n = 3$) independent replicates. (*C*) Schematic drawing of the P_{GIA1}-*GIA1*, P_{GIP1}-*GIP1*, P_{GPR-1}-*GPR-1*, P_{GIA1}-*GIP1*, P_{GIP1}-*GIA1*, and P_{GIA1}-*GPR-1* constructs. Blue boxes indicate transmembrane regions (TM). (*D*) Mating cultures of the WT, *gia1* mutant, and *gia1*/P_{GIA1}-*GIP1* transformant were examined for ascospore cirrhi (*Upper*) and ascus development (*Lower*). Bar, 20 μm. (*E*) Mating cultures of the WT, *gip1* mutant, *gip1*/P_{GIP1}-*GIA1*, and *gip1*/P_{GIP1}-*GPR-1* transformants were examined for perithecium formation. (*F*) Cultures of the WT, *gip1* mutant, *gip1/GPA1*^{DA}, and *gip1/FST7*^{DA} transformants were examined for colony growth (*Upper*) and perithecium formation (*Lower*). (*G*) Western blots of proteins isolated from vegetative hyphae of the WT, *gip1*, *gip1/GPA1*^{DA}, *gip1/FST7*^{DA}, and *gpmk1* strains were detected with an anti-TpEY (*Top*), anti-Gpmk1 (*Middle*) or anti-Tubulin2 antibody (*Lower*).

not sufficient to rescue the *gip1* mutant. Therefore, *GIA1* and *GIP1* must have different downstream signal transduction pathways in *F. graminearum*. Because binding of GPCRs with ligands results in the dissociation of Gα with Gβγ subunits that can activate downstream cAMP-PKA and MAPK pathways in yeast (27), rescue of the *gia1* but not the *gip1* mutant by $GPA1^{DA}$ suggests that Gβγ or other Gα subunits may play important roles in activating downstream signaling pathways of Gip1.

The CT Tail Plays a More Important Role in Gia1-Specific Intracellular Signaling. Both Gia1 and Gip1 proteins have typical GPCR structural features, including an extracellular NT region, seven TMs (1 to 7), three extracellular loops (EL1 to EL3), and three intracellular rings (IR1 to IR3), and a cytoplasmic CT tail (28). Because of their differences in downstream signaling, we examined the functional differences of the CT and IR3 regions between Gia1 and Gip1. The $GIA1^{CTp}$ and $GIA1^{IR3p}$ chimeric alleles with the CT and IR3 regions of *GIA1* replaced with corresponding regions of *GIP1* (Fig. 4*A*) were generated and transformed into the *gia1* mutant. Both *gia1*/$GIA1^{IR3p}$ and *gia1*/$GIA1^{CTp}$ transformants formed fertile perithecia with ascospores. In comparison with the WT, 40.2% and 15.7% asci with eight ascospores were observed in the $GIA1^{IR3p}$ transformants and $GIA1^{CTp}$ transformants, respectively (Fig. 4*B*). These results suggested that both CT and IR3 of Gia1 are involved in downstream intracellular signaling, and the CT tail plays a more important role in Gia1-specific intracellular signaling.

Sequence alignment with the CT region of Gia1 and Gip1 orthologs from closely related Hypocreales species showed that they vary significantly in amino acid sequences. Overall, neither $Gia1^{CT}$ nor $Gip1^{CT}$ is well conserved in their orthologs. However, the "RLxSRLG" motif is well conserved in Gia1 orthologs but absent in Gip1 orthologs (*SI Appendix*, Fig. S8). Based on the protein structures predicted by Alphafold v2, the RLxSRLG motif is in a region that forms an α-helix in Gip1 but random coils in Gia1 (Fig. 4*C*). In both Gia1 and Gip1, the CT region is predicted to interact with IR3 although they differ in areas involved in the CT-IR3 association (Fig. 4*C*), which may explain the difference between the *gia1*/$GIA1^{IR3p}$ and *gia1*/$GIA1^{CTp}$ transformants in ascus development and ascospore formation. IR1 and IR2 were not characterized in this study because they are short and not predicted to interact with CT.

Ligand Recognition Involves the NT Region and EL3 of Gia1. To determine which extracellular regions (ERs) are responsible for recognizing stage-specific ligands, we generated the chimeric *GIA1* alleles with its NT ($GIA1^{NTp}$) or EL1-EL3 ($GIA1^{EL1p}$, $GIA1^{EL2p}$, and $GIA1^{EL3p}$) regions replaced with corresponding regions of *GIP1* and transformed them into the *gia1* mutant (Fig. 4*A*). The $GIA1^{EL1p}$ and $GIA1^{EL2p}$ transformants were normal in perithecium formation, ascus and ascospore development (Fig. 4*D*), indicating that EL1 and EL2 of Gia1 are dispensable for ligand recognition. In contrast, the $GIA1^{NTp}$ and $GIA1^{EL3p}$ transformants were defective in ascospore cirrhus formation. Only 15.1% and 17.4% asci with eight ascospores were observed in the $GIA1^{NTp}$ and $GIA1^{EL3p}$ transformants, indicating that NT and EL3 of Gia1 may be important for ligand recognition (Fig. 4*D*). We then generated the $GIA1^{NTp\ EL3p}$ allele in which both NT and EL3 of *GIA1* were replaced with those of *GIP1*. Similar to the *gia1* mutant, the *gia1*/$GIA1^{NTp\ EL3p}$ transformants form sterile perithecia that lacked asci and ascospores (Fig. 4*D*), indicating that both NT and EL3 regions are important for Gia1 functions. In protein structures predicted with AlphaFold v2, the NT and EL3 regions of Gia1

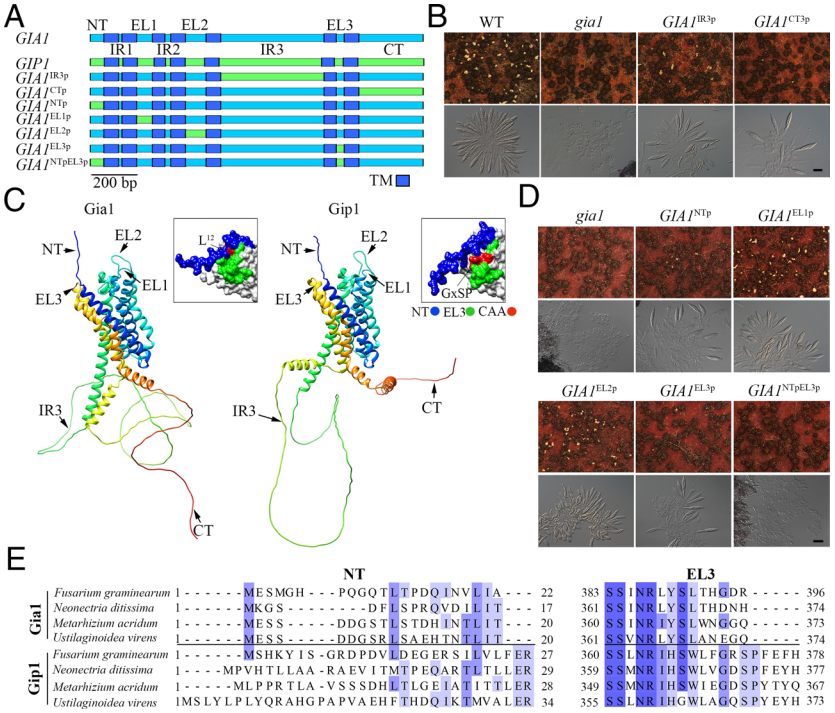

Fig. 4. Functional characterization of the intracellular and ERs of Gia1. (*A*) Schematic drawings of *GIA1, GIP1*, and marked chimeric alleles. NT and CT, NT or CT regions; EL1-3, extracellular loops; IR1-3, intracellular rings. Blue boxes indicate transmembrane regions (TM). (*B*) Mating cultures of the WT, *gia1* mutant, *gia1*/$GIA1^{IR3p}$, and *gia1*/$GIA1^{CTp}$ transformants were examined for ascospore cirrhi (*Upper*) and ascus/ascospore development (*Lower*). (*C*) Tertiary structures of Gia1 and Gip1 predicted by AlphaFold v2 and visualized using the UCSF Chimera tool. NT region, CT region, three extracellular loops (EL1-3), and the third intracellular ring (IR3) are marked with black arrows. Gray inlets indicate NT (blue area), EL3 (green area), and CAA (conserved amino acids, red area) at the interface between NT and EL3. (*D*) Mating cultures of the *gia1* mutant and its transformants expressing marked chimeric alleles were examined for ascospore cirrhi (*Upper*) and ascus/ascospore development (*Lower*). Bar, 20 μm in all the panels. (*E*) Alignments of the NT and EL3 sequences of Gia1 and Gip1 homologs. Amino acid residues shaded in dark blue, middle blue, and light blue show 100%, >50%, and > 33% identity, respectively.

are adjacent to each other, and there are topological differences between Gia1 and Gip1 in the NT-EL3 area (Fig. 4*C*), which may be responsible for binding to different ligands.

Sequence alignment showed that the amino acid sequences of NT and EL3 are not well conserved among Gia1 and Gip1 orthologs (Fig. 4*E*). For the NT region, the L12, L20, and I21 residues are conserved among Gia1 orthologs but not in Gip1 orthologs. For EL3, Gia1 and Gip1 orthologs are highly similar to each other at the region adjacent to TM6 but differ significantly in the region of TM7. Whereas the Y389, S390, and L391 residues are conserved only in Gia1 orthologs, residues I365, H366, W368, and GxSP are specifically conserved in Gip1 orthologs (Fig. 4*E*). These variations may contribute to the functional divergence between Gia1 and Gip1 in ligand recognition, particularly for residues L12 of Gia1 and GxSP of Gip1 that are located at the NT-EL3 area.

The Ligand of Gia1 Is Expressed Specifically in Developing Perithecia. Because of the similarity between the Gpmk1 MAPK and yeast pheromone response pathway, it is possible that Gia1 recognizes small peptide(s) similar to yeast mating pheromones that are cleaved by the Kex2 protease. *F. graminearum* has 11 genes predicted to encode proteins with putative Kex2-processed repeats (29, 30) and named *KEP* genes below. Seven of them, including *PPG1*, were specifically expressed or up-regulated during sexual reproduction (*SI Appendix*, Fig. S9). Because *PPG1* has been characterized (11), we generated mutants deleted of the other six *KEP* genes. Like the WT, mutants deleted of individual *KEP* genes had no defect in perithecium formation and were normal in ascus and ascospore development (Fig. 5*A*), indicating that deletion of these *KEP* genes individually has no obvious effect on sexual reproduction in *F. graminearum*.

To identify potential ligands recognized by Gia1 GPCR, we generated the chimeric yeast $STE2^{CH}$ allele with the NT ER and three extracellular loops of yeast Ste2 replaced with those of Gia1 (Fig. 5*B*). The resulting $STE2^{CH}$ allele was transformed into a yeast strain expressing the P_{FUS1}-GFP construct to generate the reporter strain YR11. We then isolated crude extracts of vegetative hyphae and 5-dpf perithecia of PH-1 as described in the materials and methods. When treated with crude extracts of perithecia, the expression of the P_{FUS1}-GFP reporter was induced in yeast cells of YR11 (Fig. 5*C*), indicating the activation of the yeast pheromone response pathway. However, GFP signals were not observed in YR11 cells treated with crude extracts of vegetative hyphae (Fig. 5*C*), suggesting that the ligand of Gia1 may be specifically expressed in developing perithecia. We then treated crude extracts with protease K for 1 h. Protease K-treated crude extracts failed to stimulate the expression of the P_{FUS1}-GFP reporter in YR11 cells (Fig. 5*C*), indicating that the ligand recognized by the chimeric Gia1-Ste2 GPCR is a peptide or protein.

With the deletion of Gia1, the ligands recognized by the Gia1 receptor may be overproduced. To test this hypothesis, we also isolated crude extracts of perithecia formed by the *gia1* mutant at 5-dpf. In comparison with those of the WT, crude extracts of *gia1* perithecia induced stronger GFP signals in yeast cells of YR11 (Fig. 5*D*). Thus, perithecia formed by the *gia1* mutant may be enriched for the Gia1 ligand. Although the ligand of Gia1 remains to be identified, our data showed that Gia1 ligand is present in developing perithecia but not in hyphae. The ligand tends to be produced by ascogenous tissues inside developing perithecia to regulate meiosis and ascus development.

Because the nature of GPCRs, the ligand recognized by Gia1 is likely to be a protein or peptide that is present on the cell surface or secreted by *F. graminearum*. Based on published RNA-seq data of the WT strain PH-1 (31), a total of 150 genes encoding proteins with a signal peptide (including secretory, transmembrane, and GPI-anchored proteins) are specially expressed during sexual reproduction (Fig. 5*E* and Dataset S2). Among them, 18 of them have orthologs in *F. verticillioides*, *N. crassa* and *Colletotrichum graminicola*, but not in *A. nidulans* and *S. cerevisiae* (Fig. 5*E*), suggesting that they may be candidate ligand genes encoding the ligand of Gia1 in *F. graminearum* and other Sordariomycetes.

Discussion

GPCRs are important for human health and the targets of over 30% of pharmaceutical compounds (32). In yeast, pheromone receptors are well characterized for their roles in regulating mating processes in response to pheromones (6). In *F. graminearum*, pheromones and pheromone receptors are not essential for perithecium formation and ascus/ascospore development (11). However, in this study we showed that Gia1, a non-pheromone GPCR, plays a stage-specific role in regulating meiosis and ascosporogenesis in *F. graminearum*. The *gia1* mutant was normal in perithecium formation and differentiation of ascogenous tissues as well as karyogamy in croziers. However, deletion of *GIA1* resulted in a never-in-meiosis defect because only a single diploid nucleus was observed in elongated, developing asci that were blocked in ascospore formation. Therefore, Gia1 GPCR likely functions as the sensor for an internal signal/ligand to regulate meiosis and ascosporogenesis. Because the *mat1-1* and *mat1-2* mutants formed smaller perithecia that lack ascogenous tissues (8) and *GIA1* expression is not affected by deletion of the *MAT* locus (33), the function of Gia1 in regulating meiosis and ascosporogenesis must be independent of the MAT TFs in *F. graminearum*.

Because of the never-in-meiosis defect of the *gia1* mutant, it is likely that deletion of *GIA1* affects the key regulator of meiosis in *F. graminearum*. However, the regulation of meiosis in ascocarp-forming filamentous ascomycetes is not well characterized but appears to differ significantly from the budding yeast. In comparison to genes known to be important for meiosis regulation in *S. cerevisiae*, *F. graminearum* lacks a distinct homolog of *IME1*, *IME4, MEI4, MEI5, RME1*, *RME2*, or *RME3* that encode the key inducers of meiosis (*IME*) and repressors of *IME1* (*RME*) in its genome (31, 34, 35). Only *IME2* has an ortholog in *F. graminearum* but an earlier study showed that *FgIME2* is dispensable for meiosis and ascospore formation (21). Therefore, the key regulators of meiosis regulated by Gia1 and its downstream Gpmk1 MAPK pathway remain to be identified and are likely to be unique to filamentous ascomycetes. Furthermore, *RME2*, *RME3,* and *IRT1* (IME1 regulatory RNA 1) in *S. cerevisiae* as well as *SME2* in *S. pombe* encode noncoding RNAs that are important for meiosis regulation (36–38). However, these yeast lncRNAs are not evolutionally conserved. Although lncRNAs with stage-specific expression during sexual development have been identified in *F. graminearum* (39), their functions in sexual reproduction remain to be characterized.

Besides *GIA1*, another non-pheromone GPCR was found to be essential for perithecium formation in *F. graminearum*. Phylogenetic and micro-synteny analyses revealed that *GIP1* is a paralog of *GIA1* in Hypocreales. In *F. verticillioides*, the *GIP1* and *GIA1* orthologs have conserved functions in perithecium formation and meiosis/ascosporogenesis, respectively. However, other Sordariomycetes have only one *GIA1* homolog, such as *GPR-1* in *N. crassa*, which partially complemented the defects of *gia1* and *gip1* mutants. Therefore, the function of *GPR-1* for

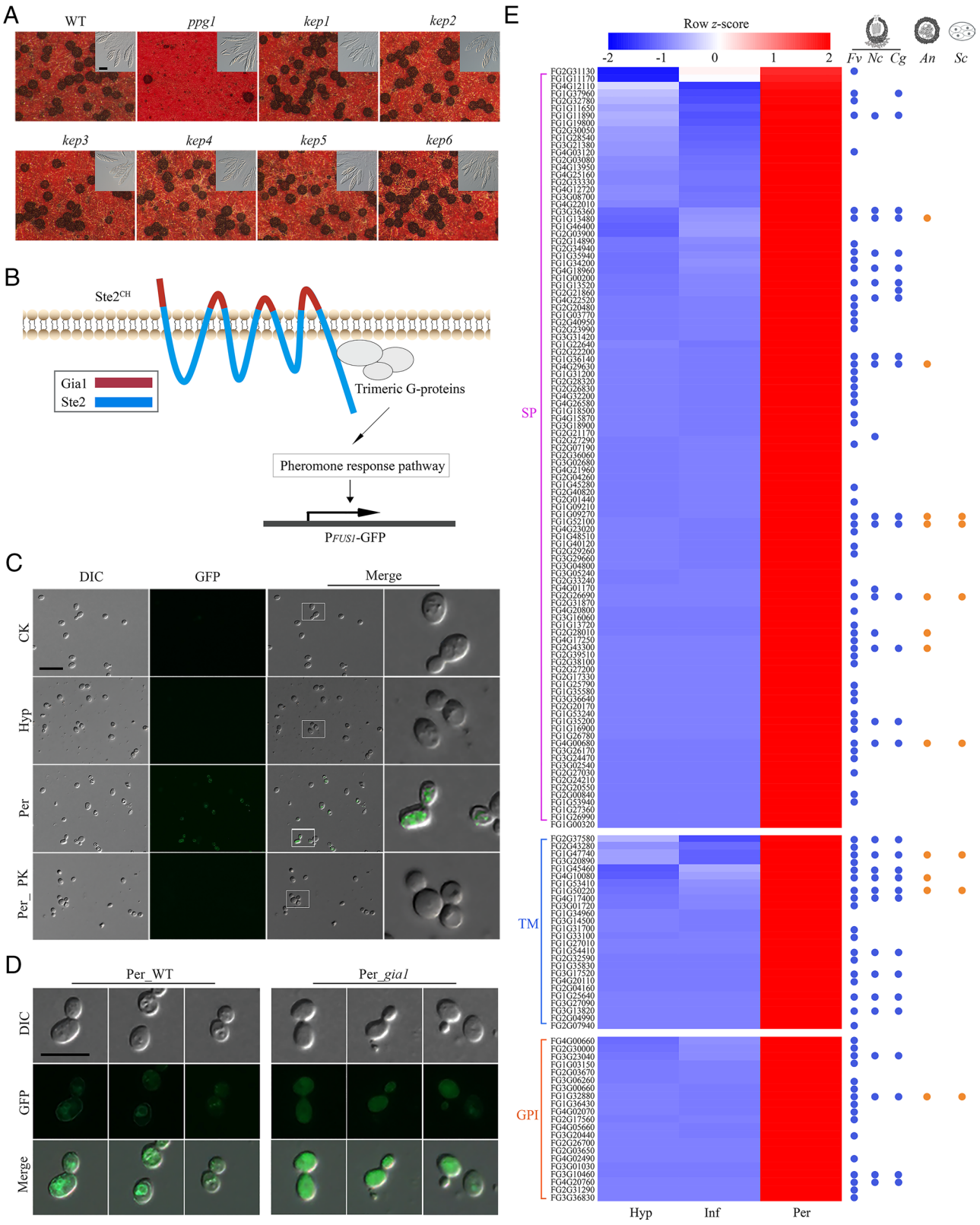

Fig. 5. Functional characterization of candidate Gia1-ligand genes and development of a yeast reporter strain for screening ligand of Gia1. (*A*) Mating cultures of the WT, *ppg1* mutant, and mutants deleted of the six *KEP* genes were examined for perithecium formation and ascus development (inlet boxes). All these mutants produced normal asci with ascospores although the *ppg1* mutant was reduced in perithecium formation. Bar, 20 μm. (*B*) A diagram of the yeast reporter strain YR11 expressing the chimeric $Ste2^{CH}$ and P_{FUS1}-GFP constructs. The NT and three extracellular loops of yeast Ste2 were replaced with those of Gia1. The endogenous *STE2* and *SST2* genes were deleted. (*C*) Yeast cells of YR11 treated with the extract buffer (CK) and crude extracts of hyphae (Hyp) or 5-dpf perithecia (Per) of the wild-type strain PH-1 were examined by DIC and epifluorescence microscopy. Crude extracts of perithecia stimulated the expression of P_{FUS1}-GFP, which was abolished by treatments with Proteinase K (Per_PK). Bar, 20 μm. (*D*) Yeast cells of YR11 were treated with extracts of 5-dpf perithecia of the WT (Per_WT) and *gia1* mutant (Per_*gia1*). Stronger GFP signals in yeast cells were observed when treated with the crude extracts of the *gia1* mutant (Per_*gia1*). Bar, 10 μm. (*E*) Heat maps showing the expression patterns of 150 genes encoding secretory proteins (SP), proteins with signal peptide and TM domain (TM) or GPI anchor (GPI) in vegetative hyphae (Hyp), infected wheat heads (Inf), and perithecia (Per). Blue and orange circles represent their homologs in the marked Sordariomycetes and non-Sordariomycetes species, respectively. *F. verticillioides* (*Fv*), *N. crassa* (*Nc*), *C. graminicola* (*Cg*), *A. nidulans* (*An*), and *S. cerevisiae* (*Sc*).

both perithecium formation and meiosis/ascosporogenesis likely represents the ancestral state of *GIP1* and *GIA1* before gene duplication. Interestingly, approximately 50% of the *GPR-1* transcripts had a missense A-to-I editing event resulting in the R693G change in the CT tail, which may contribute to Gip1- and Gia1-like functions of Gpr1 proteins during sexual reproduction. In *N. crassa*, the *gpr-1* deletion mutant is reduced in protoperithecium formation and the *gpr-1* (♀) × *GPR-1* (♂) cross produces morphologically abnormal perithecia (lacking ostioles) with fewer ascospores (16). Although it remains to be tested, the *gpr-1* × *gpr-1* cross will likely be blocked in perithecium formation. It is also worth noting that although Gpr-1 was annotated as a cAMP-receptor like GPCR, orthologs of Gia1 or Gpr-1 are unique to Sordariomycetes. In Sordariomycetes, *GPR-1* orthologs may be required to recognize stage-specific signals to regulate the formation of peridium and ostioles in coordination with the differentiation of ascogenous tissues into mature asci with ascospores. In other fungi that lack a distinct homolog of Gia1/Gip1, meiosis and sexual spore development may be regulated by other mechanisms, such as nitrogen starvation in the budding yeast. Several studies documented the involvement of non-pheromone GPCRs in sexual reproduction, such as the GprD/GprH/GprI/GprM nutrient sensing system of *A. nidulans* involved in primary metabolism, hyphal growth, and suppressing sexual development (40, 41) and CnGpr4 of *Cryptococcus neoformans* important for methionine-induced mating, cell fusion, and capsule formation (42). However, none of these non-pheromone GPCRs are similar to *GIA1* that is specifically expressed during sexual reproduction to regulate meiosis or sexual spore development.

The NT and EL3 regions of Gia1 are essential for ligand recognition because the *gia1*/*GIA1*$^{NTp\ EL3p}$ transformants had similar defects with the *gia1* mutant. Consistent with this observation, the NT and EL3 of Gia1 are predicted by AlphaFold v2 to be adjacent to each other. Gia1 also has topological differences with Gip1 in the NT-EL3 area of predicted protein structures. However, overall, the amino acid sequences of NT and EL3 are not well conserved among Gia1 orthologs. Nevertheless, residue L12 of Gia1 that is conserved in its orthologs and located at the NT-EL3 interface may contribute to ligand binding specificity. Because *GPR-1* of *N. crassa* partially complemented both *gia1* and *gip1* mutants, its NT and EL3 regions must have structural features and ligand recognition abilities similar to those of both Gia1 and Gip1. Structural prediction showed that Gpr-1 has topological differences with both Gia1 and Gip1 in the NT-EL3 area. We noticed that S390 of EL3 are conserved among Gia1, Gip1, and Gpr-1. In the EL3 region, L391 of Gia1 and H366 of Gip1 are conserved in Gpr-1. These residues may play a role in their differences in ligand recognition although they are not in the predicted NT-EL3 interface.

Because expressing the *GPA1*^{DA} allele partially rescued the *gia1* mutant but had no effect in the *gip1* mutant although Gia1 and Gip1 are paralogous GPCRs, other components of the trimeric G-proteins (other than *GPA1*) must be involved in relaying signals from Gip1 in *F. graminearum*, which has three Gα, one Gβ, and one Gγ genes. Among the mutants deleted of individual Gα genes (*GPA1, GPA2,* and *GPA3*), only the *gpa1* mutant is defective in sexual reproduction (20). One possibility is that the Gβγ heterodimer, instead of Gpa1, is responsible for relaying signals recognized by Gip1 to downstream signaling pathways. In the budding yeast, after its disassociation with Gα, the Gβγ heterodimer interacts with Ste20 and Ste5 to activate the downstream MAPK pathway (43). Another possibility is that *GPA2* and *GPA3* may have overlapping functions in relaying signals from Gip1 for perithecium formation. Although the *gpa2* and *gpa3* mutants are normal in sexual reproduction (20), it remains possible that the *gpa2 gpa3* double mutant is defective in perithecium formation.

Although Gia1 functions upstream from Gpa1 and the Gpmk1 MAPK cascade in *F. graminearum*, mutants deleted of *GPA1, GPMK1*, or its upstream MEK and MEKK genes, unlike the *gia1* mutant, have pleiotropic defects and are blocked in perithecium formation (20, 21). Therefore, Gpa1 and the Gpmk1 pathway must be involved in responses to signals recognized by other upstream receptors (other than Gia1). Although the activation of Gpmk1 may be dynamic, constitutive active mutations in *FST7* will result in the hyperactivation of Gpmk1 in hyphae, perithecia, and other stages. The defect of the *gia1* mutant in meiosis and ascosporogenesis could be partially rescued by the expression of *FST7*^{DA} and stimulation of Gpmk1 activation. The Gpmk1 MAP kinase pathway is well conserved in fungi and has been implicated in regulating various growth and developmental processes, including mating and female fertility (44). However, its regulatory role in meiosis has not been reported in fungi. In higher eukaryotes, MAP kinase pathways have been implicated in regulating various processes of meiosis. For examples, in mammalian oocytes, MAPKs not only promote arrest at metaphase II but also play compensatory roles with Cdk1 during prometaphase I to restrain APC/C activity for the completion of meiosis I (45). In *Xenopus*, a MAP kinase pathway is required during the onset of meiosis II although it is not essential for entry into meiosis I (46). In *Caenorhabditis elegans*, the conserved RAS/ERK MAP kinase pathway regulates the disassembly of the synaptonemal complex (SC) for proper chromosome segregation during meiosis (47). Nevertheless, none of these studies showed the regulation of meiosis by a MAP kinase pathway similar to the role of Gpmk1 observed in *F. graminearum* in this study.

With typical GPCR structural elements, Gia1 likely recognizes a stage-specific ligand(s) to regulate meiosis and ascosporogenesis via the Gpmk1 pathway. Progesterone is known to induce entry to meiosis in *Xenopus* (46) and some membrane progesterone receptors are GPCR proteins or have GPCR characteristics in mammals. In mouse and rat oocytes, two GPCRs are involved in maintaining meiotic arrest via the cAMP-PKA pathway but their ligands are not clear (48). In this study, we showed that crude extracts of developing perithecia but not those of vegetative hyphae contained the ligand(s) recognized by a chimeric Gia1-Ste2 protein in yeast. Furthermore, stimulating activities of perithecium extracts were higher in the *gia1* mutant and sensitive to protease K treatment. Thus, Gia1 likely recognizes a protein or peptide that is specifically produced in developing perithecia and likely overproduced in the *gia1* mutant. This ligand functions as an autocrine or "meiogen" to regulate meiosis via Gia1 and its downstream Gpa1-Gpmk1 MAPK pathway. Although the *KEP* genes characterized in this study are dispensable for ascosporogenesis, dozens of genes encoding secreted, membrane, or surface proteins in *F. graminearum* are specifically expressed during sexual reproduction and conserved in *F. verticillioides*, *N. crassa* and *C. graminicola*. It is possible that one of these candidate genes may encode the ligand of Gia1 to promote meiosis and ascosporogenesis in perithecia in *F. graminearum*.

Overall, in this study, we showed that *GIA1* encodes a non-pheromone GPCR that is specifically required for meiosis in developing asci after karyogamy in croziers. The ligand recognized by Gia1 GPCR appears to be proteinaceous and specifically produced in developing perithecia by *F. graminearum*. Upon the binding of Gia1 with its ligand, the signal is relayed to the Gpmk1

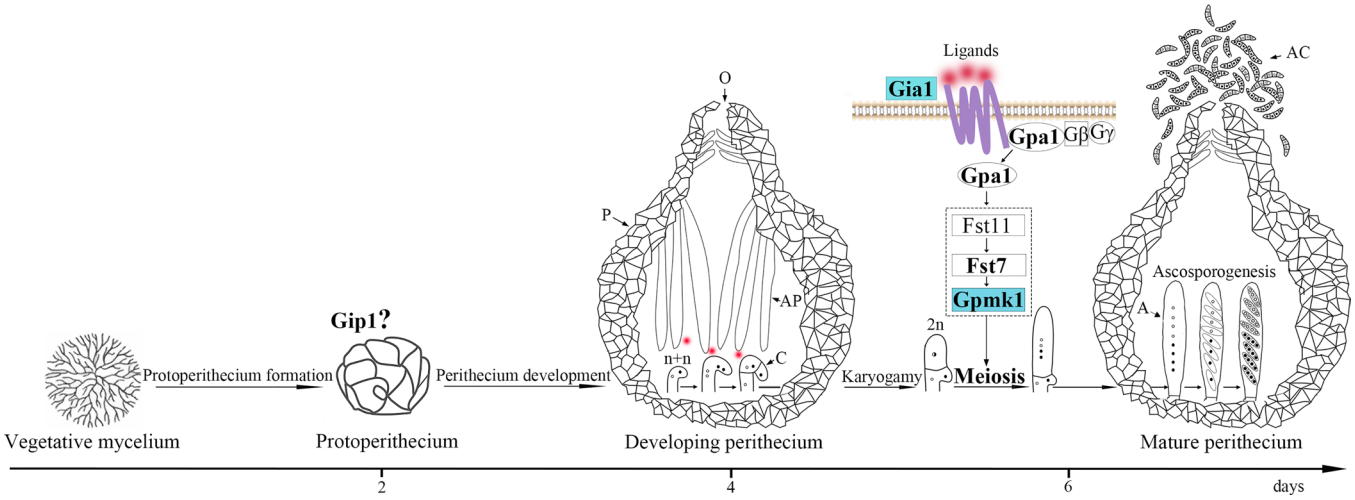

Fig. 6. A proposed model for the functions of Gia1 and Gip1 during sexual reproduction. In *F. graminearum,* a homothallic fungus, sexual reproduction can be induced by self-fertilization on mating cultures. Up fertilization, protoperithecia are formed and further developed into flask-shaped perithecia with an ostiole (O) on the top. In developing perithecia, ascogenous tissues are differentiated at the bottom and apical paraphyses (AP) are differentiated from the upper peridium (P). Karyogamy occurs in croziers (C) that are formed at the tip of dikaryotic hyphae (n+n). Meiosis occurs in the diploid (2n) nucleus moving into developing asci. After meiosis, three rounds of mitosis occur to form eight four-celled ascospores in each ascus (A). In mature perithecia, AP are disintegrated and ascospores can be released from the ostiole to form ascospore cirrhi (AC). Gip1 is essential for perithecium formation but its downstream signaling pathways remain to be characterized. Gia1 has a stage-specific function in meiosis and ascosporogenesis in developing asci. Our data showed that the ligand recognized by Gia1 is likely a protein or peptide produced inside developing perithecia. Upon binding of Gia1 with the ligand, Gpa1 is dissociated from Gβγ to activate the downstream Fst11-Fst7-Gpmk1 MAPK pathway for the regulation of meiosis and ascospore formation.

MAPK pathway via Gpa1 to regulate meiosis and ascosporogenesis (Fig. 6).

Materials and Methods

Details of the methods used in this study are provided in *SI Appendix, SI Materials and Methods*, including strain culture conditions, microscopic examinations, generation and transformation of constructs, ligand screening, MAPK phosphorylation and qRT-PCR assays, and bioinformatic analysis.

Data, Materials, and Software Availability. All study data are included in the article and/or supporting information.

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Author affiliations: [a]State Key Laboratory of Crop Stress Biology for Arid Areas, College of Plant Protection, Northwest A&F University, Xianyang, Shaanxi 712100, China; [b]Institute of Plant Protection, Jiangsu Key Laboratory for Food Quality and Safety-State Key Laboratory Cultivation Base of Ministry of Science and Technology, Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu 210014, China; and [c]Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907

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