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Identification and functional characterization of genes involved in the sexual reproduction of the ascomycete fungus *Gibberella zeae*

Jungkwan Lee [a], Chanju Park [a], Jin-Cheol Kim [b], Jung-Eun Kim [a,1], Yin-Won Lee [a,*]

[a] Department of Agricultural Biotechnology and Center for Fungal Pathogenesis, Seoul National University, Seoul 151-921, Republic of Korea
[b] Chemical Biotechnology Center, Korea Research Institute of Chemical Technology, Daejon 305-600, Republic of Korea

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ABSTRACT

We previously reported that G protein alpha subunit 1 (GPA1) is essential for sexual reproduction in the homothallic ascomycete fungus *Gibberella zeae*. In this study we performed microarray analyses on a GPA1 deletion mutant of *G. zeae* (Δ*gpa1*) to identify genes involved in the sexual reproduction of this fungus. In the Δ*gpa1* strain, 645 genes were down-regulated and 550 genes were up-regulated during sexual reproduction when compared to the wild-type strain. One hundred of the down-regulated genes were selected for further investigation based on orthologous group clusters and differences in transcript levels. Quantitative real time-PCR was used to determine transcriptional profiles of these genes at various sexual and vegetative stages. We observed that transcript levels of 78 of these genes were dramatically increased in the wild-type strain during sexual reproduction compared to levels observed during vegetative growth, and were down-regulated in Δ*gpa1* compared to the wild-type strain. We deleted 57 of these genes and found that four of the deletion mutants lost self-fertility and five produced fewer perithecia compared to the wild-type strain. Two mutants produced wild-type numbers of perithecia, but maturation of perithecia and ascospores was delayed. In all we identified 11 genes that are involved in sexual reproduction of *G. zeae* and present evidence that some of these genes function at distinct stages during sexual reproduction in the fungus.

1. Introduction

The ascomycete fungus *Gibberella zeae* (anamorph *Fusarium graminearum*) is an important pathogen of Fusarium head blight of cereal crops such as wheat and barley. When infected, crops become contaminated with mycotoxins which can lead to mycotoxicoses in humans and animals [1,2]. This fungus produces ascospores and conidia via sexual and asexual reproduction, respectively. Forcibly discharged into the air from perithecia, ascospores are the primary inoculum of the disease [3,4]. Infection is initiated in florets and can occur at the onset of anthesis until the soft dough stage of kernel development. Bleaching occurs several days after infection, and the fungus subsequently spreads from the infected floret to rachis [3].

In ascomycete fungi, sexual reproduction is controlled by mating type (*MAT*) locus [5]. In the homothallic fungus *G. zeae*, *MAT1-1* and *MAT1-2* idiomorphs are tightly linked on the same chromosome and this fungus can complete sexual reproduction without a mating partner [6,7]. *G. zeae* produces perithecia that are composed of three distinguishable layers. Within perithecia, asci are biseriately arranged and each ascus contains eight ascospores [8]. Several studies have identified genes involved in the sexual reproduction of *G. zeae*, including *MGV1*, *GPMK1*, *FBP1*, *CCH1*, *GzSNF1*, *GzCHS5*, *GzCHS7*, and *GzSYN2* [12–18]. Mutant collections generated by random insertional mutagenesis [9,10] and the availability of genome sequence [11] have provided important tools for the characterization of additional factors and signaling pathways that mediate sexual reproduction in *G. zeae* and should serve to help increase our understanding of this process.

Sexual reproduction in ascomycete fungi is initiated by the binding of pheromone peptides and pheromone receptors, which are regulated by *MAT* locus. The pheromone receptors are each coupled to a heterotrimeric G protein complex, which consists of a GDP-bound Gα subunit in complex with Gβγ subunits. Upon pheromone binding, GTP is exchanged for GDP on the Gα protein, leading to the dissociation of Gα-GTP and Gβγ subunits. This triggers the activation of a mitogen-activated protein kinase (MAPK) signal transduction pathway [19]. Several hundred genes involved in the sexual reproduction of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are regulated by MAPK signaling cascades [20,21]. Though *G. zeae* is homothallic, it retains

* Corresponding author. Fax: +82 2 873 2317.
E-mail address: lee2443@snu.ac.kr (Y.-W. Lee).
[1] Present address: Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843-2132, United States.

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functional pheromones [22] and G protein alpha subunit 1 (GPA1) is essential for sexual reproduction in this fungus [23], suggesting that GPA1-mediated signaling is also conserved in this fungus.

To further investigate sexual reproduction in *G. zeae*, we focused on genes regulated by GPA1 signaling. We hypothesized that genes regulated by this pathway are involved in sexual reproduction because the deletion of *GPA1* results in complete loss of sexual reproduction in *G. zeae*. To address this hypothesis, we analyzed RNA transcript levels of the GPA1 deletion mutant (Δ*gpa1*) and wild-type strain at various stages of sexual reproduction. We characterized the genes that were down-regulated in Δ*gpa1* by deleting these genes in *G. zeae* and observing effects of these deletions on perithecia and ascospore formation, two important phenotypes associated with sexual reproduction. Using this approach we identified 11 genes involved in the sexual reproduction of *G. zeae* and present evidence that suggests some of these genes function at distinct stages during the process.

2. Materials and methods

2.1. Strains and culture conditions

The wild-type strain Z03639 of *G. zeae* [24] and mutants derived from this strain were stored as conidia in a 20% glycerol solution at −80 °C. A Z03639-derived mutant that carries a deletion of *GPA1* gene [23] was used for microarray analysis. Standard laboratory methods and media were used [1], and conidial production was induced in carboxymethyl cellulose medium [25].

2.2. Nucleic acid preparation, primers, and PCR conditions

Fungal genomic DNA was extracted using a cetyltrimethylammonium bromide procedure [1]. PCR primers (Table S1) for this study were synthesized by Bionics (Seoul, Korea) and standard PCR was performed as previously described [22]. For total RNA extraction, the wild-type and Δ*gpa1* strains were incubated on carrot agar [1] at 25 °C. After a 5-day incubation, sexual reproduction was induced by applying 1 ml of 2.5% Tween 60 solution and knocking down the aerial mycelium with a bent glass rod. The cultures were continuously incubated under a near-UV light (wavelength 365 nm; HKiv Co., Xiamen, China) at 25 °C. Total RNA was isolated from vegetative cultures at 3, 5, and 7 days post-inoculation (non-induced cultures) and from cultures induced to undergo sexual reproduction 3, 5, and 7 days post-induction using an Easy-Spin Total RNA Extraction Kit (Intron Biotech, Seongnam, Korea). The first strand cDNA was synthesized with SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Supermix (Bio-Rad, Hercules, CA) and a 7500 real-time RCR system (Applied Biosystems) with rt1/rt2 primers (Table S1). Elongation factor 1-beta (Broad Institute ID: FGSG_01008.3) was used as an endogenous control for normalization. The PCRs were repeated two times with two replicates per run. We used the Broad Institute *Fusarium* comparative database for our analyses (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html).

2.3. cDNA synthesis and microarray

Total RNAs were isolated from carrot agar cultures of the wild-type and Δ*gpa1* strains 3 days after sexual induction. Double strand cDNAs were synthesized using Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen). For the synthesis of Cy3-labeled target DNA fragments, 1 μg of double-stranded cDNA was mixed with 40 μl of Cy3-9mer primers (Sigma–Aldrich, St. Louis, MO) and denatured at 98 °C for 10 min. Subsequently, 10 μl dNTP (10 mM each), 8 μl water, and 2 μl Klenow fragment (50 units/μl) (New England Biolabs, Ipswich, MA) were added to the reaction and incubated at 37 °C for 2 h. DNA was precipitated by adding 11.5 μl of 5 M NaCl and 110 μl isopropanol and collected by centrifugation (12 000×*g*). Precipitated samples were rehydrated in 25 μl water, of which 13 μl was used for microarray.

Microarray analysis was conducted at GreenGene Biotech (Yongin, Korea) using the *F. graminearum* 13 k Microarray, which was designed from the *F. graminearum* sequencing assembly 3 and includes 13 382 transcripts from the *Fusarium* comparative database. Ten 60-nt probes were designed for each gene. The first probe was designed such that it annealed to a sequence located within the target gene 250 bp from stop codon. Additional probes were created by shifting the sequence 10 bp, and all probes were duplicated in two blocks. In total, 267 324 probes were designed. The microarray was manufactured at NimbleGen (Madison, WI). Experiments were repeated two times with independently prepared total RNA samples. Microarrays were scanned with a Genepix 4000 B (Axon Instruments, Toronto, Canada), and signals were analyzed by Nimblescan 2.5 (NimbleGen).

2.4. Microarray data analyses

Robust multiarray average (RMA) [26] was used to normalize microarray data. The GPA1 data set refers to genes that were at least twofold up- or down-regulated in Δ*gpa1* compared to the wild-type strain. RMA results from the FG5 data sets that were previously constructed to identify genes regulated during sexual development in *G. zeae* were downloaded from the PLEXdb (http://www.plexdb.org/plex.php?database=Fusarium). RMA results from the FG5-0h data set (generated from a non-induced culture) were used as a reference for the other FG5 data sets, FG5-72h, FG5-96h, and FG5-144h. These data sets were generated from samples collected from cultures 72, 96, and 144 h after sexual induction. RankProd [27] was used to analyze the data sets and an SAS macro was used to generate overlapping gene pools among the GPA1 and FG5 data sets.

2.5. Targeted gene deletion

Targeted gene deletion was conducted using split marker recombination [28] with a slight modification. The 5′ and 3′ regions of the target gene were amplified by PCR using the F1/R2 and F3/R4 primer sets, respectively (Table S1). The geneticin resistance gene cassette (*gen*) was amplified from pII99 [29] with gen-F1/gen-R2 primers. The three amplicons (5′ region, 3′ region, and *gen*) were mixed in a 1:1:3 M ratio and fused by a second round of PCR. The fused construct was used as a template to generate split markers with the new nested primer sets, F1-NT/gen-R-NT and gen-F-NT/R4-NT. The amplification products were combined and transformed into the protoplast of the wild-type strain. Transformants were first selected on regeneration medium containing 50 μg/ml of geneticin (Sigma–Aldrich), and colonies were then selected again by transferring to complete medium containing 100 μg/ml of geneticin.

2.6. Sexual reproduction

Each strain was incubated on carrot agar [1] at 25 °C for 5 days. The cultures were mock-fertilized with 1 ml of 2.5% Tween 60 solution to induce sexual development [1] and were continuously incubated under a near-UV light at 25 °C. Seven to ten days after sexual induction, approximately twenty perithecia were dissected on glass slides in a drop of 20% glycerol and the asci were flattened under a coverslip. Asci rosettes and ascospores were observed under a DE/Axio Image A1 microscope (Carl Zeiss, Oberkochen, Germany). The experiments were performed twice with three replicates.

3. Results

3.1. Identification of genes regulated by GPA1-mediated pathways

We measured RNA transcript levels isolated from cultures of the wild-type and Δ*gpa1* strains 3 days after sexual induction. In the Δ*gpa1* mutant, 645 and 550 genes were down- and up-regulated, respectively, twofold or more than in the wild-type strain (Tables S2 and S3). We compared our GPA1 data set to microarray data previously collected in *G. zeae* (FG5 microarray data sets). Of the 1195 genes down- or up-regulated in Δ*gpa1*, 402 genes overlapped with at least one or more of the FG5 data sets, suggesting that these genes may be involved in the sexual development of *G. zeae*. Of the 168 genes that were differentially regulated in all microarray data sets (Fig. 1), 67 were down-regulated and 101 genes were up-regulated in Δ*gpa1* (Tables S2 and S3).

3.2. Transcriptional profiles of genes regulated by GPA1-mediated pathways

One hundred of the 645 genes down-regulated in Δ*gpa1* were selected for further study based on their orthologous group clusters and their overall differences in RNA transcript levels. This group of 100 genes included 22 of 67 genes that were identified in the analysis above. Levels of all 100 genes were measured via qRT-PCR to determine the transcriptional profiles during the various vegetative and sexual stages. Sixty-four genes were specifically down-regulated in Δ*gpa1* compared to the wild-type strain 3 days after sexual induction, which was consistent with the microarray analysis (Table S4). Five to seven days after sexual induction, we observed the down-regulation of an additional fourteen genes in Δ*gpa1* compared to the wild-type strain. In total, 78 of the 100 genes chosen for further study were down-regulated. The other 22 genes were not significantly down-regulated in Δ*gpa1* (Table S4), suggesting that these genes were false positives.

We observed that 18 of the 22 genes originally identified as down-regulated in the microarray and meta-analysis were identified as down-regulated in Δ*gpa1* by qRT-PCR analysis at all stages of sexual reproduction. Transcripts of three genes were not observed to be different until 5–7 days after induction in Δ*gpa1*, while one gene was not observed to be down-regulated until 7 days post-induction in Δ*gpa1* (Table S4).

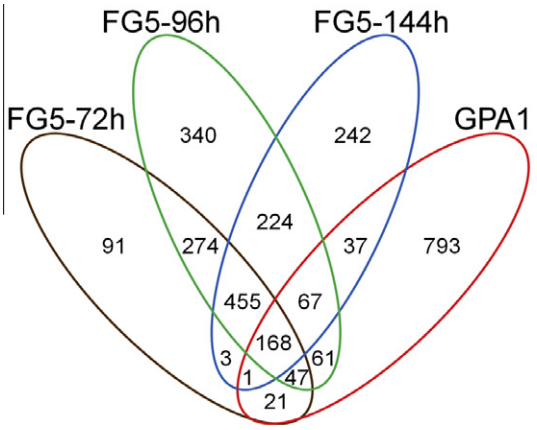

Fig. 1. Meta-analysis of microarray data sets. FG5 data sets were downloaded from PLEXdb. FG5-72h, FG5-96h, and FG5-144h include genes differentially regulated 72, 96, and 144 h, respectively, after sexual induction compared to a non-induced culture. GPA1 indicates genes significantly regulated in Δ*gpa1* compared to the wild-type strain 72 h after sexual induction. The GPA1 and FG5 data sets were generated from *G. zeae* strains Z03639 and PH-1, respectively.

3.3. Targeted gene deletions

The ORF of a target gene from the wild-type genome was replaced with the *gen* to generate deletion mutants. The mutants were first screened with PCR amplification using F1/gen-R2 primers. Deletion mutants contained 1.5–2.5 kb fragments depending on the size of the gene. Due to the primer combination used for validating insertions, mutants that carried ectopic integration did not give rise to any amplicons. A second PCR was performed to validate the deletion mutant using the rt1/rt2 qRT-PCR primers. Using these primers, the correct deletion mutants did not generate amplicons whereas strains with ectopic integrations generated 0.1–0.5 kb fragments. Using this system of validating deletions, three independent deletion mutants per gene were selected for phenotypic analysis. We were able to delete 57 of the 78 genes identified as significantly down-regulated in Δ*gpa1* (Table S2).

3.4. Phenotypes of deletion mutants

We determined that four genes, FGSG_04480, FGSG_05239, FGSG_05325, and FGSG_08320, were essential for self-fertility. Mutants carrying deletions in three of these genes did not produce protoperithecia, indicating these genes are required for perithecia production. Though FGSG_08320-deleted mutants (Δ*08320*) produced a few perithecia, they were small and did not contain ascospores (Fig. 2 and Table S5). Five mutants, Δ*03673*, Δ*06484*, Δ*07578*, Δ*07590*, and Δ*07869*, produced fewer perithecia than did the wild-type strain (Fig. 2). One of these mutants, Δ*03673*, produced perithecia of mixed sizes. The small sized perithecia never fully developed, even after the cultures were incubated for three additional weeks, whereas the large perithecia contained normal ascospores and cirrhi (Figs. 3 and 4). In the other four mutants, maturation of both perithecia and ascospores was delayed compared to that of the wild-type strain (Fig. 3 and Table S5).

Two other mutants, Δ*00348* and Δ*01862*, produced wild-type numbers of perithecia, but maturation of approximately half of the perithecia was delayed compared to the wild-type strain (Fig. 2). Dissection of the perithecia showed that ascospore formation, even in the early mature perithecia, was dramatically delayed in both mutants (Fig. 3). These results suggest that both genes function in the maturation of ascospores. While the wild-type strain oozed out ascospores and formed cirrhi, most perithecia observed in Δ*00348* and Δ*01862* did not form cirrhi at the ostiole 10 days after sexual induction (Fig. 4). Cirrhi were first observed in both mutants 12–13 days after sexual induction.

4. Discussion

G proteins are important signaling proteins that are conserved from lower eukaryotes to human [19]. The *G. zeae* genome contains three G protein alpha subunits, one beta and one gamma subunit, but GPA1 alone is involved in sexual reproduction in this fungus [23]. Therefore, we hypothesized that we would be able to identify genes involved in sexual reproduction by comparing transcription levels of genes between the wild-type and Δ*gpa1* strains. In this study, we successfully identified and characterized 11 genes that are required for proper sexual reproduction of *G. zeae*.

Identification and functional characterization of genes involved in *G. zeae* sexual reproduction has been done using forward and reverse genetics [9–18]. Since the genome sequence [11] and a whole-genome Affymetrix GeneChip [30] have become available, numerous microarray analyses have been conducted to identify genes involved in perithecium development [31–33] and to find genes regulated by *MAT1-2* [34], but large-scale functional characterization of those genes has not been performed. To date only a

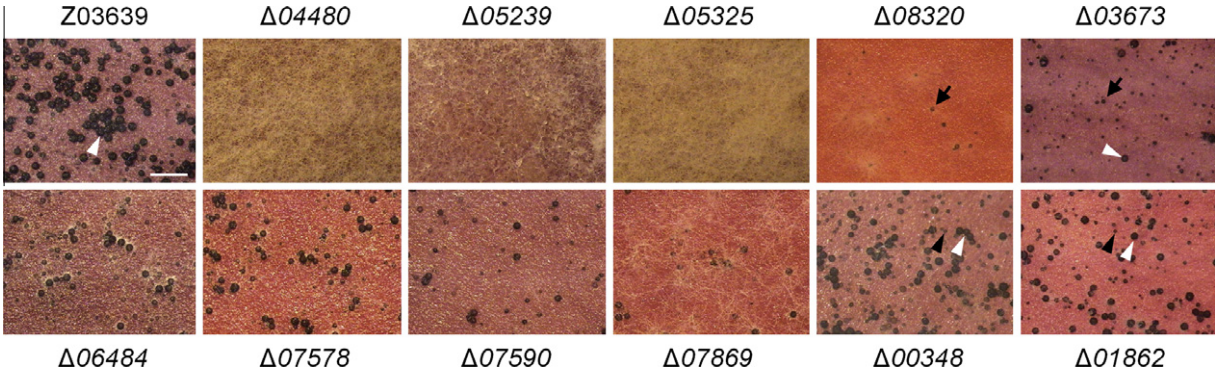

Fig. 2. Self-fertility of *G. zeae* strains. Z03639, wild-type *G. zeae* strain; the number after "Δ" indicates a locus (FGSG#) in the *G. zeae* genome database and ΔFGSG# indicates the deletion mutant of each locus. Five-day old carrot agar culture was mock-fertilized to induce sexual reproduction and incubated for an additional 7 days. Photographs were taken using a dissecting microscope. White and black arrowheads indicate mature and immature perithecia, respectively. Immature perithecia were observed to develop more fully after additional incubation. Arrows indicate protoperithecia that did not produce ascospores. Scale bar = 1 mm.

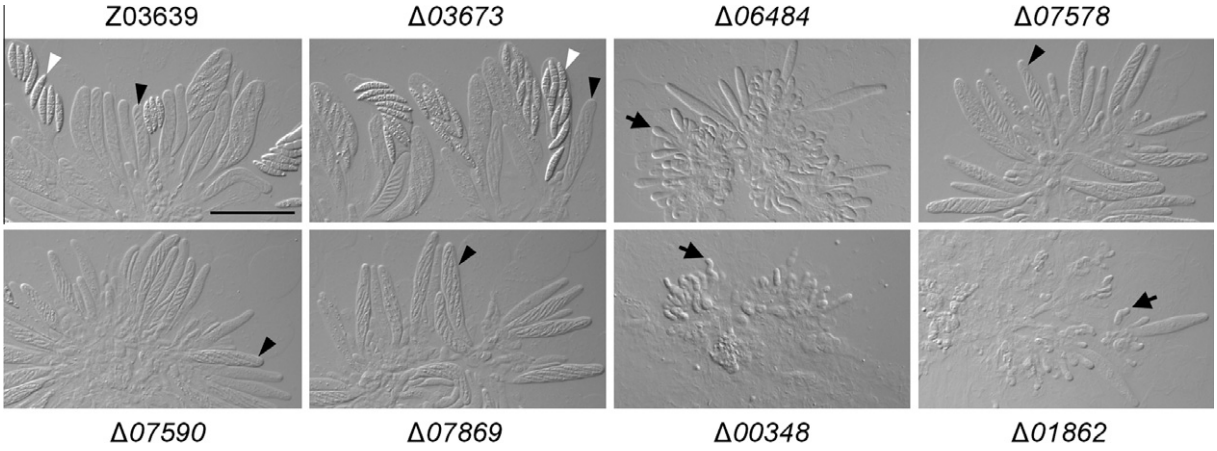

Fig. 3. Asci rosettes of *G. zeae* strains. Perithecia were dissected 7 days after sexual induction. White and black arrowheads indicate asci that contained mature and immature ascospores, respectively, and arrows indicate young asci. Scale bar = 50 μm.

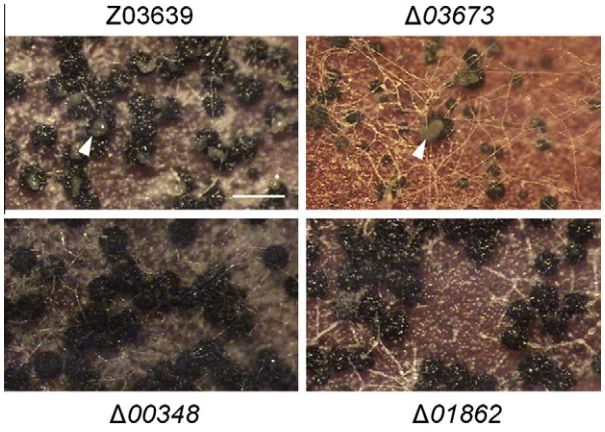

Fig. 4. Formation of cirrhi in *G. zeae* strains. Photographs were taken 10 days after sexual induction. Arrowheads indicate cirrhi formed at the ostioles of the perithecia. Scale bar = 500 μm.

few genes, including three protein kinases, MGV1 [12], GPMK1 [13], and GzSNF1 [16], have been shown to be involved in sexual reproduction. Our microarray analysis showed that five putative genes encoding protein kinases were down-regulated and three were up-regulated in Δ*gpa1* compared to the wild-type strain (Tables S2 and S3), though *MGV1*, *GPMK1*, and *GzSNF1* kinases were not identified in our studies. Other genes previously demonstrated to be involved in sexual reproduction, including *FBP1* [14], *CCH1* [15], *GzCHS5*, *GzCHS7* [17], and *GzSYN2* [18], were also not identified in our study. These observations suggest that these genes are regulated independently of GPA1-mediated pathways.

In ascomycete fungi, sexual reproduction is regulated by *MAT* genes [5]. Lee et al. [34] showed that 171 genes were significantly down-regulated in the *MAT1-2* deletion mutant during sexual reproduction, but we did not observe any of these genes to be down-regulated in Δ*gpa1*. In our study, we observed that the transcript levels of *MAT1-2* were the same in both wild-type and Δ*gpa1* strains, explaining why none of the 171 genes down-regulated in *MAT1-2* deletion mutant overlapped with our data set. This result suggests that genes downstream of the GPA1-mediated pathway perform different roles in sexual reproduction from genes regulated by *MAT1-2*, even though GPA1 is activated by the pheromone pathway. One possible explanation is that genes regulated by *MAT* genes may be responsible for early events in sexual reproduction, such as recognition of mating partner and opposite nuclei, whereas the GPA1-mediated pathway may be functional for the middle and late stages of sexual reproduction, such as maturation of perithecia and formation of ascospores.

Microarray analyses typically produce too many genes to make these analyses useful on their own. For instance, we identified 645 genes that were down-regulated in Δ*gpa1*, some of which may be responsible for the loss of fertility observed in Δ*gpa1*. To identify

the genes in this set that were potentially involved in GPA1-mediated signaling processes, we compared our GPA1 data set with the previously collected microarray FG5 data sets, and consequently identified 67 genes that are significantly up-regulated during sexual reproduction. We analyzed 22 of these genes via qRT-PCR and showed that while transcript levels for all these genes were significantly increased at the various sexual stages in the wild-type strain, they were down-regulated in Δ*gpa1*. We deleted 15 of these 22 genes and found that four of them showed defects in sexual reproduction. Our results demonstrate that the meta-analysis conducted in this study can be used to generate gene pools involved in sexual reproduction of *G. zeae*.

In conclusion, we identified 11 genes involved in the sexual reproduction of *G. zeae* by identifying and characterizing genes regulated by GPA1-mediated pathways. Considering that as many genes have been demonstrated to play a role in sexual reproduction of this fungus in the last two decades, our study has provided a much more comprehensive list of factors involved in the sexual reproduction of *G. zeae*, as well as an initial characterization of some of these factors. Future studies will be focused on in-depth analyses of each gene and elucidating the cross talk between the genes identified in this study to better establish the molecular mechanisms underlying sexual reproduction of *G. zeae*.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.09.005.

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