

G α and G β Proteins Regulate the Cyclic AMP Pathway That Is Required for Development and Pathogenicity of the Phytopathogen *Mycosphaerella graminicola*[∇]

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We identified and functionally characterized genes encoding three G α proteins and one G β protein in the dimorphic fungal wheat pathogen *Mycosphaerella graminicola*, which we designated *MgGpa1*, *MgGpa2*, *MgGpa3*, and *MgGpb1*, respectively. Sequence comparisons and phylogenetic analyses showed that MgGPA1 and MgGPA3 are most related to the mammalian G α_i and G α_s families, respectively, whereas MgGPA2 is not related to either of these families. On potato dextrose agar (PDA) and in yeast glucose broth (YGB), *MgGpa1* mutants produced significantly longer spores than those of the wild type (WT), and these developed into unique fluffy mycelia in the latter medium, indicating that this gene negatively controls filamentation. *MgGpa3* mutants showed more pronounced yeast-like growth accompanied with hampered filamentation and secreted a dark-brown pigment into YGB. Germ tubes emerging from spores of *MgGpb1* mutants were wavy on water agar and showed a nested type of growth on PDA that was due to hampered filamentation, numerous cell fusions, and increased anastomosis. Intracellular cyclic AMP (cAMP) levels of *MgGpb1* and *MgGpa3* mutants were decreased, indicating that both genes positively regulate the cAMP pathway, which was confirmed because the WT phenotype was restored by adding cAMP to these mutant cultures. The cAMP levels in *MgGpa1* mutants and the WT were not significantly different, suggesting that this gene might be dispensable for cAMP regulation. In planta assays showed that mutants of *MgGpa1*, *MgGpa3*, and *MgGpb1* are strongly reduced in pathogenicity. We concluded that the heterotrimeric G proteins encoded by *MgGpa3* and *MgGpb1* regulate the cAMP pathway that is required for development and pathogenicity in *M. graminicola*.

Signal transduction pathways are important for sensing and responding to different environmental stimuli in both lower and higher eukaryotes. The highly conserved heterotrimeric guanine nucleotide-binding proteins (G proteins) belong to a family of regulatory proteins that are crucial for the transduction of signals, which are perceived by a distinct family of cell surface receptors (4). Heterotrimeric G proteins contain three subunits (α , β , and γ) that are linked in the inactive state. Activation of a G α subunit by a transmembrane receptor leads to exchange of bound GDP with GTP on the G α subunit, resulting in dissociation of the G α and the G $\beta\gamma$ dimeric subunits, which can now interact with downstream effectors that subsequently generate changes in cellular responses (for a review, see reference 10).

Filamentous fungi have one G β - and usually three G α -encoding genes that belong to three major groups. Encoded proteins in groups I and III are related to the mammalian G α_i and G α_s families, respectively, but group II fungal G α proteins have no mammalian counterpart (1, 4, 14, 22, 33, 53). Interestingly, the corn smut fungus *Ustilago maydis* contains a unique fourth G α -encoding gene, and *Saccharomyces cerevisiae*

contains only two G α proteins (10, 57). Irrespective of the observed numerical variation, G α proteins regulate a variety of cellular and developmental responses (4). For plant-pathogenic fungi, G β -encoding genes have been characterized functionally (9, 14, 22, 27, 31, 48, 52). Apart from the fact that individual G α -encoding genes and the G β -encoding gene have been demonstrated to regulate growth, reproduction, and virulence, comparative functional characterization of all G α -encoding genes has been reported only for a few plant-pathogenic fungi, including *Magnaporthe grisea*, *Cryphonectria parasitica*, and *U. maydis* (5, 41, 57).

Mycosphaerella graminicola (anamorph *Septoria tritici*) causes septoria tritici blotch disease in bread and durum wheat in areas with high rainfall during the growing season, particularly in Western Europe, where it is considered to be the most important wheat disease (30). It is a ubiquitous phytopathogen with a lifestyle completely different from that of the aforementioned plant-pathogenic fungi. It is a dimorphic pathogen, and therefore the transition from a yeast-like to a filamentous form is important for initiation of infection (45). *M. graminicola* does not form appressoria but penetrates the leaves through stomata without forming specific infection structures. Furthermore, as a hemibiotroph, it has a biotrophic phase of about 10 days that is followed by a rapid switch to necrotrophy. The necrotic foliar lesions bear anamorphic and teleomorphic fructifications. *M. graminicola* is the model fungus for the Mycosphaerellaceae and even for the order Dothideales, an extremely large and diverse class of fungi with over 1,000 named

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TABLE 1. Generated *Mycosphaerella graminicola* G protein transformants and primers used in this study

Gene or position	<i>M. graminicola</i> transformant	Type	Primer	Primer sequence
<i>MgGpa1</i>	IPO323 Δ <i>MgGpa1-14</i>	Disruption strain	<i>MgGpa1</i> -F3	TTCAGCGAAGCCGAGAATATC
	IPO323 Δ <i>MgGpa1-15</i>	Disruption strain	<i>MgGpa1</i> -R2	CACATGCGCAGGTTCTCTTG
	IPO323 E- <i>MgGpa1-13</i>	Ectopic transformant		
<i>MgGpa2</i>	IPO323 Δ <i>MgGpa2-2</i>	Disruption strain	<i>MgGpa2</i> -F1	GGCGTACAGCTTGCGAT
	IPO323 Δ <i>MgGpa2-6</i>	Disruption strain	<i>MgGpa2</i> -R2	CATCAAGTTATGCAAGTCCCG
	IPO323 E- <i>MgGpa2-10</i>	Ectopic transformant		
<i>MgGpa3</i>	IPO323 Δ <i>MgGpa3-21</i>	Disruption strain	<i>MgGpa3</i> -F2	AGCAGTGGGAACAATGAGGAG
	IPO323 Δ <i>MgGpa3-33</i>	Disruption strain	<i>MgGpa3</i> -R4	TGAATACTGAGCTGGCCCAT
	IPO323 E- <i>MgGpa3-2</i>	Ectopic transformant		
<i>MgGpb1</i>	IPO323 Δ <i>MgGpb1-3</i>	Disruption strain	<i>MgGpb1</i> -F4	ACCACGAACAAAGTACACGCC
	IPO323 Δ <i>MgGpb1-22</i>	Disruption strain	<i>MgGpb1</i> -R4	AGTCGACCTGAGACGGAGAAAG
	IPO323 E- <i>MgGpb1-1</i>	Ectopic transformant		
Left border of transposon (Tn7L)			S	ATAATCCTTAAAAACTCCATTTCCACCCCT
Right border of transposon (Tn7R)			N	ACTTTATTGTCATAGTTTAGATCTATTTTG

species, including major plant pathogens such as the banana leaf streak fungus *Mycosphaerella fijiensis* (12, 21). Large expressed sequence tag (EST) libraries and the recently released genome sequence have been instrumental for the identification and characterization of genes involved in the development and pathogenicity of *M. graminicola* (<http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html>). Recently, we reported that genes encoding mitogen-activated protein kinases (MAPKs) (*MgFus3*, *MgSlt2*, and *MgHog1*) and the catalytic (*MgTpk2*) and regulatory (*MgBcy1*) subunits of protein kinase A (PKA) are essential pathogenicity factors and regulate specific steps during the infection process (8, 43–45). To extend our knowledge about the role of G proteins in the development and pathogenicity of *M. graminicola*, we functionally analyzed three G α -encoding genes and one G β -encoding gene of *M. graminicola*, which we designated *MgGpa1*, *MgGpa2*, *MgGpa3*, and *MgGpb1*, respectively. Our results show the requirement of *MgGpa1*, *MgGpa3*, and *MgGpb1* for pathogenicity, whereas the latter also negatively regulates cell fusion and anastomosis. Among the G protein-encoding genes characterized in this study, *MgGpa3* and *MgGpb1* positively regulate the cyclic AMP (cAMP) pathway. *MgGpa1* seems to be dispensable for cAMP regulation, whereas *MgGpa2* appears to be redundant, for none of the assays rendered altered phenotypes. Our results open new perspectives for studying the regulatory machinery of the cAMP pathway in *M. graminicola* and other plant-pathogenic fungi.

MATERIALS AND METHODS

Fungal strains and culture conditions. The Dutch field strain *M. graminicola* IPO323 is highly virulent on winter wheat cv. Obelisk and was used as the wild type (WT) and recipient strain throughout this study. Growing conditions, propagation, and maintenance were as described previously (35, 44).

DNA manipulations and analysis. Standard protocols were used for DNA manipulation (58). Plasmid DNA was isolated using a QIAprep Spin miniprep kit (Qiagen, Hilden, Germany). Genomic DNA of *M. graminicola* was extracted using a Puregene DNA isolation kit (Gentra Systems Inc., Minneapolis, MN). A DYEnamic ET dye terminator cycle sequencing kit (Amersham Biosciences, Roosendaal, The Netherlands) was used to sequence DNA on an ABI Prism 3100 capillary automated sequencer according to recommended protocols. Phylogenetic tree generation, DNA and protein sequence alignment, editing, and analysis were performed using MEGALIGN and DNA Star software (Madison, WI). Homology searches were performed with the BLAST program (2).

Construction of plasmid vectors. By in silico analyses of EST databases of *M. graminicola* IPO323 (34), we identified full-length cDNA clones of *MgGpa1*, *MgGpa2*, *MgGpa3*, and *MgGpb1*. We used the GPS-Mutagenesis system (New England Biolabs, Leusden, The Netherlands) to generate disruption constructs, using a homemade customized donor construct, pGPS3HygKan, that was described previously (44). After transposition, the transposition mixture was cloned into *Escherichia coli* strain DH10B (Invitrogen, Groningen, The Netherlands) by electroporation, using the recommended protocol of the suppliers, and subsequently plated onto LB containing gentamicin and kanamycin to select for colonies with transposon insertions in the target constructs, which were confirmed by multiplex colony PCR as described previously (44). The exact position of the transposon insertion was determined by sequencing from the right and left borders of the transposon, using primer N and primer S (Table 1), and by comparison with nondisrupted sequences. We selected constructs with transposon insertions in the central part of the WT genes, and these were designated pCGN1589 Δ *MgGpa1*, pCGN1589 Δ *MgGpa2*, pCGN1589 Δ *MgGpa3*, and pCGN1589 Δ *MgGpb1*, respectively (Fig. 1A).

Fungal transformation. The disruption constructs pCGN1589 Δ *MgGpa1*, pCGN1589 Δ *MgGpa2*, pCGN1589 Δ *MgGpa3*, and pCGN1589 Δ *MgGpb1* were cloned into *Agrobacterium tumefaciens* strain LBA1100 by electroporation. *A. tumefaciens*-mediated transformation was performed according to a standard protocol (44, 72). Homologous recombinants were identified by PCR screening (Fig. 1B), using primers that were designed upstream and downstream of the transposon insertions together with either primer S or primer N, located in the left or right border of the transposon, respectively (Table 1). Eventually, we selected two mutant strains and one ectopic transformant for each gene for phenotypic analyses (Table 1). To ensure the independence of the transformants, we performed transformation experiments over time. In cases where transformants were generated in the same experiment, they were collected mostly from different plates (at least four plates for each transformation) to prevent the selection of clones. Moreover, in contrast to polyethylene glycol transformation, *A. tumefaciens*-mediated transformation precludes the selection of clones, as cocultivation of the fungus and *Agrobacterium* is done on solid nitrocellulose filters, and hence each colony represents an independent transformation event.

Phenotyping. We phenotyped all disruptant strains along with ectopic transformants and the WT as control strains.

In vitro assays were performed on agar plates to study colony development by spotting approximately 1 μ l of spore suspension (10^7 spores ml $^{-1}$) on potato dextrose agar (PDA), which was subsequently cultured for 10 days at 20°C under continuous light, with and without 13 mM exogenous cAMP, or at 28°C in darkness, as well as in liquid yeast glucose broth (YGB) at 20°C under daylight settings. Spore length measurements were performed on 100 randomly selected spores per strain from YGB cultures.

Histological analyses were performed on water agar (WA) and PDA, and the effect of exogenous cAMP (15 mM) on the early development of the strains was examined. Samples were monitored every 24 h until 80 h after initiation of the experiment, using a Zeiss Axioskop microscope (Carl Zeiss, Jena, Germany) equipped with a Carl Zeiss AxioCam 6.08.0 digital camera connected to an IBM

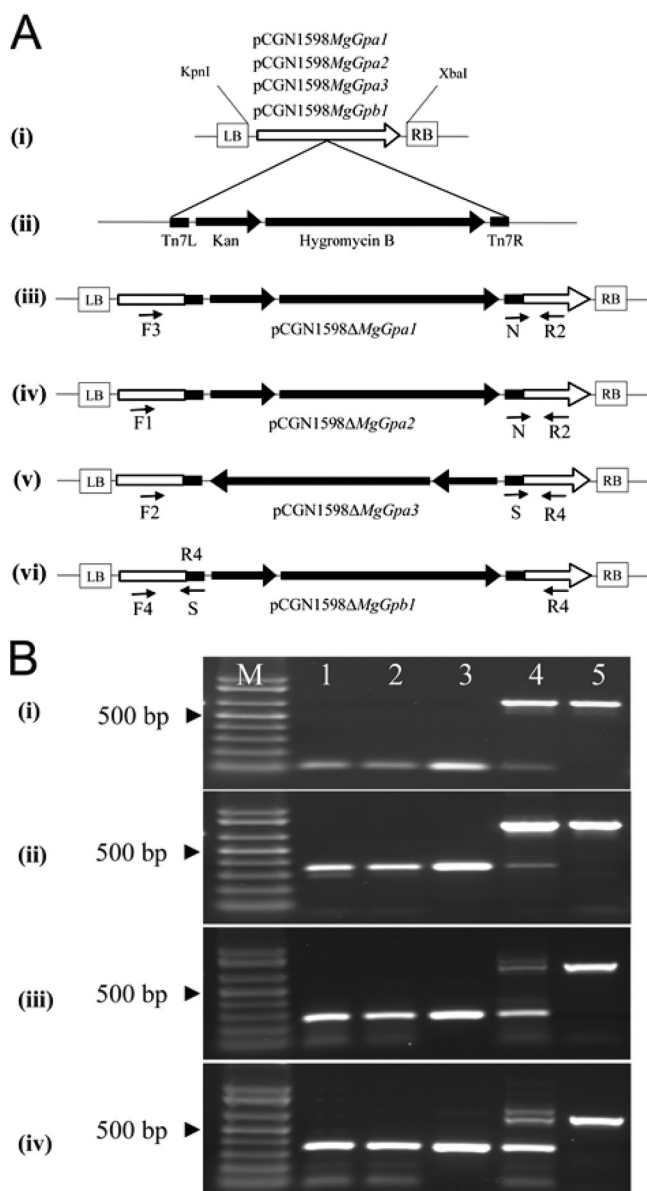


FIG. 1. Generation of disruption constructs and subsequent identification of mutants of *Mycosphaerella graminicola*. (A) (i) The cDNA fragments of *MgGpa1*, *MgGpa2*, *MgGpa3*, and *MgGpb1* were excised from the pSport1 vector, using KpnI/XbaI restriction enzymes, and inserted into the same site of the binary vector of *Agrobacterium tumefaciens* (pCGN1589), generating the target constructs pCGN1589MgGpa1, pCGN1589MgGpa2, pCGN1589MgGpa3, and pCGN1589MgGpb1, respectively. The target constructs were transposed using the customized donor construct pGPS3HygKan (ii), resulting in the disruption constructs pCGN-1598 Δ MgGpa1 (iii), pCGN1598 Δ MgGpa2 (iv), pCGN1598 Δ MgGpa3 (v), and pCGN1598 Δ MgGpb1 (vi). (B) Multiplex PCR screening to identify homologous recombinants in *MgGpa1* (i), *MgGpa2* (ii), *MgGpa3* (iii), and *MgGpb1* (iv), using two gene-specific primers (indicated in panel A) together with either primer N or primer S, located at the right or left border of the transposon, respectively. Homologous recombinants (lanes 1 and 2) amplified a small amplicon identical in size to the amplicon generated from the disruption construct (lane 3), whereas in ectopic transformants (lane 4) two bands were amplified, including a small band corresponding to the disruptant strains and a larger band of equal size to the amplicon generated from the WT strain *M. graminicola* IPO323 (lane 5). Lane M shows One Kb Plus markers.

computer. Pictures were saved with Axio Vision 4.1 software (Carl Zeiss, Jena, Germany), and composed pictures were prepared with Adobe Photoshop 7.0.

In planta phenotyping assays were performed according to a previously described protocol (44, 45). Disease progress was monitored at various time points after inoculation, and final observations were performed at 20 days postinoculation (dpi).

cAMP measurements. Quantification of intracellular cAMP was performed on 12 isolates (Table 1), including the WT, according to the protocol suggested by the manufacturer (Amersham Life Science Inc., Arlington Heights, IL). Spores were collected and lyophilized after being cultured for 5 days in YGB. Lyophilized spores (100 mg) were ground to a fine powder in a 2-ml Eppendorf tube by use of a bead beater (Ribolyser; Hybaid, Heidelberg, Germany). Powdered mycelia were resuspended in distilled water, and proteins were precipitated by adding 700 μ l of 6% tricarboxylic acid (TCA). After centrifugation, the supernatant was extracted five times with water-saturated ether to remove the TCA and subsequently lyophilized. The lyophilized sample was dissolved in phosphate buffer (10 mM, pH 7.4) and used for the cAMP assay. Before protein precipitation with TCA, two replicate samples of 100 μ l of the supernatant were taken for protein quantification using a Coomassie Plus protein assay reagent kit (Pierce Biotechnology, Inc.), with bovine serum albumin as a reference. Before cAMP quantification, the pH was measured, and if necessary, 50% KHCO₃ was added until a pH of 6 to 8 was obtained. In total, three biological replicates of each strain were assayed in duplicate by a cAMP (3H) assay system following the manufacturer's instructions (Amersham Life Science Inc., Arlington Heights, IL). After quantification, the cAMP values were normalized to the protein level for each strain (pmol cAMP/mg total protein).

Statistical analyses. cAMP (pmol cAMP/mg total protein) and spore length (μ m) data were natural log transformed and further analyzed by analysis of variance followed by pairwise comparisons among treatment means (based on *t* tests with a *P* value of 0.05) to test for significant differences between experiments, using the statistical software package Genstat (54) extended with Biometris Procedure library applications (20). The analyses did not show significant differences between control strains (WT and ectopic transformants) and between the independent mutants of the same gene, which allowed us to lump these data into groups for final analyses (controls and MgGpa1, MgGpa3, and MgGpb1 mutants).

Nucleotide sequence accession numbers. Sequence data from this article were deposited with the EMBL/GenBank data libraries under accession numbers DQ458049, DQ458050, DQ458051, and DQ458052, for *MgGpa1*, *MgGpa2*, *MgGpa3*, and *MgGpb1*, respectively.

RESULTS

Sequence comparison and phylogenetic analyses of *MgGpa1*, *MgGpa2*, *MgGpa3*, and *MgGpb1*. Screening of a large EST resource comprising 27,007 *M. graminicola* ESTs (34) enabled us to identify three G α -encoding genes and one G β -encoding gene. Assembly of individual reads of these ESTs combined with sequencing of full-length clones resulted in identification of the genes *MgGpa1*, *MgGpa2*, *MgGpa3*, and *MgGpb1*, with 1,059-, 1,065-, 1,068-, and 1,050-bp open reading frames encoding proteins consisting of 353, 355, 356, and 350 amino acids, respectively. Screening of the recently released genome sequence of *M. graminicola* IPO323 (U.S. Department of Energy Joint Genome Institute [http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html]) revealed no additional G α - and G β -encoding genes, but we identified an expected putative G γ protein-encoding gene, showing 60% identity with the G γ subunit of *Aspergillus fumigatus* (GenBank accession number XP_750270), which was not present in the EST database (34) and hence was not considered in this project.

Comparison of proteins encoded by these genes with orthologs in *M. grisea* showed that MgGPA1, MgGPA2, MgGPA3, and MgGPB1 are the orthologs of MAGB, MAGC, MAGA, and MGB1, with 89, 76, 87, and 88% similarity, respectively. Analyses of the genomic DNA sequences and the EST contigs of these genes revealed that *MgGpa1*, *MgGpa2*,

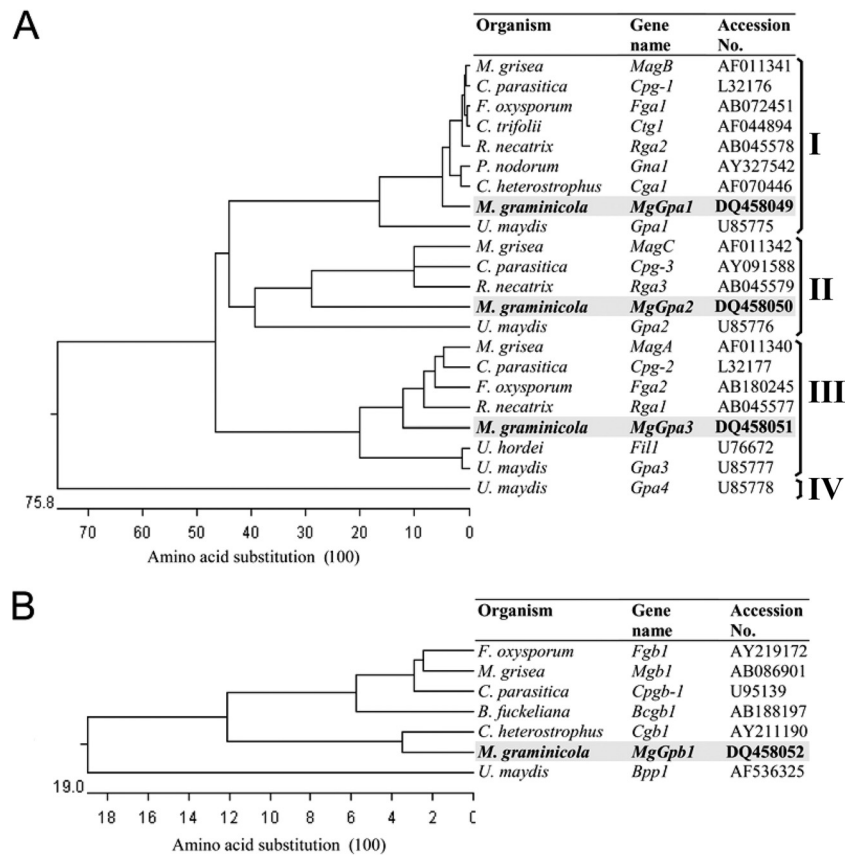


FIG. 2. Phylogenetic comparison of orthologs of $G\alpha$ proteins (A) and $G\beta$ proteins (B), based on amino acid sequence alignments. The protein sequences encoded by *MgGpa1* (GenBank accession number DQ458049), *MgGpa2* (GenBank accession number DQ458050), *MgGpa3* (GenBank accession number DQ458051), and *MgGpb1* (GenBank accession number DQ458052) from *Mycosphaerella graminicola* were compared with orthologs present in other fungi. Multiple sequence alignments and the phylogenetic tree were constructed using the MEGALIGN program (DNASar, Madison, WI).

MgGpa3, and *MgGpb1* contain one, four, five, and four introns, respectively. Multiple sequence alignments of the fungal $G\alpha$ protein sequences resulted in a phylogenetic tree with three major groups within the superfamily of the $G\alpha$ proteins (Fig. 2A). All $G\alpha$ proteins (*MgGpa1*, *MgGpa2*, and *MgGpa3*) contain the characteristic P-loop motif, which is one of the ATP/GTP binding regions (G/AXXXXGKT/S), and a G motif that stabilizes the guanine ring (24, 55). *MgGpa1* belongs to major group I and contains the consensus myristoylation site, indicated as MGX (an uncharged residue), XX (small uncharged residues), S at the N terminus, and a consensus CXXX sequence at the C terminus, which is the fingerprint of the pertussis toxin labeling site (17, 60). Multiple sequence alignments showed that *MgGpa2* has no mammalian counterpart and belongs to group II, which is a distinct group lacking the consensus myristoylation site (49). *MgGpa3* belongs to major group III, which has only the consensus myristoylation sequence, which is characteristic for adenylyl cyclase-stimulating G proteins ($G\alpha_s$).

Protein comparisons via an NCBI conserved domain search showed that *MgGpb1* contains seven WD40 domains, which are characteristic of a family of ancient regulatory proteins (51). WD40 repeats in *M. graminicola* generally contain a C-terminal GH dipeptide, an inter-WD40 region of 13 to 29

amino acids, and an N-terminal WD dipeptide, representing the WD40 signature. However, there is variation in either one or both GH/WD dipeptides. The second WD40 repeat (residues 104 to 134) in *MgGpb1* is the most variable domain among fungi and contains LR/YN at the C/N termini in *M. graminicola* instead of GH/WD. The other WD40 domains are highly conserved in one or both of the C/N termini.

Transposon-mediated vector construction and targeted gene disruption. The disruption constructs for *MgGpa1*, *MgGpa2*, *MgGpa3*, and *MgGpb1* were generated using an in vitro transposition system as described previously (44). PCR screening and sequencing enabled the selection of constructs with transposon insertions downstream of the start codon in the open reading frames of *MgGpa1*, *MgGpa2*, *MgGpa3*, and *MgGpb1*, at 666, 772, 393, and 586 bp, respectively (Fig. 1A). We used these constructs to disrupt *MgGpa1*, *MgGpa2*, *MgGpa3*, and *MgGpb1* in the WT through *A. tumefaciens*-mediated transformation (44, 72). After transformation, homologous recombinants were identified by PCR screening (Fig. 1B). Among 28, 25, 92, and 47 hygromycin-resistant transformants for *MgGpa1*, *MgGpa2*, *MgGpa3*, and *MgGpb1*, we identified 3, 14, 3, and 2 disruptant strains, respectively, and 12 transformants were selected for further studies (Table 1).

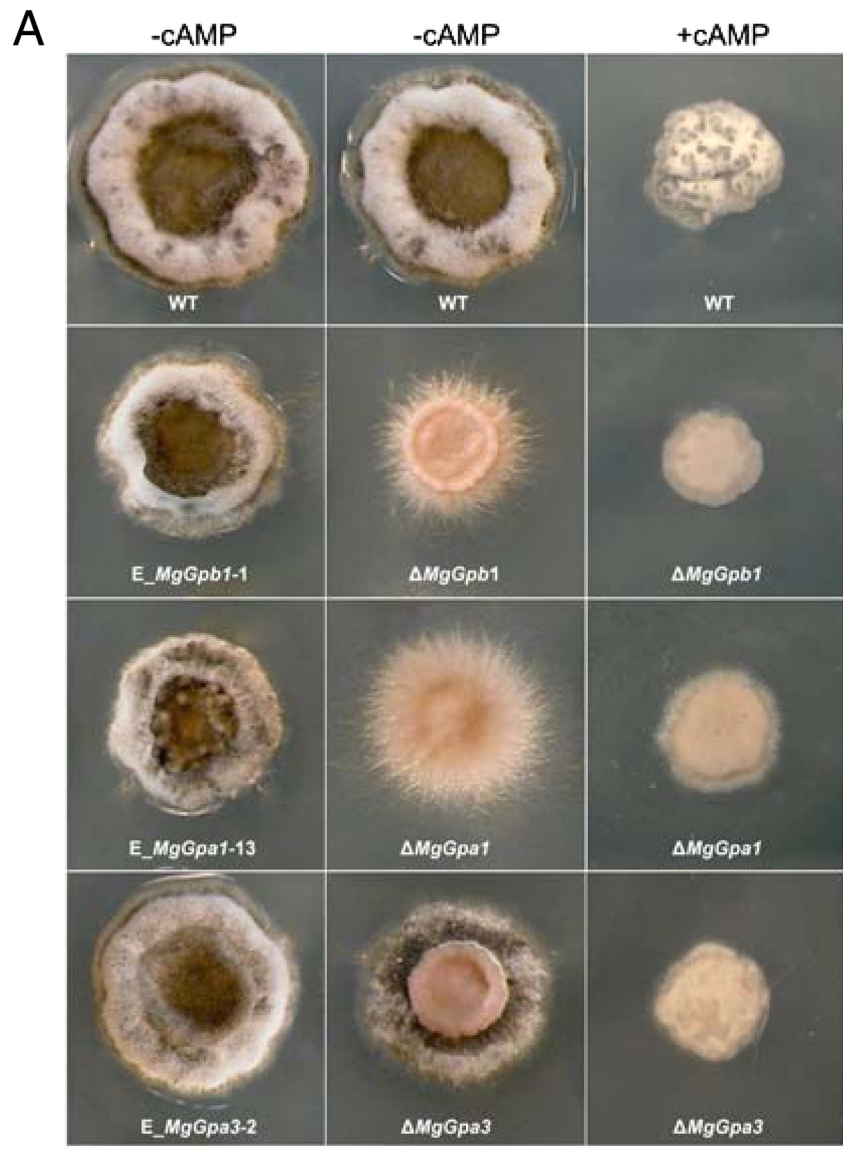


TABLE 2. Comparison of microscopic phenotypes (24 to 80 h) and intracellular cAMP levels in *Mycosphaerella graminicola* mutants of G protein-encoding genes versus the *M. graminicola* IPO323 WT and ectopic controls

<i>Mycosphaerella graminicola</i> isolate	Intracellular cAMP value (pmol cAMP/mg total protein) ^a	Phenotype on PDA	Phenotype on PDA plus cAMP
Controls			
IPO323		Short germ tubes	Short germ tubes
IPO323 E- <i>MgGpa</i>	10.17c	Budding on apical and intermediate cells of spores and secondary filaments (24 h)	Budding on apical and intermediate cells of spores and secondary filaments (24 h)
IPO323 E- <i>MgGpb</i>		Filamentation (48 h) Microconidiation in center of colony (80 h)	Reduced filamentation (48 h) Microconidiation in center of colony (80 h)
Mutants			
IPO323 Δ <i>MgGpa1</i>	9.45bc	Long germ tubes Increased filamentation, reduced branching (48 to 80 h)	Reduced filamentation
IPO323 Δ <i>MgGpb1</i>	5.48b	Fused germ tubes (48 h) Uncontrolled anastomosis (80 h)	Restored to control phenotype
IPO323 Δ <i>MgGpa3</i>	1.68a	Short germ tubes No secondary and tertiary filaments Extensive budding	Restored to control phenotype

^a Back-transformed values. Values with the same letter are not significantly different at a *P* level of 0.05.

Phenotypic characteristics. The *MgGpa1*, *MgGpa3*, and *MgGpb1* mutants showed altered phenotypes on PDA at 20°C under continuous light (Fig. 3A) and darkness (not shown), whereas the phenotypes of *MgGpa2* mutants were identical to those of the controls under all conditions tested (not shown). The colonies of the WT and ectopic transformants started to melanize after incubation for 4 to 5 days and were completely melanized and covered with a thin layer of white aerial mycelium 10 days after incubation, whereas the colonies of *MgGpa1* and *MgGpb1* mutants were nonmelanized and showed fimbriated borders (Fig. 3A). Spore size measurements showed that the *MgGpa1* disruptants produced significantly longer spores in YGB than those of the WT and ectopic controls (Table 2; Fig. 4) (WT and ectopic control spores were 32.2 μ m long, whereas transformant spores were \geq 62.8 μ m long; *P* < 0.05). The growth rate of the *MgGpb1* mutant was significantly reduced (~25% colony area, with fimbriated borders 56% of those of the WT after 10 days), whereas the *MgGpa3* mutant showed only a slight growth reduction and delayed melanization. In addition, the *MgGpa3* mutant exhibited an extensive yeast-like growth in the center of the colonies, which was characterized by an intensive microconidiation and a concatenation of budding cells (Fig. 3A). Interestingly, such a tendency to yeast-like growth and reduced filamentation was also observed in the *MgTpk2* mutant, in which the catalytic subunit of PKA was disrupted (43). At 28°C, the growth patterns and phenotypes of the WT and ectopic transformant controls and the mutants were nearly similar and were characterized by a fluffy white aerial mycelium that covered the colony (not shown). At temperatures of >29°C, all colonies stopped growing, including the WT and ectopic transformant controls (not shown). In

YGB, controls and all mutants merely produced yeast-like cells, except for the *MgGpa1* mutants, which produced fluffy mycelia (Fig. 3B). *MgGpa3* mutants secreted a dark brown pigment into YGB (Fig. 3C), similar to our previous observation with the *MgTpk2* mutant (43).

Complementation of mutants with cAMP. To test whether the G proteins are involved in the cAMP-dependent signaling pathway, we supplemented PDA with exogenous cAMP (Fig. 3A). This had pleiotropic effects on the WT and all mutants. In the WT, cAMP complementation had multiple effects on appearance, filamentation, and growth rate, resulting in a smaller colony (~36% of the WT colony area) that was covered with white aerial mycelium. Exogenous cAMP restored the phenotypes of all mutants to the above-mentioned WT phenotype (Fig. 3A).

Microscopic analyses. We monitored germination, early colony formation, and conidiation in all mutants in the absence and presence of cAMP to further explore the aforementioned phenotype restoration by exogenous cAMP (Table 2; Fig. 4 and 5). On WA, the controls initially germinated from both apical cells of the spores, producing relatively long primary germ tubes within 12 h. Subsequently, secondary germ tubes from the same or other cells of the spore were formed. Within 48 h, tertiary hyphal filaments developed from the primary and secondary germ tubes. We did not observe significantly altered initial germination patterns for the *MgGpa1*, *MgGpa2*, and *MgGpa3* mutants. However, at later stages, *MgGpa1* mutants produced longer germ tubes than the WT or ectopic transformants did. *MgGpb1* mutants grew significantly slower, with wavy germ tubes, and secondary and tertiary filaments as well

FIG. 3. Phenotypic characterization of G α - and G β -encoding gene mutants, strain *Mycosphaerella graminicola* IPO323 (WT), and ectopic transformants (named with an E prefix) grown in vitro under different culture conditions. (A) Phenotypes of 10-day-old cultures grown on PDA without and with exogenous cAMP (13 mM) under continuous light at 20°C. (B) Highly increased filamentous growth of *MgGpa1* mutants in YGB medium at 18°C after 5 days. (C) *MgGpa3* mutants secrete dark brown pigments, as shown for 5-day-old YGB cultures grown at 18°C.

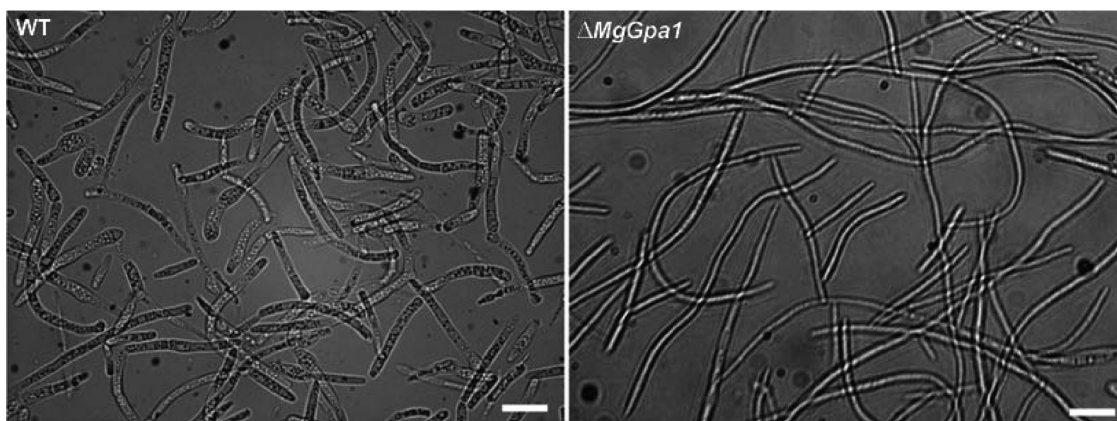


FIG. 4. Comparative phenotyping of *Mycosphaerella graminicola* mutants of the Gα-encoding gene *MgGpa1* and of *M. graminicola* IPO323 (WT) in YGB at 20°C under daylight settings. The *MgGpa1* disruptants produced significantly ($P < 0.05$) longer spores in YGB. Bar = 30 μm (also see the text for further details).

as some hyphae showed anastomosis after growth for 80 h (data not shown).

On PDA, the WT and ectopic controls formed shorter germ tubes that often showed differentiated budding cells on apical and intermediate cells of the spore as well as on secondary

filaments within 24 h. These extended into filamentous colonies at 48 h, and these showed abundant microconidiation in the colony center after 80 h. Compared to the WT, the *MgGpa1* mutants showed longer germ tubes and increased filamentation, with altered polarized growth, reduced branch-

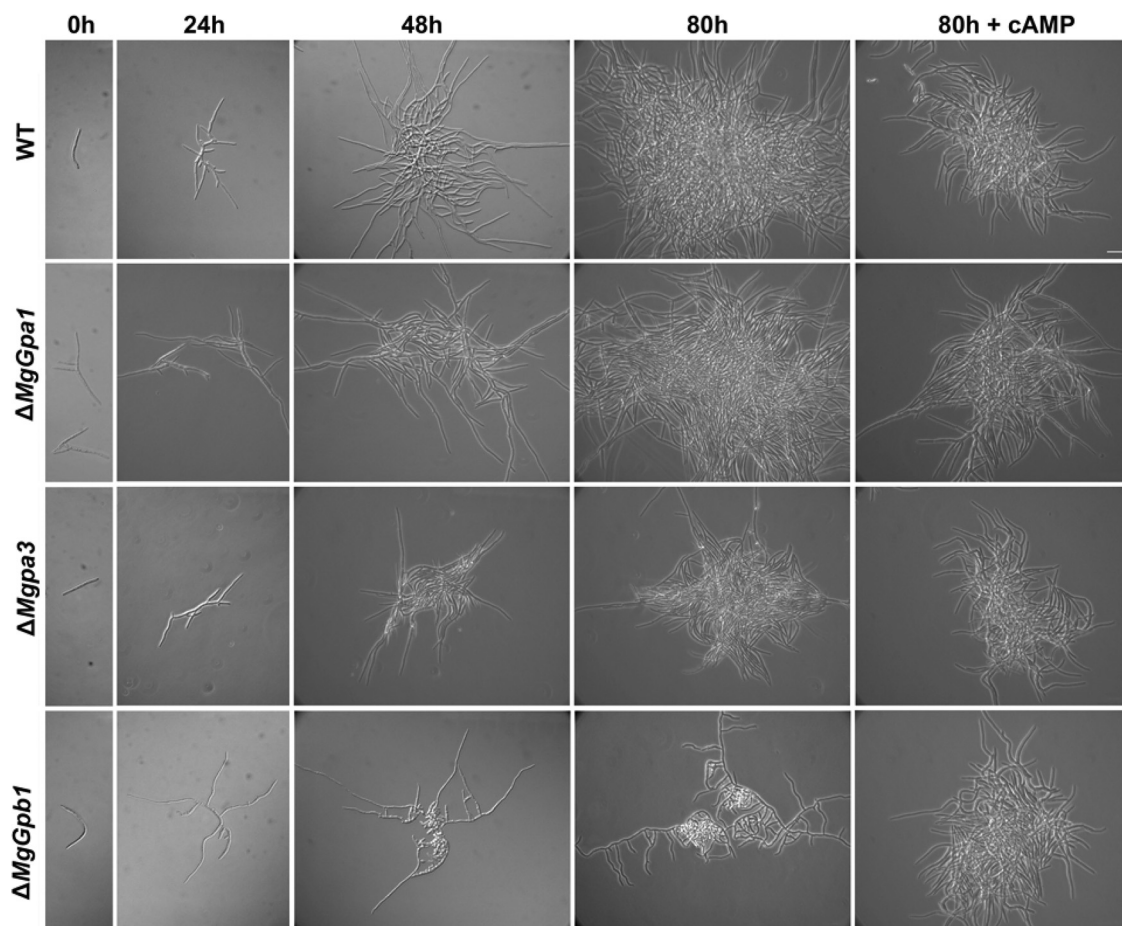


FIG. 5. Effects of disruption of the Gα- and Gβ-encoding genes *MgGpa1*, *MgGpa3*, and *MgGpb1* on germination (24 h), early colony formation (48 h), and microconidiation (80 h) and restoration of the *Mycosphaerella graminicola* IPO323 control (WT) phenotype of the Δ*MgGpa3* and Δ*MgGpb1* mutants by adding exogenous cAMP (15 mM) to PDA at 20°C in the dark. Bar = 30 μm (also see the text for further details).

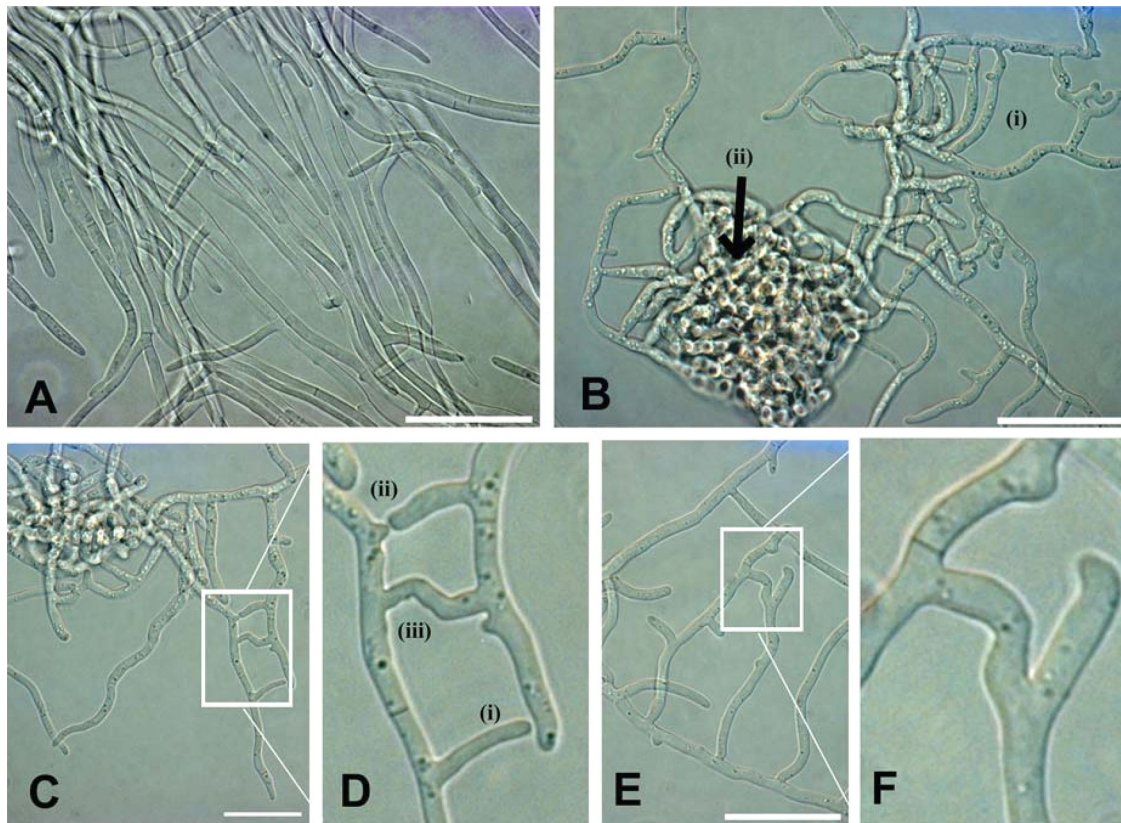


FIG. 6. Disruption of *MgGpb1* induces anastomosis. (A) Hyphae of the *Mycosphaerella graminicola* IPO323 WT strain grown on PDA for 80 h at 20°C in continuous darkness. (B) Extensive anastomosis in *MgGpb1* mutants results in a network of interconnected hyphae (i) that show uncontrolled fusions producing dense local networks of mycelium (ii). (C and D) Three distinct phases are depicted during the anastomosis process, including (i) attraction phase, (ii) contact phase, and (iii) fusion phase. Panel D shows a higher magnification of the box in panel C. (E and F) Septum development after fusion. Panel F shows a higher magnification of the box in panel E. Bar = 30 μ m.

ing (>24 h), and long uniform spores (>48 h) (Table 2; Fig. 4). *MgGpa3* disruptant spores produced short germ tubes without secondary or tertiary filaments (>24 h). Colony formation was associated with extensive budding, resulting in dense colonies of typical uniform spores (>48 h) (Table 2; Fig. 5). The *MgGpb1* mutant produced germ tubes that fused within 48 h and subsequently produced an extremely dense biomass between 48 and 80 h, which resulted from seemingly uncontrolled continuous anastomosis (Table 2; Fig. 5 and 6). This process greatly suppressed filamentous growth and significantly reduced microconidiation. The addition of cAMP to the medium reduced the colony size of the WT and *MgGpa1* mutants but hardly affected the growth characteristics of the latter. For the *MgGpa3* and *MgGpb1* mutants, exogenous cAMP resulted in the formation of colonies that were very similar to the WT, suggesting that these genes may function in the cAMP pathway.

Intracellular cAMP measurements. In order to strengthen the macroscopic (Fig. 3) and microscopic (Fig. 5 and 6) observations in the presence and absence of exogenous cAMP, we performed intracellular cAMP measurements on all mutants. The *MgGpa3* and *MgGpb1* mutants showed significantly reduced levels of cAMP. In contrast, internal cAMP measurement data for the *MgGpa1* mutant and the WT were not significantly different (Table 2).

In planta characterization of mutants. Ten-day-old seedlings of wheat cv. Obelisk inoculated with the WT or ectopic transformants showed chlorotic areas at 8 dpi that continued to expand starting from the leaf tips, which became necrotic at 12 dpi and eventually merged into large necrotic lesions containing numerous pycnidia between 16 and 18 dpi. Plants inoculated with *MgGpa1*, *MgGpa3*, or *MgGpb1* mutants showed delayed symptom development, with only limited chlorotic areas, starting from the leaf tips, between 12 and 15 dpi. These chlorotic areas sometimes became necrotic but never contained pycnidia, the typical symptom of *M. graminicola* infection. On the leaf tips of water-treated controls, some chlorosis or necrosis was also observed, most likely as a result of senescence. An overview of disease symptoms caused by the WT and various disruptants and ectopic mutants and a summary of phenotypes of mutants of G α - and G β -encoding genes in other filamentous plant-pathogenic fungi are shown in Fig. 7 and Table 3.

DISCUSSION

The availability of a large EST library and verification with the genome sequence resulted in the identification and isolation of the G α -encoding genes and the G β -encoding gene in *M. graminicola*. Gene replacement studies revealed that these

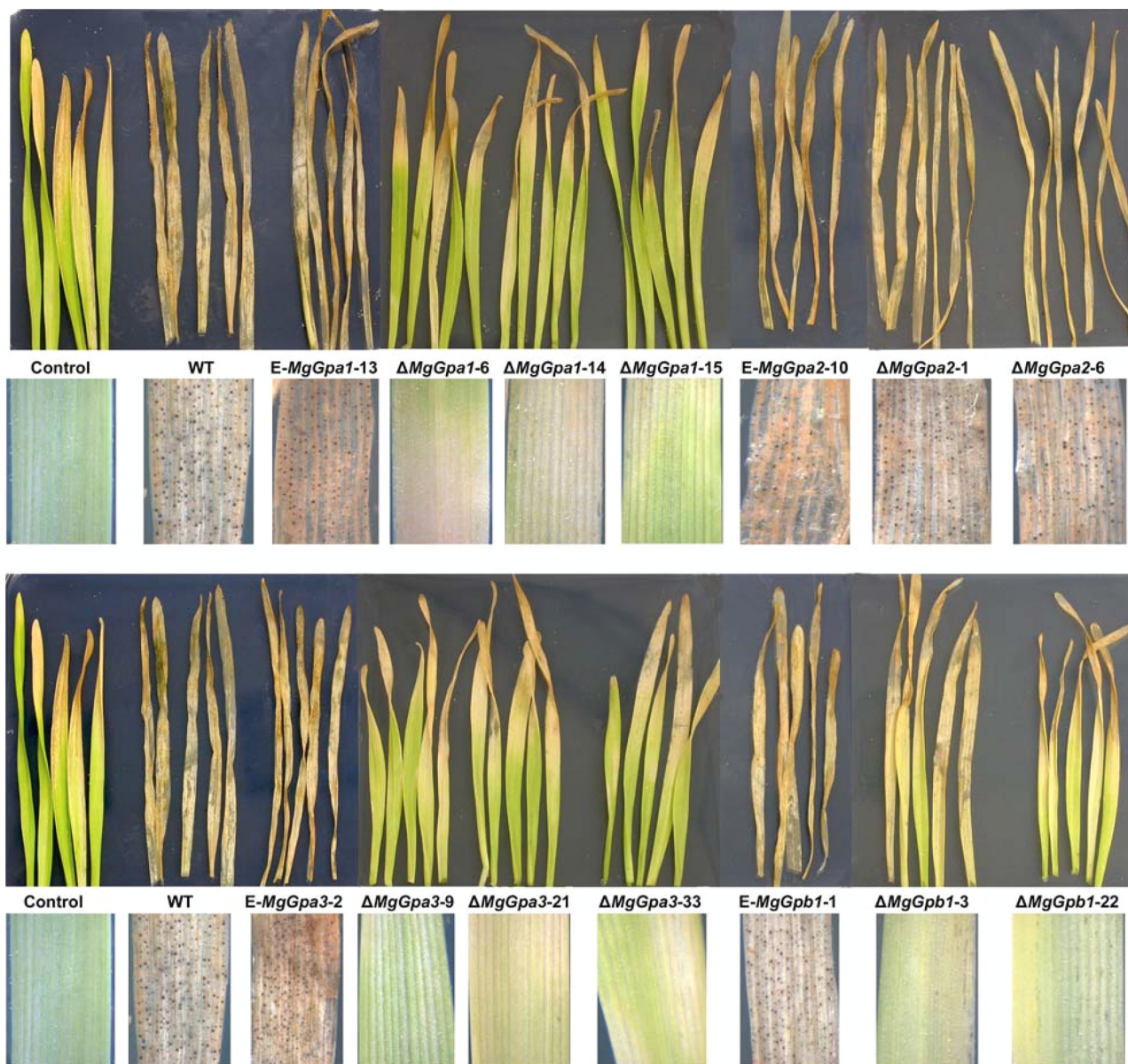


FIG. 7. In planta phenotyping of *MgGpa1*, *MgGpa2*, *MgGpa3*, and *MgGpb1* disruptants, the *Mycosphaerella graminicola* IPO323 WT strain, and ectopic transformants on 10-day-old seedlings of wheat cv. Obelisk 20 days after inoculation. Primary leaves were inoculated with either water (control) or the different *M. graminicola* strains. Note the strongly reduced pathogenicity for Δ *MgGpa1*, Δ *MgGpa3*, and Δ *MgGpb1* mutants.

G proteins are involved in the development and pathogenicity of this fungus. With the exception of *MgGpa2*, all genes are required for pathogenicity and play an important role in regulation of the dimorphic switch between filamentous and yeast-like growth, microconidiation, or melanization. Phylogenetic analyses indicated that the G α -encoding genes belong to three different groups. The mammalian orthologs of MgGPA1 and MgGPA3 are G α_i and G α_s , respectively, whereas MgGPA2 has no close relative in mammals. Activation of the G α_i protein in mammals results mostly in inhibition of adenylyl cyclase and hence increases intracellular cAMP levels (5, 6). However, in fungi, the orthologous genes encoding G α_i proteins vary substantially in their regulation of intracellular cAMP levels. For instance, the *Cpg-1* gene in *C. parasitica* negatively regulates

the cAMP level, as disruption increased intracellular cAMP levels in this fungus (5). In contrast, the orthologs *Gna1* in *Neurospora crassa*, *Fga1* in *Fusarium oxysporum*, and *MagB* in *M. grisea* positively regulate the cAMP pathway, as disruption of these genes decreased intracellular cAMP levels (26, 28). In *M. grisea*, exogenous cAMP restored appressorium formation and pathogenicity of the *MagB* mutant (41). In the budding yeast, Gpa1 regulates the MAPK pathway and does not affect the cAMP pathway (3, 59).

Our data show that *MgGpa1* is a negative regulator of filamentation, as mutants had significantly longer spores than the WT did. Moreover, phenotypes in YGB and on PDA were characterized by increased filamentous growth, and those on WA were characterized by longer germ tubes. However, the

TABLE 3. Effect of disruption of G α - and G β -encoding genes on the development and pathogenicity of fungal plant pathogens

Organism	Gene	Class	Growth	Conidiation	Germination	Virulence	Additional phenotypes	Reference
<i>C. heterostrophus</i>	<i>Cga1</i>	G α -I	Reduced		Reduced	Normal	Low appressorium formation, osmosensitive	24
<i>C. trifolii</i>	<i>Ctg1</i>	G α -I	Reduced		Reduced	Reduced	Low appressorium formation, long germ tubes bearing appressoria	65
<i>C. parasitica</i>	<i>Cpg-1</i>	G α -I	Reduced	Lost		Lost	Reduced pigmentation, increased cAMP level	16
<i>F. oxysporum</i>	<i>Fga1</i>	G α -I	Normal	Reduced		Reduced	Decreased cAMP level, thermotolerant	27
<i>M. grisea</i>	<i>MagB</i>	G α -I	Reduced	Reduced	Normal	Reduced	Reduced penetration, long germ tubes bearing appressoria that are restored by exogenous cAMP	41
<i>S. nodorum</i>	<i>Gna1</i>	G α -I	Normal	Lost		Reduced	Defect in penetration, albino, osmosensitive	61
<i>U. maydis</i>	<i>Gpa1</i>	G α -I	Normal	Normal	Normal	Normal	No phenotype in all aspects	57
<i>B. cinerea</i>	<i>Bcg1</i>	G α -I	Reduced	Normal	Normal	Reduced	Altered colony morphology, normal penetration, loss of protease secretion,	22
<i>M. graminicola</i>	<i>Gpa1</i>	G α -I	Normal	Reduced	Normal	Reduced	Altered colony morphology, hampered melanization, induced filamentation, altered polarized growth	This study
<i>C. parasitica</i>	<i>Cpg-3</i>	G α -II					Not functionally analyzed	53
<i>M. grisea</i>	<i>MagC</i>	G α -II	Normal	Reduced	Normal	Normal	Normal appressorium formation	41
<i>U. maydis</i>	<i>Gpa2</i>	G α -II	Normal	Normal	Normal	Normal	No phenotype in all aspects	57
<i>B. cinerea</i>	<i>Bcg2</i>	G α -II	Normal	Normal	Normal	Reduced	Normal colony morphology, virulence slightly reduced	22
<i>M. graminicola</i>	<i>Gpa2</i>	G α -II	Normal	Normal	Normal	Normal	No phenotype in all aspects	This study
<i>C. parasitica</i>	<i>Cpg-2</i>	G α -III	Reduced	Reduced		Normal	Decreased cAMP level	16
<i>F. oxysporum</i>	<i>Fga2</i>	G α -III	Normal	Normal		Lost	Thermotolerant	29
<i>M. grisea</i>	<i>MagA</i>	G α -III	Normal	Normal	Normal	Normal	No phenotype in all aspects	41
<i>U. maydis</i>	<i>Gpa3</i>	G α -III				Lost	Elongated cells in liquid media, defective in mating	57
<i>M. graminicola</i>	<i>Gpa3</i>	G α -III	Reduced	Increased	Normal	Reduced	Altered colony morphology, hampered filamentation, reduced melanization	This study
<i>U. maydis</i>	<i>Gpa4</i>	G α -IV	Normal	Normal	Normal	Normal	No phenotype in all aspects	57
<i>F. oxysporum</i>	<i>Fgb1</i>	G β	Reduced	Reduced	Normal	Reduced	Altered colony morphology, faster conidial germination, decreased cAMP level, thermotolerant	27
<i>M. grisea</i>	<i>Mgb1</i>	G β	Reduced	Reduced	Delayed	Lost	No appressorium formation, decreased cAMP level, more aerial hyphae, formation of fluffy colonies	52
<i>C. parasitica</i>	<i>Cpgb-1</i>	G β	Increased	Reduced		Reduced	Reduced pigmentation	31
<i>C. heterostrophus</i>	<i>Cgb1</i>	G β	Normal	Lost		Lost	Impaired formation of appressoria, increased pigmentation, straight germination	14
<i>U. maydis</i>	<i>Bpp1</i>	G β				Normal	Increased filamentation and reduced budding growth, restored by adding exogenous cAMP	48
<i>M. graminicola</i>	<i>Gpb1</i>	G β	Reduced	Reduced	Normal	Reduced	Altered colony morphology, hampered filamentation and melanization, increased anastomosis	This study

addition of exogenous cAMP hardly affected the phenotypes 80 h and 10 days after initiation of the experiments. Moreover, intracellular cAMP levels in *MgGpa1* mutants and the WT were not significantly different. This suggests that *MgGpa1* might be dispensable for cAMP regulation, similar to reports for yeast (3, 59). Alternatively, equal intracellular cAMP levels in both the *MgGpa1* mutant and the WT strain could also be due to growth characteristics that may influence cAMP extraction efficiencies. For instance, all mutants and controls showed blastic conidiogenesis in YGB, except for *MgGpa1* mutants, which showed highly increased filamentous growth. It is possible that such differences in growth pattern affected the intracellular cAMP measurements in the *MgGpa1* mutants. In sum-

mary, our data suggest that MgGPA1 is functionally different from mammalian inhibitory G α_i proteins and that fungal G α_i -like proteins comprise three subgroups that can either negatively or positively regulate adenylyl cyclase activity or, similar to what has been observed in yeast, do not interfere with the cAMP pathway. This also shows that high protein sequence identities do not necessarily imply similar biological functions.

G α proteins that belong to group III show high protein sequence identity to stimulatory G α proteins in *N. crassa*, *C. parasitica*, *S. cerevisiae*, and *Schizosaccharomyces pombe*. Unlike the G α_i -like proteins, they all positively regulate adenylyl cyclase and are functionally related to mammalian G α_s (16, 25, 33, 39, 42, 50). Significantly reduced intracellular cAMP levels

in the *MgGpa3* and *MgGpb1* mutants confirmed our observation that the addition of exogenous cAMP to these mutants restored their phenotypes to that of the WT and hence suggest that *MgGpa3* and *MgGpb1* positively regulate adenylyl cyclase activity in *M. graminicola*. In many biological systems, cAMP is generated by adenylyl cyclase and acts as a second messenger that binds to the regulatory subunit of PKA. This leads to dissociation of the regulatory and catalytic subunits of this complex and consequently activates the latter. Hence, inhibition of adenylyl cyclase by disruption of G α_s genes or the catalytic subunit-encoding gene of PKA could lead to a similar phenotype. Indeed, the *MgGpa3* and *MgTpk2* (catalytic subunit-encoding gene of PKA) mutants of *M. graminicola* showed similar phenotypes, including reduced filamentation, increased microconidiation on PDA, and secretion of a dark brown pigment into YGB (43). Therefore, we suggest that MgGPA3 acts upstream of MgTPK2, positively regulates the cAMP pathway, and functionally belongs to the G α_s protein family.

Since MgGPA2 belongs to a distinct group of G proteins (group II) with no mammalian orthologs, we cannot predict its biochemical function yet. Interestingly, the phenotypes of all *MgGpa2* mutants were identical to that of the WT or ectopic transformants under all in vitro and in planta conditions tested, which supports observations with *U. maydis*, *Botrytis cinerea*, and *M. grisea* where disruption of the *MgGpa2* orthologs did not alter or only slightly affected in vitro phenotypes and pathogenicity (22, 41, 57). The phenotypes of all fungal G α_2 -encoding gene mutants belonging to group II obtained so far indicate that this type of G α protein does not play a major role in development and/or pathogenicity of plant-pathogenic fungi. Further studies are required to understand the role of the GPA2 subunits that are both specific to and highly conserved in filamentous fungi.

Spores of *MgGpb1* mutants germinated normally, but the germ tubes had a wavy appearance and showed anastomosis on WA and PDA, which culminated on the latter medium into extensive fusions, resulting in dense areas in the colonies with little filamentous growth and strongly reduced conidiation. Although somatic cell fusion, known as homokaryon anastomosis, is common during vegetative growth of many filamentous fungi (18, 19, 23), it is very unusual for *M. graminicola*. Here we show that in *M. graminicola*, *MgGpb1* negatively regulates vegetative cell fusion, resulting in its unique phenotype. The molecular mechanism of homokaryon cell fusion is poorly understood, but *N. crassa ham-1* and *ham-2* mutants were unable to undergo both self- and nonself-hyphal fusion during vegetative growth (69, 70). In *S. cerevisiae* and *Cryptococcus neoformans*, G β positively regulates the mating pheromone response pathway. Therefore, disruption of the encoding gene inhibits the response to sex pheromones (67, 68). In contrast, in *S. pombe* pheromone signaling is mediated by G α (Gpa1) and the cAMP pathway is regulated by G α (Gpa2) and G β (Gpb1). Interestingly, deletion of G β in *S. pombe* stimulates conjugation in nutrient-rich media, which is in accordance with our observations (37, 40). We showed that exogenous cAMP restored the phenotypes of the *MgGpa3* and *MgGpb1* mutants to the WT phenotype, indicating that these genes positively regulate the cAMP pathway. A possible mechanism for cAMP pathway regulation by MgGPB1 and MgGPA3 in *M. graminicola* could be that MgGPB1 is required for the efficient release of

MgGPA3 from the heterotrimeric complex in order to stimulate adenylyl cyclase. Alternatively, or in addition, MgGPB1 could directly stimulate adenylyl cyclase, as observed for type II mammalian adenylyl cyclase, which is positively regulated by both G α and G $\beta\gamma$ subunits (13, 15, 64).

We observed that all *M. graminicola* strains were equally thermosensitive, whereas for *N. crassa* and *F. oxysporum*, thermotolerance was observed in G α -encoding mutants and was positively correlated with decreased intracellular cAMP levels (28, 29, 71). Indeed, constitutive expression of *Gna1* in *N. crassa* resulted in elevated cAMP levels and increased thermosensitivity (26, 71). The observed phenotypic differences could be due to the various lifestyles of these fungi.

Similar to MgGpa1, the orthologous group I G α proteins in *C. parasitica*, *M. grisea*, *Stagonospora nodorum*, *F. oxysporum*, *Colletotrichum trifolii*, and *B. cinerea* affect pathogenicity (7, 16, 28, 41, 61, 65), whereas the orthologous proteins CGA1 and GPA1 in *Cochliobolus heterostrophus* and *U. maydis*, respectively, are dispensable for pathogenicity (24, 57). Such a functional dichotomy was also observed for group III G α orthologs. *M. graminicola MgGpa3* mutants were strongly reduced in pathogenicity, which was also observed in *U. maydis* and *F. oxysporum* (29, 57) but not in *M. grisea* and *C. parasitica* (7, 41). Similar observations were reported for G β -encoding genes. *MgGpb1* mutants and orthologous mutants in *F. oxysporum*, *M. grisea*, *C. parasitica*, and *C. heterostrophus* were impaired in pathogenicity, whereas mutation of the G β -encoding gene in *U. maydis* did not disrupt pathogenicity (14, 27, 31, 48, 52).

Recently, we showed that impaired melanization in *M. graminicola* correlated with defects in pathogenicity for *MgFus3*, *MgSlt2*, and *MgHog1* mutants (8, 44, 45), which is in agreement with our present results, where we observed that disruption of the *MgGpa1*, *MgGpb1*, and *MgGpa3* genes affects melanization as well as pathogenicity. This is somewhat surprising, as *M. graminicola* penetrates its host by stomata, whereas melanization is reported to be very important for generating turgor pressure in the appressoria of appressorium-forming fungal plant pathogens like *M. grisea* and *Colletotrichum lagenarium* (32, 38, 46, 63). The melanin biosynthesis pathway in fungi is also well characterized (47, 66), and expression of three melanin biosynthesis genes was reduced only at the onset of germination in the *Fus3* orthologous mutants of *C. lagenarium*, suggesting the involvement of this ortholog in melanization of appressoria (62). However, unlike the case in appressorium-forming pathogens, melanization in *M. graminicola* might affect other stages during infection of plants where melanization is important. Indeed, our previous studies revealed that non-melanized mutants of *MgSlt2* and the WT strain were equally effective in penetrating the host plant (44) but differed significantly in pathogenicity, which suggests that melanization is required at the time that the fungus starts to differentiate asexual fructifications in substomatal cavities (11, 36). However, to test this hypothesis, genes involved in melanin biosynthesis need to be studied in detail. In addition, expression profiling of the mutants generated in this study could possibly reveal potential cross talk between the melanin biosynthesis and cAMP pathways in *M. graminicola*.

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