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Expression and Function of Sex Pheromones and Receptors in the Homothallic Ascomycete *Gibberella zeae*▽

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In heterothallic ascomycete fungi, idiomorphic alleles at the *MAT* locus control two sex pheromone-receptor pairs that function in the recognition and chemoattraction of strains with opposite mating types. In the ascomycete *Gibberella zeae*, the *MAT* locus is rearranged such that both alleles are adjacent on the same chromosome. Strains of *G. zeae* are self-fertile but can outcross facultatively. Our objective was to determine if pheromones retain a role in sexual reproduction in this homothallic fungus. Putative pheromone precursor genes (*ppg1* and *ppg2*) and their corresponding pheromone receptor genes (*pre2* and *pre1*) were identified in the genomic sequence of *G. zeae* by sequence similarity and microsynteny with other ascomycetes. *ppg1*, a homolog of the *Saccharomyces* α-factor pheromone precursor gene, was expressed in germinating conidia and mature ascospores. Expression of *ppg2*, a homolog of the a-factor pheromone precursor gene, was not detected in any cells. *pre2* was expressed in all cells, but *pre1* was expressed weakly and only in mature ascospores. *ppg1* or *pre2* deletion mutations reduced fertility in self-fertilization tests by approximately 50%. Δ*ppg1* reduced male fertility and Δ*pre2* reduced female fertility in outcrossing tests. In contrast, Δ*ppg2* and Δ*pre1* had no discernible effects on sexual function. Δ*ppg1*/Δ*ppg2* and Δ*pre1*/Δ*pre2* double mutants had the same phenotype as the Δ*ppg1* and Δ*pre2* single mutants. Thus, one of the putative pheromone-receptor pairs (*ppg1*/*pre2*) enhances, but is not essential for, selfing and outcrossing in *G. zeae* whereas no functional role was found for the other pair (*ppg2*/*pre1*).

Gibberella zeae (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe sensu lato) is the most important causal agent of *Fusarium* head blight (also termed scab) of wheat and barley (32) and also causes stalk rot and ear rot of maize and crown rot of carnation (30). In addition to direct yield loss, *G. zeae* can reduce grain quality and harvested grain often is contaminated with mycotoxins such as deoxynivalenol and zearalenone (13). *G. zeae* is globally distributed and comprises multiple phylogenetic lineages (34, 35).

G. zeae is a homothallic fungus, and strains originating from a single haploid nucleus can successfully complete the sexual cycle without a mating partner. This process may be advantageous for maximizing the production of ascospores, which are important as inoculum for initiating disease epidemics of wheat (17). Other species in the genus *Gibberella* are heterothallic, and strains of these species must cross with a strain of the opposite mating type to produce perithecia, complete meiosis, and produce ascospores. *G. zeae* is capable of outcrossing facultatively under laboratory conditions (7), and laboratory crosses have been used to generate genetic maps (18, 22, 27). Although the evidence is indirect, *G. zeae* apparently outcrosses at a significant rate in North American field populations (41, 51). Thus, both selfing and outcrossing are important in the life cycle of this fungus.

Sexual development in filamentous ascomycetes is controlled by the mating type (*MAT*) locus (10, 49). In heterothallic ascomycete fungi, there are two idiomorphic alleles. One allele, *MAT1-1*, encodes three proteins, MAT1-1-1, MAT1-1-2, and MAT1-1-3, while the *MAT1-2* allele encodes only a single protein, MAT1-2-1 (10, 49). In *G. zeae*, the *MAT* locus is rearranged such that both alleles are adjacent on the same chromosome and opposite mating types do not exist (28, 49). However, both idiomorphs must function for homothallic sexual reproduction to occur. Deletion of either the *MAT1-1* or the *MAT1-2* coding region in *G. zeae* results in strains that are obligately heterothallic (28). Such mutants are useful for forcing outcrossing in genetic experiments.

Heterothallic ascomycetes possess two different diffusible pheromone peptides and corresponding G-protein-coupled receptors that function in the recognition and chemoattraction of strains of opposite mating types (2, 5, 14). The particular pheromone and receptor expressed depends on the allele at the *MAT* locus, although neither the pheromone nor the receptor is encoded at this locus. The structure and sequence of the pheromones and their receptors are broadly conserved in heterothallic ascomycetes such as *Saccharomyces cerevisiae* (26), *Neurospora crassa* (4, 25), *Podospora anserina* (11), *Cryphonectria parasitica* (48, 52), and *Magnaporthe grisea* (44). The pheromone peptides are encoded by two classes of pheromone precursor genes. One class, typified by the *Saccharomyces* α-factor precursor gene, contains a secretion signal and multiple tandem copies of a short peptide that are flanked by Kex2 protease-processing sites. The other class, typified by the *Saccharomyces* **a**-factor precursor gene, contains a CAAX carbox-

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ylation and farnesylation motif and produces a lipopeptide pheromone.

Pheromones and receptors are essential for sexual fertility in the heterothallic ascomycetes that have been studied. Deletion of the *pre-1* pheromone receptor gene of *N. crassa* caused female sterility of the *mat A* mating type because trichogynes were incapable of directional growth and fusion with spermatia (23). Deletion of either pheromone precursor gene caused male sterility of the corresponding mating type because spermatia could no longer attract female trichogynes (24). Similar results were obtained with pheromone deletion mutants of *P. anserina* (11) and *C. parasitica* (48).

In the homothallic ascomycete *Sordaria macrospora*, both types of pheromones and their cognate receptors have been reported (31, 37) but no effect on fruiting body or ascospore development was produced by a single mutation of any pheromone precursor gene or receptor gene (31). However, the double pheromone mutant exhibited drastically reduced self-fertility and the double receptor mutant was completely sterile (31). In the homothallic ascomycete *Emericella nidulans* (*Aspergillus nidulans*), a single mutation of either receptor resulted in greatly reduced self-fertility and the double receptor mutant was self-sterile (42). However, the double mutants could outcross, suggesting that the pheromone receptors are required specifically for self-fertilization (42).

The mechanism of sexual fertilization and the role of pheromones are not known for *G. zeae* or any other *Gibberella* species. Information on the sex pheromones in *G. zeae* may provide insights into the mechanism of fertilization and could identify opportunities for reducing the ascospore inoculum of this economically important pathogen. Our objectives in this study were to identify and characterize the putative pheromone precursor genes and the corresponding pheromone receptor genes in *G. zeae* and to determine their roles in sexual reproduction in this homothallic fungus. Putative pheromone precursor genes and receptor genes were identified by sequence similarity and microsynteny with other ascomycetes, while the expression of the putative pheromone precursor genes and receptor genes was assayed with green fluorescent protein (GFP) reporter constructs. Deletion mutants were constructed, and their sexual function was tested in three assays: self-fertilization tests, obligate outcrossing tests using heterothallic *MAT* deletion strains, and facultative outcrossing using GFP-marked strains to monitor the outcrossing frequency.

MATERIALS AND METHODS

Fungal strains and methods. *G. zeae* (anamorph: *F. graminearum* sensu lato lineage 7) wild-type strain Z3639, isolated in Kansas (6), and mutants derived from it were stored as frozen conidial suspensions in 15% glycerol at −70°C (Table 1). Standard laboratory methods and culture media for *Fusarium* spp. were used (30). Carboxymethyl cellulose (CMC) liquid culture medium was described previously (8). The sexual stage was induced in 3- to 5-day-old cultures on 6-cm carrot agar plates by applying 500 μl of an aqueous 2.5% Tween 60 solution containing 1×10^5 propagules (spermatia) of the male strain and then knocking down the aerial mycelium with a bent glass rod while rotating the plate several times (7). Mock fertilizations were performed similarly but with sterile Tween 60 solution lacking spermatia. Plates were incubated at 24°C with a 12-h photoperiod provided by cool white fluorescent lights.

Identification of pheromone precursor and pheromone receptor genes of *G. zeae*. The pheromone precursor genes and receptors are named differently in different fungi. For *G. zeae*, we use *ppg1* for the putative homolog of the *Saccharomyces* α-factor pheromone precursor gene and *ppg2* for the homolog of the **a**-factor pheromone precursor gene. The putative cognate pheromone receptors are designated *pre2* and *pre1*, respectively. To identify *G. zeae ppg1*, the sequences of *ppg1* of *S. macrospora* (37), *ccg4* of *N. crassa* (4), and *mf2-1* of *M. grisea* (44) were used in a BlastP search of the *F. graminearum* genome database (http://www.broad.mit.edu/annotation/fungi/*Fusarium*/). The other pheromone precursor gene (*ppg2*) is less conserved across species and is too short (less than 25 amino acids in *S. macrospora*, *N. crassa*, and *M. grisea*) to be found in a BlastP search. Instead, microsynteny near *mf1-1* of *M. grisea* (44) and *mfa-1* of *N. crassa* (25) was used to identify *ppg2* of *G. zeae*. Two putative pheromone receptor genes, *pre1* and *pre2*, were identified from the database following BlastP searches with the pheromone receptor genes of *N. crassa*, *pre1* and *pre2* (38), and *E. nidulans*, *preA* and *preB* (15).

TABLE 1. *G. zeae* strains used in this study

Genotype	No. of strains	Description
Z3639	1	Wild-type Z3639
Δ*mat1-1*	1	Deletion of *mat1-1-1*
Δ*mat1-2*	1	Deletion of *mat1-2*
Δ*ppg1*	11	Deletion of *ppg1*
Δ*ppg2*	19	Deletion of *ppg2*
Δ*pre1*	11	Deletion of *pre1*
Δ*pre2*	13	Deletion of *pre2*
GFPZ3639	1	Z3639 constitutively expressing GFP
GFPΔ*ppg1*	1	Δ*ppg1* constitutively expressing GFP
GFPΔ*pre2*	1	Δ*pre2* constitutively expressing GFP
Δ*ppg1*GFPR	4	*ppg1* replaced with GFP reporter cassette for *ppg1* expression
Δ*ppg2*GFPR	9	*ppg2* replaced with GFP reporter cassette for *ppg2* expression
Δ*pre1*GFPR	4	*pre1* replaced with GFP reporter cassette for *pre1* expression
Δ*pre2*GFPR	7	*pre2* replaced with GFP reporter cassette for *pre2* expression
Δ*ppg1*/Δ*mat1-1*	1	Δ*ppg1* Δ*mat1-1-1* double mutant
Δ*ppg1*/Δ*mat1-2*	1	Δ*ppg1* Δ*mat1-2* double mutant
Δ*ppg2*/Δ*mat1-1*	1	Δ*ppg2* Δ*mat1-1-1* double mutant
Δ*ppg2*/Δ*mat1-2*	1	Δ*ppg2* Δ*mat1-2* double mutant
Δ*ppg1*/Δ*ppg2*	3	Δ*ppg1* Δ*ppg2* double mutant
Δ*ppg1*/Δ*pre2*	8	Δ*ppg1* Δ*pre2* double mutant
Δ*pre1*/Δ*pre2*	3	Δ*pre1* Δ*pre2* double mutant
ppg1-ect	1	*ppg1* ectopic insertion mutant with intact *ppg1*
CNPΔ*ppg1*	7	Complementation of *ppg1* with native promoter
CSPΔ*ppg1*	5	Complementation of *ppg1* under ICL promoter
CSP2SΔ*ppg1*	8	Complementation of *ppg1* without signal peptide under ICL promoter

DNA manipulations. DNA was extracted by a cetyltrimethylammonium bromide (CTAB) procedure (30). Standard procedures were used for restriction endonuclease digestions, agarose gel electrophoresis, Southern hybridizations, and Northern hybridizations (40).

Targeted gene deletion. *ppg1*, *ppg2*, *pre1*, *pre2*, *mat1-1-1*, and *mat1-2* were deleted by split marker recombination (9) with slight modifications. Both the 5′ and 3′ flanking regions of a target gene were amplified by PCR with the F1/R2 and F3/R4 primer sets (Table 2) and an amplification protocol of 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C and 10 min at 72°C for a final extension. The PCR products were purified with the DNA Purification System (Promega, Madison, WI) by following the manufacturer's instructions. The hygromycin phosphotransferase cassette (HYG; 1.4 kb) was amplified with the HYG-1F/2R primers from pIGPAPA (20) and purified with the same system. The HYG sequence for *ppg1* deletion was amplified from pCSN43 (46) by PCR with the GNT-F1 and GNT-R4 primers. In this construct, the hygromycin resistance gene was flanked by a *trpC* promoter and *trpC* terminator and the size of amplicon is ~2 kb. The three amplicons were fused by PCR in a 25-μl reaction mixture containing 1 μl of purified 5′ flanking amplicon (100 ng/μl), 1 μl of 3′ flanking amplicon (100 ng/μl), 3 μl of HYG amplicon (100

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5′→3′)
PPG1-F1	CGCGTCTGACAAGTAAAAGGAGAAAC
PPG1-F1-NT	GCGTCTGACAAGTAAAAGGCGAAACCAAATGGCAA
PPG1-R2	AAAAAGTGCTCCTTCAATATCATCTTCTGAGGCGGCTAGCGTCAAAATGGA
PPG1-F3	CTTGTTTAGAGGTAATCCTTCTTTCTAGAGTAAGTTTGGTTATCGACGCAGAG
PPG1-R4	CTAGCGCACAAGGCATCAACT
PPG1-R4-NT	CTGCCCCACCATCTCAGACGC
PPG1-R2-GFP	CCTCGCCCTTGCTCACCATGTTGGGCGCCGTACTTGTCG
PPG1-R-TRPCP	GCTCCTTCAATATCATCTTCTGGGATATGCTGCTTGGCGTCAAC
PPG1-F1-ICL	TTCATACCACACCTGCCCACCGCGCCCAACATGAAGTACTCC
PPG1M2-F1-ICL	TTCATACCACACCTGCCCACCATGCCCTGGTGCACCTGGAAA
PPG2-F1	GGCCGCCAAAGACCTAAGC
PPG2-F1-NT	GCGGTGGCAGCATTGTTCTACG
PPG2-R2	TTGACCTCCACTAGCTCCAGCCAAGCCGGCCAGAACGATACGTC
PPG2-F3	GAATAGAGTAGATGCCGACCGCGGGTTCCGCCACGAGGACGCCA
PPG2-R4	GCGAGGAGGGCGGTTGTGTTGTTA
PPG2-R4-NT	GGGGCTCTTCATCGTCCTCATCAT
PPG2-R2-GFP	CGCCCTTGCTCACCATTTTGAAAGTTGGGGTTGAAAGACTTAGA
PRE1-F1	GCTGAGACGCGATAGGGTAGGAA
PRE1-F1-NT	TCAACCTCACCACGTCCCTCAACA
PRE1-R2	TTGACCTCCACTAGCTCCAGCCAAGCCGCGGGAAGCGCAAGGAC
PRE1-F3	GAATAGAGTAGATGCCGACCGCGGGTTCGGCATCATCTGCGAGC
PRE1-R4	GACGAACGTATGCGAAATGGAGAC
PRE1-R4-NT	AAGGGCTTGGTTATGGCGGTTGG
PRE1-F5	CCTGGACCGGGAACAACTATCACT
PRE1-R6	AGGCCATGCGACCCAACTG
PRE1-R2-GFP	CGCCCTTGCTCACCATGTTGGGACGTCGACCGTGATGTGGAAG
PRE2-F1	GCGCAGCAGGCAGCAGAA
PRE2-F1-NT	GAATGGGCCTGGCTGCGTGAT
PRE2-R2	TTGACCTCCACTAGCTCCAGCCAAGCCGGCAAGAAGACACGGGA
PRE2-F3	GAATAGAGTAGATGCCGACCGCGGGTTCCGCCTCGATACCCCAA
PRE2-R4	TCGCCACAATTCGGTTCCTGAT
PRE2-R4-NT	GGAACCCCGGTCGCCTCACA
PRE2-F5	TGTCTTATCATGCTGGTCGTGCTC
PRE2-R6	GGGAGAATCACAGCGACAGAGGTA
PRE2-R2-GFP	CGCCCTTGCTCACCATGTTGGGGTGGTATCTGCTTTTCGACTGG
MAT11-F1	CTCCACTTGCGGCATCGTCTAC
MAT11-F1-NT	GCCCTGATGATGCTGTAAGTGTTA
MAT11-R2	TTGACCTCCACTAGCTCCAGCCAAGCCGGAGGGAAAGGGGTGTG
MAT11-F3	GAATAGAGTAGATGCCGACCGCGGGTTGCACATGTCGGGCACGG
MAT11-R4	CTCCCAACGCTTACATCCTCTACC
MAT11-R4-NT	CCCGCCGCCCAGCCTACTC
ICL-F1	GGGCCCCACACGGACTCAAAC
ICL-F1-NT	CCCCACACGGACTCAAACTGATGTTCGAGTC
ICL-R2-PPG1	GGAGTACTTCATGTTGGGCGCGGTGGGCAGGTGTGGTATGAAA
ICL-R2-PPG1M2	CCAGGTGCACCAGGGCATGGTGGGCAGGTGTGGTATGAAA
GFP-F1	GGGGCCCCACACGGACTC
GFP-F1-NT	CCAGAGGTCCGATCGCCAATGA
GFP-R2	TTGACCTCCACTAGCTCCAGCCAAGCCAGATGACACCGCGCGCG
GFP-F3	CCATGGTGAGCAAGGGCGAGGAG
GFP-F3-PPG1	ACGACAAGTACGGCGCCCAACATGGTGAGCAAGGGCGAGG
GFP-F3-PPG2	CTTTCAACCCCAACTTTCAAAATGGTGAGCAAGGGCGAGG
GFP-F3-PRE1	TCCACATCACGGTCGACGTCATGGTGAGCAAGGGCGAGG
GFP-F3-PRE2	CGAAAAGCAGATACCACCATGGTGAGCAAGGGCGAGG
GFP-R4	AGATGACACCGCGCGCGATAATTTA
HYG-F1	GGCTTGGCTGGAGCTAGTGGAGG
HYG-R2	AACCCGCGGTCGGCATCTACTCTA
HY-F3	GATGTAGGAGGGCGTGGATATGT
YG-R4	GAACCCGCTCGTCTGGCTAAG
HYG-F1-GFP	TATCGCGCGCGGTGTCATCTGGCTTGGCTGGATCTAGTGGAGG
GNT-F1	CAGAAGATGATATTGAAGGAGC
GNT-R4	CTAGAAAGAAGGATTACCTCT
GNT-F1-PPG1	CAACGTTGACGCCAAGCAGCATATCCCAGAAGATGATATTGAAAG
GNT-R4-NT	CCTGTGCATTCTGGGTAAACGAC
GNT-R4A-NT	GTACCTGTGCATTCTGGGTAAACGACTCATAGGAG
rRNA-F	CATCCGGCACGCAAACCAC
rRNA-R	CGATGTCGCCGCTGTCAATG

ng/μl), 2 μl of deoxynucleoside triphosphates (each at 2.5 mM), 2.5 μl of 10× PCR buffer including $MgCl_2$, 1 U of ExTaq polymerase (Takara Bio Inc., Japan), and 15.25 μl of water. The PCR amplification conditions were 2 min at 94°C, followed by 10 cycles of 30 s at 94°C, 20 min at 58°C, and 5 min at 72°C and 10 min at 72°C for a final extension. One microliter of this amplification mixture was reamplified as a template in a PCR with F1-NT/YG-R4 and HY-F3/R4-NT primer sets and a 50-μl reaction volume. The PCR conditions were 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at 60°C, and 90 s at 72°C and 10 min at 72°C for a final extension. The amplification products were combined and used to directly transform *G. zeae* protoplasts by a polyethylene glycol-mediated method (29). The percent deletion of the open reading frame was 100, 100, 100, 100, 86, and 100% for *mat1-1-1*, *mat1-2*, *ppg1*, *ppg2*, *pre1*, and *pre2*, respectively. The presence of single copies of each insertion was confirmed by Southern blotting. The numbers of independent transformants obtained for the deletion mutant classes are listed in Table 1. Δ*ppg1*/Δ*mat1-1*, Δ*ppg1*/Δ*mat1-2*, Δ*ppg2*/Δ*mat1-1*, Δ*ppg2*/Δ*mat1-2*, Δ*ppg1*/Δ*ppg2*, Δ*ppg1*/Δ*pre2*, and Δ*pre1*/Δ*pre2* double mutants were generated by sexual crosses between single mutants. Multiple strains in a double-mutant class were derived from the same single-mutant parents.

GFP-tagged strains for outcrossing. The DNA fragment (3.4 kb) carrying the GFP and HYG cassettes was amplified from pIGPAPA with primers ICL-F1 and HYG-F1 and transformed into Z3639. Transformant GFPZ3639 constitutively expresses GFP and was used as the spermatial parent in crosses. GFP cassettes were combined with Δ*ppg1* and Δ*pre2* mutants by crossing Δ*ppg1* and Δ*pre2* mutants with GFPZ3639 to yield GFPΔ*ppg1* and GFPΔ*pre2*. GFP fluorescence was detected with an Axioplan2 microscope (Carl Zeiss, Thornwood, NY) with 480 ± 10 nm for excitation and 510 ± 10 nm for emission.

GFP reporter constructs. We amplified a GFP sequence (0.97 kb) that has a terminator, but no promoter, from pIGPAPA. The GFP gene fragment was fused to the HYG cassette (1.4 kb), which was amplified from pIGPAPA with primers HYG-F1-GFP and HYG-R2 (GFP::HYG). The promoter region of the target gene was amplified with primers F1 and R2-GFP, and the 3′ flanking region was amplified with primers F3 and R4. After PCR purification, the promoter and the 3′ flanking region were fused with the GFP::HYG construct in a 25-μl reaction mixture, 1 μl of which was used to produce split markers. Following transformation with the two split markers, we recovered deletion mutants in which GFP expression was controlled by the native promoter of the target gene. Target gene expression was monitored by screening for GFP expression. The experiment was performed twice with four to nine independent transformants per class.

RNA isolation and reverse transcription (RT)-PCR. Total RNA was isolated from vegetative mycelia (uninduced mycelia or mycelia 3 days after induction) or ascospores of wild-type and mutant strains by using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA concentration and purity were checked with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). For RT-PCR, 1 μg of total RNA was used to synthesize first-strand cDNA with the 3′-full RACE Core Set (Takara Bio Inc., Japan) according to the manufacturer's instructions and 1 μl of the cDNA solution was used for RT-PCR. To normalize cDNA, the 60S ribosomal gene (FGSG_13664.3) was amplified with the rRNA-F/R primer set (Table 2). Twenty micrograms of total RNA was subjected to Northern hybridization with the PCR product of the target gene as a probe. The experiment was performed twice with one mutant strain per class.

Fertility tests. For qualitative self-fertilization tests, cultures were mock fertilized to induce homothallic sexual development. The number and size of perithecia were noted after incubation for 10 to 14 days following induction.

For quantitative self-fertilization tests, there were three independent transformants for each mutant class and eight replicate plates for each transformant, and the experiment was performed twice. Photographs of each plate were taken to facilitate the counting of perithecia. The percentage of mature perithecia was calculated for each plate. Percentage data were given an arcsine square root transformation to improve the homogeneity of variance, and means were back-transformed for presentation. Data from both runs of the experiment were combined and analyzed as a completely randomized design ($n = 16$) with Minitab Version 14 (Minitab, Inc., State College, PA) to compare means for each mutant to the value for the wild-type control with Dunnett's two-tailed test (family error rate = 0.05).

For obligate outcrossing tests, heterothallic strains carrying a *mat1-1* or *mat1-2* deletion were used as females so that all ascospore progeny resulted from heterozygous crosses. Plates were fertilized with a suspension of conidia from cultures grown on complete medium. Crosses were scored qualitatively as either fertile or nonfertile. The experiment was performed twice with one mutant strain per class and three replicates.

For facultative outcrossing tests, conidia from GFP-expressing males were used as spermatia to fertilize non-GFP-expressing females. Ascospores resulting from self-fertilized perithecia did not express GFP, and ascospores resulting from heterozygous perithecia segregated 1:1 for GFP expression. Ten days after fertilization, crossing plates were inverted and the ascospores ejected were collected on the underside of the lid of a petri dish overnight. Ascospores were suspended in 1 ml of water and counted with a hemocytometer to determine the total number of progeny produced and the proportion of the progeny expressing GFP. Under the conditions of this study, the spermatial parent never produced mature homothallic perithecia or ascospores. Therefore, the outcrossing percentage was estimated as two times the percentage of GFP-expressing ascospores. The experiment was performed three times with three replicate plates per cross. There was one mutant strain per class. Data from the three runs were combined and analyzed as a randomized complete block design. Data were given an arcsine square root transformation and back-transformed for presentation. Means were compared to the control (Z3639 female/GFPZ3639 male) with Dunnett's test. The experiment was also performed once with ascospores as spermatia, and the results were analyzed as a completely randomized design with three replicates.

Complementation of Δ*ppg1*. Three constructs were made to complement *ppg1*. In the first, the entire *ppg1* gene, including the native promoter and terminator, was amplified from *G. zeae* strain Z3639 with primers PPG1-F1 and PPG1-R-TRPCP and the Geneticin resistance gene cassette (GNT) was amplified from pII99 (33) with primers GNT-F1-PPG1 and GNT-R4. These two amplicons were fused (whole *ppg1* cassette::GNT) after purification as described above. In the second, the *ppg1* gene was amplified by PCR from Z3639 with PPG1-F1-ICL and PPG1-R-TRPCP as the primers. The resulting DNA fragment contained the entire *ppg1* sequence, and the 3′ flanking region including the terminator. A strong constitutive promoter, ICL from isocitrate lyase of *N. crassa*, was amplified from pIGPAPA (20) by PCR with ICL-F1 and ICL-R2-PPG1. The ICL promoter fragment, the *ppg1* genomic fragment, and the GNT cassette were fused (ICL promoter::*ppg1*::GNT). The third construct was similar to the second construct, except that the *ppg1* gene fragment, amplified with primers PPG1M2-F1-ICL and PPG1-R-TRPCP, began at the second methionine codon of *ppg1* and did not include the signal peptide. All three constructs were transformed into one Δ*ppg1* mutant, and there were five to eight transformants per class. The self-fertility phenotype was determined in two experiments with three replications.

RESULTS

Identification of *ppg1*, *ppg2*, *pre1*, and *pre2*. One *ppg1* candidate sequence (Broad Institute; FGSG_05061.3) was identified by BlastP. This protein sequence had significant identity (28, 30, and 21%, respectively) with *ppg1* of *S. macrospora*, *ccg4* of *N. crassa*, and *mf2-1* of *M. grisea*. Upstream of FGSG_05061.3, there are *MAT* binding motifs (CTTTG) at positions −434 and −483. The putative *ppg1* gene contains four repeats of one decapeptide (WCTWKGQPCW) and five repeats of a second decapeptide (WCWWKGQPCW) that differs from the first decapeptide by a single amino acid (Fig. 1). All decapeptides, except for the first two, are bordered by a putative Kex2 protease site (KR).

ppg2 could not be unambiguously identified in a BlastP search with *ppg2* of *S. macrospora*, *mfa-1* of *N. crassa*, or *mf1-1* of *M. grisea*, because the sequences are short and have relatively low levels of sequence similarity. We compared the synteny of the *N. crassa* and *M. grisea* sequences flanking the *mfa-1* and *mf1-1* genes. Both genes are between cyanate lyase and *ebp2* homologs, and both *ppg2* homologues are transcribed in the same direction and in the same open reading frame (Fig. 2). In the *Fusarium verticilloides* genomic sequence, a putative *ppg2* homolog was found in the same orientation with cyanate lyase but these two genes were inverted relative to the *ebp2* homolog. These results suggest that microsynteny in this region generally is well conserved in these related ascomycete fungi.

The 10-kb sequence flanking *mfa-1* of *N. crassa* and *mf1-1*

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1   MKYSILTLAAVASTTLAVAVPAPQPDPVAEPMPWCTWKGQPCWKEKMARREAQPEPEAVAAPEPDPVAEP
71  MPWCTWKGQPCWKEKMAKRAAQPEPVPAPQPDPVAEAEPWCTWKGQPCWKEKMVKRAAEAEAEAEPIPDP
141 VAAPQPDPVAEPMPWCTWKGQPCWKEKMAKREAKPEPWCWWKGQPCWKAKRDAAPEPWCWWKGQPCWKAK
211 RNAAPEPMPEPANEPRWCWWKGQPCWKSKSKRDASPEPWCWWKGQPCWKAKRDAGEALTVALHATRGVET
281 RSVAETEHLPRDAAHQAKRSIVELANVIALSARGSPEEYFKHLYLEEFFPEIPHNATAKRDVKTLQEDKR
351 WCWWKGQPCWKAKRAAEAVLHAVDGSDGAGAPGGPEEHFDTSHFNPQNFEAKRDLMAIKAAARSVVESLE
421 G
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FIG. 1. Deduced sequence of the protein encoded by *ppg1* (FGSG_05061.3) of *G. zeae*. This polypeptide contains two types of decapeptides. Four repeats of one type (WCTWKGQPCW) are underlined, and five repeats of a second type (WCWWKGQPCW) that differs from the first decapeptide by a single amino acid are in bold underlined type. The putative secretion signal sequence cleavage site is marked by an arrow. KR dipeptides, which are potential Kex2 protease-processing sites, are in bold type.

of *M. grisea* was blasted against the genomic sequence to find the syntenous region of *G. zeae*. Contig 1.310 contains a cyanate lyase (FGSG_07458.3) and a homolog of *ebp2* (FGSG_07457.3). A previously undescribed open reading frame was identified in the region between the two genes that contains a candidate *ppg2* gene in the same orientation as in *M. grisea* and *N. crassa* (Fig. 3). The putative *ppg2* gene encodes a peptide of 21 amino acids with a prenylation signal sequence (CAAX) at its C terminus. This signal sequence also is found in the precursors of several other fungal pheromones (1, 12, 36, 37, 44, 45). Upstream of the putative *ppg2* gene, there are a putative TATA box, two CAAT boxes, and two putative *MAT* transcription factor binding sites.

One *pre1* candidate sequence (FGSG_07270.3) was identified in the genomic sequence. It had sequence identity (30 and 23%, respectively) to the *preA* gene in *E. nidulans* (GenBank DAA01795) and the *pre1* gene of *N. crassa* (GenBank CAC86413.1). The putative *G. zeae pre1* gene has a CAAAG motif at −440 and encodes a protein with seven transmembrane domains typical of G protein-coupled receptors.

One *pre2* candidate sequence (FGSG_02655.3) was identified in the genomic sequence. It has sequence identity (34 and 22%, respectively) with the receptor gene for *preB* from *E. nidulans* (GenBank DAA01796) and *pre2* from *N. crassa* (GenBank CAC86431.1). The *G. zeae pre2* sequence has a CAAAG motif at positions −506 and −526 and seven transmembrane domains.

Gene expression. In four independent Δ*ppg1*GFPR mutants in which GFP expression was controlled by the native *ppg1* promoter, GFP was expressed strongly in induced germinating conidia and mature discharged ascospores (Table 3, Fig. 4). Mycelia and conidia from colonies >10 days old on carrot agar media had a weak GFP signal. Young mycelia, perithecia, ungerminated conidia, and ascospores inside asci had no detectable GFP signal. We never observed GFP expression in the Δ*ppg2*GFPR reporter strains (nine mutants). GFP was weakly expressed in mature ascospores of Δ*pre1*GFPR mutants (four mutants) and was more strongly expressed in Δ*pre2*GFPR strains (seven mutants) by all mycelial ages or tissues from carrot agar except young mycelia prior to induction (Table 3).

The expression of *ppg1*, *pre1*, and *pre2* was confirmed by Northern blotting and RT-PCR of RNA from induced mycelial cultures or ascospores, but *ppg2* expression was not detected (Fig. 5).

Self-fertilization tests. Δ*ppg1* mutants (11 strains) had normal colony morphology on carrot agar and complete medium. They were self-fertile but produced fewer large perithecia than

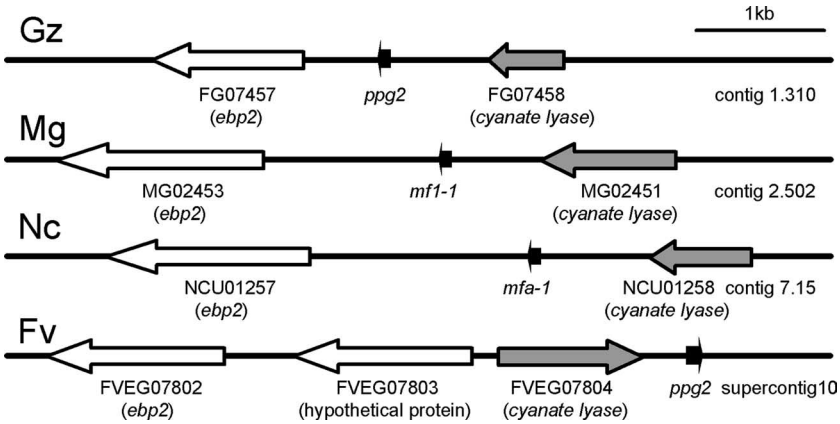

FIG. 2. Microsynteny near the *ppg2* gene among different ascomycete species. The putative *ppg2* gene of *G. zeae* (Gz) was between FG07457 (*ebp2* homolog) and FG07458 (cyanate lyase homolog) in contig 1.310 of the *Fusarium* genome database developed by the Broad Institute. In the other fungal species (Mg, *M. grisea*; Nc, *N. crassa*), the *ppg2* gene was between homologs of *ebp2* and cyanate lyase. Note that the names for the *ppg2* gene are different in these three fungal species. In the *F. verticilloides* (Fv) genomic sequence, a putative *ppg2* homolog was found downstream of cyanate lyase but these two genes were inverted relative to the *ebp2* homolog in the other fungi.

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1    ACTCATAACTAACCAACATGTTCAGGCTCCCTTTCAGTCGTTTCTAGGCATTGTTCTCTCAAGAGAATTG
71   GTTGATAGCTCGGAGTTATAACCACTCTGCATGCTGTGTCACTTTCATTAATGACTACTCAAAGCGTATT
141  TACATAACGGAAAATGCGGGTTGGAGCATCGCTAAGTTCACACAAGCCTCACTCAAGAATGACATGTATT
211  TCGAGTAAAGCGGACGGGGCAGGTTGTGGCAATGCGCGTGAAGACAATATGAAGCGAGAGGACAATACTA
281  AGCAATTTACAAACAAATACCAACATTGAGTTCTAGCGCTTCGACGTCCGTGTACCACATGACATGACAA
351  CTCGGCGCATTTCTAAAACCCGAAGGCTTCTAAAGCCTAACTTTCATCTAAAAGAAGGCATGCCAGACAG
421  TCTAGAAGACCTGTGACAAGTTGGTGGCTGCATTAGCGGCGGGTATTCTTTTGTCTGGTAGCAACACCAT
491  CTCGAGAACAATATTGAGCGATTTTGCACGGATCATGGGCTTCACGGCCAACACCAGAACTCAGTGAGTG
561  AGACGTATCGTTCTGGCCAAAATCGAGGGCATCTTGAGTCAGGCAAGTTGATCAGATTCGAGTATAAAGA
631  TGATGGCTTCTTCCTCCCAAATATCATTCTCACCAACAATCACTCACTTCACACACTCACAACACTCTTT
701  TCACCATTCTCAACACAGAACACTCTCCGACTTTTCTAAGTCTTTCAACCCCAACTTTCAAAATGCCTTC
                                                                  M  P  S
771  CACCAAGCCCACCTCTTCCCAGAAGCCCGGCTACCCCCTCAGCTGCACTGTCATGTAAAGCAGCTTTGCT
       T  K  P  T  S  S  Q  K  P  G  Y  P  L  S  C  T  V  M  *
841  TCCCTGATCACTTCTGGACTTTCTTTTCTGAGAGTGACTTGACAACCAACAACGCATGGCAAAGCGTTAG
911  CTCTCAGGCGACAAATTGGAGGAAAATGGAGATGATATGGCAAGGAGAGGATTCATTGGCTTGTTTACGT
981  TTTGTTTTTACCTGGCTCTAAGTGACGGGACACCGCCACGAGGACGCCATTTTACACTGGCTGTCCACTC
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FIG. 3. Putative nucleotide sequence of the *ppg2* gene of *G. zeae*. The sequence shown is from *Fusarium* genome database contig 1.310. The putative *MAT* transcription factor binding motif (GTTTC or CAAAG), CAAT box, and TATA box are underlined, and the prenylation signal sequence (CAAX) is boxed. The amino acid sequence is shown below the nucleotide sequence.

did the wild-type strain (Fig. 6). The Δ*pre2* (13 strains), Δ*ppg1*/Δ*ppg2* (3 strains), Δ*pre1*/Δ*pre2* (3 strains), and Δ*ppg1*/Δ*pre2* (8 strains) mutants all had the same phenotype as the Δ*ppg1* mutant. However, the Δ*ppg2* (19 strains) and Δ*pre1* (11 strains) mutants showed no obvious changes in perithecial production compared to the wild type.

Large and small perithecia of Δ*ppg1* mutant strains were measured and then crushed to examine their contents microscopically. Immature perithecia contained no asci or ascospores and ranged from 24 to 98 μm (average, 58 μm) in diameter. Mature perithecia contained normal-appearing asci and ascospores, regardless of the mutant genotype, and ranged from 146 to 293 μm (average, 235 μm) in diameter. Immature perithecia never developed further, even if the cultures were incubated for 3 additional weeks. For counting purposes, 120 μm was considered the minimum diameter of a mature perithecium. The percentage of mature perithecia was reduced by approximately 50% in the Δ*ppg1* and Δ*pre2* mutants but was not reduced significantly in the Δ*ppg2* or Δ*pre1* mutant or the *ppg1* ectopic-insertion control (Fig. 7).

Obligate outcrossing tests. All single-pheromone mutants (Δ*ppg1* and Δ*ppg2*), the double-pheromone mutant (Δ*ppg1*/

TABLE 3. Expression assays of putative pheromone precursors (*ppg1* and *ppg2*) and receptors (*pre1* and *pre2*) in different cell types of different ages on carrot agar

Sample	*ppg1*[a]	*ppg2*	*pre1*	*pre2*
3 DAI[b] (without induction[c])				
Mycelia	−[d]	−	−	−
Ungerminated conidia	−	−	−	+
6 DAI (3 days after induction)				
Mycelia	−	−	−	++
Ungerminated conidia	−	−	−	++
Germinating conidia	++++	−	−	++
Young perithecia	−	−	−	+++
13 DAI (10 days after induction)				
Mycelia	−	−	−	++
Ungerminated conidia	−	−	−	++
Germinating conidia	++++	−	−	++
Mature perithecia	−	−	−	++
Ascospores in perithecia	−	−	−	+
Ascospores discharged from perithecia	+++	−	+	+
Old culture without induction (15 DAI)				
Mycelia	+	−	−	++
Ungerminated conidia	+	−	−	++
Germinating conidia	++++	−	−	++
Mature perithecia	−	−	−	++
Ascospores in perithecia	−	−	−	+
Ascospores discharged from perithecia	+++	−	+	+

[a] *ppg1*, *ppg2*, *pre1*, and *pre2* expression was assayed with GFP reporter constructs Δ*ppg1*GFPR, Δ*ppg2*GFPR, Δ*pre1*GFPR, and Δ*pre2*GFPR, respectively.
[b] DAI, days after inoculation.
[c] Induction by knockdown of mycelia with 500 μl of 2.5% Tween 60 solution to induce sexual development.
[d] −, GFP fluorescence signal not detected; +, weak fluorescence; ++, moderate fluorescence; +++, strong fluorescence; ++++, very strong fluorescence; NT, not tested.

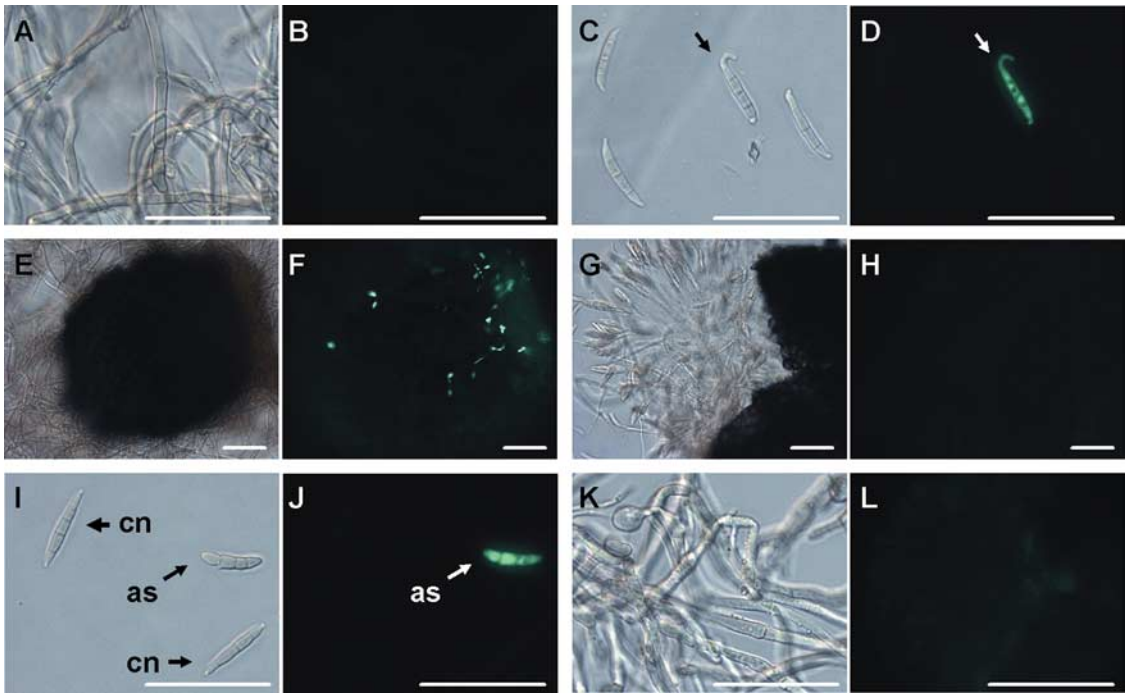

FIG. 4. GFP expression assay for *ppg1* in the Δ*ppg1*GFPR mutant strain on carrot agar. (A and B) Four-day-old mycelia without induction. (C and D) Conidia 3 days after induction. Only germinating conidia (arrow) expressed GFP. (E and F) Young perithecium 3 days after induction. GFP expression from the perithecium was not detected, but there were a few associated germinating conidia expressing GFP. (G and H) Squashed perithecium 10 days after induction. Ascospores in asci did not express GFP. (I and J) Conidia (cn) and freshly discharged ascospore (as) from a perithecium 10 days after induction. Only the ascospore expressed GFP. (K and L) Mycelia 15 days old without induction. (A, C, E, G, I, and K) Bright-field microscopy. (B, D, F, H, J, and L) Same specimen, fluorescence microscopy. Scale bars, 50 μm.

Δ*ppg2*), and double mutants with mating type deletions (Δ*ppg1*/Δ*mat1-1*, Δ*ppg1*/Δ*mat1-2*, Δ*ppg2*/Δ*mat1-1*, and Δ*ppg2*/Δ*mat1-2*) could serve as either the male or the female parent in a cross (Table 4). Crosses were infertile only in pairings that lacked a functional copy of one or the other *MAT* idiomorph.

Facultative outcrossing tests. Both conidia and ascospores functioned similarly as spermatia (Table 5). Outcrossing percentages generally were low but never zero. However, Δ*ppg1* females had a >20-fold increase in facultative outcrossing, ranging from 35 to 87%. This effect was abolished by Δ*ppg1* in

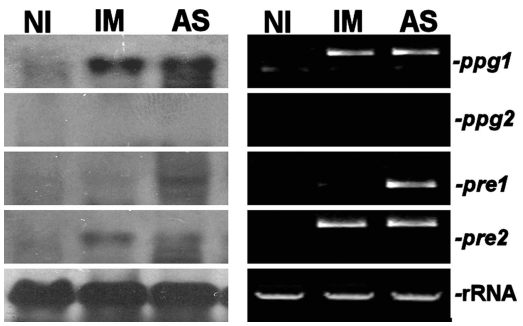

FIG. 5. Analysis of transcript levels of pheromone precursor genes *ppg1* and *ppg2* and pheromone receptor genes *pre1* and *pre2* in *G. zeae*. Total RNA was extracted from 3-day-old fresh mycelia without induction (NI), 3-day-old mycelia after induction (IM), or ascospores (AS). Twenty micrograms was subjected to Northern analysis with the PCR product of the target gene serving as a probe (left panel), and 1 μg was used to synthesize first-strand RNA cDNA for RT-PCR (right panel).

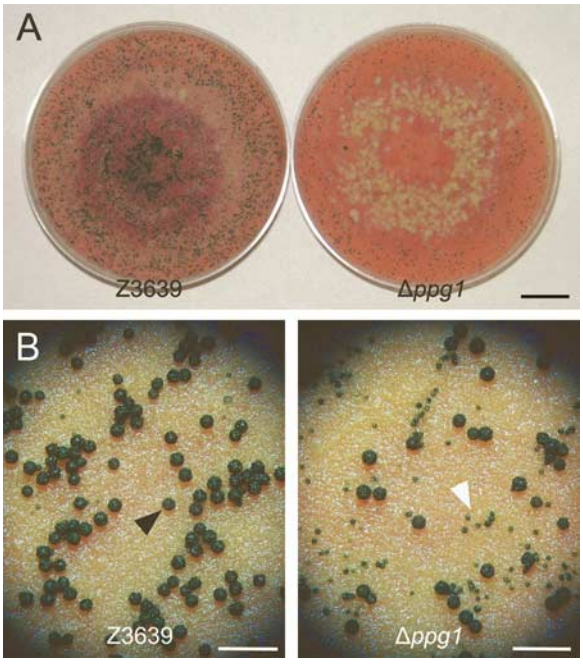

FIG. 6. Effect of *ppg1* deletion on self-fertility of *G. zeae* growing on carrot agar 10 days after induction. (A) The wild-type Z3639 control and the Δ*ppg1* mutant, with a reduced number of large mature perithecia. Scale bar, 1 cm. (B) Magnification showing fewer large mature perithecia (black arrowhead) and more small immature perithecia (white arrowhead) in the Δ*ppg1* mutant strain than in Z3639. Scale bars, 1 mm.

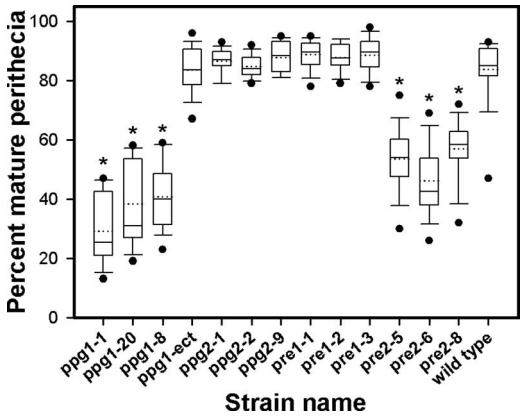

FIG. 7. Box plots of percent mature perithecia formed by self-fertilization of wild-type *G. zeae* and pheromone precursor gene *ppg1* and *ppg2* and pheromone receptor gene *pre1* and *pre2* deletion mutants of *G. zeae*. There are three independent transformants for each class. *ppg1*-ect is an ectopic-insertion mutant shown as a control. Each box plot shows the 75th and 25th percentiles (upper and lower borders of the box), the median (a solid line within a box), the mean (a dotted line within a box), the 90th and 10th percentiles (upper and lower horizontal lines outside of the box), and outliers (black circles). Box plots with asterisks indicate significant difference from the wild type by Dunnett's test.

TABLE 5. Estimated facultative outcrossing percentages of *ppg1* and *pre2* deletion mutants

Female	Male[a]	Estimated outcrossing percentage[b]	
		Conidia[c]	Ascospores
Z3639	GFPZ3639	0.3	1.7
Z3639	GFPΔ*ppg1*	0.2	0.3
Z3639	GFPΔ*pre2*	1.0	3.9
Δ*ppg1*	GFPZ3639	35.1[e]	61.7[e]
Δ*ppg1*	GFPΔ*ppg1*	1.2	0.5
Δ*ppg1*	GFPΔ*pre2*	53.1[e]	87.8[e]
Δ*pre2*	GFPZ3639	0.4	0.3
Δ*pre2*	GFPΔ*ppg1*	2.1	4.7
Δ*pre2*	GFPΔ*pre2*	0.9	1.5
Δ*ppg1*/Δ*pre2*	GFPZ3639	0.3	NT[d]
Δ*ppg1*/Δ*pre2*	GFPΔ*ppg1*	0.8	NT
Δ*ppg1*/Δ*pre2*	GFPΔ*pre2*	0.4	NT

[a] Male strains were GFP labeled so GFP segregated 1:1 in heterozygous crosses.
[b] The outcrossing frequency was estimated as twice the percentage of GFP-labeled ascospores produced.
[c] Conidia and ascospores were used to fertilize female cultures at a concentration of 1×10^5 spermatia/ml.
[d] NT, not tested.
[e] Significantly different from the control (Z3639 female/GFPZ3639 male) by Dunnett's test.

the male or the additional deletion of *pre2* (i.e., Δ*ppg1*/Δ*pre2*) in the female.

Complementation of Δ*ppg1*. Δ*ppg1* deletions were made with a construct that contained *hyg* as the selectable marker. To test for complementation, the entire *ppg1* gene, including the 5′ and 3′ flanking regions, was fused with a Geneticin resistance cassette (GNT), and Geneticin-resistant transformants were selected following transformation into a Δ*ppg1* mutant. All seven of the Geneticin-resistant transformants had a wild-type phenotype in self-fertilization tests. If the reintroduced *ppg1* sequence was controlled by the constitutive ICL promoter from *N. crassa* (ICL promoter::*ppg1*::GNT), then partial complementation (70 to 80% mature perithecia produced) occurred in each of the five transformants. Thus, the timing and/or the level of *ppg1* expression is important for full function.

Eight transformants carried the *ppg1* gene construct lacking the initial signal peptide. Strains carrying this construct did not complement the *ppg1* deletion and had the same phenotype as the Δ*ppg1* mutants. Thus, the secretion signal peptide of *ppg1* is essential for the *ppg1* gene product to function properly.

DISCUSSION

Two putative pheromone precursor genes (*ppg1* and *ppg2*) and two putative pheromone receptor genes (*pre1* and *pre2*) were identified in the genomic sequence of *G. zeae*. *ppg1*, a homolog of the *Saccharomyces* α-factor pheromone precursor gene, was expressed strongly in germinating conidia and discharged ascospores but weakly, if at all, in other cells. The cognate receptor, *pre2*, was expressed in all cells except uninduced mycelia. In fertility tests with Δ*ppg1* and Δ*pre2* mutants, the cognate pair enhanced both self-fertility and facultative outcrossing ability. Δ*ppg1* mutants were successfully complemented by transformation with an intact *ppg1* gene, thus confirming that *ppg1* retains a functional role in this homothallic ascomycete fungus.

TABLE 4. Male and female fertility of mutant strains in obligate outcrosses

Male	Female					
	Δ*mat1-1*	Δ*mat1-2*	Δ*ppg1*/Δ*mat1-1*	Δ*ppg1*/Δ*mat1-2*	Δ*ppg2*/Δ*mat1-1*	Δ*ppg2*/Δ*mat1-2*
Z3639 (wild type)	+[a]	+	+	+	+	+
Δ*ppg1*	+	+	+	+	+	+
Δ*ppg2*	+	+	+	+	+	+
Δ*ppg1*/Δ*mat1-1*	−[b]	+	−	+	−	+
Δ*ppg1*/Δ*mat1-2*	+	−	+	−	+	−
Δ*ppg2*/Δ*mat1-1*	−	+	−	+	−	+
Δ*ppg2*/Δ*mat1-2*	+	−	+	−	+	−
Δ*ppg1*/Δ*ppg2*	+	+	+	+	+	+
Mock fertilization[c]	−	−	−	−	−	−

[a] +, ascospores produced.
[b] −, no ascospores produced.
[c] Female was self-fertilized but with no exogenous male conidia.

Expression of *ppg2*, a homolog of the **a**-factor pheromone precursor gene, was not detected in any cells. The cognate receptor, *pre1*, was expressed weakly and only in mature ascospores. In fertility tests with deletion mutants, the *ppg2*/*pre1* pheromone-receptor pair had no detectable function in selfing or outcrossing. The lack of detectable function of the *ppg2*/*pre1* cognate pair probably was not due to incorrect identification of the genes. There were many candidates for *ppg2*, and we relied on microsynteny to identify the correct sequence (Fig. 2). The selected candidate had the expected regulatory and prenylation sequences (Fig. 3), which strongly supports its identification as *ppg2*. There was only one candidate for *pre1* in the genomic sequence, and it had the typical seven transmembrane domains of a G-protein-coupled receptor. Since *ppg2* and *pre1* homologs are functional in heterothallic ascomycetes (11, 23, 24), we hypothesize that the *ppg2* and *pre1* genes in *G. zeae* are nonfunctional vestiges of the evolutionarily recent change from a heterothallic to a homothallic life style in this fungus (49, 50).

Although the *ppg1*/*pre2* pheromone-receptor pair is functional in *G. zeae*, neither the pheromones nor the receptors are essential for sexual development. In obligate outcrossing tests, Δ*ppg1*, Δ*ppg2*, and Δ*ppg1*/Δ*ppp2* mutants were all fertile as males, thus demonstrating that pheromones are not absolutely required for male fertility in *G. zeae* (Table 4). Similarly, the full self-fertility of Δ*pre1* mutants and the partial self-fertility (~50%) of Δ*pre2* mutants and Δ*pre1*/Δ*pre2* double mutants demonstrate that neither of the pheromone receptors is essential for female fertility. This pattern differs from the heterothallic ascomycetes *N. crassa*, *P. anserina*, and *C. parasitica*, in which pheromones and receptors are essential for fertilization (11, 23, 24, 48). *G. zeae* also differs from the homothallic ascomycetes *S. macrospora* and *E. nidulans*, in which at least one functional pheromone receptor is required for self-fertilization (31, 42). Thus, *G. zeae* is the first ascomycete in which the dispensability of both pheromones and pheromone receptors for sexual development has been demonstrated.

The role of the *ppg1*/*pre2* pheromone-receptor pair in *G. zeae* appears to be restricted to fertilization. Aside from a reduction in the percentage of mature perithecia, Δ*ppg1* and Δ*pre2* mutants showed no consistent differences in colony morphology compared to the wild type. We tested combinations of Δ*ppg1* and Δ*ppg2* with Δ*mat1-1* or Δ*mat1-2* that might reveal postfertilization pheromone effects but found no evidence of interactions (Table 4). Most importantly, Δ*ppg1*, Δ*pre2*, Δ*ppg1*/Δ*ppg2*, Δ*pre1*/Δ*pre2*, and Δ*ppg1*/Δ*pre2* mutants all had decreased percentages of mature perithecia but the mature perithecia were apparently normal in size and fecundity (Fig. 6). These results are similar to those obtained with *P. anserina*, in which the role of pheromones also is restricted to the fertilization step (11).

In contrast, pheromones and/or receptors appear to have additional functions in *S. macrospora* and *E. nidulans*. Single pheromone or pheromone receptor of mutants *S. macrospora* have no detectable defects in self-fertilization, but double mutants in which there was no functional cognate pair, Δ*ppg1*/Δ*ppg2*, Δ*pre1*/Δ*ppg1*, and Δ*pre2*/Δ*ppg2*, showed drastically reduced numbers of mature self-fertilized perithecia (31). These results imply that both cognate pairs function interchangeably in fertilization. However, deletion of both the *pre1* and *pre2* receptor genes in *S. macrospora* leads to a complete loss of self-fertility, showing that at least one functional receptor is required for sexual development and suggesting that the receptor can be activated even when both pheromones are absent in *S. macrospora*. In *E. nidulans*, single pheromone receptor mutants produce smaller cleistothecia with a reduced number of ascospores, which suggests a postfertilization role for the receptors (42). Double-receptor mutants completely lost the ability to self-fertilize, as in *S. macrospora*. This diversity of pheromone-receptor functions in homothallic ascomycetes is not surprising since the homothallic life cycle apparently has evolved independently and uniquely multiple times from a conserved ancestral heterothallic state (50).

In *G. zeae*, *ppg1* and *pre2* appear to play conventional roles in the chemoattraction of female cells by male cells. Deletion of *ppg1* in the male strain dramatically decreased the effectiveness of spermatia for fertilizing Δ*ppg1* females, which are efficient facultative outcrossers (Table 5). Deletion of the *pre2* pheromone receptor gene in Δ*ppg1* females (i.e., Δ*ppg1*/Δ*pre2*) eliminated the ability of females to distinguish males with an intact *ppg1* gene (Table 5). These results are consistent with the classic *Neurospora* model in which spermatia secrete pheromones to attract female trichogynes (2, 3, 4, 23, 24, 25).

The high expression level of *ppg1* in induced germinating conidia and discharged ascospores, but not other cells, accords well with the chemoattraction model. The high expression in freshly discharged ascospores was unexpected and prompted us to test their ability to serve as spermatia. Interestingly, the ability of ascospores to function as spermatia was equal to or better than that of conidia (Table 5). Mature discharged ascospores expressed *ppg1* constitutively, unlike conidia, which expressed *ppg1* only when induced and germinating (Table 3). The role of ascospores as spermatia may be an underrecognized function.

We were not able to identify the female receptive structures or visually confirm chemoattraction of the female by the male. We intended to use the *pre2* reporter constructs to identify potential receptive female structures, but this approach was not possible since *pre2* is expressed in all cells except uninduced mycelia. We used methods similar to those described by Bistis (2, 3) for *N. crassa* but were unable to visually identify trichogynes associated with immature perithecia. If these structures occur at all, they are difficult to distinguish due to the relatively dense mycelial growth on carrot agar. Without a second pheromone-receptor pair for communication from the female structures to the male spermatium, it also is unknown how cell cycle synchronization is achieved prior to conjugation (2, 3, 4, 14). Further work is needed to identify the receptive female structures and how the spermatia transfer their nuclei to the female in this fungus.

The high facultative outcrossing rate in Δ*ppg1* mutant females probably is due to a decrease in the competitiveness of conidia from the female strain as spermatia. On a facultative outcrossing plate, conidia produced in situ by the female parent usually outnumber the spermatia added from the fertilizing male parent. The deletion of *ppg1* in the female parent may decrease the ability of conidia produced by the female strain to be recognized as spermatia. An alternative hypothesis is that absence of interfering pheromone peptides produced from female mycelia or perithecia increases the recognition of sper-

matia from the male strain. However, this hypothesis seems unlikely because these female structures do not appear to express *ppg1* (Table 3; Fig. 4). It also does not explain why male spermatia would be favored over conidia from the female strain. The enhancing effect of the deletion of *ppg1* in females could be useful for studies of pheromone function.

G. zeae must have a pheromone-independent alternate mechanism to activate the pheromone signal transduction pathway. Pheromone receptors are an integral upstream part of the receptor–G-protein-coupled mitogen-activated protein kinase cascade that mediates pheromone responses in ascomycetes (16). Sexual reproduction in *G. zeae* has an absolute requirement for pheromone pathway components such as both *MAT* idiomorphs (28) and for the *MGV1* MAP kinase gene (21), but not for sex pheromones or receptors. In *S. macrospora* and *E. nidulans*, pheromone receptors are required but can apparently trigger the signal transduction cascade in the absence of pheromone peptides (31, 42). In *G. zeae*, the receptors are not required, so nonspecific triggering must occur downstream of the receptors. This phenomenon could be similar to *STE5* gain-of-function mutations in *S. cerevisiae* in which pheromone pathway signaling is constitutively activated in the absence of pheromone or Gβγ (39, 43).

The pheromone-independent alternate activation mechanism clearly functions for external fertilization by spermatia, but it also might function as part of an internal self-fertilization mechanism. Trail and Common (47) reported thick, lipid-rich dikaryotic hyphae associated with perithecium production on carrot agar plates. The dikaryotic hyphae were produced in homothallic cultures, so the paired nuclei were presumed to be genetically identical. In detailed microscopic studies of homothallic production of perithecia in wheat stems, Guenther and Trail (19) again associated the production of perithecial initials with dikaryotic hyphae. Dikaryotic hyphae were produced from uninucleate hyphae within xylem vessels, pith cavities, and chlorenchyma tissues. Although the details are still unclear, these observations suggest that sexual developmental can occur without fertilization by external spermatia.

Induction of the sexual stage in *G. zeae* occurs in response to specific compounds or conditions in carrot agar cultures. Carrot agar is an excellent medium for induction of the sexual stage of *G. zeae*, and abundant perithecia usually are formed within 10 days (7). In contrast, few or no perithecia are produced on potato dextrose agar, complete medium, minimal medium, or CMC medium (data not shown). Germinating conidia and ascospores from induced cultures, i.e., cultures growing on carrot agar that have been fertilized with a spore suspension or mock fertilized with a 2.5% aqueous Tween 60 solution, expressed *ppg1* at a high level. Germinating conidia from cultures grown on water agar, complete medium (solid or liquid), minimal medium (solid or liquid), minimal medium containing 10% of the normal nitrogen amount, and CMC liquid medium did not express *ppg1* (data not shown). In *N. crassa*, the expression of these pheromone pathway genes is influenced by nitrogen starvation (4, 25). However, in *G. zeae*, low-nitrogen media such as water agar, CMC medium, and reduced-nitrogen minimal medium apparently are insufficient for induction. The GFP reporter strains for *ppg1* and *pre2* could be useful for identifying the inducing factors for the sexual stage of this fungus.

In conclusion, this study demonstrates that one of the pheromone-receptor pairs (*ppg1*/*pre2*) found in heterothallic ascomycetes enhances, but is not essential for, selfing and outcrossing in homothallic *G. zeae*, whereas the other pheromone-receptor pair (*ppg2*/*pre1*) no longer has any detectable function in sexual reproduction. Thus, a pheromone- and pheromone receptor-independent sexual triggering mechanism exists in this fungus, which makes it unique among ascomycetes. This alternate activation mechanism appears to be an evolutionarily recent adaptation since most other *Gibberella* species are heterothallic (30). Therefore, different portions of the ancestral pheromone signaling pathway in *G. zeae* may be under purifying selection pressure (*ppg1* and *pre2*), under directional selection pressure (components of the signal transduction mechanism), or under no selection pressure at all (*ppg2* and *pre1*). Characterization of recent molecular evolution in these genes may provide insights into the fundamental mechanisms underlying cell recognition and differentiation in fungi.

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