

**Signaling pathways involved in pathogenicity  
and development of the fungal wheat pathogen**

***Mycosphaerella graminicola***

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# **Signaling pathways involved in pathogenicity and development of the fungal wheat pathogen**

## ***Mycosphaerella graminicola***

Rahim Mehrabi

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This thesis is dedicated to my dear mother, my dear wife and my lovely son, Omid.

# **CHAPTER 1**

**General introduction and outline of thesis**

### *Mycosphaerella graminicola*

Septoria tritici leaf blotch is a major foliar wheat disease caused by the heterothallic ascomycete *Mycosphaerella graminicola* (Fuckel) J. Schröt. It is a major threat of bread and durum wheat in most wheat growing areas worldwide but particularly in the areas with high rainfall during the growing season (Eyal and Levy 1987; Halama 1996; Hardwick et al. 2001; Jorgensen et al. 1999). This was particularly accentuated after the rapid development and spread of resistance to strobilurin fungicides, which was also observed in the banana pathogen *M. fijiensis* (Fraaije et al. 2005; Fraaije et al. 2003; Sierotzki et al. 2000). *Mycosphaerella* is the largest genus in the Dothideales, an extremely large and diverse order of fungi with over 1,000 named species. Along with its associated asexual stages, *Mycosphaerella* contains at least 3,000 species. It is probably the largest genus of plant pathogens in the fungal kingdom, causing extensive economic losses on a range of crops including cereals, banana, citrus, soybean, sugar beet, tomato, strawberry and even tree crops (Farr et al. 1995). Direct yield losses plus the cost of control strategies contribute to the huge economical significance of this genus. The devastating *M. fijiensis*, causing black leaf streak in banana heads the global list of fungicide inputs with an annual volume of 2.5 billion US\$. *M. citri*, the cause of greasy spot of citrus, is the largest fungicide target in this crop in the US. Septoria tritici leaf blotch is the most important wheat disease in Europe and hence *M. graminicola* is the prime target for the cereal fungicide market with an estimated annual gross of 700 million US\$.

Due to its unique pathogenesis, *M. graminicola* is rapidly developing as a model fungus for the order of the Dothideales to study pathogenicity factors (Goodwin 2004). It is a dimorphic pathogen, and therefore transition from a yeast-like to a filamentous form is important for initiation of infection. In contrast to other model fungi, such as *Magnaporthe grisea* (Dean 2005), *M. graminicola* is a non-appressorium forming pathogen that penetrates the leaves through stomata without differentiating specific infection structures. As a hemibiotroph, it has a biotrophic phase of about 10 days that is followed by a rapid switch to necrotrophy. This necrotrophic stage results in the typical symptoms of the disease starting with irregular chlorotic lesions that rapidly develop into necrotic blotches suggesting an active role of toxic compounds (Cohen 1993; Duncan and Howard 2000; Eyal and Levy 1987; Kema et al. 1996d; Mehrabi et al. 2006). The necrotic foliar lesions bear the anamorphic and teleomorphic fructifications. Pycnidia are the asexual fructifications that contain the splash-born conidia, which are dispersed over short distances within the crop. The symptomatic

blotches also contain perithecia, the sexual fruiting bodies that are continuously produced throughout the year, which release the air-borne ascospores that travel over large distances within and between wheat crops. Sexual reproduction in *M. graminicola* is responsible for the early disease establishment and high genetic diversity observed in natural populations. Because of this active sexual cycle in nature, *M. graminicola* has become a standard model for the study of population genetics for many other plant pathosystems (Hunter et al. 1999; Kema et al. 1996c; Zhan 2004; Zhan et al. 2003)

Early reports on specificity were controversial and hence, the wheat-*M. graminicola* pathosystem has been traditionally thought to be controlled by quantitative characters in both the host and the pathogen (Eyal et al. 1973, 1985; Johnson 1992; Parlevliet 1993; Van Ginkel and Scharen 1988). It is a testimony to fungal genetics that this hypothesis was eventually falsified when experimental evidence showed that the host and pathogen interact according to the gene-for-gene hypothesis (Brading et al. 2002; Kema et al. 1996abc; Kema and Van Silfhout 1997; Kema et al. 2002, 2000). Since then up to eight resistance genes in the host have been identified and mapped (Adhikari et al. 2003, 2004abc; Arraiano et al. 2001; Brown et al. 2001; Chartrain et al. 2005). A map-based cloning strategy for a putative *Avr* gene started with segregation analyses using a mapping population generated by crossing isolates IPO323 (avirulent) and IPO94269 (virulent). The avirulence phenotype segregation confirmed that avirulence in *M. graminicola* strain IPO323 is controlled by a single locus (Kema et al. 2000). However, genetic and functional analyses of a 96 kb region putatively harboring this *Avr* gene has so far not lead to the isolation of this *Avr* factor (Mehrabi et al. 2003a, 2005).

A large set of genetic tools has been developed exploiting the genetic tractability of *M. graminicola* (Goodwin et al. 2004). Transformation and targeted gene disruption is efficient in this fungus (Adachi et al. 2002; Mehrabi et al. 2006; Stergiopoulos et al. 2003; Zwiers and de Waard 2001), and a detailed genetic linkage map is available (Kema et al. 2002). Recently, we generated over 30,000 ESTs from *M. graminicola* grown under a range of different cultural conditions (Kema et al. 2003). Cytological analyses and electrophoretic karyotyping indicate that the *M. graminicola* strain IPO323 contains 20 chromosomes with a genome size of approximately 40 Mbp (Mehrabi et al. 2003b). The genomes of *M. graminicola* and *M. fijiensis* are currently being sequenced and annotated at the Joint Genome Institute of the US Department of Energy. These are the foundation for unraveling the molecular mechanisms regulating growth and differentiation that coordinate pathogenicity and survival of Dothideales in general and *M. graminicola* in particular.

## Signal transduction

Like all other living organisms, fungi are able to perceive and respond to changes in the environment. Signal transduction pathways play a central role in sensing and transmission of external signals leading to altered cell responses. In general, signal transduction is any process by which a cell converts one stimulus into another that often involves a sequence of biochemical reactions inside the cell. The transduction of a signal involves the binding of a specific ligand to a specific receptor, which leads to conformational changes of the receptor and a subsequent activation of one or several downstream effectors. Eventually this changes the expression profiles of genes within the responding cells and alters cellular activity. In many signal transduction pathways, an amazing number of proteins and other molecules are engaged in the events that are initiated by the initial stimulus. Within the signal transduction pathways, mitogen-activated protein kinases (MAPKs) and cAMP-dependent pathways are very important for development and various cell functions and involve several regulatory proteins such as the G proteins (Hirt 1997; Xu 2000). There is overwhelming evidence for extensive cross talk among MAPKs and cAMP-dependent pathways that culminates into a complex signaling network in which the regulatory mechanisms are not fully understood (Cherkasova et al. 2003; O'Rourke and Herskowitz 1998; Posas et al. 1998).

### MAP kinase pathways

In eukaryotic cells, a family of serine/threonine protein kinases known as MAPKs is involved in the transduction of a variety of extracellular signals. These MAPKs are required for the regulation of cellular development and differentiation processes (Dickman and Yarden 1999; Nishida 1993; Shaeffer and Weber 1999). MAPK pathways, sometimes called extra-cellular signal-regulated kinase pathways, are integral parts of cascades generally consisting of three conserved phosphokinases: MAPK kinase kinases (MAPKKKs), MAPK kinases (MAPKKs) and MAPKs; that sequentially activate and mediate appropriate cellular responses to specific environmental stimuli (Blumer and Johnson 1994). In the model yeast *Saccharomyces cerevisiae*, five characterized MAPK pathways have been identified that regulate mating, invasive growth, cell wall biosynthesis, growth during osmotic stress and ascospore cell wall formation (Hunter and Plowman 1997). In these pathways, a set of sequentially acting proteins is involved in the transduction of the signals to activate MAPKs (Torres et al. 1991). Transmission of these signals eventually results in the activation of transcription factors that modulate the expression of specific set of genes (Fig. 1).

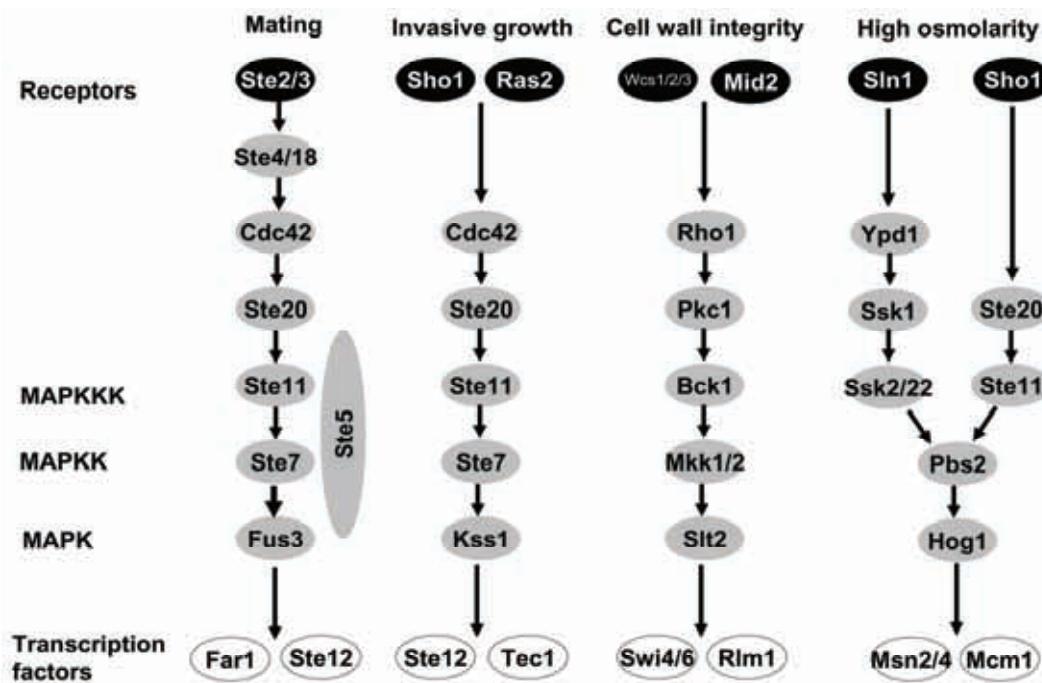


Fig. 1. MAP kinase pathways in *Saccharomyces cerevisiae*. Membrane proteins (receptors) are depicted in black, transcription factors in white and intermediate proteins in gray. For further details see explanation in text.

The Fus3 MAPK pathway in *S. cerevisiae* is a pheromone-activated signaling pathway that controls mating (Elion et al. 1990). A pheromone of one mating type binds to and activates a seven-transmembrane-domain receptor in the opposite mating type that in turn induces the dissociation of the heterotrimeric G-complex into G $\alpha$  (Gpa1) and the G $\beta\gamma$  (Ste4/Ste18) subunits. Subsequently, the G $\beta\gamma$  heterodimer activates the downstream proteins Cdc42 and Ste20, and these in turn stimulate the Ste11 and Ste7 proteins that eventually trigger the Fus3 MAPK. Subsequently, Fus3 phosphorylates downstream transcription factors, e.g., Ste12 and Far1 that mediate various responses required for successful mating [for review see (Blumer and Johnson 1994; Gustin et al. 1998)]

The Slt2 pathway is known as the cell wall integrity pathway in the budding yeast. In *S. cerevisiae*, this pathway regulates cell wall biosynthesis and responds to a range of different signals originating from the cell cycle regulation, ambient temperatures, and external osmolarity. Several membrane receptors including Wsc1, Wsc2, Wsc3 and Mid2 can activate the Slt2 pathway. The signaling proteins of this pathway downstream of the receptors include the GTP-binding protein Rho1, protein kinase C (Pkc1), a MAPKKK (Bck1), the redundant pair of MAPKKs (Mkk1 and Mkk2) and the MAPK Slt2. Slt2 activates the transcription

factors Rlm1 and Sbf (composed of the polypeptides Swi4 and Swi6) (Fig. 1). It is likely that many branches feed into this pathway (Gustin et al. 1998; Martin et al. 2000).

The Hog1 pathway in *S. cerevisiae* is known as the high osmolarity glycerol pathway and is required for growth under hyperosmotic conditions [for reviews see (Hohmann 2002; O'Rourke and Herskowitz 1998; O'Rourke et al. 2002)]. This pathway is regulated by two upstream cascades that converge to activate the MAPKK, Pbs2, and MAPK, Hog1. One branch depends on the Sho1 osmosensor transmembrane protein, while the other branch depends on the two-component histidine kinase receptor Sln1. The intermediate proteins for the Sho1 branch are Ste20 and Ste11, whereas the intermediate proteins for the Sln1 branch are Ypd1, Ssk1, Ssk2 and Ssk22 (Fig. 1). The phosphorylated Hog1 transiently accumulates in the nucleus and activates downstream target transcription factors such as Msn2, Msn4 and Mcm1. These proteins binds to the STRE consensus in the promoter of stress response genes and result in expression of genes required for survival under stress conditions.

### cAMP-dependent protein kinase A (PKA) pathway

The cAMP-dependent PKA pathway in *S. cerevisiae* involves Gpr1, a member of a family of membrane receptors commonly known as G-protein-coupled receptors (GPCRs). This receptor is coupled to Gpa2 that is a monomeric  $\text{G}\alpha$  protein (Fig. 2). The adenylyl cyclase, Cyr1, is regulated by Gpa2 as well as the transmembrane protein, Ras2 to increase the cAMP level in response to nutritional signals (Hoffman 2005). The target of cAMP in yeast is PKA that is a tetrameric protein of two cAMP-binding regulatory subunits and two catalytic subunits in the inactive holoenzyme. The regulatory subunit of PKA is encoded by *Bcy1*, and the three catalytic subunits are encoded by *Tpk1*, *Tpk2* and *Tpk3* (Pan and Heitman 1999; Toda et al. 1987). When the intracellular cAMP level increases, cAMP will bind to the regulatory subunit and trigger conformational changes that release the active catalytic subunits (Taylor et al. 1990). PKA has several targets and controls the phosphorylation state of transcription activators/repressors, kinases and metabolic enzymes (Thevelein and de Winde 1999). For example, Sfl1 and Flo8 as well as Msn2/4 transcription factors are regulated by PKA (Martinez-Pastor et al. 1996; Schmitt and McEntee 1996). In yeast, the *Pde1* and *Pde2* genes encode cAMP phosphodiesterase enzymes that hydrolyze cAMP to AMP and subsequently restore PKA to the resting, inactive state (Griffioen and Thevelein 2002; Thevelein and de Winde 1999).

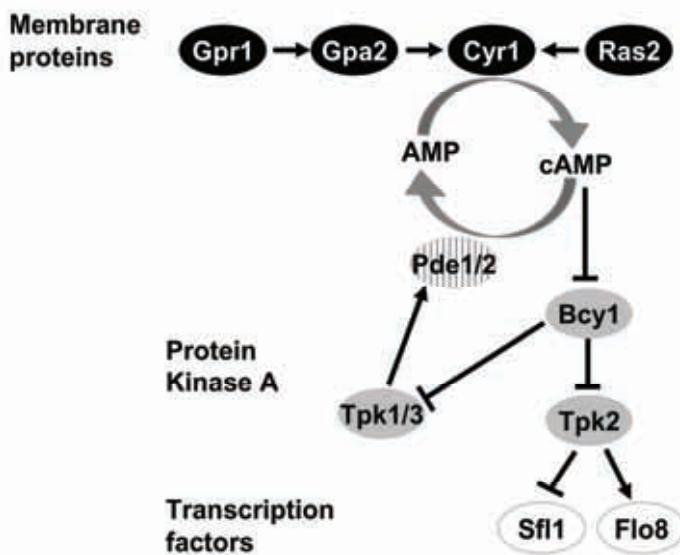


Fig. 2. The cAMP-dependent pathway in *Saccharomyces cerevisiae*. Membrane proteins are depicted in black, protein kinase A subunits in gray and transcription factors in white. For further details see explanation in text.

### Signaling through $G\alpha$ and $G\beta$ proteins

Unlike the higher eukaryotes (e.g. mammals) that contain more than three  $G\alpha$  protein-encoding genes, the genomes of most studied filamentous fungi contain one  $G\beta$  and three  $G\alpha$  protein-encoding genes (Aimi et al. 2001; Ganem et al. 2004; Gronover et al. 2001; Kays et al. 2000; Parsley et al. 2003). However, the model yeast *S. cerevisiae* contains two  $G\alpha$  protein- and one  $G\beta$  protein-encoding genes [for review see (Dohlman and Thorner 2001)]. The basic mechanism of signaling through heterotrimeric G proteins is now well established, particularly in mammalian systems. Signal transduction through G proteins often involves a three-component system i.e. receptor, G protein and effector. Activated transmembrane receptors (GPCRs) catalyze the exchange of GDP for GTP on the  $G\alpha$  protein leading to dissociation of the heterotrimeric complex into the  $G\alpha$  protein and the  $G\beta\gamma$  heterodimer. Either  $G\alpha$  or  $G\beta\gamma$ , or both can activate downstream effectors. Examples of direct effectors in mammalian cells include adenylyl cyclase, phospholipase C, exchange factors for small GTPases, some calcium and potassium channels and certain protein kinases. Activation of these effectors leads to one of the following; i) an increased level of secondary messengers (such as cAMP); ii) the stimulation of a protein kinase; or iii) the effector itself acting as a protein kinase. The resulting changes in protein phosphorylation can affect metabolism, ion fluxes, gene expression, cell morphology, cellular differentiation, and many other developmental processes. The signaling lasts until GTP is hydrolyzed to GDP, resulting in re-

association of the G $\alpha$  and G $\beta\gamma$  subunits. Therefore, the strength of the G protein-dependent signal transduction depends on the rate of nucleotide exchange, the rate of GTP hydrolysis and the rate of subunit re-association (Dohlman and Thorner 2001).

## Scope of the thesis

The experiments and results described in this thesis provide insight into the role of signal transduction pathways in the life cycle of *M. graminicola*. In this study, 10 genes encoding proteins involved in different signaling pathways were functionally analyzed via targeted gene disruption. A large EST library available from this fungus comprising over 30,000 ESTs enabled the identification of over 40 genes encoding proteins tentatively involved in signal transduction pathways. From this list, the biological function of a set of genes encoding three MAPKs, a p21-activated kinase (PAK), the regulatory and the catalytic subunit of PKA, three G $\alpha$  proteins and a G $\beta$  protein was studied in detail.

The development of the transposon mutagenesis system described in this thesis allowed us to quickly generate disruption constructs that could be used to replace the wild-type genes by *Agrobacterium tumefaciens*-mediated transformation.

**Chapter 1** describes the life cycle of *M. graminicola* that is now becoming a representative model for the order of the Dothideales. In addition, a brief overview of the signal transduction pathways is presented.

**Chapter 2** describes the functional analysis of *MgFus3*, the MAPK-encoding gene in *M. graminicola*. The role of this gene in pathogenicity and development is analyzed. Our data show that this gene is involved in the regulation of penetration and probably in the perception of the host as well as pycnidia formation.

**Chapter 3** describes the analysis of *MgSlt2* that encodes a MAPK involved in the regulation of cell wall biosynthesis. The role of this gene in the regulation of cell wall integrity, sensitivity to toxic compounds, pathogenicity and development of *M. graminicola* is investigated. The infection process of the *MgSlt2* mutants was studied *in planta* using scanning electron microscopy and light microscopy.

In **chapter 4**, the role of the MAPK-encoding gene, *MgHog1*, and the PAK-encoding gene, *MgSte20*, is studied. Early colony development experiments *in vitro* showed the importance of *MgHog1* in the (co-)regulation of the dimorphic switch from yeast-like to filamentous growth. The importance of dimorphic switch for the pathogenicity of *M.*

*graminicola* was studied by light microscopy. Generated *MgHog1* mutants were tested under different osmotic and fungicidal conditions.

**Chapter 5** describes the functional analysis of genes encoding the catalytic subunit (*MgTpk2*) and the regulatory subunit (*MgBcy1*) of PKA. The role of these genes on pathogenicity and asexual fruiting body formation is analyzed. In addition, the effect of disruption of these genes on germination, colony development and osmosensing is described.

In **chapter 6**, the functional analysis of three G $\alpha$  protein-encoding genes (*MgGpa1*, *MgGpa2* and *MgGpa3*) and a G $\beta$  protein-encoding gene (*MgGpb1*) is described. The effect of disruption of these genes on development and pathogenicity of *M. graminicola* is addressed.

**Chapter 7** provides a summarizing discussion of the thesis. The impact of the present study in understanding factors involved in the regulation of pathogenicity in *M. graminicola* and its value as a new model among plant pathogenic fungi are discussed.

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# CHAPTER 2

**The MAP kinase-encoding gene *MgFus3* of the non-appressorium phytopathogen *Mycosphaerella graminicola* is required for penetration and *in vitro* pycnidia formation**

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## ABSTRACT

In eukaryotes, a family of serine/threonine protein kinases known as mitogen-activated protein kinases (MAPKs) is involved in the transduction of a variety of extracellular signals and in the regulation of growth and development. We identified a MAPK-encoding gene in *Mycosphaerella graminicola* strain IPO323 with high homology to the orthologous *Fus3* gene of *Saccharomyces cerevisiae* and designated it *MgFus3*. Early colony development of the *MgFus3* mutants during *in vitro* growth was similar to those of the wild-type and ectopic controls, but at the later stages of growth, *MgFus3* mutants did not become melanized, showed altered polarized growth and were unable to produce aerial mycelia. The *MgFus3* mutants were non-pathogenic and detailed microscopic analyses revealed that they failed to colonize the mesophyll tissue due to the inability to penetrate stomata. Importantly, unlike the wild-type strain, *MgFus3* mutants were unable to differentiate pycnidia on plant-derived media. Thus, in addition to the crucial role of *MgFus3* in the regulation of penetration, it may also be involved in regulating in asexual fructification. Hence, *MgFus3* can be regarded as a multifunctional pathogenicity factor of *M. graminicola*.

**Additional keywords:** Gene targeting, *Agrobacterium tumefaciens*-mediated transformation, MAP kinase pathway, cytology of interaction, pycnidia formation.

## INTRODUCTION

The development of targeted gene disruption strategies has enabled the identification of a number of pathogenicity genes in plant pathogenic fungi (Adachi et al. 2002, Stergiopoulos et al. 2003). These genes have been recently categorized based on their role in the infection process (Idnurm and Howlett, 2001). Like all other living organisms, fungi are able to sense changes in the environment, respond, and adapt to new situations in an adequate manner. In eukaryotic cells, a family of serine/threonine protein kinases known as mitogen-activated protein kinases (MAPKs) is involved in the transduction of a variety of extracellular signals and in the regulation of growth and development (Dickman and Yarden, 1999; Nishida and Gotoh, 1993; Shaeffer and Weber, 1999). In *Saccharomyces cerevisiae*, several MAPK cascades have been studied extensively and, due to the high degree of conservation, homologs of MAPKs in filamentous fungi have been identified using candidate gene approaches (Xu, 2000). Xu and Hamer (1996) identified a homolog of the *S. cerevisiae Fus3* gene in the rice blast fungus *Magnaporthe grisea*, designated *PMK1*, which is essential for appressorium formation and pathogenicity. More recently, several MAPKs related to *PMK1* were shown to be essential for pathogenicity in a range of additional plant pathogenic fungi, particularly during the early stages of infection (Lev et al. 1999; Zheng et al. 2000; Takano et al. 2000; Xu 2000; Ruiz-Roldán et al. 2001; Di Pietro et al. 2001; Mey et al. 2002; Urban et al. 2003; Jenczmionka et al. 2003).

*Mycosphaerella graminicola* (Fuckel) J. Schröt in Cohn (anamorph: *Septoria tritici*), a non-appressorium forming wheat pathogen with both filamentous and yeast-like growth phases, is the causal agent of the major wheat disease septoria tritici leaf blotch. Severe epidemics of the pathogen result in considerable yield losses and have become a serious economic problem in Western Europe during the last decade. (Eyal et al. 1987; Daamen and Stol, 1992; Halama, 1996; Jorgensen et al. 1999; Hardwick et al. 2001). Therefore, *M. graminicola* has also become a major target for deployment of fungicides and for the development of novel fungicides by the agrochemical industry, particularly since the development of resistance in this fungus to strobilurin fungicides (Amand et al. 2003). *M. graminicola* penetrates the leaves through stomata without differentiating specific infection structures like appressoria. Penetration usually occurs within 24-48 h after inoculation with spores, and subsequent colonization of the mesophyll tissue remains confined to the intercellular space. The first symptoms appear on leaves about 10 days post inoculation (dpi) as chlorotic blotches. At approximately 14 dpi, these blotches turn into necrotic lesions and

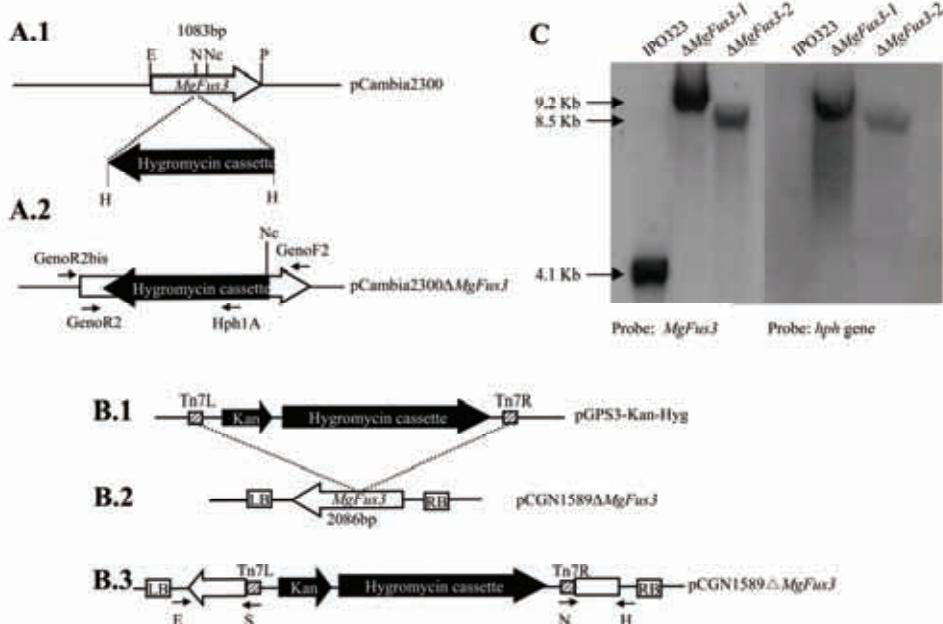
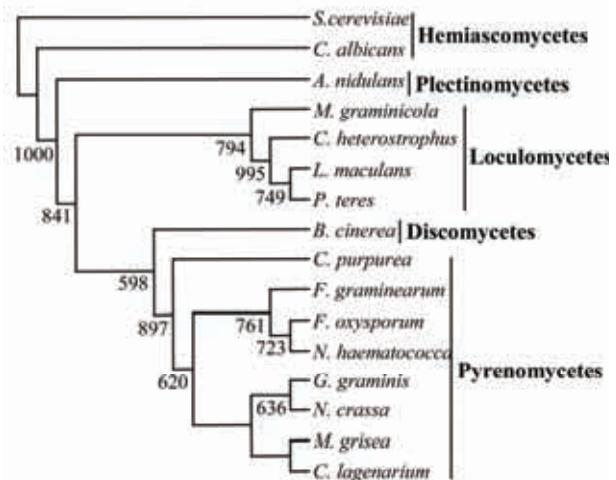
the first pycnidia emerge in sub-stomatal cavities, on both sides of the leaf (Cohen and Eyal, 1993; Kema et al. 1996; Duncan and Howard, 2000). To date, in *M. graminicola* hardly anything is known about genes encoding pathogenicity factors. We set out for a targeted gene approach and decided to search for and clone a MAPK of *M. graminicola* orthologous to *Fus3* of *S. cerevisiae* and designated it *MgFus3*. Subsequently, we functionally characterized *MgFus3* and demonstrate that it not only regulates the recognition and penetration of host tissue but that it is also required during asexual fructification, particularly during pycnidia formation.

## RESULTS

### Cloning and characterization of *MgFus3*

By using degenerated primers in combination with screening of large EST library of *M. graminicola* strain IPO323, we cloned the MAPK-encoding gene designated as *MgFus3*. This gene has an open reading frame (ORF) of 1068 bp interrupted by two introns (74 and 70 bp, respectively), with consensus fungal splicing sites. Comparisons with database entries demonstrated that the first intron of *MgFus3* is located more downstream in the ORF (behind the second MAPK subdomain), than in all other *Fus3* orthologs for which the location of introns is known. *MgFus3* encodes 356 amino-acid protein with 88 and 92% identity and 91 and 97% similarity to the *M. grisea* PMK1 and *Cochliobolus heterostrophus* CHK1 MAPKs, respectively. The MgFUS3 contains all 11 conserved protein kinase subdomains including the TEY domain, which is characteristic for MAPK phosphorylation sites (Nishida and Gotoh, 1993). Multiple sequence alignment of all predicted FUS3-like MAPKs from ascomycetes revealed that the highest variability occurs in the N-terminal region (upstream of the first characteristic sub-domain). Nevertheless, the phylogenetic classes identified from sequence comparisons of FUS3-like proteins of ascomycete fungi are in good agreement with the known taxonomic classification of these fungi (Fig. 1). Southern analysis revealed that *MgFus3* occurs as a single copy gene in the genome of *M. graminicola* (Fig. 2).

**Fig. 1.** Phylogenetic relationships of FUS3-like MAPKs from ascomycete fungi. Multiple sequence alignments and the phylogenetic tree were generated using Clustal X (Thompson et al. 1997) and Tree View (Page, 1996), respectively. Fungal classes are shown in bold face. The tree is rooted using the FUS3 amino acid sequence of *S. cerevisiae* as the out-group. Maximum bootstrap values are 1,000. Bootstrap scores of less than 500 are not shown. Accession numbers of sequences used to generate this tree are the following: *Saccharomyces cerevisiae*: FUS3 (S28548); *Magnaporthe grisea*: PMK1 (AAC49521); *Nectria haematococca*: FsMAPK (AAB72017); *Candida albicans*: CEK1 (A47211); *Cochliobolus heterostrophus*: CHK1 (AF178977); *Aspergillus nidulans*: unnamed protein (AAF12815); *Gaeumannomyces graminis*: GMK1 (AF258529); *Colletotrichum lagenarium*: CMK1 (AF174649); *Botrytis cinerea*: BMP1 (AF205375); *Neurospora crassa*: unnamed protein (AAK25816); *Fusarium oxysporum*: FMK1 (AF286533); *Pyrenophora teres*: PTK1 (AF272831); *Leptosphaeria maculans*: HYP2 (AY118109); *Claviceps purpurea*: CPMK1 (AJ318517); *F. graminearum*: GPMK1 (AF448230); *Mycosphaerella graminicola*: MgFus3 (AY877346).



**Fig. 2.** Diagram of *MgFus3* disruption strategy in the *Mycosphaerella graminicola* strain IPO323. **A.1**, Physical map of the *MgFus3* cDNA inserted into the binary vector pCambia2300 and the 1.4 kb fragment of pCB1003 containing the bacterial *hph* gene encoding hygromycin B phosphotransferase under the control of the *Aspergillus nidulans trpC* promoter. The restriction enzymes are: (E) *EcoRI*; (H) *HpaI*; (N) *NruI*; (P) *PvuII*; (Nc) *NcoI*. The arrows indicate the directions of *MgFus3* and *hph* genes, and primers used to identify mutants (GenoR2, GenoR2bis, GenoF2 and Hph1A). **A.2**, Physical map of disrupted *MgFus3*. **B.1**, The customized transplicer donor (pGPS3-Hyg-Kan) containing a kanamycin and hygromycin cassette was used to transpose the targeted construct, pCGN1589*MgFus3* (**B.2**). The new construct called pCGN1589 $\Delta$ *MgFus3* (**B.3**). The insertion position and the orientation of the insertion were identified using primers located at the end of the transposon (primers S and N) and downstream and upstream of *MgFus3* (primers E and H). **C**, Southern blot of wild-type *M. graminicola* IPO323 and two independent *MgFus3* mutants ( $\Delta$ *MgFus3*-1 and  $\Delta$ *MgFus3*-2). All DNA samples were digested with *NcoI*. The blot was probed with a fragment from *MgFus3* (downstream of the *NcoI* restriction site), then stripped and reprobed with the *hph* gene.

### Disruption of *MgFus3*

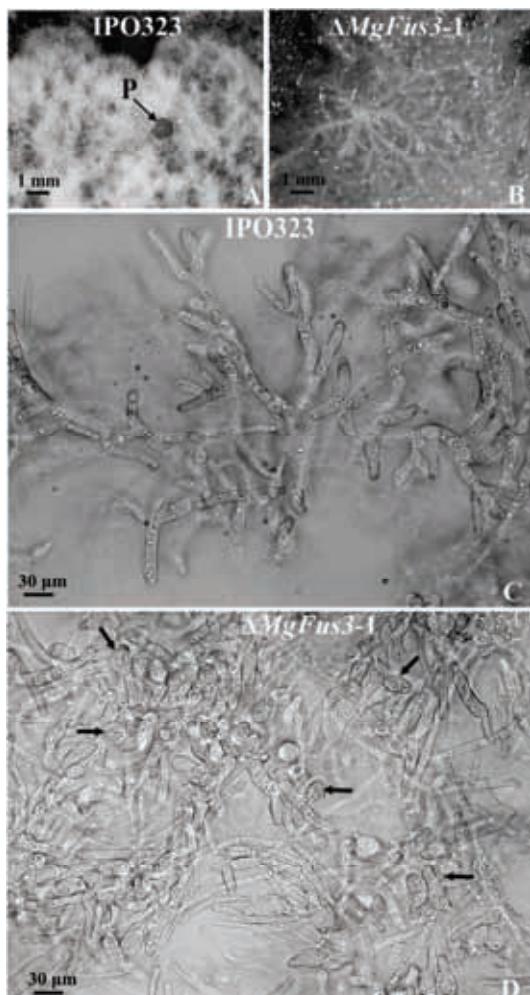
To analyze the functionality of *MgFus3* in *M. graminicola* strain IPO323, we generated two disruption constructs and successfully transformed them into *M. graminicola* by *Agrobacterium tumefaciens*-mediated transformation (Zwiers and de Waard 2001) in order to get gene replacement mutants. With the first disruption construct, gene replacement mutant  $\Delta MgFus3$ -1 was identified out of 288 hygromycin-resistant transformants, while the second disruption construct resulted in mutant  $\Delta MgFus3$ -2 out of 64 analyzed transformants. Both gene replacement events were confirmed by Southern blot analysis (Fig. 2C) and both mutants were subsequently used for phenotype analyses.

### *MgFus3* is required for pycnidia formation on plant-derived media

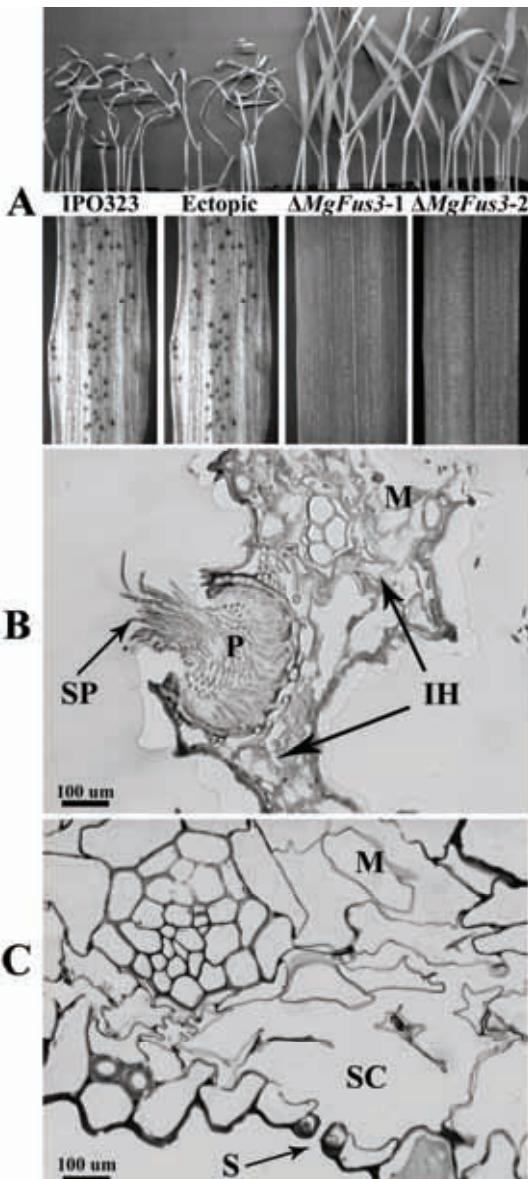
We studied germination of spores and early colony development in the IPO323 $\Delta MgFus3$  strains, the wild-type IPO323 and ectopic transformant controls *in vitro*. All strains showed normal yeast-like growth, septation and polarized growth during early colony development. Germination percentage of spores was  $75 \pm 2\%$  and did not differ significantly (data not shown). Colony formation on potato dextrose agar (PDA) and yeast-like growth on V8 medium and minimal medium (MM) was similar for all strains tested. However, at the later stages of colony development ( $>10$  days), unlike the wild-type and ectopic transformant strains, *MgFus3* mutants did not become melanized, showed altered polarized growth and were unable to produce aerial mycelia. We mimicked *in planta* growth by transferring fungal colonies from water agar (WA) or WA supplemented with wheat decoction (WA+) to V8 agar and could distinguish three distinct stages; the first stage including germination and filamentation; the second stage including mycelium maturation followed by aggregation of melanized hyphae; the third stage including initiation and formation of pycnidia. *M. graminicola* strain IPO323 developed fertile pycnidia 20 days after transfer from WA+ to rich V8 medium. In contrast, under similar conditions, the *MgFus3* mutants neither showed melanization of hyphae nor did they produce pycnidia (Fig. 3).

### *MgFus3* is essential for penetration

In the detached leaf pathogenicity assay, the susceptible wheat cultivars Scipion and Obelisk, became necrotic and produced many pycnidia emerging from the stomata 11 dpi with *M. graminicola* strain IPO323. In contrast, *MgFus3* mutants failed to produce any lesions even after prolonged incubations up to 34 days. At that time, only faint chlorosis was observed. These symptoms were also observed in whole plant assays (Fig. 4).



**Fig. 3.** Comparative analysis of *Mycosphaerella graminicola* IPO323 and  $\Delta M g F u s 3$  strains under the *in vitro* test conditions. Panels A and B: *in vitro* melanization and pycnidia formation of IPO323 (A) and IPO323 $\Delta M g F u s 3$  (B). IPO323 $\Delta M g F u s 3$  did not become melanized and was unable to differentiate pycnidia (P). Panels C-D: Normal hyphal development of IPO323 (C) contrasts with the non-specific development of buds (arrows) of IPO323 $\Delta M g F u s 3$  (D).

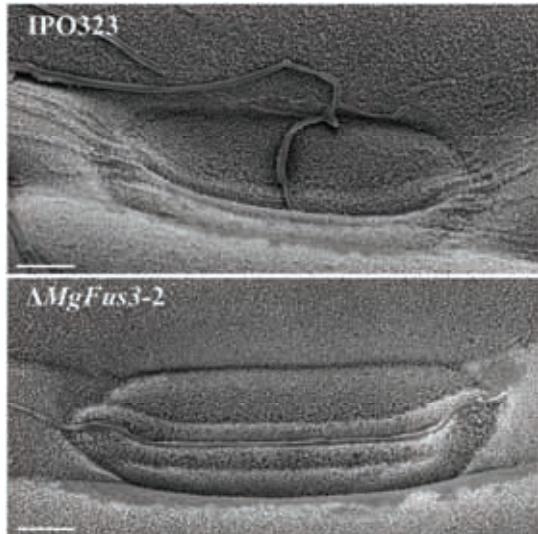


**Fig. 4.** Effect of *MgFus3* disruption on pathogenicity. A, Panels show wheat seedlings of cv. Obelisk 20 days post inoculation (dpi) with *Mycosphaerella graminicola* IPO323, an ectopic transformant and the two independent knock-outs of *MgFus3* at two magnifications. Panels B and C, show transverse sections of primary wheat leaves (cv. Scipion) infected (30 dpi) with IPO323 and  $\Delta M g F u s 3$ , respectively. The mesophyll (M) infected by IPO323 is completely colonized and collapsed (IH: infectious hyphae) and a sporulating (SP) mature pycnidium has formed in the substomatal cavity. IPO323 $\Delta M g F u s 3$  cannot colonize the mesophyll; (SC) substomatal cavity, (S) stomata.

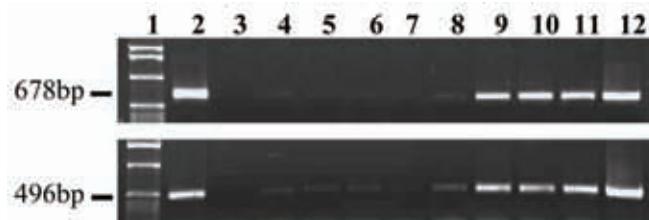
Histological observations showed that *MgFus3* mutants failed to penetrate stomata, which was due to an impaired recognition as germ tubes grew along stomatal slits without penetrating them and, hence, mesophyll colonization did not occur (Figs. 4-5).

### Expression analyses

The *MgFus3* expression analyses *in vitro* and *in planta* resulted in a characteristic 678 bp RT-PCR product and the expression pattern of *MgFus3* was similar to that of the  $\beta$ -tubulin-encoding gene, indicating that *MgFus3* is constitutively expressed (Fig. 6). In addition, comparative gene expression analysis ( $N=209$ ) of *M. graminicola* strain IPO323 and *MgFus3* mutants after five days growth in YGB revealed no significant differences in gene expression patterns on macro-arrays (data not shown).



**Fig. 5.** Scanning electron micrographs of penetration behavior of *Mycosphaerella graminicola* strain IPO323 (A) and  $\Delta M g F u s 3$  (B) on primary leaves of cv. Obelisk 48 h after inoculation. The regular successful penetration of IPO323 strongly contrasts with the typical penetration failure of the *MgFus3* mutant. The germ tubes of IPO323 $\Delta M g F u s 3$  strains reach the stomata but continue to grow on the leaf surface without penetration



**Fig. 6.** *MgFus3* expression analysis. RT-PCR products were generated using primers specific for *MgFus3* (upper panel) and for the  $\beta$ -tubulin gene (lower panel) (see Table 1 and Materials and Methods). Lane 1: 1-kb ladder. Lane 2: first-strand cDNAs generated from total RNA from *Mycosphaerella graminicola* strain IPO323 grown on PDA. Lane 3: water-inoculated wheat leaves. Lanes 4-11: total RNA from wheat inoculated with IPO323 strain at 2, 3, 4, 9, 11, 14, 16 and 24 dpi, respectively. Lane 12: positive PCR controls of *MgFus3* and  $\beta$ -tubulin.

## DISCUSSION

In this study, we cloned and characterized the MAPK-encoding gene *MgFus3* from *M. graminicola* the causal agent of septoria tritici leaf blotch on wheat. Independent transformation experiments generated only two *MgFus3* mutant strains. Previous results from targeted gene disruption experiments using PEG-based transformation methods suggested that *M. graminicola* has a low competency for homologous recombination (Keon and Hargreaves, 1998; Zwiers and De Waard, 2000, 2001). The relatively high efficiency of targeted gene disruption in *M. graminicola* using TAGKO (transposon-arrayed gene knock-out) reported by Adachi et al. (2002) is likely due to the large homologous flanking regions, which were about 40 times larger than those used in our study. Alternatively, the low frequency of gene targeting could also be due to locus-specific effects, as was previously demonstrated in other filamentous fungi (Bird and Bradshaw, 1997; Hamer et al. 2001). This hypothesis is supported by data demonstrating that the frequency of homologous recombination for different genes in *M. graminicola* is highly variable, despite the use of constructs with similarly sized flanking regions (This thesis, chapter 7).

*MgFus3* belongs to a sub-group of yeast- and fungus-specific MAPKs, referred to as extracellular signal-regulated MAPKs that are involved in extracellular signal perception (Xu, 2000). *MgFus3* closely resembles homologs from other fungal pathogens (Lev et al. 1999; Zheng et al. 2000; Urban et al. 2003; Takano et al. 2000; Ruiz-Roldán et al. 2001; Di Pietro et al. 2001; Mey et al. 2002; Xu 2000; Jenczmionka et al. 2003). The number of introns and their respective positions are identical in the majority of *Fus3* orthologs and, hence, suggest that these genes evolved from one ancestral gene. The observed variations probably represent modifications/changes accumulated during later stages of evolution. In *M. graminicola*, a rearrangement of *MgFus3* may be responsible for the deviation in the position of the first intron.

The *in planta* assays showed that *MgFus3* is required for pathogenicity as *MgFus3* mutants of *M. graminicola* showed impaired penetration of stomata even when the germ tubes were in very close contact with the stomatal guard cells. This observation is similar to those obtained with mutants of *MgFus3* orthologs that also regulate several early morphological and developmental processes essential for plant infection (Xu and Hamer 1996; Lev et al. 1999; Takano et al. 2000; Zheng et al. 2000; Di Pietro et al. 2001; Ruiz-Roldán et al. 2001; Mey et al. 2002; Jenczmionka et al. 2003; Urban et al. 2003; Talbot, 2003). However, *M. graminicola* does not develop specific infection structures like appressoria (Kema et al. 1996). Therefore,

*MgFus3* might be involved in recognition of stomatal cells or host tissue, a phenomenon that is crucial during penetration of plants by several rust species (Hoch et al. 1987). In appressorium-forming pathogens such as *M. grisea* and *Colletotrichum lagenarium* melanization is necessary to generate a high turgor pressure in appressoria, which is required for mechanical penetration of epidermis cells (Howard and Valent 1996; Money and Howard 1996; Perpetua et al. 1996). Since *M. graminicola* penetrates its host through stomata, melanization is probably not required for penetration. Indeed, we recently demonstrated that non-melanized *MgSlt2* mutants (the ortholog of *Slt2* in *S. cerevisiae*) and ectopic transformant and the wild-type controls of *M. graminicola* did not show different penetration patterns on wheat leaves, confirming that melanization is not crucial for penetration (Mehrabi et al. 2006).

We demonstrated that *MgFus3* is also crucial for the latter stages of infection, a conclusion that is based on *in vitro* analyses of the *MgFus3* mutants, which were unable to produce pycnidia on plant-derived media. This might be due to impaired differentiation that is essential for fructification, or hampered melanization. Indeed, it has been shown that pycnidia are extensively melanized, suggesting that melanin is required for asexual fructification in *M. graminicola* (Kema and Annone, 1991; Kema et al. 1996; Duncan and Howard, 2000; Mehrabi, et al. 2006). This may explain the failure of the *MgFus3* mutants to produce pycnidia *in vitro*. Unlike *M. graminicola*, in other filamentous fungi, *Fus3* orthologs are involved in various developmental stages including vegetative growth, conidiation and germination of conidia (Lev et al. 1999; Takano et al. 2000; Zheng et al. 2000), suggesting overlapping and distinct functions among *Fus3* orthologs.

Overall, our data indicate that *MgFus3* is involved in the regulation of both early and late stages of infection, particularly in stomatal recognition, penetration and asexual fructification. We conclude that MgFUS3 is a new member of a highly conserved MAPK family present in plant pathogenic fungi. Although *Fus3* orthologs were shown to have functional redundancy during initial stages of infection, our results show they might also regulate late stages of infection, suggesting that particular functions of MAPKs can relate to specific lifestyles of these fungi.

## MATERIALS AND METHODS

### Fungal strains and growth conditions

*M. graminicola* strain IPO323 (Kema and Van Silfhout, 1997, Kema et al. 2002) was used as the recipient strain in the gene disruption experiments. *M. graminicola* strain STA was used to generate a genomic library. Approximately  $10^6$  spores from the -20 °C silicagel stock were plated on V8 medium (water/vegetable juice [1/4; vol/vol] and 2 g.l<sup>-1</sup> of CaCO<sub>3</sub>) complemented with streptomycin sulfate and penicillin (both at a final concentration of 50 µg.ml<sup>-1</sup>), and incubated for seven days at 18 °C. These plates were used to start small-scale production of spores on fresh PDA plates, whereas for large-scale production of spores, *M. graminicola* was grown in yeast glucose broth (YGB; 1% yeast extract, 3% glucose) at 18 °C on an orbital shaker for five days at 120 rpm. Inoculum for pathogenicity assays with a final density of  $10^7$  spores.ml<sup>-1</sup> was produced by multiple steps of washing and concentrating the spore suspension in distilled water by centrifugation.

### Germination of spores

Two hundred spores of *MgFus3* mutants, ectopic transformant and the wild-type IPO323 controls were spread on V8 agar medium to evaluate germination after 12 h at 20 °C. For each strain, three independent experiments were conducted.

### Fungal growth tests

Three different solid media were used for *in vitro* tests mimicking the nutritional changes occurring during plant infection. Firstly, filamentous growth was checked by spreading 50 spores either on water agar (WA, 1.5% agar w/v) or on water agar supplemented with 4% (v/v) of a wheat decoction (WA+) that was obtained by boiling 15 g of wheat leaves in 300 ml water for 10 min and filtration through a 0.45 µm filter. After three weeks of growth, separate filamentous colonies that were obtained on both media were transferred onto a fresh plate of V8 agar medium. Further development of the filamentous colony and potential differentiation of asexual fructifications (pycnidia) was monitored. For each strain, two independent experiments were conducted with four replicates each.

### DNA isolation and manipulation

Plasmid DNA was isolated with a QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany). The Prep-A-Gene®DNA kit (BIO-RAD, California, USA) was used to purify DNA fragments from agarose gels. Fungal DNA was isolated from five- to six-day-old mycelia grown in yeast saccharose medium (1% saccharose, 1% yeast extract) complemented with streptomycin sulfate and penicillin, both at a final concentration of 50 µg.ml<sup>-1</sup>, and

ground in liquid nitrogen. DNA extraction was carried out using the QIAGEN Genomic DNA Purification Kit (QIAGEN, Hilden, Germany). Southern blots were performed according to Sambrook et al. (1989) and were hybridized with  $^{32}\text{P}$ -labeled probes generated by random prime labeling using the Oligolabelling Kit (Amersham Biosciences, Saclay, France) or Roche Applied Science Kit (Roche, Meylan, France).

### **Construction of *M. graminicola* genomic DNA and cDNA libraries**

A cDNA library was prepared in the ZAP Express<sup>®</sup> Vector (Stratagene, Amsterdam, The Netherlands), according to manufacturer's instructions with poly(A)+ RNA purified with magnetic Oligo(dT)<sub>25</sub> Dynabeads (Dynal, Compiègne, France) from total RNA of *M. graminicola* strain STA grown in yeast saccharose medium and *Aspergillus nidulans* minimal medium (MM). The titers of the libraries were  $2.8 \times 10^9$  and  $6.08 \times 10^9$  phages.ml<sup>-1</sup>, respectively. The ZAP Express<sup>®</sup> Vector was used to construct all libraries, including the *Sau3AI* partially digested fragments of the genomic DNA of *M. graminicola* strain STA into the *BamHI* site of the polylinker. The titer of the genomic DNA library was  $3.12 \times 10^9$  phages.ml<sup>-1</sup>, which corresponds to approximately  $5.8 \times 10^5$  times the *M. graminicola* genome.

### **Isolation, cloning and sequencing of the *MgFus3* gene**

We used *M. graminicola* strain IPO323 to clone and characterize the *MgFus3* gene because this strain has been adopted as the standard strain for molecular genetics in *M. graminicola* research. We used two approaches to clone *M. graminicola MgFus3*. In the first approach, four degenerate primers were used to amplify the *MgFus3* gene that subsequently was disrupted by a number of digestion/ligation steps. Sense primers (MKF1 and MKF2) and antisense primers (MAK4MYC and MEK3MYC) were designed based on the highly conserved amino acid sequence among *Fus3* homologs in filamentous fungi (Table 1). The antisense primers were previously used to isolate the *CHK1* gene of *Cochliobolus heterostrophus* (Lev et al. 1999).

Primary PCR was conducted with primers MKF1 and MAK4MYC (Table 1) using 50 ng of genomic DNA of IPO323 as a template under the following PCR conditions: 2 min at 94 °C, followed by 30 cycles at 94 °C for 1 min, 53 °C for 2 min and 72 °C for 1 min with a final extension of 5 min at 72 °C. Nested PCR was performed using primers MKF2 and MEK3MYC (Table 2) and 1 µl of the first amplification reaction as a template under PCR conditions 2 min at 94 °C, followed by 35 cycles (94 °C for 1 min, 48 °C for 2 min and 72 °C for 30 sec) with a final extension of 5 min at 72 °C. The PCR product was purified and cloned

Table 1. Primers used in this study

Name	Sequence (5'-3')	Location
MKF1	GCNATHAARAARATHACNCC	<i>MgFus3</i> (151-170)
MKF2	CCYTTYGAYCAYAGYATG	<i>MgFus3</i> (169-186)
MAK4MYC	TCNGGNGCNCKRTACC	<i>MgFus3</i> (661-646)
MEC3MYC	GGYTTNARTCNCKRTG	<i>MgFus3</i> (527-510)
GenoF2	CCGCATGATCTCTCGTAAATC	<i>MgFus3</i> (1212-1191)
MGMK1R2	CCAGACCCCTCCGCGCGCTGAAAGCC	<i>MgFus3</i> (464-488)
<i>MgFus3</i> -F1	AACGTGTCGGAGCAGTATGATA	<i>MgFus3</i> (55-76)
<i>MgFus3</i> -R1	GGTACCAACGAGTCGCGA	<i>MgFus3</i> (634-651)
GenoR2bis	GCCCAGCTCTGCGATCGACGAG	<i>MgFus3</i> 5' UTR
Hph1A	CAGCGAGAGCCTGACCTATTGC	Hygromycin
Sp6	TATTTAGGTGACACTATAG	pSport border
T7	TAATACGACTCACTATAAGG	pSport border
Primer S	ATAATCCTTAAAAACTCCATTCCACCCCT	Left border of transposon (Tn7L)
Primer N	ACTTTATTGTCATAGTTAGATCTATTG	Right border of transposon (Tn7R)
Primer E	CAGGAAACAGCTATGACATG	Left side of MCS in pCGN1589
Primer H	GATTAAGTTGGTAACGCCAGG	Right side of MCS of pCGN1589

into the pGEM®-T vector (Promega, Charbonnières-les-Bains, France) following the manufacturer's instructions. DNA sequences were determined on an ABI-prism 310 analyzer using the BigDye™ Terminator reaction mix (Applied Biosystems, Courtaboeuf, France). In the second approach *MgFus3* sequence was confirmed with a full-length cDNA clone obtained by screening the existing EST libraries of *M. graminicola* IPO323 (Kema et al. 2003). This cDNA clone carries 5' and 3'-UTR regions of 680 and 187 bp, respectively (data not shown).

Homology searches were performed with the BLAST program (Altschul et al. 1997) and alignments and similarity trees were generated using CLUSTAL X (Thompson et al. 1997).

### ***MgFus3* gene disruption**

Two disruption constructs were successfully generated. The first disruption vector was generated by two successive steps (Fig. 1A.1). The first step consisted of ligating a 1,083 bp *Eco*RI-*Pvu*II restriction fragment from the *MgFus3* cDNA into the binary vector pCAMBIA2300 predigested with restriction enzymes *Eco*RI and *Sma*I. The resulting construct was further digested with *Nru*I, cutting once within the *MgFus3* cDNA sequence, and ligated to a hygromycin resistance cassette consisting of a 1.4 kbp *Hpa*I fragment

obtained from the pCB1003 vector (Carroll et al. 1994). The second disruption construct was made using an *in vitro* transposon mutagenesis system (New England Biolabs Inc.) (Fig. 1B), which is described in detail by Mehrabi et al. (2006).

*A. tumefaciens*-mediated transformation enabled the disruption of *MgFus3* in IPO323 using both disruption constructs as described previously (Zwiers and de Waard, 2001; Mehrabi et al. 2006). Strains that were generated using the first disruption construct were selected by two PCR reactions. We first used GenoF2 and MGK1R2 (Table 1 and Fig. 1A.2) followed by GenoR2bis and Hph1A (Table 1 and Fig. 1A.2); the latter being homologous to the hygromycin gene sequence (Fig. 1A.2). Using the first primer pairs, strains resulting from a gene replacement event were expected to give a 2,148 bp amplicon while amplification, from genomic DNA carrying the wild-type *MgFus3* gene resulted in a 748 bp PCR product. The second primer pairs allowed amplification only if gene replacement had occurred, leading to a 1,809 bp PCR product. Mutants that had been generated using the second construct were screened by PCR using primers *MgFus3*-F1 and *MgFus3*-R1 (Table 1). Clones that did not show a 597 bp amplicon were considered to result from homologous recombination.

### Expression analysis

Expression analysis of the *MgFus3* gene was carried out by Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis at different time points during infection. Total RNA was extracted from infected leaves using the TRIzol® reagent (Invitrogen, Leek, The Netherlands), according to the manufacturer's instructions. Total RNA (1 µg) from each sample was treated with RNase-free DNase I (Invitrogen, Leek, The Netherlands) and reactions were performed using Oligo (dT)12-18 and the Superscript™ RNase H- Reverse Transcriptase (Invitrogen), following the manufacturer's instructions. PCR reactions were then carried out with two µl aliquots of this reaction mixture. Two primers were designed for specific amplification of the *MgFus3* gene transcript. Both primers, GenoF2 and MGK1R2 (Table 1), flank the second intron of *MgFus3* and were used in PCR reactions consisting of 2 min at 94 °C, subsequently followed by 35 cycles at 94 °C for 2 min, 53 °C for 1 min, and 72 °C for 1 min and a final extension step at 72 °C for 5 min. Specific *M. graminicola* β-tubulin primers defined by Rohel et al. (2001), E1 and STSP2R, were used as controls for constitutive gene expression. PCR amplification conditions for these controls were similar to *MgFus3* conditions except for the annealing step, which was conducted at 65 °C. The same experiments were carried out on wheat leaves infected with strain IPO323 at different time

points after inoculation. Aliquots of 10 µl of the PCR products were analyzed on 2% agarose gels.

### ***In planta* pathogenicity assays**

Fifteen seeds of the susceptible bread wheat (*Triticum aestivum*) cultivars Scipion and Obelisk were sown per plastic pot (5x5 cm) filled with moist vermiculite and autoclaved soil. Plantlets were grown in controlled climate chambers with a light intensity of 166 µE.sec<sup>-1</sup>.m<sup>-2</sup> and 16-h day length. The temperature and relative humidity (RH) conditions were 23 °C and 60%, respectively. Ten-day-old seedlings were inoculated when the first leaves were fully unfolded. About 10 ml of spore suspension (10<sup>7</sup> spores.ml<sup>-1</sup>) were applied per pot of 15 seedlings using a hand-operated sprayer. After inoculation, pots were placed in transparent polyethylene boxes (6x22x34 cm) and incubated at 18-20 °C in the dark for 48 h and then incubated using the above mentioned light conditions. The first symptoms were visible between 14 and 16 dpi.

### **Detached leaf pathogenicity assays**

A detached leaf assay was developed to study the pathogenicity of *M. graminicola* strains on wheat. Approximately 3-cm-leaf segments (either first or second leaf) were placed onto kinetin agar medium (1 mg.l<sup>-1</sup>) in 90 mm-diameter Petri dishes. The spore suspension was brushed onto the adaxial face of the leaf fragments and each Petri dish lid was sprayed with distilled water before sealing with Parafilm® to achieve humidity saturation. Inoculated leaf fragments were incubated at 18 °C in the dark for 48 h and were subsequently transferred to an incubator with the aforementioned conditions. The first symptoms usually appeared at 10 dpi.

### **Scanning electron microscopy**

Scanning electron microscopy (SEM) was performed to monitor timing of germination and penetration more precisely. Plants were inoculated as described above in the pathogenicity assay on leaf segments, and a small part of the leaf was cut and placed in the SEM chamber. Penetration events were analyzed on a Hitachi S-3000N (Tokyo, Japan), a variable pressure SEM. In the variable pressure range (1-270 Pa), non-conducting specimens can be imaged without a conductive film coating. Observations were carried out 48 h after inoculation with *M. graminicola* strain IPO323. Two independent experiments were conducted.

### **Experimental procedures and conditions for *in planta* cytology**

All microscopic studies were performed on the second leaves that were sampled randomly at different time points after inoculation. For cytological examination, samples were fixed under low vacuum for 15 min and incubated overnight at 4 °C in FAA fluid (formaldehyde/acetic acid/95 % ethanol/water [2/1/10/7; vol/vol/vol/vol]). The samples were dehydrated in a graded ethanol series, embedded in historesin (LEICA historesin embedding kit, Nussloch/Heidelberg, Germany) and sectioned (2 µm sections) with a microtome (Leica) equipped with glass knives. The sections were collected on glass slides, stained with toluidine blue (0.5% v/v in water) for 10 min and observed using a Zeiss Axioskop microscope (Zeiss-Jena, Oberkochen, Germany). Two independent experiments were carried out, each on three different wheat leaves inoculated with *M. graminicola* IPO323,  $\Delta MgFus3$ -1 or  $\Delta MgFus3$ -2 strains. Leaf sections were taken at different time points after inoculation and at different positions of each leaf.

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# CHAPTER 3

***MgSl2*, a cellular integrity MAP kinase gene of the fungal wheat pathogen  
*Mycosphaerella graminicola*, is dispensable for penetration  
but essential for invasive growth**

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## ABSTRACT

Among EST libraries of *Mycosphaerella graminicola* IPO323, we identified a full-length cDNA clone with high homology to the mitogen-activated protein kinase (MAPK) gene *Slt2* in *Saccharomyces cerevisiae*. This MAPK-encoding gene consists of a 1,242 bp open reading frame, and encodes a 414 aa protein. We designated this homolog *MgSlt2* and generated *MgSlt2* knockout strains in *M. graminicola* IPO323 and found several altered phenotypes *in vitro* as well as *in planta*. In yeast glucose broth, *MgSlt2* disruptants showed a defective polarized growth in the tip cells, upon aging, causing substantial local enlargements culminating in large swollen cells containing two to four nuclei. The *MgSlt2* disruptants showed a significantly increased sensitivity to several fungicides, including miconazole (2x), bifonazole (>4x), imazalil (5x) and cyproconazole (10x) and were hypersensitive to glucanase. Unlike the wild-type, *MgSlt2* disruptants did not produce aerial mycelia and did not melanize on potato dextrose agar. Although cytological analysis *in planta* showed normal penetration of wheat stomata by the germ tubes of the *MgSlt2* disruptants, subsequently formed hyphal filaments were frequently unable to branch out and establish invasive growth resulting in highly reduced virulence, and prevented pycnidia formation. We therefore conclude that *MgSlt2* is a new pathogenicity factor in *M. graminicola*.

**Additional keywords:** *Agrobacterium tumefaciens*-mediated transformation, septoria tritici leaf blotch, transposon.

## INTRODUCTION

*Mycosphaerella* is the largest genus in the Dothideales, an extremely large and diverse class of fungi. With over 1,000 named species, but along with its associated asexual stages the genus *Mycosphaerella* contains at least 3,000 species, it probably is the largest genus of plant pathogens in the fungal kingdom, causing extensive economic losses on a range of crops including cereals, banana, citrus, soybean, sugar beet, tomato, strawberry and even tree crops (Farr et al. 1995). Direct losses plus the cost of control strategies contribute to the huge agricultural significance of this genus. The devastating *M. fijiensis*, causing black leaf streak disease – commonly known as Black Sigatoka disease – in banana headlines the global list of fungicide requirements with an annual volume of 2.5 billion U.S.\$ *M. citri*, the cause of greasy spot of citrus, is the largest fungicide target in this crop in the U.S. septoria tritici leaf blotch is the most important wheat disease in Europe. The causal agent *M. graminicola* (Fuckel) J. Schröt., is the prime target for the cereal fungicide market with an estimated annual volume of 700 million U.S.\$.

*M. graminicola* is rapidly developing as a model fungus for the order of the Dothideales (Goodwin et al. 2004). In contrast to other model fungi, such as *Magnaporthe grisea* (Dean et al. 2005), the penetration of the host by *Mycosphaerella* species occurs through stomata. In compatible responses, subsequent development is intercellular, in close contact with mesophyll cells and depending on the pathosystem, symptomless for 10-20 days, before rapid cell collapse, suggesting an active role of toxic compounds (Kema et al. 1996d; Duncan and Howard 2000). The resulting necrotic foliar blotches bear numerous pycnidia, the asexual fructifications that contain the splash-born conidia, which are dispersed over short distances within the crop. The symptomatic blotches also contain perithecia, the sexual fruiting bodies that are continuously produced throughout the year, which release the air-borne ascospores that travel over large distances within and between wheat crops. These are the propagules that are responsible for early disease establishment and the observed complex genetic structure in natural populations, making *M. graminicola* population genetics the recognized standard for many other plant pathosystems (Kema et al. 1996c; Hunter et al. 1999; Zhan et al. 2003, 2004; Zhan and McDonald 2004). Early evidence for specificity was controversial and hence, the wheat-*M. graminicola* pathosystem has been traditionally thought to be controlled by quantitative characters in host and pathogen (Eyal et al. 1973, 1985; Van Ginkel 1988; Johnson 1992; Parlevliet 1993). It is a testimony to fungal genetics

that this hypothesis was eventually falsified and experimental evidence showed that host and pathogen interact according to the gene-for-gene hypothesis (Kema et al. 1996abc, 2000, 2002; Kema and van Silfhout 1997; Brading et al. 2002). Since then up to eight resistance genes in the host were identified and mapped (Arraiano et al. 2001; Brown et al. 2001; Adhikari et al. 2003, 2004abc; Chartrain et al. 2004, 2005). An impressive set of genetic tools has been developed exploiting the genetic tractability of *M. graminicola* (Goodwin et al. 2004). Transformation and targeted gene disruption is highly efficient in this fungus (Adachi et al. 2002; Zwiers and De Waard 2001; Stergiopoulos et al. 2003) and a detailed genetic linkage map is available (Kema et al. 2002). Five ABC transporters have been identified, including *MgAtr4*, the first pathogenicity factor described in the *M. graminicola*-wheat pathosystem (Stergiopoulos et al. 2002, 2003; Zwiers and De Waard 2000). Recently, we generated over 30,000 ESTs from a range of cultural conditions (Kema et al. 2003). The genomes of *M. graminicola* and *M. fijiensis* are currently being sequenced at the Joint Genome Institute of the US Department of Energy.

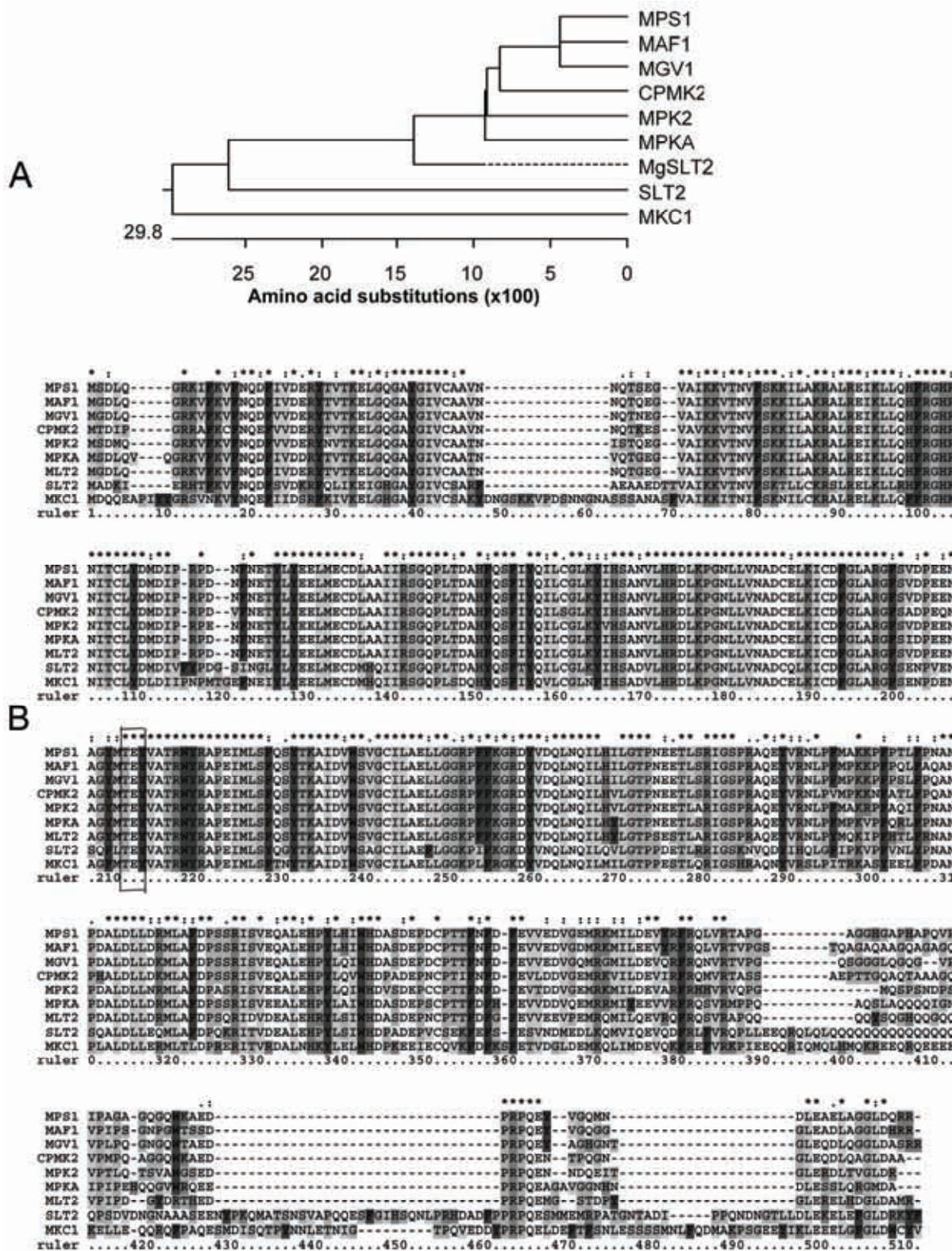
We recently identified the first *M. graminicola* mitogen-activated protein kinase (MAPK)-encoding gene *MgFus3*, a homolog of *Saccharomyces cerevisiae Fus3*, which is required for penetration (Mehrabi et al. 2004). The MAPK pathways in eukaryotes represent important processes for the regulation of cell functions (Hirt 1997; Xu, 2000). In *M. graminicola*, *MgFus3* is the first representative of this class of genes. To analyze these pathways in detail, we have started a coherent approach to decipher them, using the extensive EST resources as well as the genomic sequence that is currently being generated (Kema et al. 2003). MAPK pathways, sometimes called extra cellular-signal regulated kinase pathways, are well known for their involvement in the transduction of a variety of extra cellular signals. MAPKs belong to the family of serine/threonine protein kinases and they are integral parts of gene cascades generally consisting of three conserved phosphokinases; MAPK kinase kinases (MAPKKKs), MAPK kinases (MAPKKs) and MAPKs that sequentially activate and mediate appropriate cellular responses to specific environmental stimuli (Blumer and Johnson 1994). In the model yeast *S. cerevisiae*, five characterized MAPK pathways were identified that regulate mating, cell wall biosynthesis, pseudohyphal growth, growth under osmostress conditions and ascospore cell wall formation (Hunter and Plowman 1997). In these pathways, a set of sequentially acting proteins is involved in the transduction of the signals causing activation of serine/threonine MAPKs (Torres et al. 1991). Transmission of these signals eventually results in the activation of transcription factors that modulate the expression of

specific gene sets. In *S. cerevisiae*, the MAPK-encoding genes *Fus3* and *Sl2* control mating and cell wall integrity, respectively (Costigan et al. 1992; Davenport et al. 1995; Elion et al. 1990). Fungal cell walls play diverse roles for fungal growth, development and stress survival (Kraus et al. 2003). They form an essential barrier protecting cells from external threats, act as morphogenic elements constituting the fungal cell exoskeleton, and rather than being an inert outer layer, they play a dynamic role in many aspects of fungal physiology, e.g., morphogenesis, metabolite transport, protein secretion, signaling, cell adhesion, and cell to cell contact. The most significant components of the fungal cell wall are the polysaccharides chitin and  $\beta(1,3)$ -D-glucan. These components are absent in higher eukaryotes and therefore the fungal cell wall is an attractive target for designing specific antifungal agents with selective toxicity. We are extending our knowledge to unravel the mechanisms that connect fungal cell wall biosynthesis and integrity processes. In this report, we describe the second MAPK-encoding gene in *M. graminicola* that we designated as *MgSl2* since it is a homolog of the *S. cerevisiae Sl2* gene (Torres et al. 1991). We isolated and functionally characterized the role of *MgSl2* in the dimorphic lifestyle of *M. graminicola*, since hardly anything is known about the relation between pathogenicity, cell growth and morphogenesis in this important plant pathogenic fungus.

## RESULTS

### ***MgSl2* is a homolog of the *S. cerevisiae* MAPK-encoding gene *Sl2***

Among EST libraries of *M. graminicola*, we identified a full-length cDNA clone with 62% identity and 75% similarity at protein level to *Sl2*. *MgSl2* possesses a 1,242 bp open reading frame (ORF) encoding a 414 aa sequence with a calculated molecular weight of 47.6 kDa. The protein contains all conserved protein kinase subdomains including the kinase dual phosphorylation sites TEY (residues 185–187) (Fig. 1). Phylogenetic studies revealed that MgSLT2 clusters with SLT2 homologs in the phytopathogenic fungi *Magnaporthe grisea*, *Colletotrichum lagenarium*, *Claviceps purpurea*, *Fusarium graminearum*, *Blumeria graminis* as well as the non-phytopathogenic fungus *Aspergillus nidulans* (Fig. 1).



**Fig. 1. A,** Phylogenetic comparison of MgSLT2 MAPK homologs based on amino acid sequence alignments. The deduced peptide sequences of MgSLT2 from *M. graminicola* was compared with the MAP kinases MPS1 (AF020316) from *Magnaporthe grisea*, MAF1 (AY064246) from *Colletotrichum lagenarium*, CPMK2 (AJ320496) from *Claviceps purpurea*, MPK2 (AF301166) from *Blumeria graminis*, MGV1 (AF492766) from *Fusarium graminearum*, MPKA (U59214) from *Aspergillus nidulans*, SLT2 (X59262) from *Saccharomyces cerevisiae* and MKC1 (P43068) from *Candida albicans*. **B,** Alignment of the deduced MgSLT2 amino acid sequence with amino acid sequences of MgSLT2 homologs from other related fungi. The conserved dual phosphorylation site TEY in all MAPKs is boxed (residue 185-187). Gaps introduced for the alignment are indicated by hyphens.

### ***MgSl2* is constitutively expressed *in planta* and *in vitro***

The expression of *MgSl2* was studied, with β-tubulin-encoding gene as control, by reverse transcription (RT)-PCR under *in vitro* and *in planta* conditions resulting in a characteristic 757 bp amplification product. The expression pattern was similar for both genes (Fig. 2).

**Fig. 2.** *In vitro* and *in planta* expression analysis of *MgSl2*. Reverse transcription-polymerase chain reaction (RT-PCR) products were generated using gene specific primers for the *MgSl2* gene (upper panel) and β-tubulin gene as control (lower panel). Lane M, one kb plus ladder marker. Lanes 1-6, first-strand cDNA generated from total RNA isolated from wheat inoculated with *Mycosphaerella graminicola* IPO323 harvested at 0, 4, 8, 12, 16 and 20 days post inoculation, respectively. Lane 7, first-strand cDNAs generated from total RNA isolated from *M. graminicola* IPO323 grown in YGB after 100 h. Lane C, PCR control using genomic DNA of *M. graminicola* IPO323.

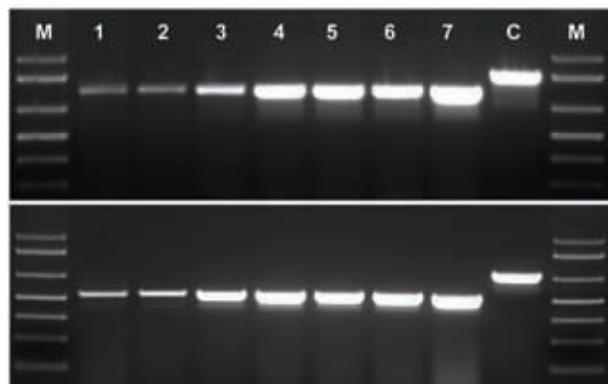
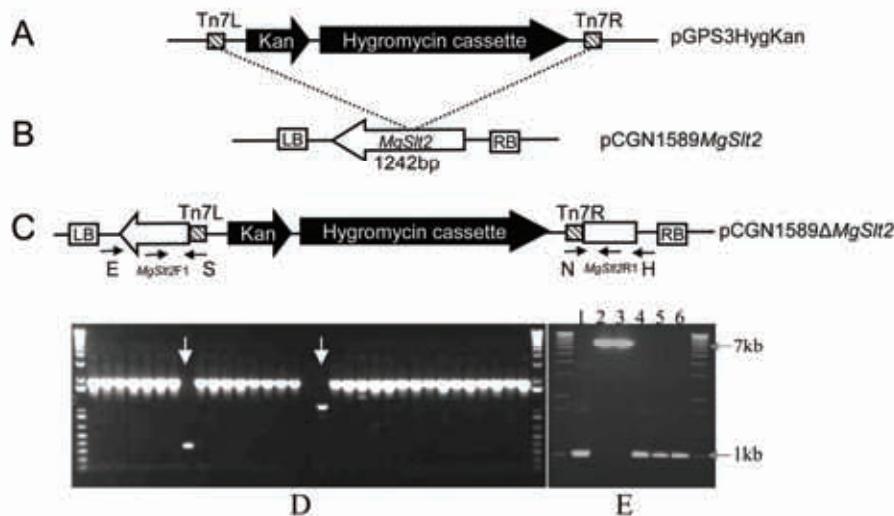


Table 1. Primers used in this study

Name	Sequence (5'-3')	Location
<i>MgSl2R1</i>	CTCCTCCACAACCTCGAA	<i>MgSl2</i> gene
<i>MgSl2F1</i>	TGACATGGACATTCCGAGA	<i>MgSl2</i> gene
Sp6	TATTTAGGTGACACTATAG	pSport1 border
T7	TAATACGACTCACTATAGGG	pSport1 border
Primer S	ATAATCCTAAAAACTCCATTCCACCCCT	Left border of transposon (Tn7L)
Primer N	ACTTTATTGTCATAGTTAGATCTATTG	Right border of transposon (Tn7R)
Primer E	CAGGAAACAGCTATGACATG	Left side of MCS in pCGN1589
Primer H	GATTAAGTTGGGTAACGCCAGG	Right side of MCS of pCGN1589

### **Isolation of *MgSl2* disruptants**

Two *M. graminicola* transformants lacking an intact copy of *MgSl2* (IPO323Δ*MgSl2*) were identified among 30 hygromycin-resistant transformants by PCR screening using primers *MgSl2F1/MgSl2R1* (Table 1), designed upstream and downstream of the transposon insertion. Replacement of the original copy of *MgSl2* by the disruption construct was further confirmed by large-size PCR amplification using Herculase DNA polymerase (Fig. 3).

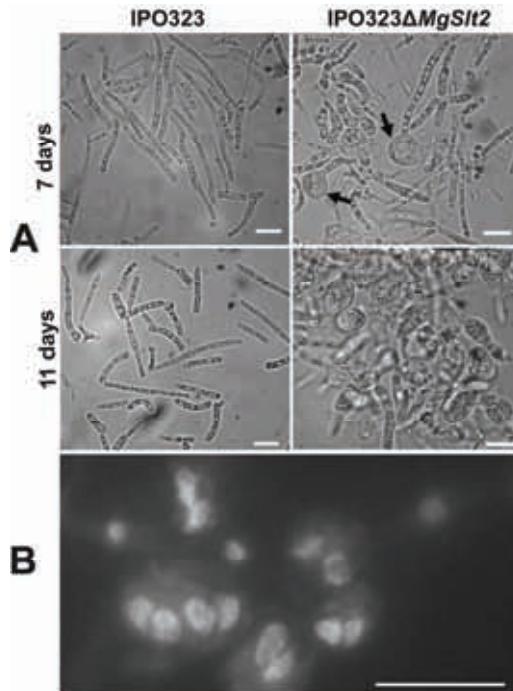


**Fig. 3.** Diagram of the disruption strategy. **A**, The customized donor construct (pGPS3HygKan) containing a kanamycin and hygromycin cassette was used for transposition into the target construct pCGN1589MgSlt2 (**B**) resulting in the disruption construct pCGN1589 $\Delta$ MgSlt2 (**C**). The position and the orientation of the insertion were identified using primers located at the end of the transposon (primers S and N) and downstream and upstream of  $\Delta$ MgSlt2 (primers E and H). **D**, After transposition, the mixture was used to transform *Escherichia coli* DH10 $\beta$ . By screening 32 *E. coli* colonies in a multiplex colony PCR using primers E, H and N, two colonies containing the transposon in the MgSlt2 gene were identified (indicated by arrows). Constructs with the insertion in the backbone of pCGN1589MgSlt2 show an amplicon size equal to the size of MgSlt2 (~2kb). **E**, PCR identification of homologous recombinants. The two *M. graminicola* IPO323 $\Delta$ MgSlt2 disruptant strains that were selected from the first PCR screening were used for large-size PCR amplification using primers MgSlt2F1/MgSlt2R1. Lane 1, *M. graminicola* IPO323. Lane 2 and 3, two homologous recombinants IPO323 $\Delta$ MgSlt2-1 and IPO323 $\Delta$ MgSlt2-2. Lane 3, 4, 5, three ectopic transformants.

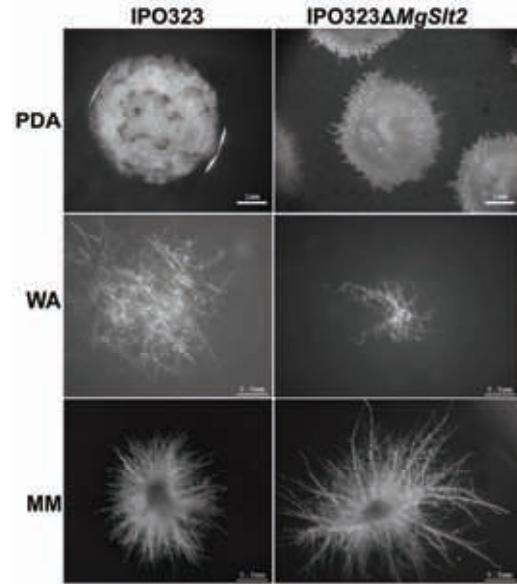
### The effect of MgSlt2 on vegetative properties

The *M. graminicola* IPO323 $\Delta$ MgSlt2 disruptants and the wild-type IPO323 strain showed identical growth and maintained the same cell shape and growth rate until five days post inoculation (dpi) in yeast glucose broth (YGB; 1% yeast extract, 3% glucose) medium. From that point onward, the cells of the IPO323 $\Delta$ MgSlt2 strains started to show a defect in polarized growth of the tip cells causing an enlarged and aberrant shape at seven days. This defect in cytokinesis eventually resulted in the formation of large and swollen cells at 11 days (Fig. 4A). DAPI staining revealed a progressive autolysing process characterized by swollen cells containing up to four nuclei (Fig. 4B), indicating that, despite hampered cell wall formation, the nuclear division continued. This process gradually started at 11 days and continued until massive cell collapse at 18 days.

The germination frequency of *M. graminicola* IPO323, IPO323 $\Delta$ MgSlt2 and ectopic control strains was similar on PDA medium (data not shown), and colony shape remained similar until three to four days after plating. Subsequently, the IPO323 $\Delta$ MgSlt2 strains grew



**Fig. 4.** Effect of *MgSl2* on vegetative reproduction of *Mycosphaerella graminicola* in yeast glucose broth (YGB). **A**, Progressive autolysis of IPO323Δ*MgSl2* cells compared to the regular cell shape of the wild-type strain IPO323. Cells show defects in polarized growth in the tip cells (arrows)



**Fig. 5.** Effect of *MgSl2* disruption on morphological characteristics of *Mycosphaerella graminicola*. Left and right panels show the wild-type strain *M. graminicola* IPO323 and the *MgSl2* disruptant, respectively, growing on potato dextrose agar (PDA), water agar (WA) and *A. nidulans* minimal medium (MM) at 11 days at 20 °C.

slower resulting in colonies half the diameter of the wild-type IPO323 at 15 days. Moreover, IPO323 also produced substantial aerial mycelium that was absent on the IPO323Δ*MgSl2* colonies, even after prolonged growth on PDA (Fig. 5). In addition, *M. graminicola* IPO323 started to melanize after four to five days and was completely melanized at 11 days, whereas melanization did not occur in the IPO323Δ*MgSl2* strains.

On water agar (1%), the growth rate of *M. graminicola* IPO323 and the IPO323Δ*MgSl2* strains was similar until five days, after which the IPO323Δ*MgSl2* strains showed a reduced growth rate resulting in colony sizes half the diameter of the wild-type IPO323 at 11 days (Fig. 5). In contrast, the colony size and shape of the IPO323Δ*MgSl2* strains was similar to IPO323 on *Aspergillus nidulans* minimal medium, but the colony shape of the IPO323Δ*MgSl2* strains appeared less compact with longer extending hyphae (Fig. 5).

#### ***MgSl2* is involved in cell wall strengthening and protects the cells from toxic compounds**

We have tested the sensitivity of *M. graminicola* IPO323, the IPO323Δ*MgSl2* and the ectopic control strains to glucanase, chitinase and 11 chemical compounds representing

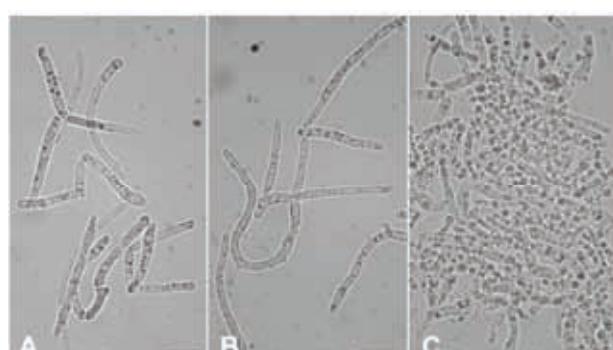
several classes of fungicides (Table 2). Treatment of the yeast-like spores of IPO323 and the ectopic transformants with glucanase did not show a phenotype, whereas those of the IPO323 $\Delta$ MgSl2 strains showed (partially) digested cell walls in the majority of cells, leading to significant aggregation after 30 min. incubation, suggesting hypersensitivity to this compound (Fig. 6). None of the strains was affected by chitinase, even after prolonged incubation (24 h, not shown).

Table 2. Minimal inhibitory concentrations (MIC) of 11 compounds that were used in toxicity assays on *Mycosphaerella graminicola* IPO323 and the disruptant IPO323 $\Delta$ MgSl2 strains as well as the derived relative increase of sensitivity (Q value) of the latter to these compounds.

Compounds	MIC ( $\mu\text{g.ml}^{-1}$ )		Q value
	IPO323 <sup>a</sup>	IPO323 $\Delta$ MgSl2 <sup>b</sup>	
<b>Antibiotics</b>			
Cycloheximide	500	500	1
<b>Fungicides</b>			
Kresoxim-methyl	>0.25	>0.25	1
Fenpiclonil	2.5	2.5	1
Trifloxim	> 0.05	> 0.05	1
Miconazole	0.025	0.005	2
Bifonazole	0.1	< 0.025	>4
Imazalil	0.5	0.1	5
Cyproconazole	0.5	0.05	10
<b>Plant metabolites</b>			
Berberine	>500	>500	1
Camptothecin	>500	>500	1
<b>Other</b>			
Rhodamine 6G	25	25	1

<sup>a</sup>*M. graminicola* IPO323 and the ectopic transformant showed similar MICs values.

<sup>b</sup>Both independent IPO323 $\Delta$ MgSl2 disruptant strains showed similar MICs values.

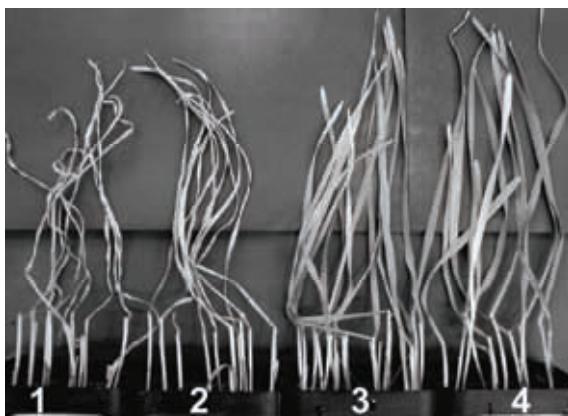


**Fig. 6.** Sensitivity of MgSl2 mutants to the cell-wall-digesting enzyme glucanase. The wild-type strain *Mycosphaerella graminicola* IPO323 (A) and ectopic transformant (B) did not release many spheroplasts whereas the cell walls of the MgSl2 disruptant (C) were completely or partially digested under the same conditions. Scale bar = 10  $\mu\text{m}$

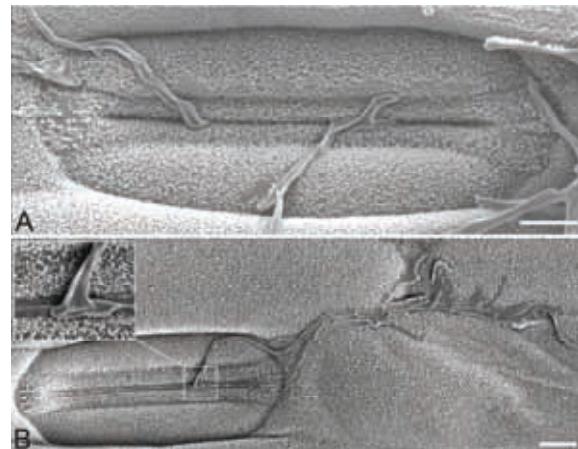
We determined the minimum inhibitory concentrations (MICs) of the 11 toxic compounds on yeast-like cells of *M. graminicola* IPO323, and the IPO323 $\Delta$ *MgSlt2* and ectopic control strains. Compared to IPO323 and the ectopic transformant, the IPO323 $\Delta$ *MgSlt2* disruptants showed a significantly increased sensitivity to miconazole (2x), bifonazole (>4x), imazalil (5x) and cyproconazole (10x) (Table 2). All strains showed a similar sensitivity to cycloheximide, kresoxim-methyl, fenpiclonil, trifloxim, berberine, camptothecin and rhodamine 6G.

### ***MgSlt2* disruptants are highly reduced in virulence**

In order to determine whether *MgSlt2* plays a role in pathogenicity, we compared the pathogenicity of the two independent IPO323 $\Delta$ *MgSlt2* strains and both controls, viz. *M. graminicola* IPO323 and the ectopic transformant, by inoculation experiments on fully unfolded first leaves of the susceptible wheat cv. Obelisk. Both controls (*M. graminicola* IPO323 and the ectopic transformant) showed small chlorotic flecks already at 7 dpi that continued to expand into large areas at 12 dpi and eventually developed into large necrotic areas, especially from the tips of the leaves, containing numerous pycnidia at 16-18 dpi (Fig. 7). However, the IPO323 $\Delta$ *MgSlt2* inoculated plants did not develop any symptom until 16-18



**Fig. 7.** Effect of the disruption of *MgSlt2* on the disease development of *Mycosphaerella graminicola* on cv. Obelisk 20 days after inoculation. The first leaves were inoculated with the wild-type strain *M. graminicola* IPO323 (1), an ectopic transformant (2), and two independent disruptive strains, IPO323 $\Delta$ *MgSlt2*-1(3) and IPO323 $\Delta$ *MgSlt2*-2(4).

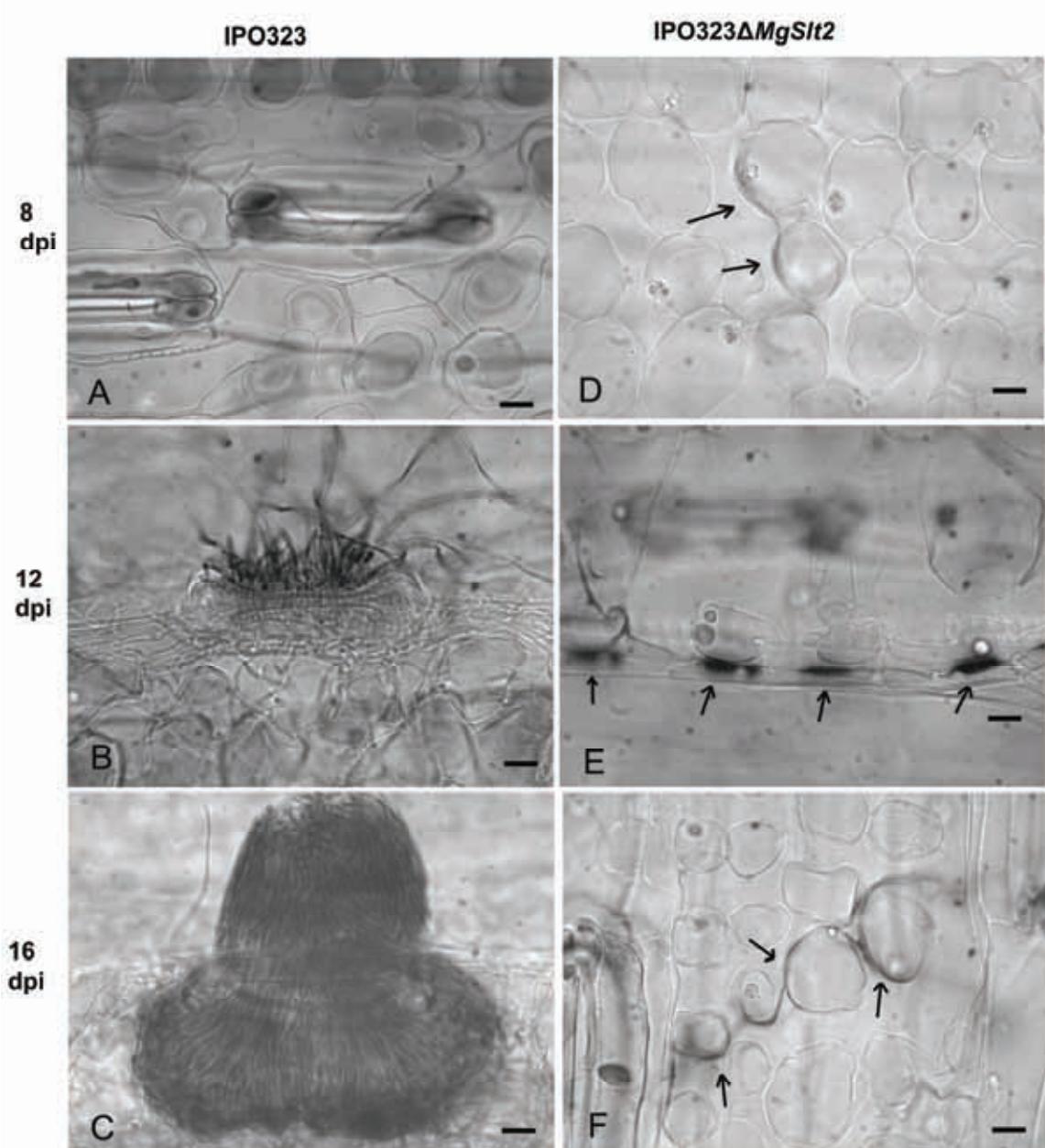


**Fig. 8.** Scanning electron microscopy images of *Mycosphaerella graminicola* penetrating leaves of the susceptible wheat cv. Obelisk at 72 h post inoculation. **A**, Penetration of the wild-type strain IPO323. **B**, Penetration of IPO323 $\Delta$ *MgSlt2* strain. The germ tube appears to have a thickened tip (magnification) bending along the stomatal guard cell lips to form an appressorium-like structure. Scale bar = 10  $\mu$ m.

dpi when limited chlorotic areas developed that gradually increased into larger chlorotic areas at the tips of the leaves, which became necrotic at 20 dpi. Pycnidia were never observed in these limited necrotic areas even after long incubation times when physiological chlorosis and necrosis were observed on other parts of these leaves.

### ***MgSlt2* is dispensable for penetration but is essential for invasive growth in wheat leaves**

To compare the behavior of *M. graminicola* IPO323 and the IPO323 $\Delta$ *MgSlt2* strains *in planta* we monitored the infection process over time on the susceptible wheat cv. Obelisk. Examination of growth *in planta* using traditional staining and light microscopy as well as scanning electron microscopy revealed no significant differences in the germination patterns during the first four dpi. The majority of IPO323 $\Delta$ *MgSlt2* spores initiated growth and developed germ tubes like IPO323. However, some (1-5%) of the germinated spores showed a shape similar to those that we observed on synthetic media (Fig. 8). Using scanning electron microscopy, we observed that some of these spores and/or their germ tube initiations had a non-characteristic shape. However, despite these abnormal phenotypes, these spores eventually were able to develop infectious germ tubes (Figs. 8, 9) that entered the wheat leaves through the stomata, similar to the control IPO323. We also observed the characteristic appressorium-like structures, which are dispensable for penetration (Kema et al. 1996d), at the tips of germ tubes that were in contact with the ridges of the stomatal guard cell lips (Fig. 8B). Further evidence for successful penetration of IPO323 $\Delta$ *MgSlt2* germ tubes came from infectious hyphae in the substomatal cavities and between mesophyll cells at later stages (Fig. 9D-F). However, unlike IPO323 in which infectious hyphae branched out into invasive mycelium, the IPO323 $\Delta$ *MgSlt2* strains failed to do so and did not colonize the substomatal cavities and intercellular spaces of the mesophyll (Fig. 9D-F). Further detailed observations revealed that the infectious hyphae of the IPO323 $\Delta$ *MgSlt2* strains had an aberrant shape in close contact with mesophyll cells. The shape and the thickness of IPO323 $\Delta$ *MgSlt2* hyphae were similar to IPO323 when growing in the open space of the apoplast, but in direct contact with the mesophyll cells, these hyphae were abnormally swollen or collapsed at the contact area (Fig. 9E-F).



**Fig. 9.** Comparative pathogenesis of *Mycosphaerella graminicola* IPO323 (panels A-C) and the disruptant strain IPO323 $\Delta$ MgSl2 (panels D-F) on the susceptible wheat cv. Obelisk at 8, 12, and 16 days post inoculation. **A**, Penetration of stomata. **B**, Early pycnidium development in a heavily colonized substomatal cavity. **C**, Mature pycnidium with exuding cyrillus, containing the conidia, from the stomatal aperture. **D-F**, Reduced mesophyll colonization. Hyphae grow in close contact with mesophyll cell walls, and stain intense at later stages (arrows in E and F). Scale bar = 10  $\mu$ m

## DISCUSSION

We have isolated and characterized *MgSl2*, a homolog of the *Sl2* MAPK-encoding gene that regulates cell wall integrity in *S. cerevisiae*. As the observed expression pattern was similar to the expression pattern of  $\beta$ -tubulin-encoding gene, we conclude that *MgSl2* is constitutively expressed *in planta* as well as *in vitro*. In *M. graminicola* and other fungal

pathogens, homologs of the *S. cerevisiae* *Fus3* are essential for virulence and other functions (Xu, 2000). We have recently shown that disruptants of *MgFus3* are non-pathogenic and demonstrated that this is due to the inability of these mutants to penetrate the stomata of wheat leaves (Mehrabi et al. 2004). In this study, we cloned and characterized *MgSlt2*, another MAPK-encoding gene of *M. graminicola*, and investigated its function in the life cycle and infection process of this important plant pathogen. *Slt2* orthologs were functionally characterized in the plant pathogens *M. grisea*, *C. lagenarium*, *C. purpurea* and *F. graminearum*, but these have significantly different pathogenic lifestyles and represent an entirely different part of the fungal kingdom (Goodwin et al. 2004; Goodwin 2004). In *C. lagenarium*, *MAF1* is required for the early differentiation phase of appressorium formation as mutants failed to form appressoria (Kojima et al. 2002). Contrastingly, *mps1* mutants of *M. grisea* could form appressoria but were non-pathogenic because of the inability of these appressoria to penetrate the plant cell surfaces (Xu et al. 1998). In the non-appressorium forming fungus *C. purpurea*, both *CMPK1* and *CMPK2* are necessary for penetration (Mey et al. 2002). In *M. graminicola*, *MgFus3* is essential for penetration, but *MgSlt2* knockouts penetrate equally well as the wild-type. Our detailed cytological analysis *in planta* showed that these attempts resulted in successful penetration since infectious hyphae were present in substomatal cavities and in the intercellular areas of the mesophyll. This suggests that *MgSlt2* is not involved in early recognition events. Nevertheless, despite successful penetration, infectious hyphae of IPO323Δ*MgSlt2* strains were not able to branch out and colonize the mesophyll. A possible explanation for this observation is that plant metabolites, phytoalexins or PR proteins are detrimental to IPO323Δ*MgSlt2* and inhibit the growth of infectious hyphae. Thus, like in *M. grisea*, *C. lagenarium*, *C. purpurea* and *F. graminearum*, *MgSlt2* affects pathogenicity in *M. graminicola*, but the mechanism and the time frame significantly differ from these other plant pathogens.

Furthermore, conidiation is significantly reduced or even abolished in *M. grisea*, *C. lagenarium* and *C. purpurea* whereas *MgSlt2* knockouts in *M. graminicola* do not show this defect on either nutrient agar or in liquid culture until aging at a later stage (Xu et al. 1998; Kojima et al. 2002; Mey et al. 2002). This might be an effect of the dimorphism that is recognized in this pathogen, which as such is intriguing since *Slt2* has an effect on budding in *S. cerevisiae*, which is not observed in *M. graminicola* (Mazzoni et al. 1993).

*Slt2* homologs have been cloned and characterized also in some non-plant pathogenic fungi. In *S. cerevisiae*, the cell integrity *Slt2* MAPK pathway controls cell wall integrity and

promotes cell wall remodeling under stress conditions. *Sl2* disruptants showed defects in cell-wall integrity, sensitivity to cell wall degrading enzymes and an inability to grow at elevated temperatures in the absence of osmotic stabilizers (Costigan et al. 1992; Davenport et al. 1995). *Mpk1*, the homolog of *Sl2* in *Cryptococcus neoformans* is required for *in vitro* growth at 37 °C and the mutants had attenuated virulence in the mouse model of cryptococcosis (Kraus et al. 2003). In *A. nidulans*, the *Sl2* homolog *MPKA* is involved in spore germination and polarized growth at several stages of colony formation (Bussink and Osmani 1999). The cell wall aspects of this gene in the abovementioned plant pathogenic fungi comply partly with our findings. Like in *C. purpurea*, *M. grisea* and *F. graminearum* (Mey et al. 2002; Xu et al. 1998; Hou et al. 2002), *MgSl2* knockouts of *M. graminicola* have an increased sensitivity to cell wall degrading enzymes and show auto-sphaeroplasting, but still undergo nuclear division. This latter phenomenon was only observed and reported in *Sl2*-orthologue studies in non-pathogenic fungi. Contrastingly, *MAF1* did not affect cell wall strength in *C. lagenarium* as shown by lytic enzyme studies (Kojima et al. 2002). We extended the information on cell wall integrity aspects of *Sl2* orthologs and showed that the IPO323Δ*MgSl2* strains are also much more sensitive to a number of fungicides and xenobiotic compounds. We conclusively showed that IPO323Δ*MgSl2* strains are more vulnerable to some antifungal compounds than IPO323 and have significantly weaker cell walls than this wild-type strain. Similar results were found by Bahmed et al. (2003) who showed that yeasts without a cell wall were 7 to 20 times more sensitive to amphotericin B, suggesting that they are an important barrier against this compound. Also, the *Mpk1* mutants in *C. neoformans* displayed enhanced sensitivity to nikkomycin Z and caspofungin (Kraus et al. 2003). During the infection process, the fungal cell wall is probably under continuous attack of plant defense compounds. In *F. oxysporum*, over-expression of a cell wall glycoprotein increased virulence and reduced the sensitivity to the pathogenesis-related protein osmotin (Narasimhan et al. 2003). Our results indicate that *MgSl2* is dispensable for penetration but is essential for invasive hyphal growth. We provided direct evidence that *MgSl2* is involved in cell wall strengthening, suggesting that the attenuated pathogenicity is due to an increased sensitivity to hitherto unknown plant defense compounds, whose identification is of eminent interest to increase our understanding of the wheat-*M. graminicola* pathosystem.

## MATERIALS AND METHODS

### Strains and growth conditions

We used *M. graminicola* IPO323 throughout this study and cultivated it on PDA media (3.9% [wt/vol] PDA; Difco, Detroit, MI) at 18 °C to produce yeast-like spores that were collected by gently scraping cultures incubated for three to four days and that were subsequently stored at -80 °C (Kema and Van Silfhout, 1997). *M. graminicola* IPO323 was grown in YGB at 18 °C in an orbital incubated shaker (Innova 4430, New Brunswick Scientific, The Netherlands) adjusted at 120 rpm for five days to produce inoculum that was collected by centrifugation, washed with water and adjusted at 10<sup>7</sup> spores.ml<sup>-1</sup> for inoculation and 10<sup>8</sup> spores.ml<sup>-1</sup> for transformation. *A. nidulans* minimal medium was prepared and used based on Barratt et al. (1965) with minor modifications.

### DNA manipulation and analysis

Basic DNA manipulations were according to standard protocols (Sambrook et al. 1989). Plasmid DNA was isolated using the QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany). Genomic DNA of *M. graminicola* IPO323 was prepared from freeze-dried spores using the Puregene DNA Isolation Kit (Gentra systems Inc., Minneapolis, MN, USA). DNA sequences were obtained on an ABI-prism 3100 capillary automated sequencer using the BigDye™ terminator reaction mix (Applied Biosystems, The Netherlands). Phylogenetic tree generation, DNA and protein sequence alignment, editing and analysis were performed using MEGALIGN and DNA Star software, (Madison, WI, USA), whereas homology searches were performed with the BLAST program (Altschul et al. 1997) and amino acid sequence alignment was performed using CLUSTAL X (Thompson et al. 1997).

### Preparation of disruption construct

A full-length 2 kb cDNA clone containing *MgSlc2* was identified in the cDNA libraries of *M. graminicola* IPO323 (Kema et al. 2003). The cDNA insert was excised from the pSport1-*MgSlc2* plasmid using *Kpn*I/*Xba*I and cloned into pCGN1589. We used the GPS™-Mutagenesis system (New England Biolabs, Leusden, The Netherlands) to make a disruption construct of *MgSlc2*. A new customized donor construct was made by insertion of the kanamycin resistance gene into pGPS3Hyg, which was previously described by Zwiers and de Waard (2001). The 1,259 bp kanamycin resistance gene was excised from pGPS3 that was supplied with the kit and inserted into the *Eco*RV site of pGPS3Hyg, resulting in the new

donor construct pGPS3HygKan that was used for transposition. By using two selective markers (hygromycin B and kanamycin) located between the two Tn7-based transposon borders (Tn7L and Tn7R), we were able to select *Escherichia coli* colonies containing the construct with a copy of the transposon into the target construct, pCGN1589*MgSlt2*. The target construct pCGN1589*MgSlt2* was transposed by the donor construct pGPS3HygKan according to the manufacturer's instruction. The transposition mixture was then cloned to *E. coli* strain DH10 $\beta$  that was subsequently grown on selective gentamycin/kanamycin media. A colony PCR was performed by using primer H and primer E (located on the right and left side of the multiple cloning site of pCGN1589, respectively) together with Primer N (located on the Tn7R) in a multiplex colony PCR to identify clones carrying a construct with the insertion of the transposon into the *MgSlt2* gene. The PCR was initiated by denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 57 °C for 30 sec, and 72 °C for 90 sec with a final extension at 72 °C for 5 min and a cooling step to 10 °C. The constructs with the transposon insertion in the backbone of the plasmid (pCGN1589) showed the amplicon size of the cDNA insert, *MgSlt2*, whereas constructs with the disrupted *MgSlt2* generated smaller fragments. We selected a construct (named as pCGN1589Δ*MgSlt2*) in which the transposon was inserted almost in the middle of the *MgSlt2* open reading frame and determined the exact position of the transposon insertion by sequencing from the right and the left borders of the transposon using Primer N and Primer S. Subsequent comparison with the *MgSlt2* sequence revealed that the transposon inserted 623 bp downstream of the *MgSlt2* starting codon. This construct was used to disrupt *MgSlt2* in *M. graminicola* IPO323 through *A. tumefaciens*-mediated transformation according to Zwiers and de Waard (2001).

### Transformation of *M. graminicola* IPO323 and screening for *MgSlt2* mutants

Construct pCGNΔ*MgSlt2* was cloned into *A. tumefaciens* strain LBA1100 by electroporation and *A. tumefaciens*-mediated transformation was carried out according to Zwiers and de Waard (2001). After three weeks, individual *M. graminicola* transformant colonies were collected and transferred to PDA containing 100 µg hygromycin.ml<sup>-1</sup> and 200 µg cefatoxime.ml<sup>-1</sup>. The stability of the putative transformants was verified by transferring them three times to fresh PDA medium containing 100 µg hygromycin.ml<sup>-1</sup> and one time to PDA without selection for purification. Finally, a hygromycin specific PCR was performed to test whether the construct integrated into the genome of the *M. graminicola* IPO323 recipient strain (data not shown). Homologous recombinants were selected by PCR using primers

*MgSl2F1* and *MgSl2R1* (Table 1), which were designed in the flanking regions of the inserted transposon that disrupts the *MgSl2* gene, and did no render an amplicon in case homologous recombination took place. Such clones were selected and analyzed using additional large amplification PCR with the same primers (*MgSl2F1* and *MgSl2R1*) and Herculase® Enhanced DNA Polymerase (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's instructions.

### Sensitivity assay to cell wall degrading enzymes and toxic compounds

Aliquots of spore suspensions from all *M. graminicola* strains (500 µl, 10<sup>7</sup> spores.ml<sup>-1</sup>) were centrifuged and spores were re-suspended in either 500µl (1 mg.ml<sup>-1</sup>) 1,3(1,3:1,4)-β-D-glucan 3(4) glucanohydrolase (Sigma, Zwijndrecht, The Netherlands) or chitinase (250 µg.ml<sup>-1</sup>) (Sigma, Zwijndrecht, The Netherlands) and incubated at 37 °C and 25 °C, respectively. The activity of the cell wall degrading enzyme was monitored microscopically every 30 min.

In Table 2 we listed the 11 compounds comprising fungicides, plant metabolites, an antibiotic and rhodamine 6G that we used for toxicity assays. Spore suspensions (5 µl; 4.10<sup>4</sup> spores.ml<sup>-1</sup>) harvested five days after inoculating YGB, were spotted on nine cm PDA (39 g.l<sup>-1</sup>) plates amended with different concentration of these compounds. Minimum inhibitory concentrations (MICs) were assessed for yeast-like cells of *M. graminicola* IPO323, the IPO323Δ*MgSl2* and ectopic control strains after 10 days of incubation at 18 °C in the dark. The relative increase of sensitivity (Q value) of the IPO323Δ*MgSl2* strains was calculated by dividing the MIC value of IPO323 by the MIC value of IPO323Δ*MgSl2*.

### Virulence assays

Fifteen seeds of the susceptible wheat cv. Obelisk were planted in square five cm plastic pots filled with autoclaved soil. The pots were transferred to a compartment providing growth conditions as described previously (Kema et al. 1996a). Ten-day-old seedlings were inoculated by spraying a spore suspension (10<sup>7</sup> spores.ml<sup>-1</sup>) supplemented with 0.15% Tween 20® as a surfactant. After inoculation, pots were placed in transparent polyethylene boxes (6x22x34 cm) and incubated at 20 °C in the dark for 48 h and subsequently incubated at 16 h light (166 µE.sec<sup>-1</sup>.m<sup>-2</sup>) and eight h darkness per day. Disease severity was assayed after 20 dpi.

### Microscopy

Spores of *M. graminicola* IPO323 produced in YGB were harvested by centrifugation and suspended in YGB at a concentration of  $4\text{-}5 \times 10^5$  spores.ml $^{-1}$ . About 200  $\mu\text{l}$  spore suspension was placed on a sterile slide and incubated under humid conditions at 20 °C for 24 h. Excessive YGB was blotted and slides were immersed in a fixative solution (methanol: acetic acid 17:3 (v:v) for 30 min, and flame dried. Samples were stained with 1 mg.ml $^{-1}$  4',6-diamidino-2-phenylindole (DAPI) dissolved in anti-fading mounting solution (Johnson & Araujo, 1981). Observations were made using an epifluorescence microscope (Zeiss, Axioscop, Germany) equipped with a Nikon CoolSNAP digital camera (RS Photometrics, Roper Scientific Inc., USA).

For cytological analysis leaves from inoculated plantlets were harvested and cut into two to three cm segments and immediately immersed in 20 ml of 0.05% trypan blue dissolved in lactophenol-ethanol (1:2, v/v) and boiled for 15 min. Samples were destained overnight in a saturated chloral hydrate solution (5:2, w/v) and then stored in 87% glycerol. For light microscopy observations, specimens (adaxial side up) were placed between a glass slide and a cover slip and observed using a Zeiss Axioscop (Germany) microscope equipped with a Nikon CoolSNAP digital camera.

Scanning electron microscopy was performed to precisely record germination and penetration events. Inoculation and maintenance of the plants was similar to the aforementioned procedures (virulence assays). A small part of the leaf was cut and glued on a sample holder with conductive carbon cement (Leit-C, Neubauer Chemicalien, Germany) and subsequently frozen in liquid nitrogen. The sample was transferred under vacuum to a dedicated cryo-preparation chamber (Oxford cryo-system, CT 1500 HF, Eynsham, UK) onto a sample stage at -90 °C. Cryo-fracture was conducted at approximately -90 °C using a cold (-196 °C) scalpel blade. The fractured sample was freeze dried for three min. at -90 °C in vacuum at  $3 \times 10^{-7}$  Pa to remove water vapor contamination. After the sample surface was sputter-coated with 10 nm platinum it was transferred to the cold sample stage (-190 °C) inside the Cryo-FESEM of the scanning electron microscope (JEOL 6300F Field Emission SEM, Japan, Tokyo) and subsequently analyzed with an accelerating voltage of 5 kV. Images were digitally recorded (Orion, Belgium).

### Expression analysis

Expression analysis of the *MgSl2* gene was performed *in vitro* and *in planta* at different time points using reverse-transcription polymerase chain reaction (RT-PCR). We

collected 10 leaves per sample from inoculated plants that were subsequently flash-frozen and ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted from 50 mg of ground leaves using the RNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) and potential DNA contamination was removed using the DNA-free™ kit (Ambion, UK), both procedures according to the manufacturer's instructions. First-strand cDNA was conducted using Expand Reverse Transcriptase (Roche Applied Science, Almere, The Netherlands) following the manufacturer's instructions. PCR reactions were subsequently carried out with five  $\mu$ l aliquots of this reaction in a 50  $\mu$ l PCR reaction volume, using the same primers and conditions as described above for the detection of homologous recombinants, and amplicons were finally analyzed on 1% agarose gels using 10  $\mu$ l aliquots of the PCR products.

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# CHAPTER 4

***MgHog1 has an imperative role in dimorphic transition and pathogenicity of the fungal wheat pathogen *Mycosphaerella graminicola****

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## ABSTRACT

The dimorphic ascomycete pathogen *Mycosphaerella graminicola* switches from a yeast-like form to an infectious filamentous form that penetrates the host foliage through stomata. Our earlier findings revealed the requirement of the mitogen-activated protein kinase (MAPK) encoding gene *MgFus3* for penetration of the host and dependency on the MAPK gene *MgSlt2* for colonization. Here, we examined the biological function of the MAPK-encoding gene *MgHog1* and the p21-activated kinase (PAK) encoding gene *MgSte20* in *M. graminicola*. No altered phenotypes of *MgSte20* mutants were observed. In contrast, compared to the wild-type strain, *MgHog1* mutants exhibited several altered phenotypes. *MgHog1* mutants were osmosensitive and highly resistant to fungicides fludioxonil, fenpiclonil, and iprodione. Moreover, the *MgHog1* mutants did not melanize and were unable to switch to filamentous growth on water agar that mimics the nutritionally poor conditions on the foliar surface and hence exclusively developed by a yeast-like budding process. Consequently, due to impaired initiation of infectious germ tubes as revealed by detailed *in planta* cytological analyses, the *MgHog1* mutants failed to infect wheat leaves. Therefore, we conclude that *MgHog1* is a novel pathogenicity factor involved in the dimorphic transition in *M. graminicola*.

**Additional keywords:** *Agrobacterium tumefaciens*-mediated transformation, virulence, transposon, dimorphism, differentiation.

## INTRODUCTION

The ascomycete *Mycosphaerella graminicola* (Fuckel) J. Schröt. (anamorph *Septoria tritici*) is a major threat of bread and durum wheat in most wheat growing areas world wide, but particularly in areas with high rainfall during the growing season. During the last decades severe epidemics of the pathogen have caused substantial yield losses in Western Europe. Recently, the importance of the disease even increased after the rapid evolution of resistance to strobilurin fungicides in this pathogen (Eyal et al. 1987; Halama, 1996; Jorgensen et al. 1999; Hardwick et al. 2001; Fraaije et al. 2003, 2005). Due to its economical importance, *M. graminicola* has been studied extensively with an emphasis on epidemiology (Royle et al. 1986; Royle 1994; Sanderson et al. 1983; Shaw et al. 1993; Thomas et al. 1989), population genetics (McDonald and Martinez 1990; McDonald et al. 1999, Chen and McDonald. 1996, Zhan et al. 1998; Cowger et al. 2002), genetics of resistance (Arraiano et al. 2001; Brading et al. 2002; McCartney et al. 2002; Rosielle and Brown 1979; Somasco et al. 1996) and host-pathogen interactions (Cohen and Eyal 1993; Duncan and Howard 2000 ; Kema et al. 1996abcd, Kema and Van Silfhout 1997; Kema et al. 2000, 2002, Stergiopoulos et al. 2003). In addition, we generated over 30,000 ESTs from a range of cultural conditions (Kema et al. 2003) and the genome of *M. graminicola* is currently being sequenced at the Joint Genome Institute of the US Department of Energy. This is the foundation for an increased understanding of the molecular mechanisms regulating growth and differentiation. *M. graminicola* has a unique pathogenesis that makes it an interesting model to study various aspects of pathogenicity. It is a dimorphic pathogen with a yeast-like form and a filamentous form that invades the host by penetrating the foliage through stomata and subsequently intercellularly colonizes the wheat mesophyll tissue. Differentiation during the fungal life cycle is very important for accomplishment of successful penetration, colonization and fructification. In some fungi such as *Ustilago maydis* it has been shown that the ability to switch from yeast-like to filamentous growth is also critical for successful infection (Klose and Kronstad 2004). In contrast to many other cereal fungal pathogens, *M. graminicola* does not form appressoria but penetrates wheat leaves through stomata without differentiating infection structures (Kema et al 1996d, Duncan and Howard 2000). *M. graminicola* is a hemibiotroph, which has a biotrophic phase that lasts for about ten days, followed by a rapid switch to necrotrophy. This results in the typical symptoms of the disease, irregular chlorotic lesions that develop to necrotic blotches bearing the anamorphic and teleomorphic

fructifications (Eyal et al. 1987; Cohen and Eyal, 1993; Kema et al. 1996b; Duncan and Howard, 2000, Mehrabi et al. 2006).

We have studied the role of mitogen-activated protein kinase (MAPK) encoding genes in pathogenicity of *M. graminicola* (Mehrabi et al. 2004, 2005, 2006). These genes are involved in many different aspects of fungal development and the encoded proteins are activated or deactivated by a wide variety of extracellular stimuli through upstream components and, hence, are major components of signal transduction cascades that transduce extracellular signals to the nucleus (Xu, 2000). In the budding yeast *Saccharomyces cerevisiae*, five well-characterized MAPK pathways regulate developmental processes such as mating, cell wall integrity, osmoregulation, pseudohyphal growth and ascospore formation (Gustin et al. 1998). Recently, we characterized *MgFus3* and *MgSl2*, the orthologous MAPK-encoding genes of the *S. cerevisiae* *Fus3* and *Sl2* that control mating and cell wall integrity, respectively. We demonstrated that *MgFus3* is required for penetration and *MgSl2* for invasive colonization of the mesophyll tissue (Mehrabi et al. 2004, 2005, 2006). Here, we describe the MAPK-encoding gene *MgHog1* and the p21-activated kinase (PAK) encoding gene *MgSte20*, the orthologs of *S. cerevisiae* *Hog1* and *Ste20* (Brewster et al. 1993).

The HOG1 pathway in *S. cerevisiae* (for reviews see Hohmann, 2002; O'Rourke et al. 2002) is regulated by at least two upstream cascades that activate HOG1 MAPK. One branch depends on the SHO1 osmosensor transmembrane protein, while the other branch depends on SLN1, a two component histidine kinase receptor. These two cascades converge to activate the MAPK kinase (MAPKK) PBS2 through phosphorylation by STE11 in the SHO1 cascade, and SSK2 and SSK22 in the SLN1 cascade. In the SHO1 cascade, under hyperosmotic conditions STE20 phosphorylates STE11, which in turn activates PBS2. *Ste20* was originally discovered in the FUS3 and KSS1 pathways but was also shown to be one of the intermediate components between SHO1 and STE11 in the HOG1 pathway (Raitt et al. 2000; O'Rourke and Herskowitz, 1998). Activated PBS2 phosphorylates MAPK HOG1, which subsequently transiently accumulates in the nucleus and activates downstream target transcription factors that eventually leads to activation of stress response genes. In turn, this induces a set of osmoadaptive responses and increases the transcription of genes encoding enzymes such as glycerol-3-phosphate dehydrogenase (GPD1) and glycerol-3-phosphatase (HOR2) involved in glycerol biosynthesis. Hyperosmotic stress also reduces the permeability of the plasma membrane for glycerol, primarily by inhibiting the activity of the FPS1 glycerol transporter. Glycerol accumulation eventually leads to increased internal osmolarity and maintains a high cellular turgor. These responses restore the osmotic gradient between cells and their

environment required for survival under hyperosmotic stresses (Gustin et al. 1998; Albertyn et al. 1994; Hirayama et al. 1995; Norbeck et al. 1996).

*MgSte20* is the first PAK-encoding gene described in *M. graminicola*. In *S. cerevisiae* the *Ste20*-encoded protein is involved in FUS3, KSS1 and HOG1 pathways. The role of this gene is extensively studied in *S. cerevisiae* where it fulfills multiple functions during mating (Leberer et al. 1992), pseudohyphae formation (Liu et al. 1993), invasive growth (Robert and Fink 1994), cytokinesis (Cvrckova et al. 1995), and polarized morphogenesis during bud emergence (Ottlie et al. 1995).

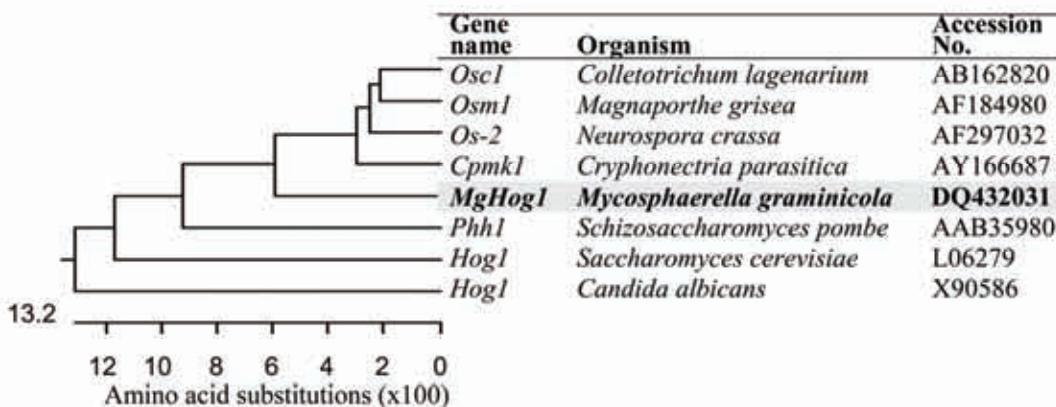
We are interested in the identification of genes involved in pathogenicity and in the relation with the dimorphic switch in *M. graminicola*. Therefore, we analyzed the function of the *M. graminicola* homologs of both *Ste20* and *Hog1*. The results presented in this chapter indicate that *MgSte20* is dispensable for these functions. In contrast, *MgHog1* is required for the morphogenic transition from yeast-like to filamentous growth and can therefore be considered a new pathogenicity factor in *M. graminicola*.

## RESULTS

### DNA and protein analysis and phylogenetic relationships

Screening of 30,000 *M. graminicola* ESTs identified twelve ESTs homologous to *S. cerevisiae Hog1*. Assembly of these ESTs resulted in a contig designated as *MgHog1* with a 1,071 bp open reading frame (ORF) encoding a 357 amino acid (aa) protein sequence. The deduced protein has a calculated molecular weight of 40.74 kDa and shows 62% identity and 96% similarity at protein level with OSM1 of the rice blast pathogen *Magnaporthe grisea*. Results of a multiple sequence alignment of MgHOG1 with other HOG1 orthologs indicated that these proteins are highly conserved except for the C-terminus. Multiple sequence alignment and phylogenetic analysis revealed that MgHOG1 groups within HOG1 orthologs of other filamentous ascomycetes (Fig.1). A dual phosphorylation motif (TGY), characteristic of hyperosmolarity-activated MAPKs (Cano and Mahadevan, 1995), is present (amino acids 171-173) in the MgHOG1 polypeptide.

Comparison of the genomic DNA sequence with the EST contig of *MgHog1* showed that the structural region is interrupted by eight introns. A putative polyadenylation signal with a stretch of 65 As is found 389 bp downstream of the stop codon. A GC-rich region with nucleotide A at nt-3 (GCCACCATG), characteristic of Kozak's consensus signature (Kozak 1987), was identified upstream of the start codon (ATG).



**Fig. 1.** Phylogenetic comparison of *MgHog1* and its orthologs based on amino acid sequence alignments. The gene name and accession number for each fungus is indicated in the figure. The phylogram was constructed with the MEGALIGN program (DNA Star, Madison, WI, USA).

In fungi the N-terminus of the orthologs of STE20 is extremely diverse and, therefore, we did not find strong evidence favoring one starting codon over the other for the putative *MgSte20*. However, the putative protein encoded by *MgSte20* contains two domains that are very characteristic for PAK proteins. The consensus domain (I-S-X-P-X-X-F-X-H-X-X-H-X-G-X-D) located at the N-terminus of MgSTE20 is a **PAK binding domain** (PBD), also known as the **Cdc42/Rac interactive binding** (CRIB) domain that is required for binding of small GTPases. The second domain is located at the C-terminus of the putative MgSTE20 protein. This is a serine/threonine protein kinase domain containing a number of conserved regions such as an ATP binding pocket, a catalytic loop, and an activation loop that are all needed for serine/threonine protein kinase activity.

### Isolation of *MgHog1* and *MgSte20* mutants

The disruption constructs for *MgHog1* (pCGN1589 $\Delta$ *MgHog1*) and *MgSte20* (pCGN1589 $\Delta$ *MgSte20*) were generated using an *in vitro* transposition system (Mehrabi et al. 2006) (Fig. 2). By PCR screening and sequencing, two constructs were selected in which the transposon was inserted upstream of the stop codon in the ORFs of *MgSte20* and *MgHog1* at 810 and 507 bp, respectively. We used these constructs to disrupt *MgSte20* and *MgHog1* in *M. graminicola* strain IPO323 (hereafter referred to as WT) through *Agrobacterium tumefaciens*-mediated transformation (Zwiers and de Waard 2001, Mehrabi et al. 2006). To identify homologous recombinants for *MgSte20* and *MgHog1*, we used PCR screening using primers *MgSte20-F2/MgSte20-R3* for *MgSte20* and *MgHog1-F2/MgHog1-R2* for *MgHog1* designed upstream and downstream of the transposon insertion (Table 1). Out of 24

hygromycin-resistant transformants obtained per transformation we identified four and eight mutant strains lacking an intact copy of *MgSte20* and *MgHog1*, respectively.

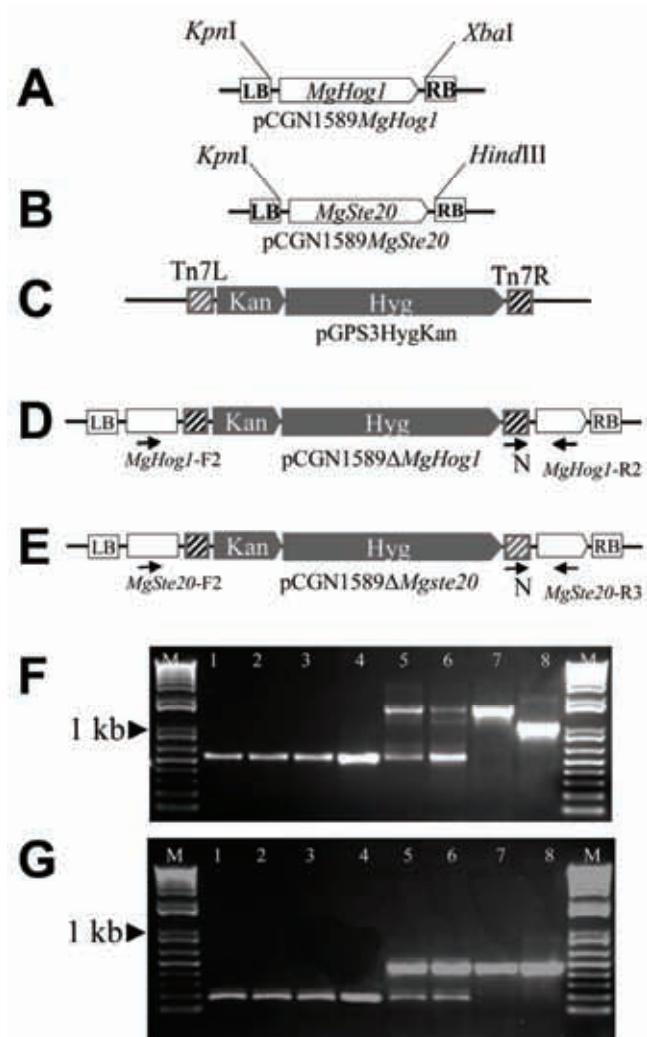


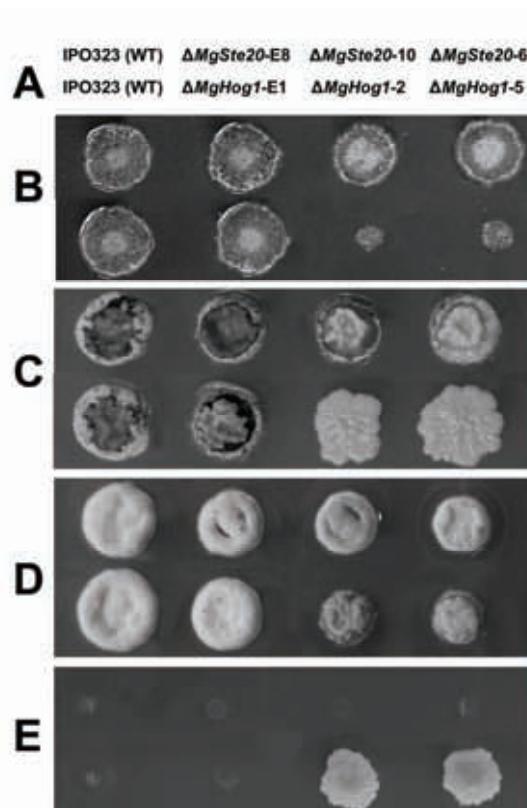
Table 1. Primers used in this study.

Name	Sequence (5'-3')	Location
<i>MgHog1</i> -F2	ATGGCCGAATTCTGTGCG	<i>MgHog1</i>
<i>MgHog1</i> -R2	TCCTGGCCCTCTCCGTT	<i>MgHog1</i>
<i>MgSte20</i> -F2	ACCTCAAGCTCCGTATCGT	<i>MgSte20</i>
<i>MgSte20</i> -R3	GTCCTTCATGACCAGAACTCG	<i>MgSte20</i>

## Disruption of *MgHog1* leads to osmotic sensitivity, promotes yeast-like growth and results in fungicide resistance

To test whether inactivation of the *MgSte20* and *MgHog1* genes would result in sensitivity to hyperosmolarity, the *MgSte20* and *MgHog1* mutants, as well as the WT and ectopic controls were grown on potato dextrose agar (PDA) plates supplemented with different concentrations of KCl, NaCl or sorbitol. The growth of *MgHog1* mutants was inhibited under all osmotic stress conditions tested. For instance, on 1.5 M sorbitol the controls grew normally but the growth of *MgHog1* mutants was reduced to approximately 10% compared to the WT (Fig. 3). We did not observe any altered phenotype for the *MgSte20* mutants compared to the WT and ectopic controls.

When grown on PDA at 20 °C, the *MgHog1* mutants exclusively developed as a yeast-like colony and did not melanize until prolonged incubation (>20 days), which was in clear contrast to the phenotype of the WT (Fig. 3C). However, when grown on PDA at 28 °C the melanization of the *MgHog1* mutants was similar to the WT and ectopic strains, and filamentous growth was also restored (Fig. 3D). The mutants and the controls were equally thermosensitive since development was inhibited at temperatures >29 °C (data not shown).



**Fig. 3.** *In vitro* phenotyping of *Mycosphaerella graminicola* IPO323,  $\Delta M\text{g}H\text{o}g1$ ,  $\Delta M\text{g}S\text{te}20$  and ectopic control strains under different growth conditions. **A**, Lay-out of the experiment. Approximately 1  $\mu\text{l}$  of spore suspension ( $10^7$  spore. $\text{ml}^{-1}$ ) was spotted on PDA and grown for ten days at 20 °C except for treatment D that was incubated at 28 °C. **B**, *MgHog1* mutants are osmosensitive on PDA supplemented with 1.5 M sorbitol. **C**, Abolishment of filamentous growth of *MgHog1* mutants on normal PDA. **D**, Recovery of the albino phenotype and filamentous growth of *MgHog1* on PDA at 28 °C. **E**, *MgHog1* mutants are resistant to the phenylpyrrole fungicide fludioxonil (30  $\mu\text{g.ml}^{-1}$ ) on PDA.

Table 2. Minimal inhibitory concentrations (MIC) of compounds that were used in toxicity assays with *Mycosphaerella graminicola* IPO323, *MgSte20* and *MgHog1* mutants.

Class	Compounds	MIC ( $\mu\text{g.ml}^{-1}$ )		Q value <sup>b</sup>
		IPO323 <sup>a</sup>	$\Delta MgHog1$	
Azole	Cyproconazole	0.15	0.15	1
	Imazalil	0.5	0.5	1
	Tricyclazole	>60	>60	1
Strobilurin	Kresoxim-methyl	0.3-1	0.3-1	1
	Azoxystrobin	1	1	1
Pyrimidine	Fenarimol	0.25	0.25	1
Dicarboximide	Iprodione	20	>100	>5
	Vinclozolin	>100	>100	1
Aromatic hydrocarbon	Chloroneb	>100	>100	1
	DNOC	10	10	1
Phenylpyrrole	Fludioxonil	1	>30	>30
	Fenpiclonil	1	>30	>30

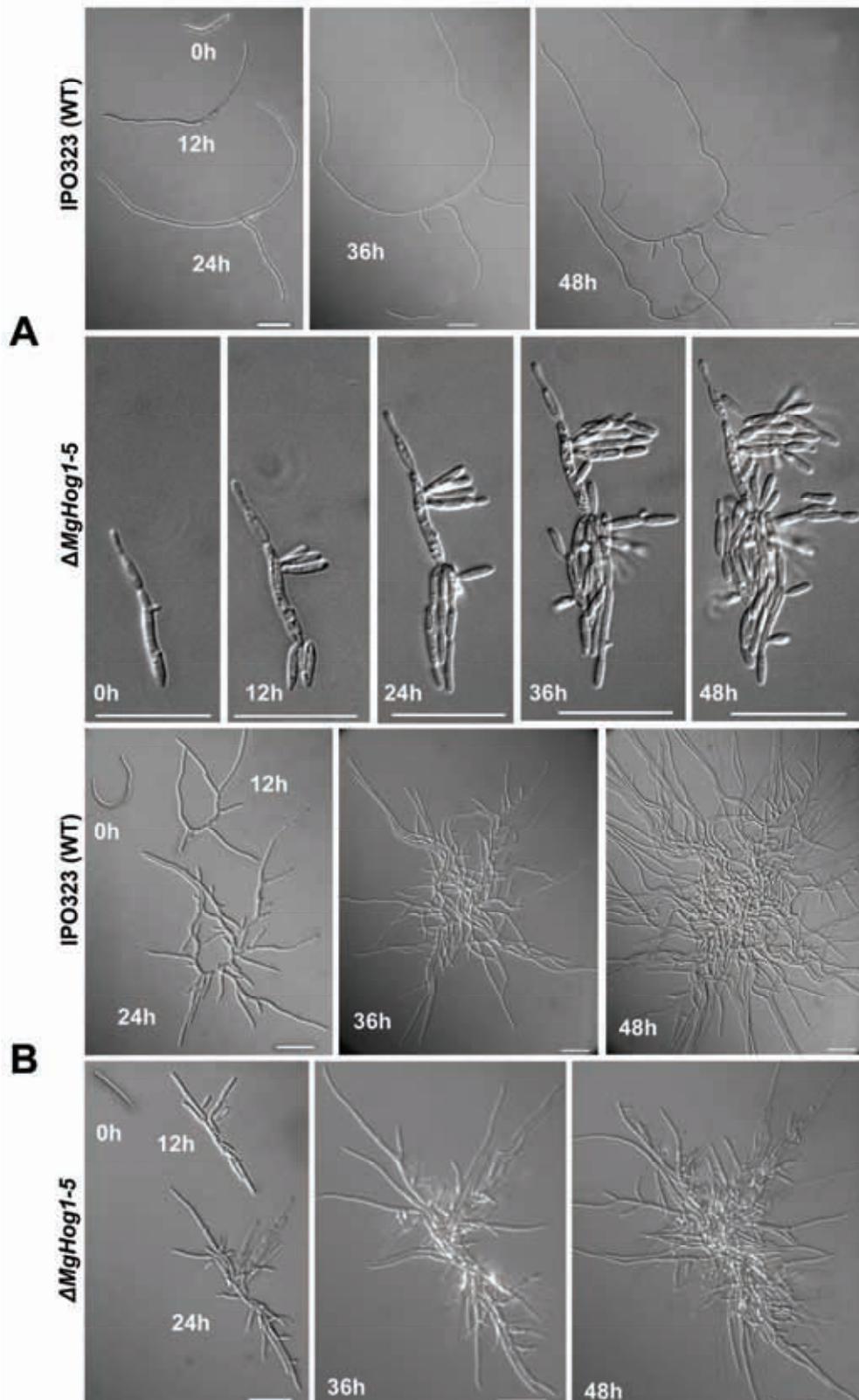
<sup>a</sup>*M. graminicola* IPO323, *MgSte20* mutants and the ectopic transformants showed similar MICs values.

<sup>b</sup>Q value: ratio between MIC values of  $\Delta MgHog1$  and IPO323

It has been reported that osmosensitive mutants of *Neurospora crassa* and *Colletotrichum lagenarium* are resistant to phenylpyrrole fungicides (Zhang et al. 2002; Kojima et al. 2004). Therefore, we also tested the sensitivity of *MgSte20* and *MgHog1* mutants to various fungicides, including phenylpyrroles. Compared to the WT and ectopic controls, the *MgHog1* mutants were highly resistant to fludioxonil (Fig. 3E), fenpiclonil (>30x) and iprodione (>5x) (Table 2). We did not observe increased sensitivity of either mutant to any of the other fungicides tested. The phenotypes of the *MgSte20* mutants were identical to the controls with regard to fungicide sensitivity (not shown).

### ***MgHog1* regulates dimorphism in *M. graminicola***

Since we noticed that *MgHog1* mutants exclusively developed as a yeast-like colony, we decided to test this phenomenon in more detail on poor water agar (WA) and PDA media. WA induces filamentous growth, as it stimulates spores to germinate and develop a superficial filamentous colony, similar to that observed on foliar wheat tissue before penetration (Kema et al. 1996, 2003). On WA, the WT and ectopic controls initially germinate from both apical cells of the spores producing relatively long primary germ tubes within 12 h, which is followed by the development of secondary germ tubes from the same or other cells of the spore. Within 48 h, tertiary hyphal filaments develop from the primary and secondary germ tubes, indicating that a poor medium promotes filamentous growth most



**Fig. 4.** Comparative analysis of germination, early colony development and micro-conidiation of *Mycosphaerella graminicola* IPO323 compared with those of the *MgHog1* mutants over time. **A**, Strains grown on WA at 20 °C in darkness. The WT strain germinates quickly and develops into a superficial filamentous web whereas *MgHog1* mutants are unable to switch from the yeast-like form to filamentous growth and propagate only by budding **B**, Strains grown on PDA at 20 °C in darkness. The WT strain grows filamentously with localized micro-conidiation, but the *MgHog1* mutants have a much higher tendency for micro-conidiation. All ectopic controls and the *MgSte20* mutants showed the same phenotype pattern as the WT strain. Scale bar = 30 µm.

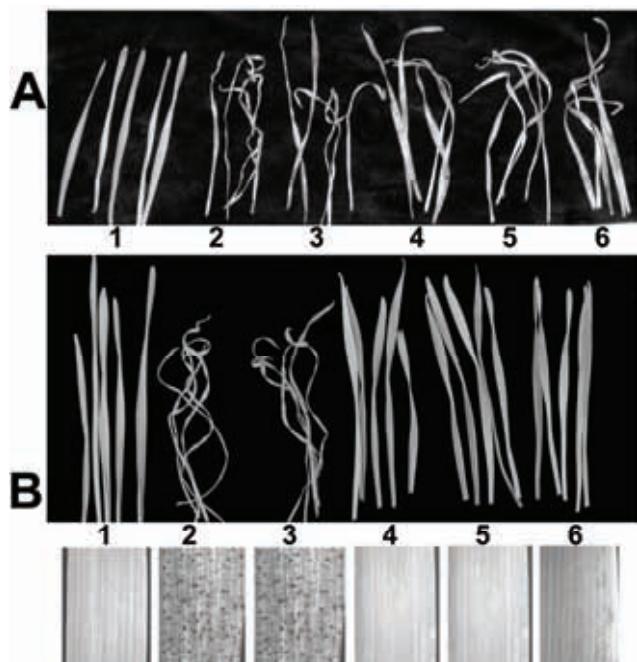
probably similar to conditions encountered on the leaf surface of the host (Mehrabi et al. 2006). Interestingly, the *MgHog1* mutants showed a significantly different germination pattern. Initial elongation of *MgHog1* mutant spores stopped within 12 h and was followed by a process of extensive budding without filamentous growth. This resulted in dense localized yeast-like colonies, a process that we characterize as micro-conidiation (Fig. 4A). Early colony development on PDA is significantly different. On PDA, germ tubes do not grow extensively in length but rather differentiate budding cells that form simultaneously on intermediate cells of the spore and on secondary filaments within 24 h (Fig. 4B). This is followed by a process of intense localized production of additional budding cells that partly elongate in relatively short filamentous hyphae at 36 h, and accumulate in the centre of the dense colony at 48 h (Fig. 4B). The budding process in *MgHog1* mutants immediately started, usually from both apical cells of the spores but sometimes also from other cells of the spores. This process was preceded by a short elongation of the spores but, unlike the WT, these spores did not develop filaments. Instead, budding continued even more intensively visualized by the bright spots in Fig. 4B at 36 and 48 hpi. The phenotypes of the *MgSte20* mutants and the WT and ectopic controls did not differ significantly in these assays (not shown).

### ***MgHog1* is required for virulence**

In order to evaluate the pathogenicity of the mutants, we inoculated the susceptible wheat cv. Obelisk with the WT, ectopic controls, and *MgSte20* and *MgHog1* mutants. Already at 8 dpi, plants inoculated with the WT and ectopic controls, as well as the *MgSte20* mutants showed small chlorotic flecks that were expanded into necrotic lesions at 12 dpi. At 16-18 dpi, these lesions developed into large necrotic areas containing numerous pycnidia. This suggests that *MgSte20* is dispensable for virulence (Fig. 5A). However, plants inoculated with the *MgHog1* mutants did not develop symptoms until 15 dpi when limited chlorotic areas started at the tips of the leaves. These chlorotic areas were also observed in water-treated control plants and apparently were due to natural senescence and aging, although it appeared that the chlorotic area was slightly larger on plants inoculated with the mutants (Fig. 5B).

### ***In planta* filamentation is hampered in *MgHog1* mutants**

Germination, penetration, colonization and pycnidia formation of *M. graminicola* were cytologically examined *in planta* over time using the *MgSte20* and *MgHog1* mutants, as well as the ectopic and WT controls. We observed no significant difference in the germination pattern, penetration, colonization and pycnidia formation among the WT, the ectopic controls



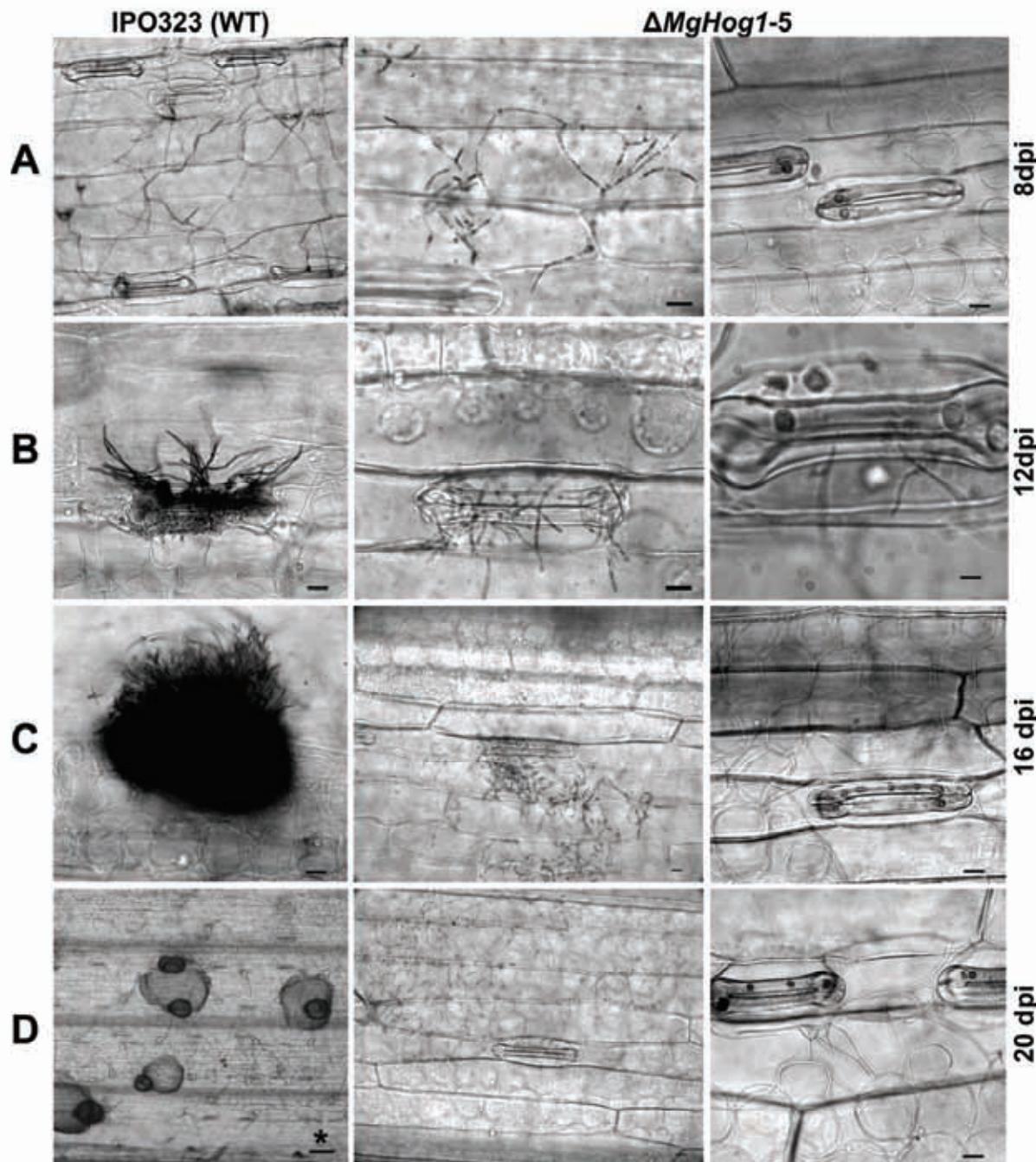
**Fig. 5.** Effects of the disruption of *MgSte20* (A) and *MgHog1* (B) on disease development of *Mycosphaerella graminicola* on the susceptible wheat cv. Obelisk 20 days after inoculation. The first leaves were inoculated with water as control (1), the wild-type strain *M. graminicola* IPO323 (2), an ectopic control (3), and three independent mutant strains (4, 5 and 6).

and *MgSte20* mutants. However, spores of *MgHog1* mutants were unable to develop infectious germ tubes that are required for penetration (Fig. 6). Some spores were able to generate significantly smaller germ tubes that were generally unable to penetrate the host. Only at very late stages of infection (> 16 dpi), we sometimes observed infectious hyphae of *MgHog1* mutants in the tip of already senescent leaves (Fig. 6).

## DISCUSSION

### Different roles for *Hog1* orthologs in monomorphic and dimorphic fungal plant and human pathogens

In genetic research, EST libraries serve as a gateway for discovery and characterization of new genes. We have generated a range of extensive EST libraries as a resource for genomic research and gene identification (Kema et al. 2003), and we have used them in a consistent strategy to identify genes involved in pathogenicity in *M. graminicola* (Mehabi et al. 2004, 2005, 2006). In order to determine the roles of MAPK pathways in the life cycle of the ascomycete *M. graminicola*, we previously characterized the MAPK-encoding genes *MgFus3* and *MgSlc2* and demonstrated that both genes are required for



**Fig. 6.** Comparative pathogenesis of *Mycosphaerella graminicola* IPO323 (WT) and *MgHog1* mutants on the susceptible wheat cv. Obelisk at 8, 12, 16 and 20 days post inoculation (dpi). **A**, Spores of the WT strain generate the infectious germ tubes that penetrate via stomata but development of infectious filamentation in the *MgHog1* mutants is hampered. **B**, Colonized stomatal cavity by the WT strain. The spores of the *MgHog1* mutants only propagate by budding and are unable to infect the tissue. Rare penetration attempts of *MgHog1* mutants are caused by individual budding cells that are positioned on top of the stomatal slit. **C** and **D**, Fructification of the WT strain at 16 dpi with conidia exuding in a cyrrhus from the stomatal opening. *MgHog1* mutants only sometimes infect the uncharacteristic tissue at the tip of the leaves. All ectopic strains and the *MgSte20* mutants showed the same pathogenesis as the WT strain. Scale bar = 10  $\mu$ m; marked (\*) scale bar is 100  $\mu$ m.

pathogenicity. *MgFus3* is required for penetration (Mehrabi et al. 2004), whereas *MgSl2* is required for colonization of the mesophyll tissue (Mehrabi et al. 2006). In this report, we characterized the role of the MAPK-encoding gene *MgHog1* and the PAK-encoding gene *MgSte20* and showed that *MgHog1* mutants are osmosensitive, similar to orthologous *Hog1* mutants of *S. cerevisiae* and other yeasts and filamentous fungi (Brewster et al. 1993; Degols et al. 1996; San Jose et al. 1996; Dixon et al. 1999; Iwaki et al. 1999; Zhang et al. 2002; Park et al. 2004). In contrast, mutants of *hogA* in *Aspergillus nidulans* did not show severe growth defects (Han and Prade, 2002; Kawasaki et al. 2002), suggesting that in this fungus, either another mechanism regulates growth under hyperosmotic stress or an additional *Hog1* homolog is present. We also tested the sensitivity of *MgHog1* mutants to hydrogen peroxide and found that they are slightly more sensitive to hydrogen peroxide compared to the WT and ectopic controls (data not shown). A similar phenotype was reported recently in *S. cerevisiae* and *Candida albicans* *Hog1* mutants (Alonso-Monge et al. 2003; Haghnazari and Heyer 2004), indicating a role for the *Hog1* orthologs in response to hydrogen peroxide.

We showed that disruption of *MgHog1* compromised the ability of *M. graminicola* to infect wheat leaves. *Hog1* mutants of the dimorphic human fungal pathogens *C. albicans* (Alonso-Monge et al. 1999) and *Cryptococcus neoformans* (Bahn, et al. 2005) also showed reduced pathogenicity, and in the chestnut blight pathogen, *Cryphonectria parasitica*, *Cpmk1* mutants showed slightly reduced canker areas, indicating the involvement of this MAPK in pathogenicity (Park et al. 2004). These observations are in contrast to those obtained with *Hog1* mutants in the rice blast pathogen *M. grisea* and the cucumber anthracnose pathogen *C. lagenarium* that remained fully pathogenic (Dixon et al. 1999; Kojima et al. 2004). These results may suggest that *Hog1* plays an important role in pathogenicity of dimorphic fungal pathogens but not in those lacking this phenomenon. We therefore investigated the role of *Hog1* in the dimorphic life cycle of *M. graminicola* in more detail. Indeed, the *MgHog1* mutants failed to switch from yeast-like to filamentous growth on WA that presumably mimics the stressful conditions on the leaf surface. *In planta*, we hardly observed germinated yeast-like spores for *MgHog1* mutants, which contrasts with reports for the orthologous *Hog1* mutants of the appressorium-forming pathogens *M. grisea* and *C. lagenarium*, for which no severe effect on germination and appressorium differentiation was found (Dixon et al. 1999; Kojima et al. 2004). *Hog1* is also involved in the dimorphic transition of *C. albicans* (Alonso-Monge et al 1999), but the role of the *Hog1* ortholog on the dimorphic transition in *M. graminicola* seems much more pronounced. In *M. graminicola*, filamentous growth of the *MgHog1* mutants on poor nutrient media is completely impaired, and growth is exclusively by

budding, whereas in low serum containing media *Hog1* mutants of *C. albicans* grow filamentously, with several cells appearing as long filamentous hyphae. On rich nutrient media, *M. graminicola* *MgHog1* mutants grow through an intensified budding process compared to the WT and ectopic control strains, but *C. albicans* WT and *Hog1* mutants show filamentous growth on rich serum media (100%). In conclusion, *MgHog1* can be considered a new pathogenicity factor of *M. graminicola*. Mutants are impaired in filamentous growth and consequently neither penetrate nor colonize the host, which clearly distinguishes *MgHog1* from *MgFus3* that is not required for filamentous growth but is essential for the recognition of the stomata (Mehrabi et al. 2004).

### Fungicide resistance through interference with the MgHOG1

After the initial publication on the effect of phenylpyrroles on glycerol synthesis in *N. crassa* (Pillonel et al. 1997), a negative correlation between osmo-sensitivity and resistance to phenylpyrrole fungicides was reported when the *Os2* and *Osc1* genes in *N. crassa* and *C. lagenarium*, respectively, were disrupted (Zhang et al. 2002; Kojima et al. 2004; Irmler et al. 2006). We extended studies on the role of *MgSte20* and *MgHog1* in fungicide sensitivity by testing the effects of different classes of fungicides on the growth of *MgSte20* and *MgHog1* mutants. Interestingly, we identified that *MgHog1* mutants are highly resistant to phenylpyrrole fungicides. It has been shown that fungicidal activity of fludioxonil is due to activation of a HOG1-related MAPK in the fungal pathogen *C. lagenarium* (Kojima et al. 2004). The finding that *MgHog1* mutants are resistant to fludioxonil and fenpiclonil might suggest that the mode of action of these fungicides is similar in both fungi. In addition, phenylpyrrole resistant *N. crassa* *Os-2* mutants are resistant to dicarboximide fungicides to which also *MgHog1* mutants showed increased resistance (>5 times), suggesting a common mechanism against iprodione and fludioxonil in *N. crassa* and *M. graminicola*. Altogether, these results indicate that interference in cell signaling networks seriously affects various cellular functions, including pathogenicity and sensitivity to fungicides. Detailed studies on these signaling mechanisms are important as they may provide target sites for novel fungicides.

### Divergent role of PAK orthologs in yeast and filamentous fungi

The PAK-encoding gene *Ste20* was originally discovered in the FUS3 and KSS1 pathways, but it was also identified as a major component that connects SHO1 and STE11 in

the HOG1 pathway (O'Rourke and Herskowitz, 1998; Raitt et al. 2000). In *S. cerevisiae*, STE20 is crucial in activating the pheromone and filamentation pathways via the MAPKs FUS3 and KSS1, respectively (Eby et al. 1998; Breitkreutz and Tyers 2002). Therefore, disruption of *Ste20*, *Fus3* or *Hog1* in *S. cerevisiae* shows both distinct and also overlapping phenotypes. Surprisingly, *MgSte20* is dispensable for pathogenicity as well as for vegetative growth *in vitro* under a range of conditions tested in this study. We have previously shown that the *Fus3* ortholog in *M. graminicola* is essential for penetration and *in vitro* pycnidia formation (Mehrabi, 2004). The observation that the disruption of *MgSte20* shows phenotypes different from the *MgHog1* and *MgFus3* mutants may indicate that these MAPK pathways have alternative upstream elements, probably with a redundant function. In *S. cerevisiae*, there are two additional PAK-encoding genes, *Cla4* and *Skm1*. *Cla4* is involved primarily in budding and cytokinesis, whereas *Skm1* appears to be dispensable for growth and differentiation (Cvrckova et al. 1995; Martin et al. 1997). The fission yeast, *S. pombe*, has two PAK-encoding genes, *Pak1* and *Pak2*, which are homologous to *S. cerevisiae Ste20* and *Cla4*, respectively. *Pak1* is required for proper cell polarity, mating and cell cycle control, but *Pak2* is dispensable for mating but required for typical cell morphology and growth (Marcus et al. 1995; Qyang et al. 2002; Merla and Johnson 2001). The ortholog of *Ste20* (*Cst20*) in the dimorphic human pathogen *C. albicans* is involved in lateral formation of mycelia on solid synthetic media and is also involved in virulence (Leberer et al. 1996, 1997). In contrast, disruption of the ortholog of *Ste20* (*Mst20*) in *M. grisea* only slightly influenced conidiation and aerial hyphae formation, but other aspects of morphogenesis were similarly affected as in *M. graminicola* with no obvious defect in conidium morphology, appressorium formation, or plant infection (Li et al. 2004). However, despite the minor effect of the disruption of *Ste20* ortholog in *M. grisea*, disruption of the *Cla4* ortholog (*Chm1*) strongly affected vegetative growth, conidiation, appressorium formation and pathogenicity (Li et al. 2004). Therefore it is of interest to search for any additional PAKs in the transcriptome database and the genome of *M. graminicola* IPO323 once its sequence is annotated.

## MATERIALS AND METHODS

### Fungal strains and cultural conditions

We used *M. graminicola* strain IPO323, which is highly virulent on seedlings of the susceptible wheat cultivar Obelisk, as the WT and recipient strain for gene disruption.

Growing conditions, propagation and maintenance were as described previously (Kema and Van Silfhout, 1997; Mehrabi et al. 2006).

### DNA manipulations and analysis

Standard protocols were used for DNA and RNA manipulation (Sambrook et al. 1989). Plasmid DNA was isolated using the QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany). Genomic DNA of *M. graminicola* was extracted using the Puregene DNA Isolation Kit (Gentra systems Inc., Minneapolis, MN, USA). The 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, USA). Homology searches were performed with the BLAST program (Altschul et al. 1997)

### Construction of plasmid vectors

Through *in silico* analyses of EST databases of *M. graminicola* IPO323 (Kema et al. 2003), we identified cDNA clones of *MgSte20* and *MgHog1* and used the GPS™-Mutagenesis system (New England Biolabs, Leusden, The Netherlands) to produce disruption constructs using a home-made customized donor construct, pGPS3HygKan (Mehrabi et al. 2006). The transposition mixture was then cloned to *Escherichia coli* strain DH10 $\beta$  that was subsequently plated on selective gentamycin/kanamycin media. The diagnostic multiplex colony PCR reactions were performed as described previously (Mehrabi et al. 2006). Two constructs with the transposon inserted in the middle of the gene were selected and designated pCGN1589 $\Delta$ *MgHog1* and pCGN1589 $\Delta$ *MgSte20*. The exact position of the transposon insertion was determined by sequencing from the right and the left borders of the transposon using primers N and S (Mehrabi et al. 2006).

### Fungal transformation

The disruption constructs pCGN $\Delta$ *MgHog1* and pCGN $\Delta$ *MgSte20* were cloned into *A. tumefaciens* strain LBA1100 by electroporation. *A. tumefaciens*-mediated transformation was performed basically according to Zwiers and de Waard (2001) with minor modifications that we have described previously (Mehrabi et al. 2006).

## Phenotyping

Phenotypic characterizations were performed with at least two independent disruptants, along with one ectopic and the WT control. Pathogenicity assays were performed as previously described with minor modifications (Mehrabi et al. 2006). After inoculation, pots were placed in transparent polyethylene boxes (30x45x30cm) and incubated at 20 °C in the dark for 48 h and subsequently incubated at 16 h light ( $166 \mu\text{E}.\text{sec}^{-1}.\text{m}^{-2}$ ) per day. Disease severity was monitored every four days, and final disease assessment was performed at 20 days post inoculation (dpi).

Cell biology assays were performed by growing spores of two independent mutant strains of both *MgSte20* and *MgHog1* and the WT and ectopic control strains in yeast glucose broth medium at 18 °C for five days that were collected by centrifugation, washed once with water and adjusted to  $10^5$  spores.ml<sup>-1</sup>. Ten µl of the diluted spore suspension were spotted on a plug of WA or PDA that was placed on a microscopic slide and then covered with a cover slip. The samples were incubated in an empty Petri dish at 20 °C in darkness, and at least two spores of each sample were monitored using a Zeiss Axioskop microscope (Carl Zeiss, Jena, Germany) equipped with a Carl Zeiss AxioCam 6.08.0 digital camera (Carl Zeiss, Jena, Germany), and pictures were processed with Axio Vision 4.1 software (Carl Zeiss, Jena, Germany) every 12 h until 48 h.

To monitor the germination and penetration behavior of *M. graminicola*, we harvested leaves from inoculated plants at 8, 12, 16 and 20 dpi and placed them on filter paper saturated with a mixture of 100% ethanol: glacial acetic acid (3:1, v/v) that was refreshed three to four times until the leaves were completely cleared. They were eventually transferred to filter paper saturated with lactoglycerol (1:1:1, lactic acid: glycerol: water, v/v/v) until cytological observation (Shetty et al. 2003). To monitor the colonization of the mesophyll, the harvested leaves were immediately immersed and boiled for 15 min. in 20 ml of 0.05% trypan blue dissolved in lactophenol-ethanol (1:2, v/v). Samples were destained at least overnight in a saturated chloral hydrate solution (5:2, w/v) and then stored in 87% (v/v) glycerol until analysis. For all light microscopy observations, specimens (adaxial side up) were placed on a glass slide, covered with a cover slip, observed and documented using the aforementioned microscopical equipment, camera and software. For colonization monitoring, samples were additionally stained with 0.1% trypan blue.

Osmosensitivity was determined by growing the *MgSte20* and *MgHog1* mutants and the WT and ectopic controls at 20 °C in darkness on PDA plates supplemented with 1, 1.5, 2.0 and 2.5 M of KCl, NaCl or sorbitol at 20 °C in darkness. Colony development and size

were visually examined at 10 dpi. Minimum inhibitory concentrations (MICs) of fungicides (Table 2) were determined by inoculating PDA plates supplemented with different concentrations of these compounds with spores harvested from 5-day-old liquid yeast glucose medium in a final concentration of  $10^7$  spores.ml $^{-1}$ . Plates were evaluated after 10 days incubation at 20 °C in darkness.

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# **CHAPTER 5**

**Protein Kinase A subunits of the ascomycete pathogen *Mycosphaerella graminicola*  
regulate asexual fructification, filamentation, melanization and osmosensing**

Rahim Mehrabi and Gert H. J. Kema  
Submitted for publication.

## ABSTRACT

As in many fungi, asexual reproduction of *Mycosphaerella graminicola in planta* is a complex process that requires proper differentiation of the infectious hyphae in the substomatal cavities of foliar tissue before pycnidia with conidia can be formed. In this study, we have investigated the role of the cAMP signaling pathway in development and pathogenicity of this pathogen by disruption of the genes encoding the catalytic (designated *MgTpk2*) and regulatory subunit (designated *MgBcy1*) of protein kinase A. The *MgTpk2* and *MgBcy1* mutants showed altered phenotypes *in vitro* when grown under different growth conditions. On potato dextrose agar (PDA), *MgBcy1* mutants showed altered osmosensitivity and delayed melanization, whereas the *MgTpk2* showed accelerated melanization when compared to the *M. graminicola* IPO323 wild-type strain and ectopic transformants. *MgTpk2* mutants also secreted a dark-brown pigment into yeast glucose broth medium. In germination and micro-conidiation assays, both mutants showed a germination pattern similar to that of the controls on water agar, whereas on PDA filamentous growth of *MgTpk2* mutants was impaired. Pathogenicity assays showed that the *MgTpk2* and *MgBcy1* mutants were less virulent as they caused only limited chlorotic and necrotic symptoms at the tips of the inoculated leaves. Further analyses of the infection process showed that *MgTpk2* and *MgBcy1* mutants were able to germinate, penetrate and colonize mesophyll tissue, but were unable to produce the asexual fructifications.

**Additional keywords:** *Septoria tritici*, functional genomics, signal transduction, differentiation, fruiting body formation.

## INTRODUCTION

In fungi, there is an enormous diversity in morphology and function of sexual and asexual fructifications that has always been instrumental for classifying species (Agrios, 2005; Taylor et al. 1999). Fungal fruiting body formation is a multifaceted fine-tuned developmental process that requires complex reprogramming of mycelial cells through changes in polarized growth, cell morphology and anastomosis. Differentiated cells have distinctive characteristics and perform specific functions. The process of fructification can be affected by a variety of external abiotic stimuli such as nutrients, CO<sub>2</sub>, light, and temperature, and biotic stimuli such as transcription factors, enzymes, and metabolites and proteins (Roger and Tivoli 1996; Vedenyapina et al. 1996; Vogelgsang and Shamoun 2002).

In many plant pathogenic fungi, asexual reproduction is a major driving force in development and epidemiology of plant disease affecting natural plant communities and crops. Sporulation can be either monocyclic or polycyclic and is programmed for the survival of a fungal species through the dissemination of anamorphic and/or teleomorphic propagules (Agrios, 2005). Pycnidia, the sub-epidermal asexual fructifications of the foliar wheat pathogen *Mycosphaerella graminicola* (Fuckel) J. Schröt in Cohn (anamorph; *Septoria tritici*), mark the successful completion of its life cycle that requires successful penetration, colonization, reprogramming and differentiation of the infectious hyphae in the substomatal cavity of the foliage (Kema et al. 1996). Regulation of these different developmental stages presumably involves sophisticated signal transduction pathways to coordinate these processes.

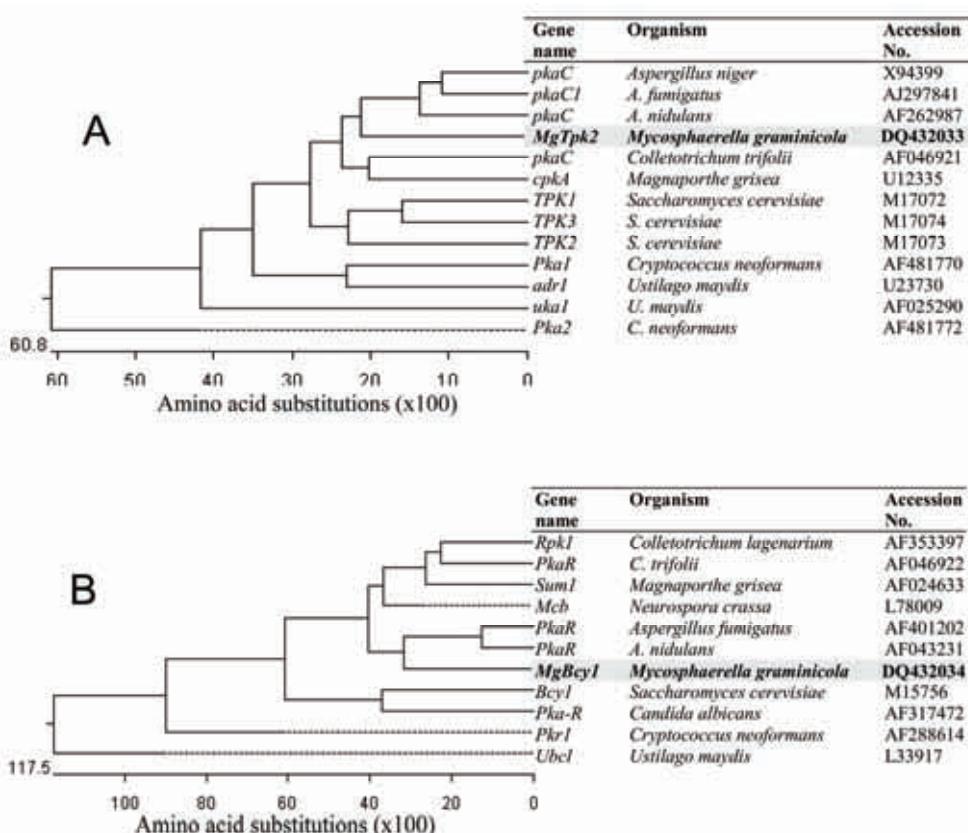
Signal transduction pathways were shown to be important for various cell functions during morphogenesis and development (Hirt 1997; Xu, 2000). The cAMP-dependent signaling pathway is involved in multiple processes in pathogenic fungi, including virulence and development (Kronstad, 1997; Wang and Heitman, 1999). The cAMP-dependent protein kinase A (PKA) is the major downstream effector of cAMP. Usually, PKA is a tetrameric protein (holoenzyme) consisting of two cAMP-binding regulatory subunits (hereafter PKAr) and two catalytic subunits (hereafter PKAc). After binding of cAMP to PKAr, the holoenzyme undergoes a conformational change, resulting in the release of the two active PKAc's (Taylor et al. 1990). In *Saccharomyces cerevisiae*, three catalytic subunit-encoding genes, *Tpk1*, *Tpk2*, and *Tpk3*, have been identified. Although these genes may have some redundancy in function, it has been shown that different catalytic subunits have distinct roles in the regulation of pseudohyphal growth. *Tpk2* promotes, whereas *Tpk1* and *Tpk3* inhibit filamentous growth (Toda et al. 1987; Pan and Heitman 1999). In *Colletotrichum lagenarium*

(Yamauchi et al. 2004; Takano et al. 2001), *C. trifolii* (Yang and Dickman 1999) and *Magnaporthe grisea* (Mitchell and Dean 1995; Xu et al. 1997) the cAMP-dependent pathway is required for either the functionality or formation of appressoria, as disruption of components of this pathway results in non-pathogenic mutants. Thus functional analysis of this pathway has provided experimental evidence that appressorium is essential for successful infection of plants for pathogenic fungi that penetrate plants directly (Kubo et al. 1981; Wolkow et al. 1983; Perpetua et al. 1994). *M. graminicola*, however, penetrates its wheat host through stomata and does not require appressoria for successful infection. It is a major representative of the Dothideales, an order that is phylogenetically relatively distant from the aforementioned ascomycetous plant pathogenic fungi (Kema et al. 1996; Duncan and Howard 2000; Goodwin et al. 2004; Goodwin, 2004; Mehrabi et al. 2006). *M. graminicola* is a hemibiotrophic pathogen that is characterized by an intercellular biotrophic phase, where hyphae grow in close contact with mesophyll cells to obtain nutrients from the plant. This phase is followed by a rapid switch to a necrotrophic phase, which is associated with an extensive collapse of mesophyll cells resulting in necrotic lesions on the leaf surface in which the fungus produces many pycnidia.

To deepen our understanding of the genetic factors controlling morphological and developmental process that occur during the infection process and life cycle of *M. graminicola* in wheat, we started a coherent strategy to dissect genes involved in the different stages of its infection process. We used an extensive EST resource to identify and functionally characterize genes in signal transduction pathways that regulate mating, cell wall integrity, and osmosensing. Recently we characterized three genes (*MgFus3*, *MgSlc2* and *MgHog1*) all encoding mitogen-activated protein kinases (MAPKs). *MgFus3* is required for penetration (Mehrabi et al. 2004), *MgSlc2* for colonization of the mesophyll tissue ((Mehrabi et al. 2006) and *MgHog1* for filamentous growth (This thesis chapter 4). In this study we describe the identification of genes involved in morphogenic transitions of *M. graminicola*. Our analysis of the cAMP-dependent signaling pathway shows that both regulatory and catalytic subunits of PKA are dispensable for germination, penetration and initial colonization, but are required for proper differentiation and aggregation of mycelium in the substomatal cavities, which are required for pycnidia formation.

## RESULTS

### Identification and annotation of *MgTpk2* and *MgBcy1*



**Fig. 1.** Phylogenetic comparison of the *Mycosphaerella graminicola* MgTPK2 (**A**) and MgBCY1 (**B**) orthologs based on amino acid sequence alignments. The deduced peptide sequences encoded by *MgTpk2* and *MgBcy1* from *M. graminicola* were compared with the orthologous proteins from other fungi.

We identified two full-length cDNA clones encoding the catalytic and regulatory subunits of PKA in extensive EST libraries of *M. graminicola* strain IPO323 (Kema et al. 2003). These genes were designated as *MgTpk2* and *MgBcy1* and are the orthologs of *Tpk2* and *Bcy1* in *S. cerevisiae* with 80% and 52% similarity at the protein level to TPK2 and BCY1, respectively.

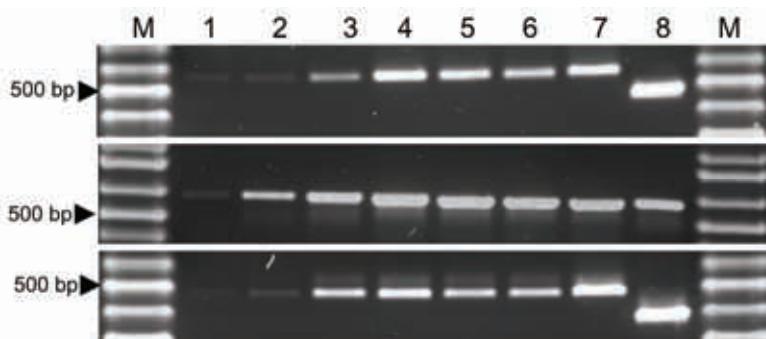
*MgTpk2* has a 1383 bp open reading frame (ORF) encoding a 461 amino acid (aa) sequence with a calculated molecular weight of 51.85 kDa. The structural region is interrupted by three short introns of 56, 57 and 52 bp. A putative polyadenylation region with a stretch of 16 As is found 58 bp downstream of the stop codon. Amino acid sequence analyses and phylogenetic studies revealed that the MgTPK2 protein grouped within catalytic subunits of PKA of other ascomycetes. As is common for PKAc, the N-terminal extension of MgTPK2 shows low homology with the other PKAc of fungi, but the overall homology is

reasonably high as exemplified by the 84% similarity at protein level to CPKA of the fungal pathogen *M. grisea* (Fig. 1).

*MgBcy1* has a 1,380 bp ORF without any intron and encodes a protein of 460 aa with a calculated molecular weight of 50.07 kDa. The predicted protein contains the two cAMP-binding sites at the C-terminus and an RII $\alpha$  domain at the N-terminus that is required for the dimerisation interface and a binding site for A-kinase-anchoring proteins (AKAPs). MgBCY1 shows 64% similarity at the protein level to SUM1 of the rice blast pathogen *M. grisea*. However, phylogenetic analysis indicated that MgBCY1 has a higher similarity to the PKAr of *Aspergillus* spp. than to PKAr from other plant pathogenic fungi (Fig. 1).

### ***MgTpk2* and *MgBcy1* are constitutively expressed**

The expression of *MgTpk2*, *MgBcy1* and the  $\beta$ -tubulin-encoding gene (as control) were studied by reverse transcription-polymerase chain reaction (RT-PCR) *in vitro* and *in planta* resulting in characteristic 363, 621 and 522 bp amplification products for *MgTpk2*, *MgBcy1* and  $\beta$ -tubulin-encoding gene, respectively. The expression analyses indicated that these genes are constitutively expressed, although *MgBcy1* is expressed at a slightly earlier stage and a higher level than *MgTpk2* (Fig. 2).

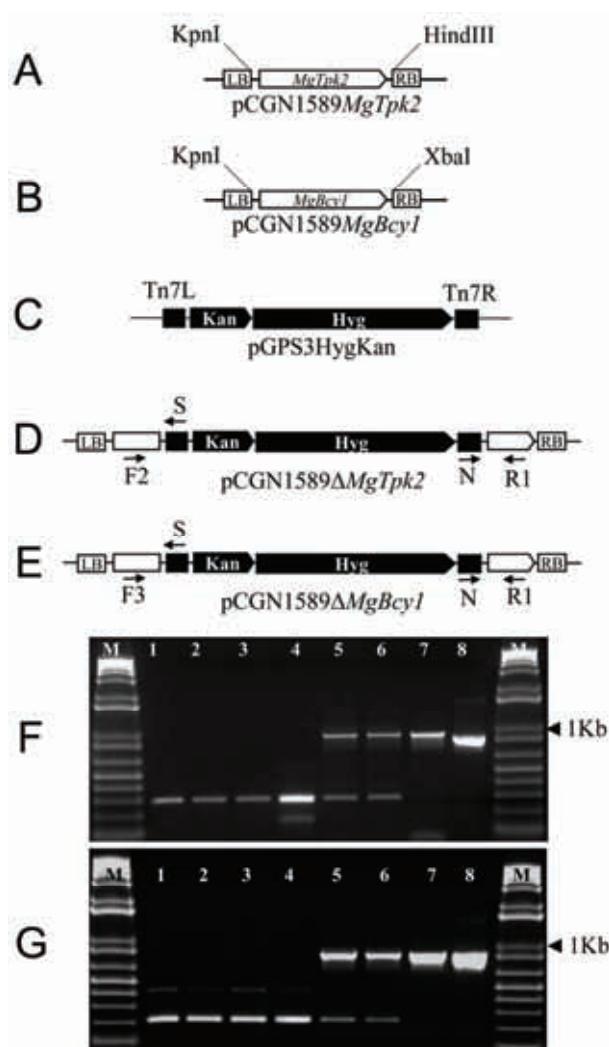


**Fig. 2.** *In vitro* and *in planta* expression profile of the *Mycosphaerella graminicola* *MgTpk2* and *MgBcy1* genes. Reverse transcription-polymerase chain reaction (RT-PCR) products were generated using gene-specific primers for the *MgTpk2* gene (top panel), the *MgBcy1* gene (middle panel) and the  $\beta$ -tubulin gene (control; lower panel). Lane M, one kb plus marker. Lanes 1-6, first-strand cDNA generated from total RNA isolated from wheat inoculated with *M. graminicola* strain IPO323, harvested at 0, 4, 8, 12, 16 and 20 days post inoculation, respectively. Lane 7, first-strand cDNA generated from total RNA isolated from *M. graminicola* IPO323 grown in YGB for 100 h. Lane 8, PCR control using genomic DNA of *M. graminicola* IPO323.

### Generation of *MgTpk2* and *MgBcy1* mutants

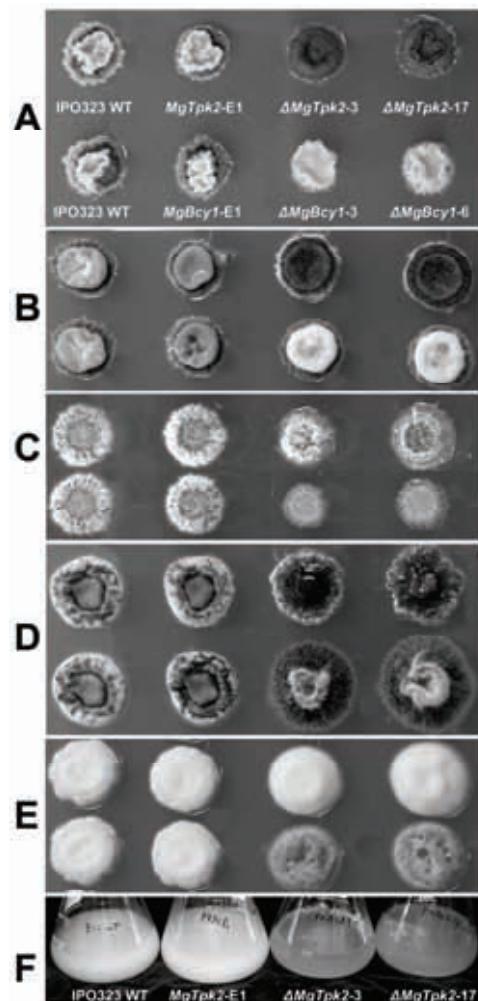
The *MgTpk2* and *MgBcy1* gene disruption constructs, pCGN1589 $\Delta$ *MgTpk2* and pCGN1589 $\Delta$ *MgBcy1* (Fig. 3), were generated using an *in vitro* transposition system that we have described previously (Mehrabi et al. 2006). In *MgTpk2*, the transposon was inserted 644 bp downstream of the start codon, while *MgBcy1* had a transposon insertion 618 bp downstream of the start codon. These constructs were used to disrupt *MgTpk2* and *MgBcy1* in *M. graminicola* strain IPO323 through *Agrobacterium tumefaciens*-mediated transformation (Mehrabi et al. 2006; Zwiers and de Waard 2001). For each disruption, 24 hygromycin-resistant transformants were tested for homologous recombination. We identified eight and five transformants for *MgTpk2* and *MgBcy1*, respectively, all lacking intact copies of these genes. Four of these transformants were characterized in detail, i.e.  $\Delta$ *MgTpk2*-3,  $\Delta$ *MgTpk2*-17,  $\Delta$ *MgBcy1*-3 and  $\Delta$ *MgBcy1*-6.

**Fig. 3.** Diagram representing the strategy to disrupt *MgTpk2* and *MgBcy1* genes of *Mycosphaerella graminicola*. The cDNAs of *MgTpk2* and *MgBcy1* were excised from the pSport1 vector using the *Kpn*I/*Hind*III and *Xba*I/*Kpn*I restriction enzymes and were inserted into pCGN1589, generating the pCGN1589*MgTpk2* (**A**) and pCGN1589*MgBcy1* (**B**) constructs, respectively. The customized donor construct pGPS3HgKan (**C**) containing kanamycin and hygromycin cassettes was used for transposition into target constructs pCGN1589*MgTpk2* and pCGN1589*MgBcy1*, resulting in the disruption constructs pCGN1589 $\Delta$ *MgTpk2* (**D**) and pCGN1589 $\Delta$ *MgBcy1* (**E**). The exact position of the transposon insertion was determined by sequencing from both right and the left borders of the transposon using primers N and S and subsequent comparison with non-disrupted sequences. To identify the homologous recombinants for *MgTpk2* (**F**) and *MgBcy1* (**G**), we used multiplex PCR screening with the gene-specific primers *MgTpk2*-F2/*MgTpk2*-R1 for *MgTpk2* and *MgBcy1*-F3/*MgBcy1*-R1 for *MgBcy1* together with primer N, which is located at the right border of the transposon. A small amplicon of equal size was amplified in homologous recombinants (lanes 1-3) and the disruption construct (lane 4). Ectopic transforms (lanes 5 and 6) two bands: a small band corresponding with the amplicon of the ectopic transforms and a larger one similar to the amplicon generated in *M. graminicola* IPO323 (lane 7). Lane 8 shows the amplicon using the cDNA clone as template. M indicates the one Kb plus marker.



### Characterization of phenotypes of *MgTpk2* and *MgBcy1* mutants *in vitro*

The *MgTpk2* and *MgBcy1* mutants grown under different conditions and their phenotypes were compared with the *M. graminicola* IPO323 and ectopic transformants. Disruption of *MgTpk2* and *MgBcy1* had a pronounced effect on the *in vitro* colony morphology when temperature, illumination and nutrients were varied (Fig. 4). We did not observe different growth rates among *M. graminicola* IPO323, the *MgTpk2* and *MgBcy1* mutants, and ectopic transformants when grown under these different conditions on potato dextrose agar (PDA) for ten days. However, the growth rate of the *MgBcy1* mutants was significantly lower on media with high osmolarity (Fig. 4C). We observed significant differences in melanization patterns between the *MgBcy1* and *MgTpk2* mutants under several growth conditions. *MgTpk2* mutants became faster melanized and accumulated much more dark pigment in darkness at 16 °C and 20 °C compared to the controls, but under continuous light *MgBcy1* mutants became equally melanized (Fig. 4A, B, D). At 28 °C, both the *MgBcy1* and *MgTpk2* mutants became already melanized after 5 days, but colonies of the latter did not become covered with a layer of white mycelium as observed for the other strains (Fig. 4E). Unlike *MgBcy1* mutants, *MgTpk2* mutants secreted dark-brown pigment into YGB (Fig. 4F). The growth of all strains stopped at temperatures higher than 29 °C, indicating that the mutants and the controls were equally thermosensitive.



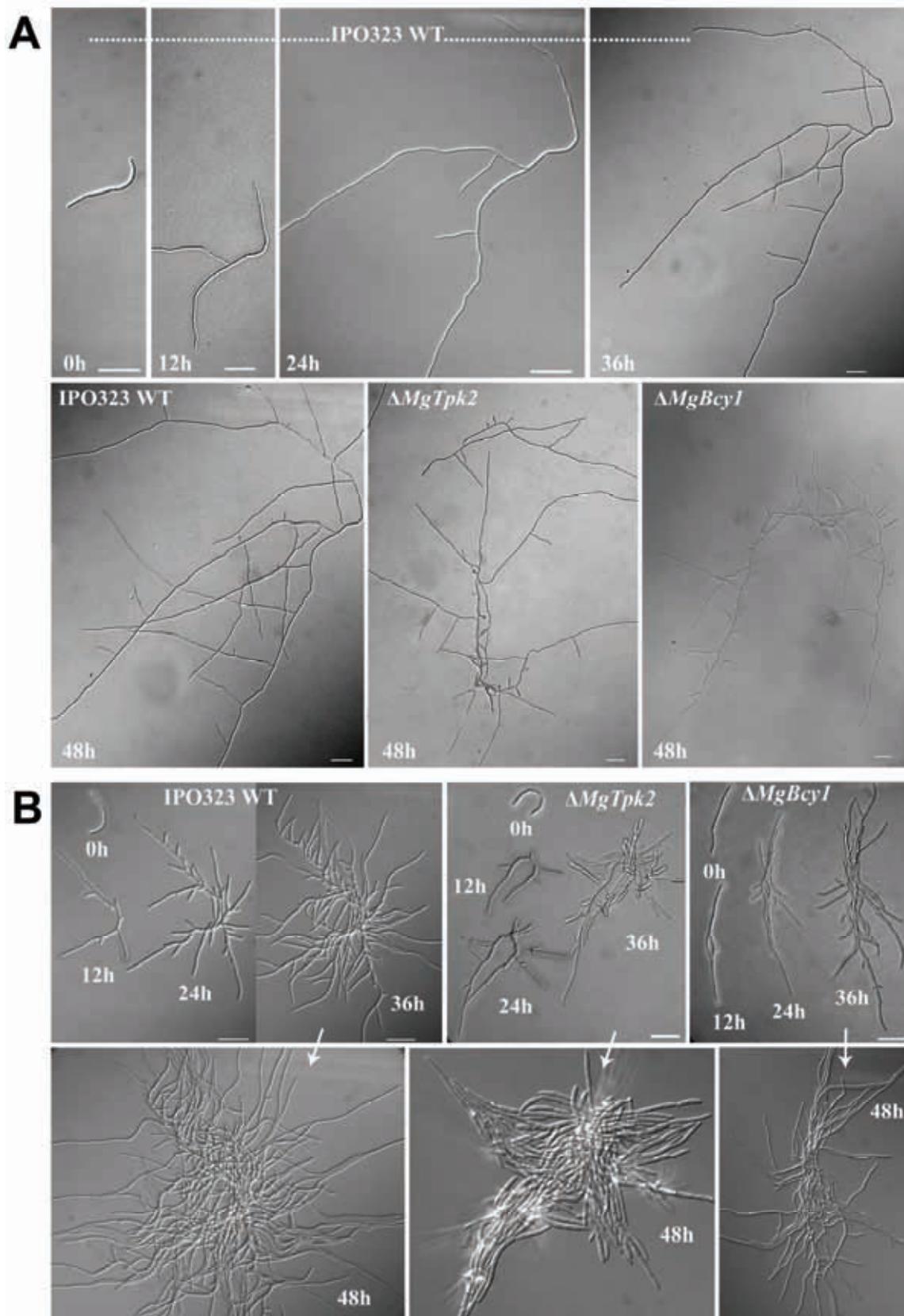
**Fig. 4.** Macroscopic analysis of the morphology of *Mycosphaerella graminicola* IPO323 (WT), two independent mutant strains for both *MgTpk2* ( $\Delta$ *MgTpk2-2* and  $\Delta$ *MgTpk2-17*) and *MgBcy1* ( $\Delta$ *MgBcy1-3* and  $\Delta$ *MgBcy1-6*) and ectopic transformants (*MgTpk2-E1* and *MgBcy1-E5*) grown on different artificial media. Approximately 1  $\mu$ l of spore suspension ( $10^7$  spores.ml $^{-1}$ ) was spotted on PDA according to the lay-out shown in Fig. 4A and cultured for 10 days at 16 °C in darkness (A), 20 °C in darkness (B), 20 °C in darkness supplemented with 1.5 M sorbitol (C), 20 °C in continuous light (D) and 28 °C in darkness (E). *MgTpk2* mutants secreting a dark pigment into YGB (F).

(data not shown). Minimum inhibitory concentrations (MICs) of the fungicides cyproconazole, imazalil, tricyclazole, kresoxim-methyl, azoxystrobin, fenarimol, iprodione, vinclozolin, chloroneb, DNOC, fludioxonil and fenpiclonil were similar for the *MgBcy1* and *MgTpk2* mutants and the control strains on PDA plates supplemented with different concentrations of these compounds (data not shown).

### Germination, early colony formation and micro-conidiation

The first essential step of infection is the differentiation of germ tubes that develop into infectious hyphae on the surface of wheat leaves. The leaf surface is a hostile environment with high UV light intensity and little availability of nutrients when compared to the mesophyll tissue. We, therefore, compared the germination pattern, early colony development and micro-conidiation of *M. graminicola* IPO323, the *MgTpk2* and *MgBcy1* mutants, and ectopic transformants on nutrient-rich PDA medium or water agar (WA). WA is supposed to mimics the leaf surface condition since it stimulates *M. graminicola* to develop germ tubes in a similar way as observed on wheat leaves (Kema et al. 1996). On WA, spores of *M. graminicola* IPO323 and the ectopic transformants usually germinate initially from both apical cells of the spores and produce relatively long primary germ tubes within 12 h (Fig. 5A). From thereon, secondary germ tubes develop from the same or other cells of the spore and form, along with the initially produced germ tubes, a thin superficial filamentous colony that is at least 5x larger than the original spore within 24 h. Within 48 h, tertiary hyphal filaments develop from the primary and secondary germ tubes, indicating that a poor medium promotes filamentous growth. The *MgTpk2* and *MgBcy1* mutants did not show significantly different germination patterns on WA compared to the control strains (Fig 5A).

Early colony development on PDA is significantly different. After germination, germ tubes do not grow extensively in length but rather differentiate budding cells that form simultaneously on other parts of the spore and on secondary filaments within 24 h (Fig. 5B). This is followed by a process of intense localized production of these budding cells that partially elongate into relatively short filamentous hyphae at 36 h and accumulate in the center of the dense colony at 48 h. The dimorphic growth of *M. graminicola* is clearly affected by nutrition, and we designated the extensive budding process on PDA as micro-conidiation. The development of the *MgTpk2* mutants was similar to the controls until 12 h, after which filamentous growth was significantly reduced and the colony merely developed by an intensive budding process, which we designated as micro-conidiation. (Fig. 5B). In

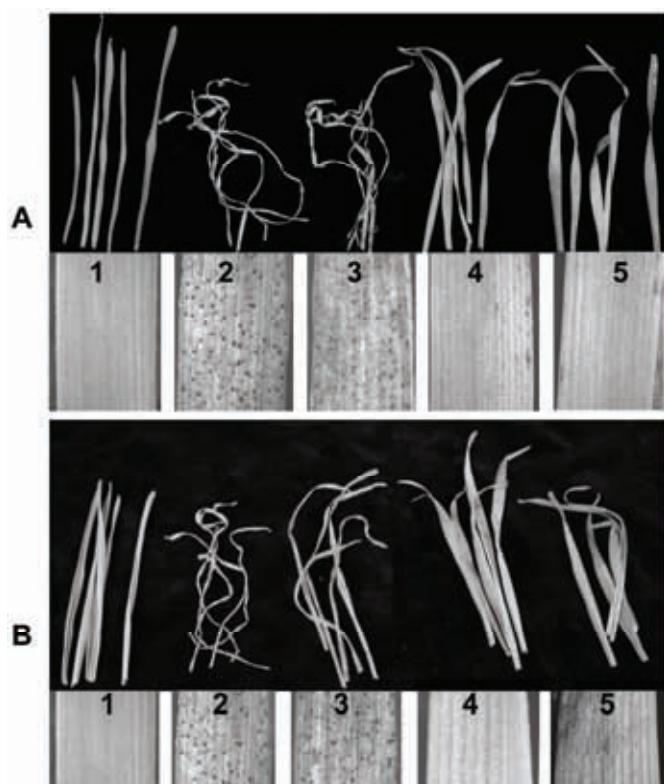


**Fig. 5.** Comparative analysis of germination, early colony development and micro-conidiation of *Mycosphaerella graminicola* IPO323 and the *MgTpk2* and *MgBcy1* mutants over time on WA (A) and PDA (B) at 20 °C grown in continuous darkness. Scale bar = 30  $\mu$ m.

early colony development (until 48 h) *MgBcy1* and *MgTpk2* mutants showed a slightly reduced growth rate compared to the controls.

### ***MgTpk2* and *MgBcy1* are required for pathogenicity**

In order to assess the pathogenicity of the *MgTpk2* and *MgBcy1* mutants, susceptible wheat cv. Obelisk was inoculated with the mutant strains and the control strains (*M. graminicola* IPO323 and ectopic transformants). Both control strains showed small chlorotic flecks at 7 dpi that continued to expand into large chlorotic areas at 12 dpi and eventually developed into large necrotic blotches containing numerous pycnidia at 16-18 dpi (Fig. 6). In contrast, the *MgTpk2* and *MgBcy1* mutants did not induce symptoms until 12-14 dpi when small chlorotic lesions developed that gradually coalesced into larger ones along with development of chlorotic areas at the leaf tips that became necrotic at 20 dpi but never contained pycnidia, indicating that both mutants are significantly reduced in pathogenicity.



**Fig. 6.** Symptoms caused by *Mycosphaerella graminicola* strains on the primary leaves after inoculation of 10-day-old seedlings of the susceptible wheat cv. Obelisk observed 20 dpi. Analysis of *MgTpk2* and *MgBcy1* disruptants, (A) and (B), respectively, with water control (1), *M. graminicola* IPO323 (2), an ectopic transformants (3), and two independent mutants (4 and 5).

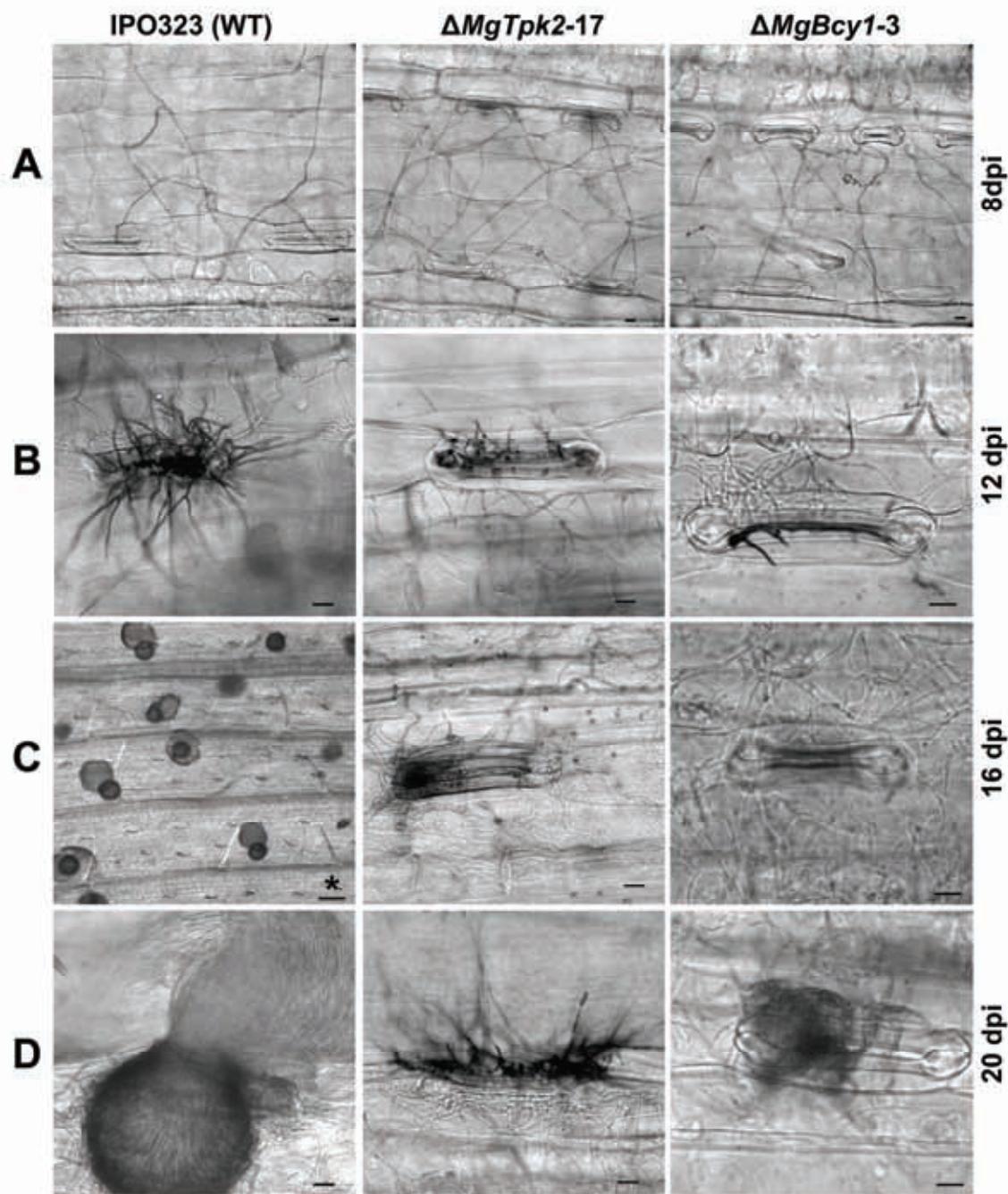
***MgTpk2* and *MgBcy1* are dispensable for germination, penetration and mesophyll colonization but are required for pycnidia formation**

In order to more precisely characterize the stage in the infection process in which *MgTpk2* and *MgBcy1* are required, we performed detailed comparative cytological studies on the susceptible wheat cv. Obelisk including *M. graminicola* IPO323 and the ectopic transformants. No distinct differences in the germination pattern of spores were observed in all treatments (Fig. 7). The spores of both *MgTpk2* and *MgBcy1* mutants germinated and developed infectious germ tubes similar to those of the controls (Fig. 7). Successful penetration of stomata and subsequent growth of the infectious hyphae in the substomatal cavities were observed that was followed by the development of invasive mycelium, which colonized the mesophyll tissue. At later stages, many additional substomatal cavities were colonized, but mature pycnidia did not develop (Fig. 7).

## DISCUSSION

In order to determine the importance of signal transduction pathways in the life cycle of the non-appressorium forming pathogen *M. graminicola*, we recently characterized three MAPK-encoding genes (*MgFus3*, *MgSlt2* and *MgHog1*), and demonstrated that they regulate different stages of the infection process (Mehrabi et al. 2004; 2005, 2006; This thesis, chapter 4). In this report, we functionally characterized the role of PKA in the life cycle of *M. graminicola*. We therefore generated *M. graminicola* strains lacking the functional regulatory subunit, MgBCY1, and the catalytic subunit, MgTPK2, of PKA and observed a range of distinctly altered phenotypes, which suggests that both genes have distinct functions during fungal development *in vitro* and *in planta*.

In contrast to the *MgTpk2* mutants, *MgBcy1* mutants are more sensitive to hyperosmotic conditions compared to the controls. It has been reported that high PKA activity in *S. cerevisiae* reverses the nuclear localization of the transcription factors MSN2 and MSN4 that are required for activation of stress response genes and hence leads to higher stress sensitivity (Görner et al. 1998). This is in agreement with the finding of Schmitt and McEntee (1996) and Martinez-Pastor et al. (1996) who reported that translocation of these transcription factors to the nucleus enhances resistance to stressful conditions. In *S. cerevisiae*, disruption of *Bcy1* increases PKA activity, and consequently, sensitivity to osmotic stresses (Thevelein, 1994), which is in agreement with our results obtained with the *MgBcy1* mutants. In *C. albicans*, however, the orthologs of *Msn4* and *Msn2* are not involved in stress responses (Nicholls et al.



**Fig. 7.** Histopathological analyses of *Mycosphaerella graminicola* IPO323 and the *MgTpk2* and *MgBcy1* mutants on the susceptible wheat cv. Obelisk. **A**, Germination and penetration pattern at eight days post inoculation (dpi). **B**, Colonization of substomatal cavity at 12 dpi. **C** and **D**, Fructification at 16 and 20 dpi, respectively. IPO323 produces pycnidia already at 16 dpi with pycnidiospores exuding in a cyrrhus from the stomatal opening. Note that *MgTpk2* and *MgBcy1* mutants do not produce pycnidia. Scale bar = 10  $\mu$ m, marked scale bar (\*) is 100  $\mu$ m.

2004), and it is therefore interesting to characterize the *M. graminicola* orthologs of these genes to examine whether the hypersensitivity of *MgBcy1* mutants is modulated by nuclear localization of the orthologs of MSN4 and MSN2.

We also observed that the melanization pattern of the *MgTpk2* and *MgBcy1* mutants was significantly altered. Mutation of *MgTpk2* resulted in higher levels of melanization, whereas mutation of *MgBcy1* resulted in decreased levels of melanization, compared to the controls. This is in agreement with our finding that high levels of exogenous cAMP also inhibited melanin production in all strains (data not shown). Therefore, we suggest that a high level of PKA activity negatively regulates melanin synthesis and, hence, disruption of the regulatory subunit-encoding gene (*MgBcy1*) increases PKA activity and reduces melanin synthesis. We further suggest that disruption of the catalytic subunit-encoding gene (*MgTpk2*) decreases PKA activity and therefore results in increased melanization. This is in agreement with the data obtained by Takano et al. (2001) who showed that disruption of the regulatory subunit-encoding gene, *rpk1*, in *C. lagenarium* also reduced melanization. Furthermore, constitutive or increased PKA activity partially repressed melanization in *Cryptococcus neoformans* (D'souza et al. 2001). In our study, *MgBcy1* mutants became equally melanized under continuous light, which rules out direct regulation through the PKA pathway and suggests other factors to be involved. The effect of light on various morphological and physiological processes during fungal development has been reported by others (Chern and Ko 1993; Kritsky et al. 2002; Tsror 2004). Recently it was shown that three melanin biosynthesis genes are upregulated under near-UV light (Kihara 2004; Kihara et al. 2004; Moriwaki et al. 2004). We observed impaired melanization for MAPK-encoding gene mutants (*MgFus3*, *MgSlt2* and *MgHog1*), as well as in mutants where G $\alpha$  and G $\beta$  subunit-encoding genes were disrupted (Mehrabi et al. 2004, 2005, 2006; This thesis chapter 4, 6), which demonstrates that the mechanism of melanization in *M. graminicola* remains poorly understood and certainly is under complex control involving several signal transduction pathways.

Early colony development experiments revealed that the accumulation of fungal biomass in *MgTpk2* and *MgBcy1* mutants of *M. graminicola* was only slightly reduced compared to the controls until 48 h of incubation on PDA. However, at later stages macroscopical differences were no longer observed, which could be due to the intrinsic slow growth rate of *M. graminicola* compared to other filamentous fungi. The catalytic subunit of PKA is involved in germination and *in vitro* biomass accumulation in most filamentous fungi, including *Aspergillus fumigatus*, *A. nidulans*, *A. niger*, *C. trifolii* and *C. lagenarium* (Liebmann et al. 2004; Shimizu and Keller 2001; Fillinger et al. 2002; Staudohar et al. 2002;

Yang and Dickman 1999; Yamauchi et al. 2004). However, in *M. grisea*, mutants of *cpkA* germinated normally (Xu et al. 1997), and growth and development, including asexual and sexual competence, were unaffected (Mitchell and Dean 1995). Disruption of the regulatory subunit-encoding genes in *M. graminicola* and many other fungi apparently had no suppressive effect on germination of conidia, with the exception of disruption of the *rpk1* ortholog in *C. lagenarium* that causes abnormal germination (Takano et al. 2001). However, regulatory subunit-encoding genes have been reported to be involved in biomass production *in vitro*. Disruption of the *MgBcy1* orthologs *pkaR* in *A. niger* (Staudohar et al. 2002) and *rpk1* in *C. lagenarium* (Takano et al. 2001) also resulted in reduced growth. In *S. cerevisiae*, a direct relationship between high PKA levels and reduced growth rate was observed, and in *C. albicans* overexpression of *Tpk2* caused a twofold reduction in growth rate (Thevelein et al. 2000; Bockmuhl et al. 2001).

Dimorphism is important in the life cycle of several fungi. We have shown that the role of signal transduction pathways on the dimorphic switch can be readily addressed in *M. graminicola*, since this fungus has a yeast-like growth pattern on rich media and a filamentous growth pattern on poor media and *in planta* (This thesis, chapter 4). This is an important aspect of *M. graminicola* compared to other model organisms such as *M. grisea*, *C. lagenarium*, *C. trifolii* that lack dimorphism in their life cycles. In this report, we show *MgTpk2* mutants strictly grow by an intensive budding process. Therefore, we suggest the cAMP pathway positively regulate filamentation via *MgTpk2*, which is in agreement with observations made for its orthologs in *C. albicans* and *C. neoformans* that are also involved in filamentation (Sonneborn et al. 2000; Hicks et al. 2004). In *S. cerevisiae* *Tpk2* also promotes filamentation, whereas the two other catalytic subunit-encoding genes (*Tpk1* and *Tpk3*) inhibit filamentation (Pan and Heitman 1999). In *Ustilago maydis*, two genes encoding catalytic subunits (*uka1* and *adr1*) of PKA have been characterized. Disruption of *uka1* had no effect on cell morphology, but disruption of *adr1* resulted in constitutive filamentous growth that is in contrast to the impaired filamentous growth of *MgTpk2* mutants (Durrenberger et al. 1998). In conclusion, the analyses of *in vitro* growth of the *M. graminicola* *MgBcy1* and *MgTpk2* mutants indicate that these genes play an important role in the life cycle of *M. graminicola*, as their disruption affects development, filamentation, melanization and osmoregulation.

Since we observed no effect on germination and filamentation of *MgTpk2* and *MgBcy1* mutants on WA, we did not expect to observe an *in planta* phenotype during the onset of pathogenesis. Indeed, the *MgTpk2* and *MgBcy1* mutants were not affected in germination, penetration and colonization of mesophyll tissue, but further analyses revealed that they were

unable to develop mature asexual fructifications. We monitored the fruiting body formation in a time course experiment and found that this requires a drastic developmental change at the final stages of infection. The observation that *MgTpk2* is not required in the early stages of infection is in agreement with data obtained for the appressorium-forming pathogens *C. lagenarium* (Yamauchi et al. 2004), *C. trifolii* (Yang and Dickman 1999) and *M. grisea* (Mitchell and Dean 1995; Xu et al. 1997), in which the orthologs of PKAc are dispensable for development of the appressorium but are required for its function. In these fungi, the catalytic subunit is clearly not involved in the final stages of infection, because mutants were able to complete their life cycle and fructified normally upon inoculation of wounded tissue (Yamauchi et al. 2004; Yang and Dickman 1999; Mitchell and Dean 1995; Xu et al. 1997).

Interestingly, our findings are also in agreement with observations made for the dimorphic corn pathogen, the basidiomycete *U. maydis*, in which disruption of regulatory (*ubc1*) and catalytic (*adr1*) subunit genes did also affect the morphogenetic transition at the final stages of infection. These mutants can still cause symptoms initially but are unable to produce galls with teliospores. However, disruption of the other catalytic subunit-encoding gene (*uka1*) in this fungus showed little effect on pathogenicity (Gold et al. 1997; Durrenberger et al. 1998).

Our studies have shown that in addition to at least three MAPK-encoding genes, the catalytic and regulatory subunit-encoding genes of PKA are also required for pathogenicity of *M. graminicola*. Our studies have also shown that, despite extensive analyses of signal transduction pathways in many different plant pathogens, detailed characterization of *M. graminicola* still adds new information to the functions of these genes in the life cycle of dimorphic pathogens in particular.

## MATERIALS AND METHODS

### Strains, growth conditions and pathogenicity assay

*M. graminicola* strain IPO323 was used as a recipient strain for transformation in this study. The growing conditions, inoculum preparation, and maintenance of the transformants were as previously described (Kema and Van Silfhout, 1997; Mehrabi et al. 2006). Virulence assays were performed as previously described with minor modifications (Mehrabi et al. 2006). After inoculation, plants were placed in transparent polyethylene boxes (30x30x45cm) and incubated at 20 °C in darkness for 48 h and subsequently incubated at 16 h light (166 µE. sec<sup>-1</sup>.m<sup>-2</sup>) and 8 h darkness per day. Disease severity was assayed at 20 dpi.

### Nucleic acid preparation and analysis

Standard protocols were used for DNA and RNA manipulation (Sambrook et al. 1989). Plasmid DNA was prepared using the QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany). Genomic DNA of *M. graminicola* was extracted from approximately 10 mg lyophilized spores or mycelium using the Puregene DNA Isolation Kit (Gentra systems Inc., Minneapolis, MN, USA). The 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 the recommended protocols. Phylogenetic tree generation, DNA and protein sequence alignment, editing, and analysis were performed using MEGALIGN and DNA Star software (Madison, WI, USA). Homology searches were performed with the BLAST program, and prediction of domains was performed at NCBI (Altschul et al. 1997; Marchler-Bauer and Bryant, 2004).

### Construction of gene disruption vectors

Through *in silico* analyses of EST databases of *M. graminicola* IPO323 (Kema et al. 2003), we identified full-length cDNA clones of the PKA catalytic and regulatory subunit-encoding genes, *MgTpk2* and *MgBcy1*, respectively. The cDNA of *MgTpk2* and *MgBcy1* were excised from the pSport1 vector using *Kpn*I/*Hind*III and *Xba*I/*Kpn*I restriction enzymes, and the fragments were inserted into pCGN1589 to generate pCGN1589*MgTpk2* and pCGN1589*MgBcy1*, respectively. We used the GPS™-Mutagenesis system (New England Biolabs, Leusden, The Netherlands) to make disruption constructs by using the home-made customized donor construct pGPS3HygKan that we described previously (Mehrabi et al. 2006). The target constructs pCGN1589*MgTpk2* and pCGN1589*MgBcy1* were transposed *in vitro* according to the manufacturer's instruction using pGPS3HygKan as the donor, and the transposition mixture was subsequently cloned into *Escherichia coli* strain DH10 $\beta$  that was afterwards grown on selective gentamycin/kanamycin medium. The multiplex colony PCR reactions to identify disruption of the target genes were performed as described previously (Mehrabi et al. 2006). Two constructs with the transposon in the middle of the gene were selected and designated pCGN1589Δ*MgTpk2* and pCGN1589Δ*MgBcy1*. The exact position of the transposon insertion was determined by sequencing from the right and the left borders of the transposon using primer N and primer S and comparison with non-disrupted sequences (Mehrabi et al. 2006).

**Table 1.** Primers used in this study.

Name	Sequence (5'-3')	Used for	Location
<i>MgTpk2</i> -R1	AATCTTGAATGTGGCCGT	Screening	<i>MgTpk2</i>
<i>MgTpk2</i> -F3	TGAGCAGCAAAGGCTACAACA	RT-PCR	<i>MgTpk2</i>
<i>MgTpk2</i> -R2	AAAGTCGGGAACAACGTC	RT-PCR	<i>MgTpk2</i>
<i>MgTpk2</i> -F2	TCGCTGGGCTTCCTCAA	Screening	<i>MgTpk2</i>
<i>MgBcy1</i> -F1	CAGTCCCGCGCAGGTCTT	Screening and RT-PCR	<i>MgBcy1</i>
<i>MgBcy1</i> -R1	GCTCAACACACTCGCCG	Screening and RT-PCR	<i>MgBcy1</i>
<i>MgBcy1</i> -F3	GCGCCATGGATCAACCT	Screening	<i>MgBcy1</i>
β-tubulin-F2	ACCATCTCCGGCGAACAT	RT-PCR	β-tubulin
β-tubulin-R2	GGAATTCTCGACCAGCTGG	RT-PCR	β-tubulin

### *A. tumefaciens*-mediated gene disruption

The disruption constructs pCGN1589Δ*MgTpk2* and pCGN1589Δ*MgBcy1* were cloned into *A. tumefaciens* strain LBA1100 by electroporation. *A. tumefaciens*-mediated transformation was performed according to Zwiers and de Waard (2001) and Mehrabi et al. (2006). After three weeks, individual *M. graminicola* transformant colonies were collected and transferred to PDA containing 100 µg hygromycin.ml<sup>-1</sup> and 200 µg cefatoxime.ml<sup>-1</sup>. The DNA of the transformants was isolated according to the abovementioned procedure. A hygromycin-specific PCR was performed to confirm the integration of the constructs into the genome of the *M. graminicola* IPO323 recipient strain (data not shown). Homologous recombinants were selected by a multiplex PCR screening using primer N, which is located at the right border of the transposon, together with two gene specific primers, *MgTpk2*-F2/*MgTpk2*-R1 in the case of *MgTpk2* and *MgBcy1*-F3/*MgBcy1*-R1 in the case of *MgBcy1* (Table 1). The gene-specific primers were designed in the flanking regions of the inserted transposon and did not render amplicons of the intact gene when homologous recombination occurred.

### Expression analysis

The *in planta* expression pattern of the *MgTpk2*, *MgBcy1* and the β-tubulin-encoding gene (as a control) was investigated using RNA isolated from primary leaf samples of the susceptible wheat cv. Obelisk that was inoculated with *M. graminicola* strain IPO323. These leaves were harvested at 0, 4, 8, 12, 16 and 20 days post inoculation. Ten leaves per sample were collected and immediately frozen in liquid nitrogen and ground using a mortar and

pestle. Total RNA was extracted from 50 mg of ground leaves using the RNeasy<sup>®</sup> Plant Mini Kit (QIAGEN, Hilden, Germany), and potential DNA contamination was removed using the DNA-free<sup>TM</sup> kit (Ambion, UK); both procedures according to the manufacturers' instructions. RT-PCR was conducted using 1 µl of RNA as the template using SuperScript<sup>TM</sup> One-Step RT-PCR with Platinum<sup>®</sup> Taq Kit (Invitrogen Groningen, the Netherlands) following the manufacturer's instructions. *In vivo* analysis was performed on cDNA generated from total RNA isolated from *M. graminicola* IPO323 grown in YGB for 100 h. The first strand of cDNA was synthesized for 30 min at 50 °C, and PCR was initiated by denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 20s, 60 °C for 30s and 70 °C for 60s with a final extension at 70 °C for 10 min and a cooling step to 10 °C using the primers *MgTpk2*-F3/*MgTpk2*-R2, *MgBcy1*-F1/*MgBcy1*-R1 and β-tubulin-F2/β-tubulin-R2). Amplicons were analyzed on 1% agarose gels using 5 µl aliquots of the PCR products.

### **Germination, early colony formation and micro-conidiation assay**

We used the independent mutant strains  $\Delta$ *MgTpk2*-2,  $\Delta$ *MgTpk2*-17,  $\Delta$ *MgBcy1*-3 and  $\Delta$ *MgBcy1*-6, as well as the ectopic transformants *MgTpk2*-E1 and *MgBcy1*-E5 and the *M. graminicola* IPO323 that were grown in YGB at 18 °C for 5 days until application and analysis of at least two spores/colonies per strain in the assay described previously (This thesis, chapter 4).

### **Cytological examination**

Germination, penetration and colonization of the *M. graminicola* IPO323 and the aforementioned ectopic transformants and mutant strains for *MgTpk2* and *MgBcy1* were studied according to the procedures described previously (Mehrabi et. al. 2006; This thesis chapter 4). For each time point, at least three specimens of each strain inoculated on the susceptible wheat cv. Obelisk were analyzed.

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# CHAPTER 6

**G $\alpha$  and G $\beta$  proteins regulate the cAMP pathway required for development and pathogenicity of the fungal wheat pathogen**

*Mycosphaerella graminicola*

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Submitted for publication.

**ABSTRACT**

We identified and functionally characterized three G $\alpha$ -encoding genes and one G $\beta$ -encoding gene present 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 $\alpha_i$  and G $\alpha_s$  families, respectively, whereas MgGPA2 is not related to either of the two families. Under continuous light strains disrupted in *MgGpa1*, *MgGpa3* or *MgGpb1* showed altered phenotypes on potato dextrose agar (PDA) at 20 °C but not at 28 °C. On PDA, *MgGpa1* mutants produced spores that were significantly longer than the controls, but in yeast glucose broth (YGB) they produced few spores and formed fluffy mycelia indicating that this gene has a negative effect on filamentation. In contrast, *MgGpa3* mutants showed more pronounced yeast-like growth accompanied with hampered filamentation and secretion of dark-brown pigment into YGB medium. Germ tubes emerging from spores of *MgGpb1* mutants were wavy on water agar (WA) and showed a nested-type of growth on PDA that was due to hampered filamentation, numerous cell fusions and increased anastomosis. The hampered filamentation of *MgGpb1* and *MgGpb3* mutants could be reversed to the wild-type by adding cAMP, indicating both genes positively regulate the cAMP pathway and filamentation. *In planta* assays showed that *MgGpa1*, *MgGpa3* and *MgGpb1* are strongly reduced in pathogenicity, whereas *MgGpa2* mutants are not affected. These results provide evidence for the role of heterotrimeric G proteins in the regulation of the cAMP pathway that is required for development and pathogenicity of the fungal wheat pathogen *Mycosphaerella graminicola*.

**Additional keywords:** *Agrobacterium tumefaciens*-mediated transformation, transposon mutagenesis, functional genomics, cell biology, hyphal fusion, adenylyl cyclase, anastomosis

## INTRODUCTION

Signal transduction pathways are important for sensing and responding to various 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 (Boelker 1998). The heterotrimeric G proteins contain three subunits ( $\alpha$ ,  $\beta$  and  $\gamma$ ) that are linked in the inactive state. Activation of a G $\alpha$  subunit by a transmembrane receptor leads to exchange of bound GDP for GTP on the G $\alpha$  subunit resulting in dissociation of the G $\alpha$  and the G $\beta\gamma$  dimeric subunits that can interact with downstream effectors, which subsequently generate changes in cellular responses (for review see (Dohlman and Thorner 2001)).

Filamentous fungi have one G $\beta$ - and usually three G $\alpha$ -encoding genes that belong to three major groups. Encoded proteins in groups I and III are related to the mammalian G $\alpha_i$  and G $\alpha_s$  families, respectively, but group II fungal G $\alpha$  proteins have no mammalian counterpart (Aimi et al. 2001; Boelker 1998; Ganem et al. 2004; Gronover et al. 2001; Kays et al. 2000; Parsley et al. 2003). Interestingly, the corn smut fungus *Ustilago maydis* contains a unique fourth G $\alpha$ -encoding gene and *Saccharomyces cerevisiae* contains only two G $\alpha$  proteins (Dohlman and Thorner 2001; Regenfelder et al. 1997). Irrespective of the observed numerical variation, G $\alpha$  proteins regulate a variety of cellular and developmental responses (Boelker 1998). In plant pathogenic fungi G $\beta$ -encoding genes have been functionally characterized (Delgado-Jarana et al. 2005; Ganem et al. 2004; Gronover et al. 2001; Jain et al. 2003; Kasahara and Nuss 1997; Muller et al. 2004; Nishimura et al. 2003). Apart from the fact that individual G $\alpha$ -encoding genes and G $\beta$ -encoding gene have been demonstrated to regulate growth, reproduction and virulence, comparative functional characterizations of all G $\alpha$ -encoding genes have only been reported for *Magnaporthe grisea*, *Cryphonectria parasitica* and *U. maydis* (Chen et al. 1996; Liu and Dean 1997; Regenfelder et al. 1997). Here we describe the cloning, characterization and targeted disruption of three G $\alpha$ -encoding genes and one G $\beta$ -encoding gene, which we designated as *MgGpa1*, *MgGpa2*, *MgGpa3* and *MgGpb1*, respectively, in the ascomycete *Mycosphaerella graminicola* (Fuckel) J. Schröt, a dimorphic fungal wheat pathogen with a lifestyle completely different from the aforementioned fungi (Mehrabi et al. 2006). *M. graminicola* (anamorph *Septoria tritici*) causes septoria tritici leaf 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 (Jorgensen et al. 1999). Apart from its economic importance, *M. graminicola* is the model fungus for the genus *Mycosphaerella* that is the largest genus within the Dothideales, an extremely large and diverse class of fungi with over 1,000 named species including major plant pathogens such as the banana leaf streak fungus *M. fijiensis* (Farr et al. 1995; Goodwin 2004). Large EST libraries and the availability of parts of the genome have been instrumental for identification and characterization of genes involved in development and pathogenicity of *M. graminicola* (Kema et al. 2003; <http://www.jgi.doe.gov/sequencing/why/CSP2005/mycosphaerella.html>). Recently we have 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 or regulate specific phases during the infection process (Mehrabi et al. 2006; Mehrabi et al. 2005; Mehrabi et al. 2004). For example, *MgFus3* and *MgHog1* are essential during penetration where the first is required for stomatal recognition and the latter is necessary for the dimorphic switch to pathogenic filamentous growth. Here we report on the requirement of *MgGpa1*, *MgGpa3* and *MgGpb1* for pathogenicity of which the latter is also a negative regulator of cell fusion. The phenotypes of mutants disrupted in *MgGpa3* and *MgGpb1* can be restored to the wild-type by exogenous cAMP, suggesting these genes act upstream of the cAMP pathway.

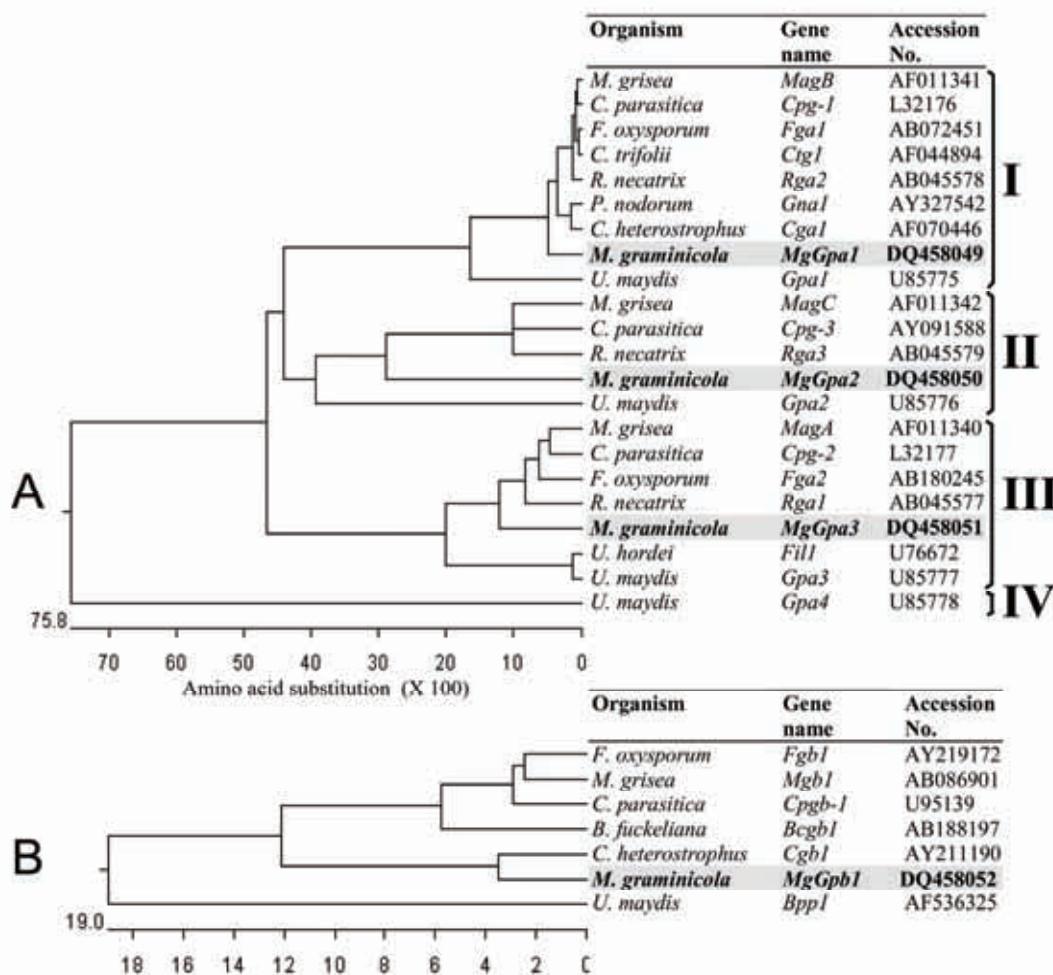
## RESULTS

### Sequence comparison and phylogenetic analyses

Screening of a large EST resource comprising over 30,000 *M. graminicola* ESTs (Kema et al. 2003) enabled us to identify three G $\alpha$ -encoding genes and one G $\beta$ -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 1059, 1065, 1068 and 1050 bp open reading frames (ORFs) encoding proteins consisting of 353, 355, 358 and 350 amino acids, respectively. Screening of the recently released non-annotated genome sequence of *M. graminicola* IPO323 (U.S. Department of Energy-Joint Genome Institute: <http://www.jgi.doe.gov/sequencing/why/CSP2005/mycosphaerella.html>) did not provide additional G protein-encoding genes.

Comparison of proteins encoded by these genes with the orthologs in *M. grisea* shows 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



**Fig. 1.** Phylogenetic comparison of orthologs of G $\alpha$  (A) and G $\beta$  proteins (B) based on amino acid sequence alignments. The encoded protein sequences of *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 the orthologs in other fungi.

DNA sequences and the EST contigs of these genes revealed that *MgGpa1*, *MgGpa2*, *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. 1A). 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 (Horwitz et al. 1999; Pennington 1994). *MgGPA1* belongs to major group I and contains

the consensus myristoylation site indicated as MGX (an uncharged residue), XX (a small uncharged residue), S at the N-terminus, and a consensus CXXX sequence at the C-terminus, which is the fingerprint of the pertussis toxin-labeling site (Gao and Nuss 1998; Simon et al. 1991). 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 that is required for N-terminal modification (Mumby et al. 1994). MgGPA3 belongs to major group III that only has the consensus myristoylation sequence, which is characteristic for adenylyl cyclase-stimulating G proteins ( $G\alpha_s$ ).

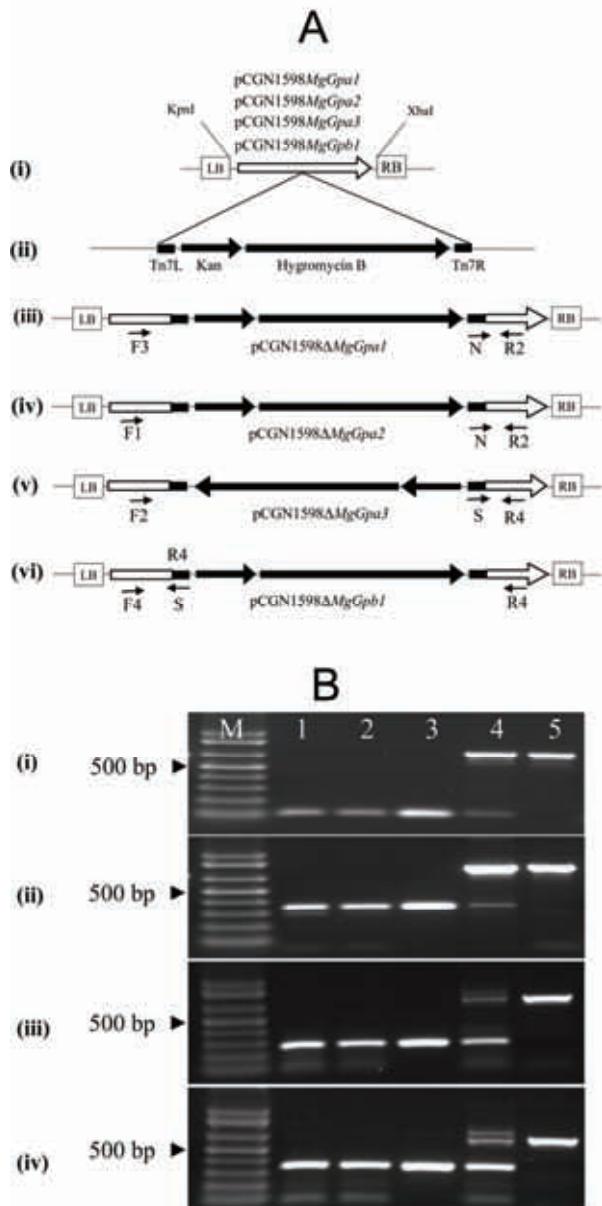
Protein comparisons via the NCBI Conserved Domain Search showed that MgGPB1 contains seven WD40 domains that are characteristic for a family of ancient regulatory proteins (Neer et al. 1994). WD40 repeats in *M. graminicola* generally contain a C-terminus GH dipeptide, an inter-WD40 region of 13-29 amino acids and an N-terminus WD dipeptide, representing the WD40 signature. However, there is variation in either one or both GH/WD dipeptides. The second WD40 repeat (residues 104-134) 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 either both or one of the C/N-termini.

### **Transposon-mediated vector construction and targeted gene disruption**

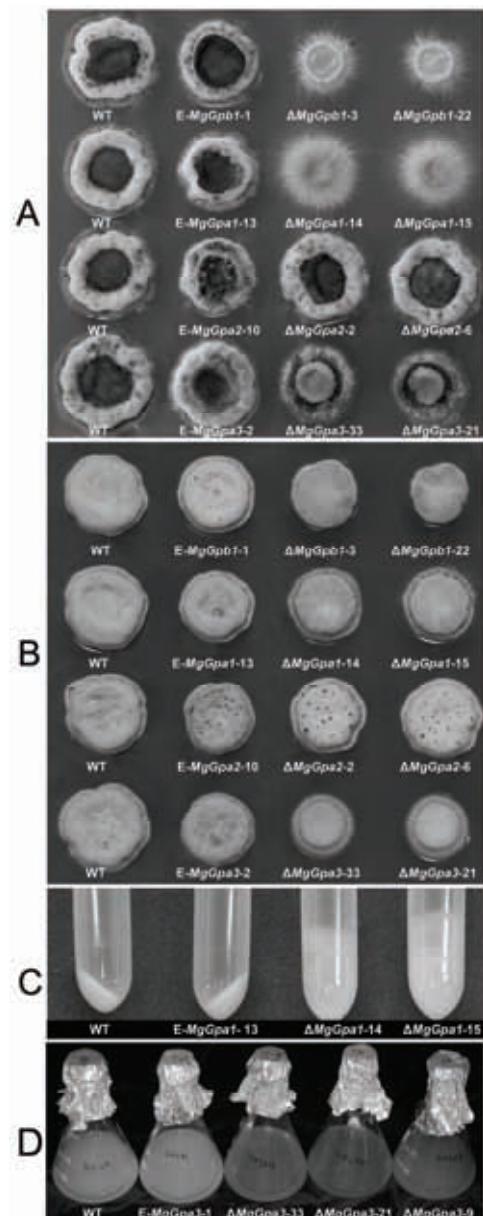
The disruption constructs for *MgGpa1*, *MgGpa2*, *MgGpa3* and *MgGpb1* were generated using the *in vitro* transposition system that we described previously (Mehrabi et al. 2006) (Fig. 2). PCR screening and sequencing enabled the selection of constructs with transposon insertions downstream the start codon in the ORFs of *MgGpa1*, *MgGpa2*, *MgGpa3* and *MgGpb1* at 666, 772, 393 and 586 bp, respectively (Fig. 2A). We used these constructs to disrupt *MgGpa1*, *MgGpa2*, *MgGpa3* and *MgGpb1* in *M. graminicola* IPO323 through *Agrobacterium tumefaciens*-mediated transformation (Mehrabi et al. 2006; Zwiers and de Waard 2001). After transformation, homologous recombinants were identified by PCR screening (Fig. 2B). Among 28, 25, 92 and 47 hygromycin-resistant transformants for *MgGpa1*, *MgGpa2*, *MgGpa3* and *MgGpb1* we identified 3, 14, 3, and 2 disruptive strains, respectively.

### ***In vitro* phenotypic characterization**

*Culture characteristics.* The *MgGpa1*, *MgGpa3* and *MgGpb1* mutants showed pronounced altered phenotypes on PDA at 20 °C under continuous light (Fig. 3A) and



**Fig. 2.** Generation of disruption constructs and subsequent identification of mutants in *Mycosphaerella graminicola*. **A**, The cDNA fragments of *MgGpa1*, *MgGpa2*, *MgGpa3* and *MgGpb1* were excised from the pSport1 vector using *Kpn*I/*Xba*I restriction enzymes and inserted into the same site of the binary vector of *Agrobacterium tumefaciens* (pCGN1589) generating the target constructs pCGN1589*MgGpa1*, pCGN1589*MgGpa2*, pCGN1589-*MgGpa3* and pCGN1589*MgGpb1* (i), respectively. The target constructs were transposed using the customized donor construct pGPS3HgKan (ii) resulting in the disruption constructs pCGN-1598Δ*MgGpa1* (iii) pCGN1598Δ*MgGpa2* (iv), pCGN1598Δ*MgGpa3* (v) and pCGN1598Δ*MgGpb1* (vi). **B**, Multiplex PCR screening to identify homologous recombinants for *MgGpa1* (i), *MgGpa2* (ii), *MgGpa3* (iii) and *MgGpb1* (iv), respectively using two gene specific primers (indicated in A panel) together with either primer N or primer S, which are located at the right and left borders of the transposon, respectively. Homologous recombinants (lanes 1-2) amplified a small amplicon of a size identical to the amplicon generated from the disruption construct (lane 3), whereas in ectopic transformants (lane 4) two bands were amplified: a small band corresponding to the disruptant strains and a larger band of equal size as the amplicon generated from the wild-type strain *M. graminicola* IPO323 (lane 5). M indicates one Kb plus markers (lane 1).

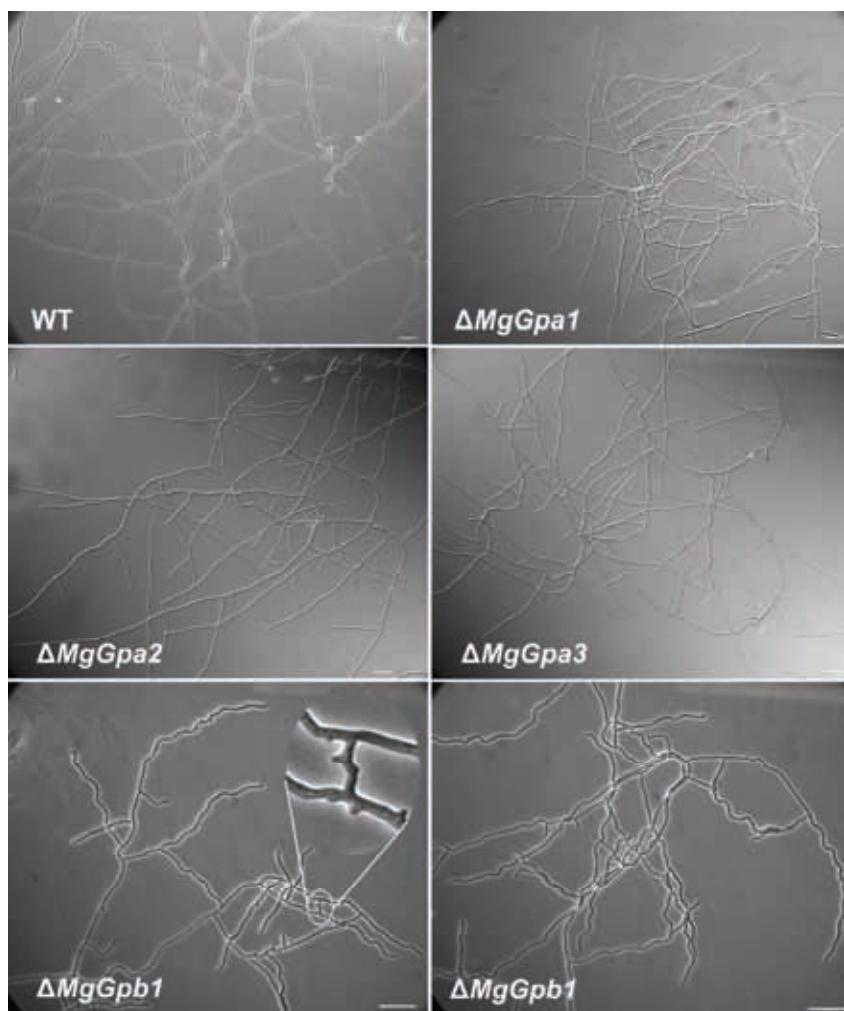


**Fig. 3.** *In vitro* phenotypic characterization of G $\alpha$ - and G $\beta$ -encoding gene mutants, strain *Mycosphaerella graminicola* IPO323 (WT) and ectopic transformants (E-prefix) under different culturing conditions. Phenotypes of ten-day-old cultures grown on PDA in continuous light at 20 °C (**A**) or at 28 °C in darkness (**B**). Highly increased filamentous growth of *MgGpa1* mutants in YGB medium at 18 °C (**C**). *MgGpa3* mutants secrete dark-brown pigment as shown for five-day-old YGB cultures grown at 18 °C (**D**).

darkness (not shown), whereas the phenotypes of *MgGpa2* mutants were identical to the controls under all conditions tested (Fig. 3A). The colonies of the *M. graminicola* IPO323 wild-type (hereafter WT) and ectopic transformants started to melanize after four to five days and were completely melanized and covered with a thin layer of white aerial mycelium at 10 days after incubation, whereas the colonies of *MgGpa1* and *MgGpb1* mutants were non-melanized and showed fimbriated borders (Fig. 3A). The growth rate of *MgGpb1* mutant was significantly reduced (~50% colony diameter at 10 days), whereas the *MgGpa3* mutant showed only a slight growth reduction, delayed melanization and extensive yeast-like growth in the center of the colonies (Fig. 3A). At 28 °C the phenotypes were nearly similar to those of the controls (Fig. 3B). At temperatures >29 °C all colonies stopped growing including the controls (not shown). In yeast glucose broth (YGB), controls and all mutants merely produced yeast-like cells, except *MgGpa1* mutants that produced fluffy mycelia. *MgGpa3* mutants secreted a dark-brown pigment into YGB (Fig. 3C-D). All mutants and controls showed the same minimum inhibitory concentrations to the fungicides cyproconazole, imazalil, kresoxim-methyl, azoxystrobin, fenarimol, iprodione, vinclozolin, chloroneb, DNOC, fludioxonil and fenpiclonil, and were equally sensitive to hydrogen peroxide (data not shown).

*Microscopical analysis.* In order to define the effect of the disruption of G $\alpha$ - and G $\beta$ -encoding genes on development of *M. graminicola* we microscopically monitored germination, early colony formation and conidiation in all mutants. On water agar (WA), the controls initially germinated from both apical cells of the spores producing relatively long primary germ tubes within 12 h and subsequently, secondary germ tubes from the same or other cells of the spore. Within 48 h, tertiary hyphal filaments developed from the primary and secondary germ tubes. We did not observe significant altered germination patterns for the *MgGpa1*, *MgGpa2* and *MgGpa3* mutants although the *MgGpa1* mutants produced longer germ tubes compared to the WT and ectopic transformants (data not shown). However, *MgGpb1* mutants grew significantly slower with wavy germ tubes, secondary and tertiary filaments of which some cells showed typical anastomosis after growth for 80 h on WA (Fig. 4).

Early colony development on PDA showed shorter germ tubes that often differentiated budding cells that were also produced on the intermediate cells of the spore as well as on secondary filaments within 24 h. These extended into filamentous colonies at 48 h that showed abundant micro-conidiation in the colony center by 80 h. On PDA, the *MgGpa1* mutants showed altered polarized growth with reduced branching and production of exceptionally long spores (Fig. 5). In *MgGpa3* mutants individual cells of multicellular spores

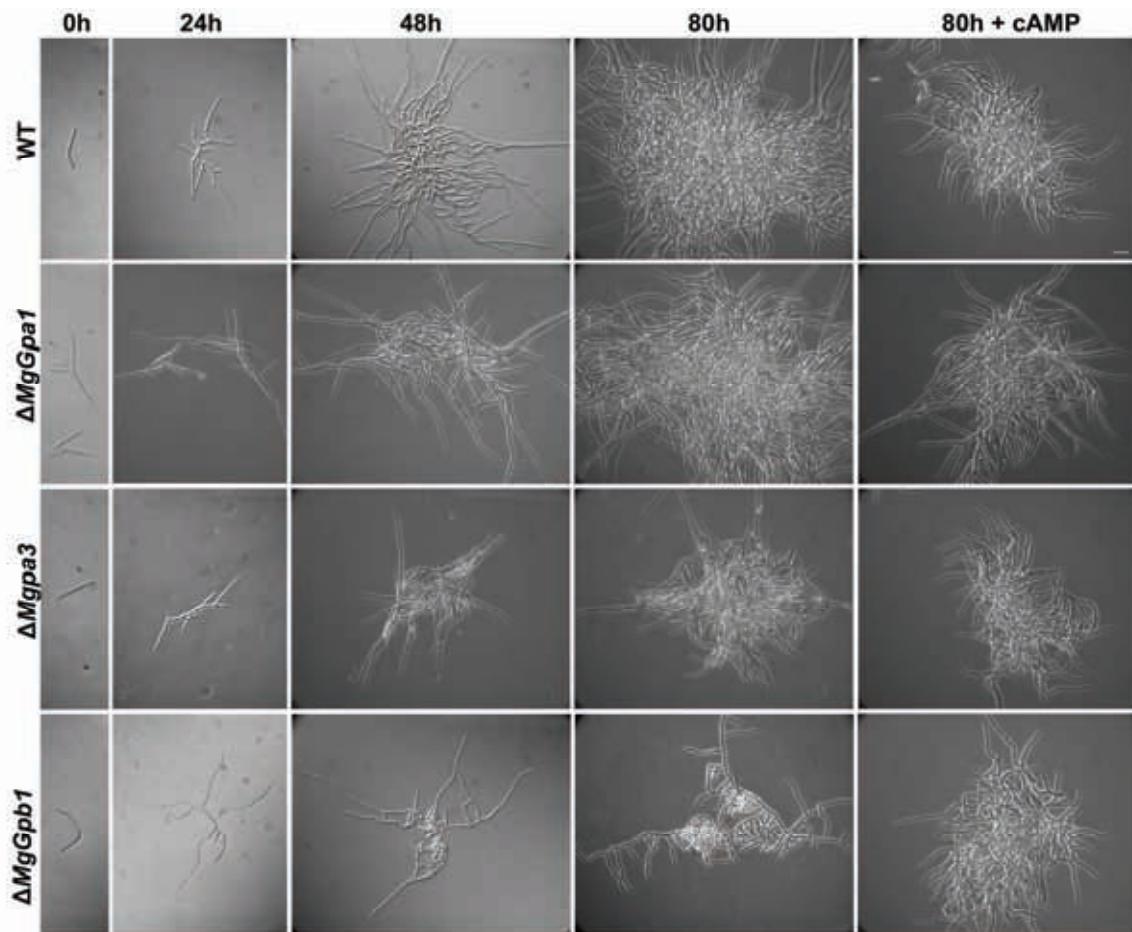


**Fig. 4.** Germination of the *MgGpa1*, *MgGpa2*, *MgGpa3* and *MgGpb1* mutants and the *Mycosphaerella graminicola* IPO323 control strain (WT) grown for 80 h at 20 °C on water agar. The mutants *ΔMgGpa1*, *ΔMgGpa2*, *ΔMgGpa3* and WT germinate equally well and switch to filamentous growth resulting in a superficial filamentous colony. Mutant *ΔMgGpb1* is reduced in growth of germ tubes that have a wavy appearance and show anastomosis. Scale bar = 30  $\mu$ m.

produced short germ tubes that did not develop secondary or tertiary filaments but propagated by budding resulting in dense colonies with abundant homogeneous spores (Fig. 5). The *MgGpb1* mutants initiated germ tubes that fused already within 24 h (Fig. 4-6) and subsequently produced extremely dense areas between 48-80 h (Fig. 5), which resulted from seemingly uncontrolled continuous anastomosis (Fig. 6). This process greatly suppressed filamentous growth and impaired micro-conidiation.

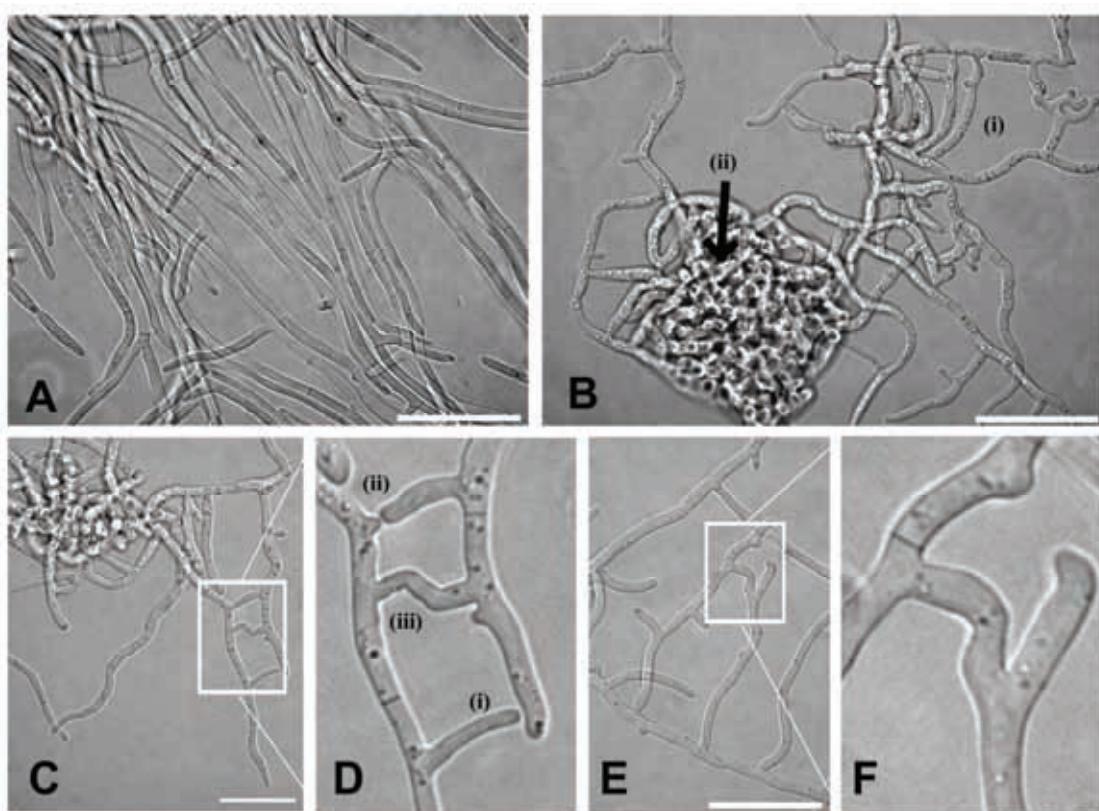
Sequence comparison and phylogenetic analyses suggested that *MgGpa1* and *MgGpa3* might regulate the cAMP-dependent signaling pathway. This hypothesis could be confirmed by the fact that phenotypes of *MgGpb1* and *MgGpa3* mutants could be reversed to the WT by adding cAMP to PDA medium. However, this was not true for the *MgGpa1* mutants (Fig. 5).

*In planta characterization of mutants.* Ten-day-old seedlings of wheat cv. Obelisk



**Fig. 5.** The effect of the disruption of the G $\alpha$ - and G $\beta$ -encoding genes *MgGpa1*, *MgGpa3* and *MgGpb1* on germination (24 h), early colony formation (48 h) and micro-conidiation (80 h) and restoration to *Mycosphaerella graminicola* IPO323 control (WT) of the  $\Delta M g G p a 3$  and  $\Delta M g G p b 1$  mutants by adding exogenous cAMP (80 h + cAMP) on PDA at 20 °C in darkness. The spores produced by *MgGpa1* mutants are significantly longer than those produced by the WT. Scale bar = 30  $\mu$ m.

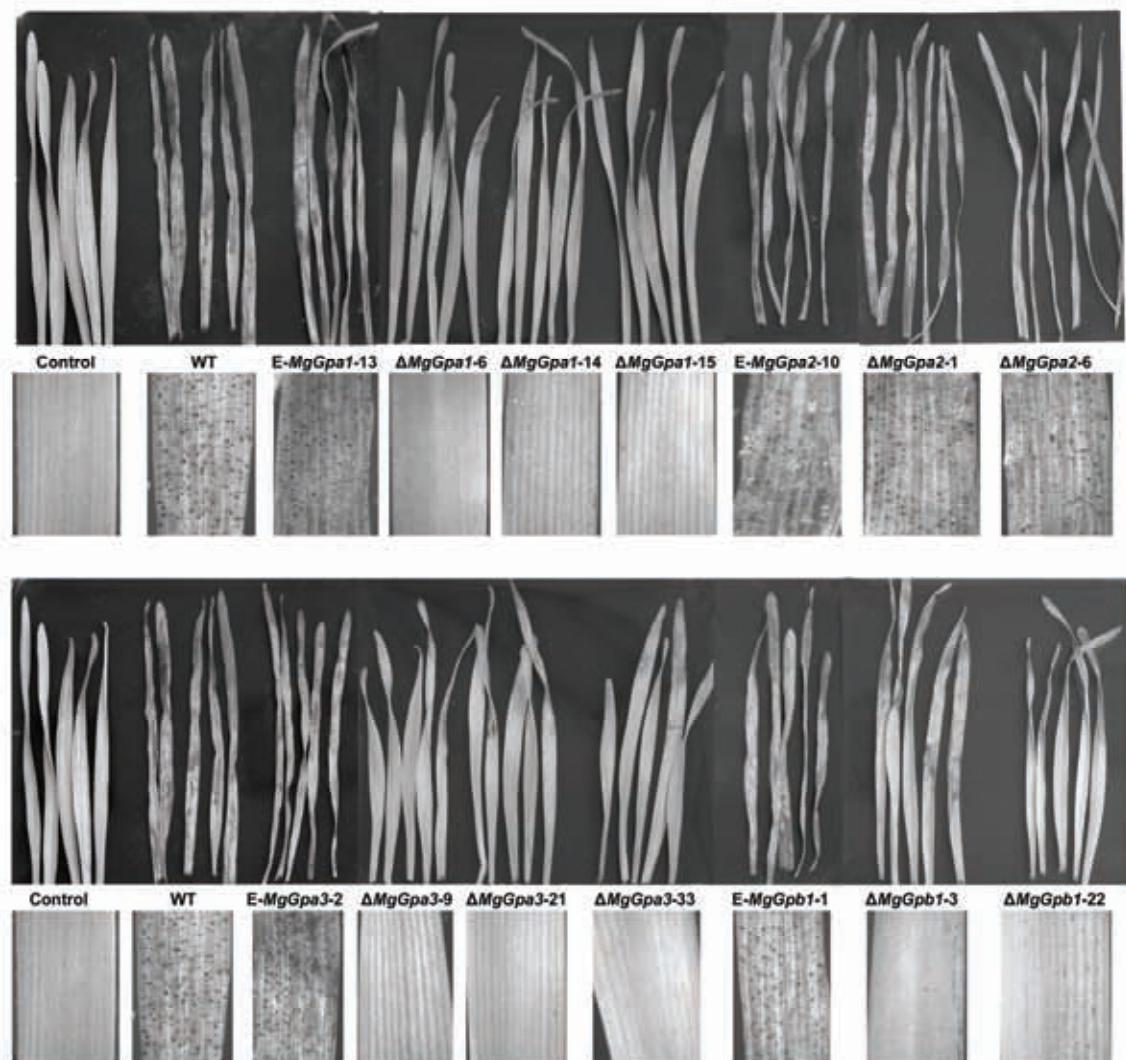
inoculated with the WT or ectopic transformants caused chlorotic areas at 8 dpi that continued to expand into larger lesions starting from the leaf tips, which became necrotic at 12 dpi and eventually merged into large necrotic blotches containing numerous pycnidia between 16 and 18 dpi. Plants inoculated with *MgGpa1*, *MgGpa3* or *MgGpb1* mutants showed delayed symptom expression and only developed limited chlorotic areas starting from the leaf tips between 12 and 15 dpi, which sometimes became necrotic but never contained pycnidia. Some chlorosis or necrosis was also observed in the leaf tips of water-treated controls, but this was most likely a result of natural senescence (Fig. 7). An overview of phenotypes of mutants of G $\alpha$ - and G $\beta$ -encoding genes in a range of filamentous fungi observed *in vitro* and *in planta* is presented in Table 1.



**Fig. 6.** Disruption of *MgGpb1* induces anastomosis. **A**, Hyphae of the *Mycosphaerella graminicola* IPO323 control 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 eventually show seemingly uncontrolled fusions producing dense local networks of mycelium (ii). **C-D** (**D** = magnification of box in **C**). Three distinct phases depicted during the anastomosis process; (i) attraction phase, (ii) contact phase and (iii) fusion phase. **E-F** (**F** = magnification of box in **E**). Septum development after fusion. Scale bar = 30 µM.

## DISCUSSION

The availability of a large EST library and the recently released yet non-annotated genome sequence of *M. graminicola* IPO323 resulted in the identification and isolation of three G $\alpha$ -encoding genes and one G $\beta$ -encoding gene. Gene replacement studies revealed that these heterotrimeric G protein-encoding genes are involved in 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 dimorphic switching, filamentation, micro-conidiation and melanization. Phylogenetic analyses indicated that the G $\alpha$ -encoding genes belong to three different groups. The mammalian orthologs of MgGPA1 and MgGPA3 are G $\alpha_i$  and G $\alpha_s$ , respectively, whereas MgGPA2 has no close relative in mammals. Activation of the G $\alpha_i$  protein, mostly by dissociation from the trimeric complex, inhibits adenylyl cyclase activity



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

and negatively regulates intracellular cAMP levels (Chen et al. 1996; Childers and Deadwyler 1996). We observed that *MgGpa1* mutants did not respond to exogenous cAMP, indicating that MgGPA1 is functionally similar to mammalian inhibitory G $\alpha_i$  proteins and the ortholog *Cpg-1* in *C. parasitica*; disruption of the latter gene increased intracellular cAMP levels in this fungus (Chen et al. 1996). In contrast, the orthologs *Gna1* in *N. crassa*, *Fga1* in *Fusarium oxysporum* and *MagB* in *M. grisea* positively regulate the cAMP pathway as disruption of these genes decreased intracellular cAMP levels (Ivey et al. 1999; Jain et al. 2002). In *M. grisea* exogenous cAMP restored appressoria formation and

Table 1. Effect of disruption of Gα- and Gβ-encoding genes on the development and pathogenicity of fungal plant pathogens.

<i>Organism</i>	<i>Gene</i>	<i>Class</i>	<i>Growth</i>	<i>Conidi- ation</i>	<i>Germi- nation</i>	<i>Virulence</i>	<i>Additional phenotypes</i>	<i>Reference</i>
<i>C. heterostrophus</i>	<i>Cgal</i>	Gα-I	Reduced	-	Reduced	Normal	Low appressorium formation, osmosensitive	Horwitz et al. 1999
<i>C. trifolii</i>	<i>Ctg1</i>	Gα-I	Reduced	-	Reduced	Reduced	Low appressorium formation, long germ tubes bearing appressoria	Truesdell et al. 2000
<i>C. parasitica</i>	<i>Cpg-1</i>	Gα-I	Reduced	Lost	-	Lost	Reduced pigmentation, increased cAMP level	Gao and Nuss 1996
<i>F. oxysporum</i>	<i>Fgal</i>	Gα-I	Normal	Reduced	Reduced	Reduced	Decreased cAMP level, thermo-tolerant	Jain et al. 2003
<i>M. grisea</i>	<i>MagB</i>	Gα-I	Reduced	Reduced	Normal	Reduced	Reduced penetration, long germ tubes bearing appressoria that are restored by exogenous cAMP	Liu and Dean 1997
<i>S. nodorum</i>	<i>Gna1</i>	Gα-I	Normal	Lost	-	Reduced	Defect in penetration, albino, osmosensitive	Solomon et al. 2004
<i>U. maydis</i>	<i>Gpa1</i>	Gα-I	Normal	Normal	Normal	Normal	No phenotype in all aspects	Regenfelder et al. 1997
<i>B. cinerea</i>	<i>Bcg1</i>	Gα-I	Reduced	Normal	Normal	Reduced	Altered colony morphology, normal penetration, loss of protease secretion,	Gronover et al. 2001
<i>M. graminicola</i>	<i>Gpa1</i>	Gα-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>	Gα-II	-	-	-	-	Not functionally analyzed	Parsley et al. 2003
<i>M. grisea</i>	<i>MagC</i>	Gα-II	Normal	Reduced	Normal	Normal	Normal appressorium formation	Liu and Dean 1997
<i>U. maydis</i>	<i>Gpa2</i>	Gα-II	Normal	Normal	Normal	Normal	No phenotype in all aspects	Regenfelder et al. 1997
<i>B. cinerea</i>	<i>Bcg2</i>	Gα-II	Normal	Normal	Normal	Reduced	Normal colony morphology, virulence slightly reduced	Gronover et al. 2001
<i>M. graminicola</i>	<i>Gpa2</i>	Gα-II	Normal	Normal	Normal	Normal	No phenotype in all aspects	This study
<i>C. parasitica</i>	<i>Cpg-2</i>	Gα-III	Reduced	Reduced	-	Normal	Decreased cAMP level	Gao and Nuss 1996
<i>F. oxysporum</i>	<i>Fga2</i>	Gα-III	Normal	Normal	-	Lost	Thermo-tolerant	Jain et al. 2003
<i>M. grisea</i>	<i>MagA</i>	Gα-III	Normal	Normal	Normal	Normal	No phenotype in all aspects	Liu and Dean 1997
<i>U. maydis</i>	<i>Gpa3</i>	Gα-III	-	-	-	Lost	Elongated cells in liquid media, defective in mating	Regenfelder et al. 1997
<i>M. graminicola</i>	<i>Gpa3</i>	Gα-III	Reduced	Normal	Normal	Reduced	Altered colony morphology, hampered filamentation, reduced melanization	This study
<i>U. maydis</i>	<i>Gpa4</i>	Gα-IV	Normal	Normal	Normal	Normal	No phenotype in all aspects	Regenfelder et al. 1997
<i>F. oxysporum</i>	<i>Fgb1</i>	Gβ	Reduced	Reduced	Normal	Reduced	Altered colony morphology, faster conidial germination, decreased cAMP level, thermo-tolerant	Jain et al. 2003
<i>M. grisea</i>	<i>Mgb1</i>	Gβ	Reduced	Reduced	Delayed	Lost	No appressorium formation, decreased cAMP level, more aerial hyphae, formation of fluffy colonies	Nishimura et al. 2003
<i>C. parasitica</i>	<i>Cpgb-1</i>	Gβ	Increased	Reduced	-	Reduced	Reduced pigmentation	Kasahara and Nuss 1997
<i>C. heterostrophus</i>	<i>Cgb1</i>	Gβ	Normal	Lost	-	Lost	Impaired formation of appressoria, increased pigmentation, straight germination	Ganem et al. 2004
<i>U. maydis</i>	<i>Bpp1</i>	Gβ	-	-	Normal	Normal	Increased filamentation and reduced budding growth, restored by adding exogenous cAMP	Muller et al. 2004
<i>M. graminicola</i>	<i>Gpb1</i>	Gβ	Reduced	Reduced	Normal	Reduced	Altered colony morphology, hampered filamentation and melanization, increased anastomosis	This study

pathogenicity of the *MagB* mutant (Liu and Dean 1997). High similarity of MgGPA1 to G $\alpha_i$  proteins, the enhanced filamentation of the *MgGpa1* mutants in YGB and the non-responsiveness to exogenous cAMP suggest that MgGPA1 acts upstream of the cAMP pathway, an observation that also fits our recent finding that the cAMP pathway regulates filamentation (This thesis, chapter 5). These data suggest that (i) fungal G $\alpha_i$  proteins comprise two subgroups that can either negatively or positively regulate adenylyl cyclase activity, and (ii) that high protein sequence identities do not necessarily imply similar biological functions. In contrast, the G $\alpha$  proteins that belong to Group III show high protein sequence identity in *N. crassa*, *C. parasitica*, *S. cerevisiae* and *Schizosaccharomyces pombe*. They all positively regulate adenylyl cyclase and are functionally related to mammalian G $\alpha_s$  (Gao and Nuss 1996; Isshiki et al. 1992; Kays et al. 2000; Kuebler et al. 1997; Lorenz and Heitman 1997; Nakafuku et al. 1988).

In many biological systems cAMP is generated by adenylyl cyclase and acts as a secondary 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 $\alpha_s$  genes or the catalytic subunit-encoding gene of PKA could lead to a similar phenotype. Indeed, *MgGpa3* and *MgTpk2* (catalytic subunit-encoding gene of PKA) mutants of *M. graminicola* both secreted a dark-brown pigment into YGB, stimulated yeast-like growth and affected filamentation on PDA (This thesis, chapter 5). Therefore, we suggest that MgGPA3 acts upstream of MgTPK2, positively regulates the cAMP pathway, and, based on its activity, functionally belongs to the G $\alpha_s$  protein family.

Since MgGPA2 belongs to a distinct group (group II) with no mammalian orthologs, we cannot predict its biochemical function. Interestingly, the phenotype of *MgGpa2* mutants was identical to the WT and ectopic transformants under all *in vitro* and *in planta* conditions tested, which supports observations in *U. maydis*, *Botrytis cinerea* and *M. grisea* where disruption of the *MgGpa2* orthologs did not or only slightly affected *in vitro* phenotypes and pathogenicity (Gronover et al. 2001; Liu and Dean 1997; Regenfelder et al. 1997). The phenotypes of all fungal G $\alpha$ -encoding gene mutants belonging to group II obtained so far, indicate that this type of G $\alpha$  protein does not play a major role in development and pathogenicity of plant pathogenic fungi.

Spores of *MgGpb1* mutants germinated normally but the germ tubes had a wavy appearance and showed anastomosis on WA, which culminated into extensive fusions on PDA, resulting in dense areas in the colonies with little filamentous growth and impaired conidiation. Although somatic cell fusion, known as homokaryon anastomosis, is common during vegetative growth of filamentous fungi (Glass et al. 2000; Glass et al. 2004; Hickey et al. 2002), it is unusual for *M. graminicola* and hence *MgGpb1* apparently negatively regulates vegetative cell fusion in this fungus resulting in a unique phenotype. The molecular mechanism of homokaryon cell fusion is poorly understood, but in *N. crassa ham-1* and *ham-2* mutants were unable to undergo both self- and non-self-hyphal fusion during vegetative growth (Wilson and Dempsey 1999; Xiang et al. 2002). In *S. cerevisiae* and *C. neoformans* G $\beta$  positively regulates the mating pheromone response pathway. Therefore, disruption of the encoding gene inhibits the response to sex pheromones (Wang et al. 2000; Whiteway et al. 1989). In contrast, in *S. pombe* pheromone signaling is mediated by G $\alpha$  (*Gpa1*) and the cAMP pathway is regulated by G $\alpha$  (*Gpa2*) and G $\beta$  (*Gpb1*). Interestingly, deletion of G $\beta$  in *S. pombe* stimulates conjugation in nutrient-rich media, which is in accordance with our observations (Kim et al. 1996; Landry et al. 2000). Furthermore, we were able to restore the phenotypes of *MgGpa3* and *MgGpb1* mutants to the WT by adding cAMP, indicating that both genes positively regulate the cAMP pathway. One explanation why both MgGPB1 and MgGPA3 regulate the cAMP pathway in *M. graminicola* might be that MgGPB1 is required for the efficient release of MgGPA3 from the heterotrimeric complex 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 $\alpha$  and G $\beta\gamma$  subunits (Federman et al. 1992; Gao and Gilman 1991; Tang and Gilman 1991). Altogether, our data favor the hypothesis that the regulation of the cAMP pathway in *M. graminicola* is similar to that of *S. pombe* but different from that of *S. cerevisiae* where one monomeric G $\alpha$  protein regulates the cAMP pathway without a contribution of the G $\beta$  protein.

We observed that all *M. graminicola* strains were equally thermosensitive, whereas in *N. crassa* and *F. oxysporum*, thermotolerance was observed in a few G $\alpha$ -encoding mutants and was positively correlated with decreased intracellular cAMP levels (Jain et al. 2002; Jain et al. 2005; Yang and Borkovich 1999). Indeed constitutive expression of *Gna1* in *N. crassa* resulted in elevated cAMP levels and increased thermosensitivity (Ivey et al. 1999; Yang and Borkovich 1999). The observed phenotypic differences could be due to the various lifestyles of these fungi.

Similar to *MgGpa1*, the orthologous group I G $\alpha$ -encoding genes in *C. parasitica*, *M. grisea*, *Stagonospora nodorum*, *F. oxysporum*, *Colletotrichum trifolii* and *B. cinerea* affect pathogenicity (Choi et al. 1995; Gao and Nuss 1996; Jain et al. 2002; Liu and Dean 1997; Solomon et al. 2004; Truesdell et al. 2000), whereas the orthologous genes *Cga1* and *Gpa1* in *Cochliobolus heterostrophus* and *U. maydis*, respectively, are dispensable for pathogenicity (Horwitz et al. 1999; Regenfelder et al. 1997). Such a functional discrepancy has also been observed for group III G $\alpha$  orthologs. *M. graminicola MgGpa3* mutants showed strongly reduced pathogenicity, which was also observed in *U. maydis* and *F. oxysporum* (Jain et al. 2005; Regenfelder et al. 1997), but not in *M. grisea* and *C. parasitica* (Choi et al. 1995; Liu and Dean 1997). Similar seeming discrepancies have also been observed for G $\beta$ -encoding genes. *MgGpb1* mutants and orthologous mutants in *F. oxysporum*, *M. grisea*, *C. parasitica* and *C. heterostrophus* were impaired in pathogenicity, whereas the mutants of the G $\beta$ -encoding gene in *U. maydis* were still pathogenic (Ganem et al. 2004; Jain et al. 2003; Kasahara and Nuss 1997; Muller et al. 2004; Nishimura et al. 2003).

We also observed that the *MgGpa1*, *MgGpb1* and *MgGpa3* mutants were not only affected in pathogenicity but also in melanization. The importance of melanization of hyphae and appressoria for pathogenicity of plant pathogenic fungi has been frequently reported (Kawamura et al. 1997; Kubo et al. 1991; Talbot 2003), and the melanin biosynthesis pathway is well characterized (Moriwaki et al. 2004; Tsuji et al. 2003). Recently, we showed that impaired melanization correlated well with defects in pathogenicity for *MgFus3*, *MgSlt2* and *MgHog1* mutants (Mehrabi et al. 2004, 2005, 2006). Expression of three melanin biosynthesis genes was only reduced at the onset of germination in the *Fus3* orthologous mutants of *C. lagenarium* suggesting the involvement of this ortholog in melanization of appressoria (Takano et al. 2000). In appressorium-forming pathogens, such as *M. grisea* and *C. lagenarium*, melanization is extremely important for generating turgor pressure in appressoria prior to direct penetration of epidermis cells (Money and Howard 1996; Perpetua et al. 1996; Talbot 2003). These results may suggest the requirement of melanization for pathogenicity in *M. graminicola*. However, unlike appressorium-forming pathogens, *M. graminicola* enters its host through stomata and, hence, impaired melanization probably does not affect penetration. Indeed, our previous study revealed that non-melanized mutants of *MgSlt2* and the wild-type strain were equally effective in entry of the host (Mehrabi et al. 2006), which suggests that melanization is a not crucial factor for penetration. Therefore, we anticipate that melanization is required at the time that the fungus starts to differentiate asexual fructifications in sub-stomatal cavities (Duncan and Howard 2000; Kema et al.

1996). 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*.

## MATERIALS AND METHODS

### Fungal strains and cultural conditions

The Dutch field strain *M. graminicola* IPO323 is highly virulent on winter wheat cv. Obelisk and was used as a recipient throughout this study. Growing conditions, propagation and maintenance were as described previously (Kema and Van Silfhout 1997; Mehrabi et al. 2006).

### DNA manipulations and analysis

Standard protocols were used for DNA manipulation (Sambrook et al. 1989). Plasmid DNA was isolated using the QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany). Genomic DNA of *M. graminicola* was extracted using the Puregene DNA Isolation Kit (Genta systems Inc., Minneapolis, MN, USA). The 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, USA). Homology searches were performed with the BLAST program (Altschul et al. 1997).

### Construction of plasmid vectors

By *in silico* analyses of EST databases of *M. graminicola* IPO323 (Kema et al. 2003), 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 home-made customized donor construct pGPS3HygKan that was described previously (Mehrabi et al. 2006). After transposition, the transposition mixture was cloned to *Escherichia coli* strain DH10 $\beta$  (Invitrogen, Groningen, The Netherlands) by electroporation using the recommended protocol, which was subsequently plated onto gentamycin/kanamycin media to select colonies with transposon insertions into the target constructs, which was confirmed by multiplex colony PCR as

Table 2. Primers used in this study.

Name	Sequence (5'-3')	Location
<i>MgGpa1</i> -F3	TTCAGCGAAGCCGAGAATATC	<i>MgGpa1</i>
<i>MgGpa1</i> -R2	CACATGCGCAGGTTCTCTTG	<i>MgGpa1</i>
<i>MgGpa2</i> -F1	GGCGTACAGCTTGCAT	<i>MgGpa2</i>
<i>MgGpa2</i> -R2	CATCAAGTTATGCAAGTTCCGC	<i>MgGpa2</i>
<i>MgGpa3</i> -F2	AGCAGTGGAACAAATGAGGAG	<i>MgGpa3</i>
<i>MgGpa3</i> -R4	TGAATACTGAGCTGGCCCAT	<i>MgGpa3</i>
<i>MgGpb1</i> -F4	ACCACGAACAAAGTACACGCC	<i>MgGpb1</i>
<i>MgGpb1</i> -R4	AGTCGACCTGAGACGGAGAAAG	<i>MgGpb1</i>
Primer S	ATAATCCTTAAAAACTCCATTCCACCCCT	Left border of transposon (Tn7L)
Primer N	ACTTTATTGTCATAGTTAGATCTATTTG	Right border of transposon (Tn7R)

described previously (Mehrabi et al. 2006). The exact position of the transposon insertion was determined by sequencing from the right and the left borders of the transposon using primer N and primer S and comparison with non-disrupted sequences. We selected constructs with transposon insertions in the central part of the WT genes that were designated pCGN1589Δ*MgGpa1*, pCGN1589Δ*MgGpa2*, pCGN1589Δ*MgGpa3* and pCGN1589Δ*MgGpb1*, respectively.

### Fungal transformation

The disruption constructs pCGN1589Δ*MgGpa1*, pCGN1589Δ*MgGpa2*, pCGN1589-Δ*MgGpa3* and pCGN1589Δ*MgGpb1* were cloned into *A. tumefaciens* strain LBA1100 by electroporation. *A. tumefaciens*-mediated transformation was performed according to the standard protocol (Zwiers and de Waard 2001; Mehrabi et al. 2006). Homologous recombinants were identified by PCR screening (Fig. 2B) using primers that were designed upstream and downstream of the transposon insertions together with either primer S or primer N that were located in left and right border of the transposon, respectively (Table 2).

### Phenotyping

We phenotyped each gene disruptant using two or three independent mutants and ectopic transformants and IPO323 (WT) as control strains. *In vitro* colony development assays were performed by spotting approximately 1 µl of spore suspension ( $10^7$  spores.ml<sup>-1</sup>)

on PDA, which was subsequently cultured for ten days at 20 °C under continuous light or 28 °C in darkness. Histological analyses were performed on WA and PDA (This thesis, chapter 4 and 5) and the effect of cAMP on the development of the strains was examined by adding cAMP (at final concentration of 15 mM) to PDA. Samples were monitored every 24 h and observed with a Zeiss Axioskop microscope (Carl Zeiss, Jena, Germany) equipped with a Carl Zeiss AxioCam 6.08.0 digital camera connected to an IBM PC. 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 carried out according to a previously described protocol with a minor modification (This thesis, chapter 4 and 5). Disease progress was monitored at various time points after inoculation and final observations were performed at 20 dpi.

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## Chapter 6

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# **CHAPTER 7**

**General discussion**

## Introduction

The interaction between a plant and a pathogen is a balanced process that requires complex fine regulations and physiological adaptations. Prior to invasion of a host by a pathogen, distant communications lead to activation or suppression of numerous genes that eventually determine whether a plant is susceptible or resistant. In a compatible interaction, pathogens are able to circumvent or avoid the resistance responses of plants, resulting in invasive colonization of the host tissue.

These dynamic interactions require proper regulation of perception and transduction of signals. The aim of our research was to establish a better understanding of the role of signaling pathways in the life cycle and pathogenesis of *M. graminicola*. In this chapter we discuss the roles of various kinases as well as G proteins during development *in vitro* and *in planta* for fungal pathogens in general and for *Mycosphaerella graminicola* in particular.

### Targeted gene disruption in *M. graminicola*

High throughput functional analysis of genes requires the efficient generation of targeted mutants. Although efficient gene disruption in *M. graminicola* was described previously (Adachi et al. 2002; Zwiers and de Waard 2001), efficiency of generation of disruption constructs is often gene-dependent and is very time consuming. Development of the transposon mutagenesis system described in this thesis made the generation of disruption constructs more efficient and enabled the disruption of several genes in a coherent strategy (Mehrabi et al. 2005a). We directly transposed cDNAs that were inserted in a binary vector. After transposition, disruption constructs were identified using a general PCR screening protocol. The cloning steps and PCRs were independent of the targeted gene, which greatly increased efficiency of the procedure.

An efficient protocol to generate disruption constructs in combination with *Agrobacterium tumefaciens*-mediated transformation enabled us to simultaneously disrupt several genes in *M. graminicola*. The frequency of homologous recombination achieved in this study was strongly dependent on the targeted gene and ranged between ~0.5-56% (Table 1). It was shown previously that targeted gene disruption in *M. graminicola* is efficient as the homologous recombination frequencies for *MgAtr1*, *MgAtr2* and *MgAtr4* were 25, 44, and 75%, respectively (Stergiopoulos et al. 2003). In filamentous fungi and other organisms, relatively long flanking regions usually positively affect the frequency of homologous

Table 1. Homologous recombination frequency of the genes disrupted in *Mycosphaerella graminicola* in this study.

Gene	Number of transformants tested	Homologous recombination (%)
<i>MgFus3</i>	352	0.5
<i>MgSlt2</i>	30	7
<i>MgHog1</i>	24	33
<i>MgSte20</i>	24	17
<i>MgTpk2</i>	24	33
<i>MgBcy1</i>	24	20
<i>MgGpa1</i>	28	11
<i>MgGpa2</i>	25	56
<i>MgGpa3</i>	92	3
<i>MgGpb1</i>	47	4

recombination (Papadopoulou and Dumas 1997). Since all genes used in this study contained an almost equally sized flanking region (about 2 Kb), it is likely that additional factors affect homologous recombination. The divergent homologous recombination frequencies observed in our studies might be due to locus-specific effects as observed for other fungi and organisms (Bird and Bradshaw 1997; Papadopoulou and Dumas 1997). In addition, impaired growth caused by disruption of a particular gene may result in very small colonies that are masked during picking of transformants.

### Regulation of filamentation and dimorphic transition by signaling pathways

In some dimorphic fungal pathogens such as *Ustilago maydis* and *Candida albicans* the ability to switch from budding to filamentous growth is important for successful infection (Klose et al. 2004; Sonneborn et al. 2000). *M. graminicola* is a dimorphic pathogen with yeast-like and filamentous growth phases. Filamentous growth is regulated to invade the host through stomata. This unique dimorphic lifestyle, which is absent in most other important plant pathogenic fungi, makes *M. graminicola* an interesting model for studying the genes involved in dimorphic transition. In order to study the function of signaling genes in the regulation of the dimorphic transition, early colony development of mutants generated in this study was monitored over time. Our results showed that genes encoding the G $\alpha$  protein, *MgGpa2*, the p21-activated kinase (PAK), *MgSte20*, and the regulatory subunit of PKA, *MgBcy1*, are all dispensable for dimorphic transition. Although we did not examine the effect of disruption of the *MgFus3* and *MgSlt2* on the dimorphic switch, *in vitro*, scanning electron

microscopy and *in planta* light microscopy revealed no alternation in germination patterns, indicating that these genes are most likely not involved in the dimorphic transition.

Disruption of several genes affected the dimorphic switch and polarized growth either by inhibition or induction of yeast-like growth. Mutants of the G $\alpha$  protein-encoding genes (*MgGpa1* and *MgGpa3*) and the catalytic subunit-encoding gene of PKA (*MgTpk2*), displayed a high tendency for yeast-like growth on potato dextrose agar (PDA), but germinated like the wild-type strain on water agar (WA). All mutants abundantly produced yeast-like cells when grown in yeast glucose broth medium (YGB), except the *MgGpa1* mutants that grew filamentously. In YGB induced hyphal tip extension strongly suppressed the generation of yeast-like cells and resulted in a fluffy mycelium, indicating that *MgGpa1* is involved in polarized growth. The fact that the wild-type strain is able to quickly switch from yeast-like to filamentous growth and *vice versa* and that *MgGpa1* mutants have difficulty in these transitions could indicate that *MgGpa1* is distantly involved in the dimorphic switch.

Although the yeast-like cells of the *MgGpb1* mutants could switch to filamentous growth, the hyphae of *MgGpb1* grow very wavy on WA. The growth pattern of *MgGpb1* mutants on PDA is very unique when compared to all other mutants and the wild-type strain. Mutation of *MgGpb1* enhanced hyphal anastomosis resulting in a dense interconnected hyphal web that eventually developed into one or several mycelial clumps. Although both vegetative and sexual cell fusions occur in fungi, it is unclear whether these fusion processes share common mechanisms. In *Neurospora crassa*, *ham-1* and *ham-2* mutants were unable to undergo homokaryon and heterokaryon fusion (Wilson and Dempsey 1999; Xiang et al. 2002). The observed results on increased conjugation and anastomosis in *MgGpb1* mutants are similar to those observed with the deletion mutants of the G $\beta$  encoding gene in *Schizosaccharomyces pombe* that also stimulated conjugation under nutrient-rich conditions (Kim et al. 1996; Landry et al. 2000), indicating that G $\beta$  negatively regulates cell fusion in both fungi.

Unlike all other mutants and the wild-type strain, *MgHog1* mutants were unable to switch to filamentous growth on WA and exclusively grew via a yeast-like budding process that we designated micro-conidiation. Similar to our results, the *Hog1* orthologs in the dimorphic pathogens *C. albicans* and *Cryptococcus neoformans* were also involved in differentiation and virulence (Alonso et al. 1999; Bahn et al. 2005). However, disruption of the *Hog1* genes in *M. grisea* and *Colletotrichum lagenarium*, which have exclusively filamentous growth, had no severe effect on morphological differentiation and virulence

(Dixon et al. 1999; Kojima et al. 2004). These results suggest that *Hog1* orthologs play an important role in virulence and differentiation of dimorphic fungal pathogens.

## Signal transduction and virulence

The role of signaling pathways in virulence of plant pathogenic fungi such as *M. grisea*, *C. lagenarium* and *U. maydis* is well documented (Lengeler et al. 2000; Xu 2000). In the model plant pathogenic fungus *M. grisea* several genes in the MAP kinase (MAPK) and cAMP-dependent pathways are shown to be crucial in pathogenicity (Table 2). Most of these genes are either involved in appressorium formation or are required for the functionality of the appressorium. Since a functional appressorium is essential for direct penetration of the host plants, disruption of these genes resulted in non-pathogenic mutants. Therefore, the function of these genes in later stages of infection can be masked due to the fact that the penetration is blocked. Thus, to investigate whether a particular gene is exclusively required for appressorium formation, virulence assays using wounded tissue are required.

Unlike appressorium-forming pathogens, *M. graminicola* penetrates through stomata without differentiating specific infection structures (Duncan and Howard 2000; Kema et al. 1996; Mehrabi et al. 2006) and, hence, dissection of genes involved in the different stages of

Table 2. Comparison of *in planta* phenotypes of mutants in which orthologous genes were disrupted in *Mycosphaerella graminicola* and *Magnaporthe grisea*.

<i>Mycosphaerella graminicola</i>				<i>Magnaporthe grisea</i>			
Gene	Function	Pathogenicity	Reference	Gene	Function	Pathogenicity	Reference
<i>MgFus3</i> <sup>1</sup>	Stomatal Penetration	Non-pathogenic	This thesis	<i>Pmk1</i>	AP <sup>2</sup> formation	Non-pathogenic	Xu and Hamer 1996
<i>MgSlt2</i>	Invasive growth	Reduced	This thesis	<i>Mps1</i>	AP function	Non-pathogenic	Xu et al. 1998
<i>MgHog1</i>	Dimorphic switch	Highly-Reduced	This thesis	<i>Osm1</i>	Not studied	Normal	Dixon et al. 1999
<i>MgSte20</i>	No role in virulence	Normal	This thesis	<i>Mst20</i>	No role in virulence	Normal	Li et al. 2004
<i>MgTpk2</i>	Pycnidia formation	Reduced	This thesis	<i>CpkA</i>	AP function	Non-pathogenic	Xu et al. 1997
<i>MgBcy1</i>	Pycnidia formation	Reduced	This thesis	<i>Sum1</i> <sup>3</sup>	-	-	Adachi and Hamer 1998
<i>MgGpa1</i>	Not studied	Reduced	This thesis	<i>MagB</i>	AP formation <sup>4</sup>	Reduced	Fang and Dean 2000
<i>MgGpa2</i>	No role in virulence	Normal	This thesis	<i>MagC</i>	No role in virulence	Normal	Liu and Dean 1997
<i>MgGpa3</i>	Not studied	Reduced	This thesis	<i>MagA</i>	No role in virulence	Normal	Liu and Dean 1997
<i>MgGpb1</i>	Not studied	Reduced	This thesis	<i>Mgb1</i>	AP formation	Non-pathogenic	Nishimura et al. 2003

1. The orthologous genes for *M. graminicola* and *M. grisea* are presented in the same row.

2. AP= appressorium.

3. The regulatory subunit of PKA, *Sum1*, was mutated in the genetic background where the catalytic subunit-encoding gene (*CpkA*) was also disrupted. No single gene mutant for *Sum1* has been reported.

4. Reduced and disturbed appressorium formation.

infection can be studied without using wounded tissue. This makes *M. graminicola* an appropriate model to study pathogenicity factors, particularly in the Dothideomycetes. Therefore, we were interested in the identification and characterization of genes involved in different stages of infection. Our results showed that several genes, including three MAPK-encoding genes (*MgFus3*, *MgSl2* and *MgHog1*), the regulatory and the catalytic subunit PKA-encoding genes (*MgTpk2* and *MgBcy1*), two G $\alpha$  protein-encoding genes (*MgGpa1* and *MgGpa3*) and the G $\beta$  protein-encoding gene (*MgGpb1*) are required for pathogenicity, whereas the PAK-encoding gene, *MgSte20*, and the G $\alpha$  protein-encoding gene, *MgGpa2*, are dispensable for pathogenicity in *M. graminicola*.

In addition, we showed that these genes are involved in different stages of the infection process, as revealed by detailed *in planta* cytological analyses (Fig. 1). The *MgHog1* mutants failed to infect wheat leaves, due to impaired initiation of infectious germ tubes, indicating the essential role of *MgHog1* in dimorphic transition and pathogenicity (This thesis, chapter 4). Similar to the wild-type strain, the *MgFus3* mutants germinated on the leaf surface but could not penetrate the stomata and, hence, did not colonize the mesophyll tissue. The loss of pathogenicity in *MgFus3* mutants was due to an impaired penetration, suggesting a role for this gene in the perception of host tissue (Mehrabi et al. 2004).

*MgSl2* mutants germinated and penetrated wheat stomata normally, but infectious germ tubes were unable to establish invasive growth in the mesophyll, resulting in highly reduced virulence. The fact that *MgSl2* is also involved in cell wall strengthening may suggest that the

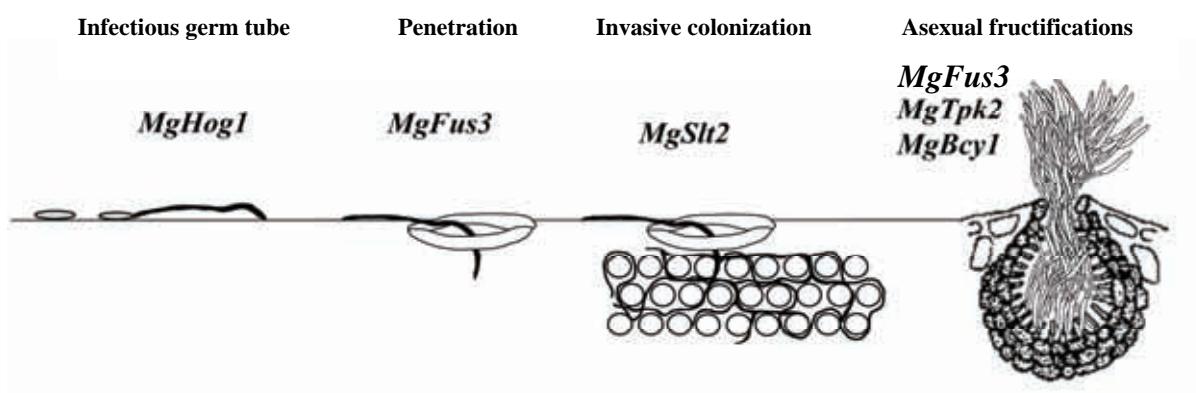


Fig. 1. Genes that are implicated in the regulation of different stages during the infection process.

attenuated virulence is due to an increased sensitivity to hitherto unknown plant defense compounds (Mehrabi et al. 2006; Mehrabi et al. 2005b).

Mutants of the catalytic and regulatory subunit-encoding genes of PKA (*MgTpk2* and *MgBcy1*) showed reduced virulence. Mutants of *MgTpk2* and *MgBcy1* were able to germinate, penetrate and colonize mesophyll tissue, but were unable to produce the asexual fructifications (This thesis, chapter 5). Virulence assays showed that the *MgGpa1*, *MgGpa3* and *MgGpb1* mutants were highly reduced in virulence but the temporal aspects were not studied.

### The cAMP-dependent PKA pathway is regulated by G proteins

Regulation of the cAMP pathway in *Saccharomyces cerevisiae* and *S. pombe* is well documented (for review see (Hoffman 2005). In *S. cerevisiae* the main components of the cAMP pathway are a G protein-coupled receptor (Gpr1), the monomeric G $\alpha$  protein (Gpa2), the Ras2 protein, adenylyl cyclase (Cyr1), and the regulatory and catalytic subunits of PKA (Bcy1 and Tpk1/2/3). The *S. pombe* cAMP signaling pathway involves the receptor Git3, a heterotrimeric G protein composed of the G $\alpha$  (Gpa2), the G $\beta$  (Git5), and the G $\gamma$  (Git11) proteins. In *S. pombe*, adenylyl cyclase and the regulatory and catalytic subunit of PKA (Cgs1 and Pka1, respectively) are downstream effectors of the G proteins and regulate Ste11 and Fbp1 (Fig. 2). Despite the fact that these two yeasts are phylogenetically closely related, their cAMP pathways are substantially different. In *S. cerevisiae*, cAMP signaling is mediated by a monomeric G $\alpha$  protein (Gpa2) and the Ras2 protein. However, in *S. pombe* the G $\alpha$  and G $\beta$  proteins (in a heterotrimeric composition) co-operatively activate the downstream effector adenylyl cyclase, and the Ras2 protein does not play a role in the regulation of adenylyl cyclase (Fig. 2).

Unlike *S. pombe* and *S. cerevisiae*, the genome of *M. graminicola* contains three genes encoding G $\alpha$  proteins: MgGPA1, MgGPA2 and MgGPA3. MgGPA1 contains a consensus myristoylation site at the N-terminus and a consensus pertussis toxin-labeling site at the C-terminus (This thesis, chapter 6). These are characteristic features of members of a specific class of G $\alpha$  proteins in mammals known as inhibitory G (G $\alpha_i$ ) proteins (Boelker 1998). The fact that *MgGpa1* mutants did not respond to cAMP may indicate that MgGPA1 is functionally similar to the mammalian G $\alpha_i$  family and is likely a negative regulator of adenylyl cyclase. More importantly, highly enhanced filamentation observed for the *MgGpa1*

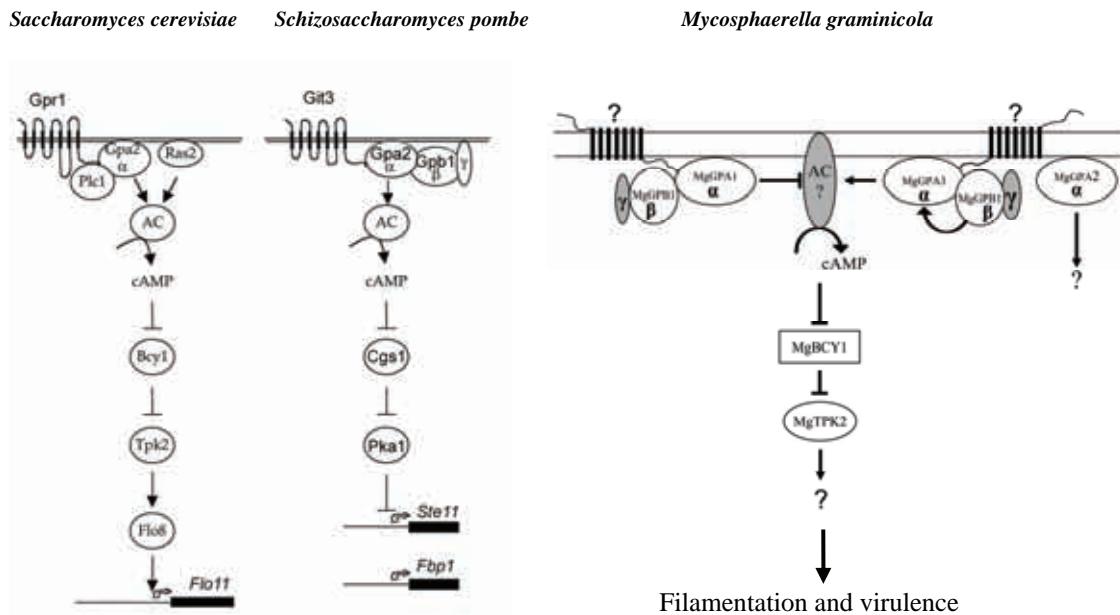


Fig. 2. Regulation of the cAMP pathway in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [adapted from Lengeler et al. (2000)] and proposed cAMP pathway in *Mycosphaerella graminicola*. The genes encoding G $\gamma$  and adenylyl cyclase (gray boxes) have not been characterized yet. In contrast to MgGPA3 and MgGPB1, MgGPA1 is a negative regulator of the cAMP pathway.

mutants suggests that MgGPA1 negatively regulates filamentation, which is in agreement with our finding that the cAMP pathway controls filamentation in *M. graminicola* (Fig. 2).

MgGPA2 has no mammalian counterpart and the mutants of this gene did no show any phenotype indicating that this gene most probably has no significant role in the life cycle of *M. graminicola*.

MgGPA3 is similar to mammalian stimulating G $\alpha$  proteins commonly known as G $\alpha_s$  proteins that positively regulate adenylyl cyclase and increase the internal cAMP level. Compatible with the stimulating function of mammalian G $\alpha_s$  proteins, disruption of *Gna3* in *N. crassa* (Kays et al. 2000) and of *Cpg-2* in *C. parasitica* (Gao and Nuss 1996) decreased adenylyl cyclase activity and consequently reduced intracellular cAMP levels. *MgGpa3* mutants showed a phenotype similar to mutants of the PKA catalytic subunit-encoding gene (*MgTpk2*) of *M. graminicola* (This thesis, chapter 5). Disruption of *MgGpa3* and *MgTpk2* resulted in secretion of dark-brown pigment into YGB and hampered filamentation on PDA. In addition, filamentation in *MgGpa3* mutants could be restored to the wild-type by exogenous cAMP. The presence of an adenylyl cyclase-stimulating domain in the MgGPA3

protein, the response of *MgGpa3* mutants to exogenous cAMP, the high phenotypic similarity of *MgGpa3* and *MgTpk2* mutants and the hampered filamentation of *MgGpa3* mutants suggest that MgGPA3 functionally and evolutionary belongs to the class of G $\alpha_s$  proteins (Fig. 2). Furthermore, mutation of *MgGpb1* hampered filamentation, which could be restored by exogenous cAMP suggesting that MgGPB1 positively regulates the cAMP pathway co-operatively with MgGPA3. In *S. pombe*, G $\alpha$  (Gpa2) and G $\beta$  (Git5) are also required for activation of adenylyl cyclase (Landry et al. 2000). Therefore, we suggest that a putative adenylyl cyclase in *M. graminicola* is positively regulated by heterotrimeric G $\alpha$  and G $\beta$  proteins, in a similar way as in *S. pombe* (Fig. 2). This hypothesis is also supported by our finding that *MgGpb1* mutants had increased cell conjugation, a phenomenon that was also observed for G $\beta$  encoding-gene mutants of *S. pombe* (Kim et al. 1996; Landry et al. 2000).

### The complex regulation of different signaling pathways in melanin biosynthesis

Melanin is a dark-pigmented polymer and one of the well-known secondary metabolites produced by many fungi that usually accumulates in fungal cell walls and appressoria. Melanin is considered to provide protection from various environmental stress factors such as UV radiation (Bell and Wheeler 1986; Butler and Day 1998; Henson et al. 1999). The melanin biosynthesis pathway starts with production of pentaketide resulting in 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN). The following steps include synthesis of scytalone, 1,3,8-trihydroxynaphthalene (1,3,8-THN), vermelone and 1,8-dihydroxynaphthalene (DHN) (Fig. 3). DHN is then polymerized and oxidized to melanin (Moriwaki et al. 2004; Perpetua et al. 1996; Tsuji et al. 2003; Wang and Breuil 2002) (Fig. 3). Although disruption of a polyketide synthase (*PKS*) gene in *M. graminicola* did not influence melanization, we speculate that this is due to redundancy of *PKS* genes in *M. graminicola* (Mehrabi et al. 2006, unpublished).

In this thesis we revealed impaired melanization upon disruption of three MAPK-encoding genes (*MgFus3*, *MgSl2* and *MgHog1*) and two G protein-encoding genes (*MgGpa1* and *MgGpb1*). In addition, the melanization pattern of *MgGpa3*, *MgTpk2* and *MgBcy1* mutants was significantly altered compared to the wild-type strain under several different experimental conditions. These results may suggest that signaling pathways in *M. graminicola* regulate melanin production.

The expression of three melanin biosynthesis genes during early germination of conidia in the *Fus3* ortholog mutants of *C. lagenarium* was significantly reduced, suggesting the

involvement of the *Fus3* ortholog in appressorial melanization. Expression of these genes during mycelial melanization in the mutants was not affected, suggesting differential or

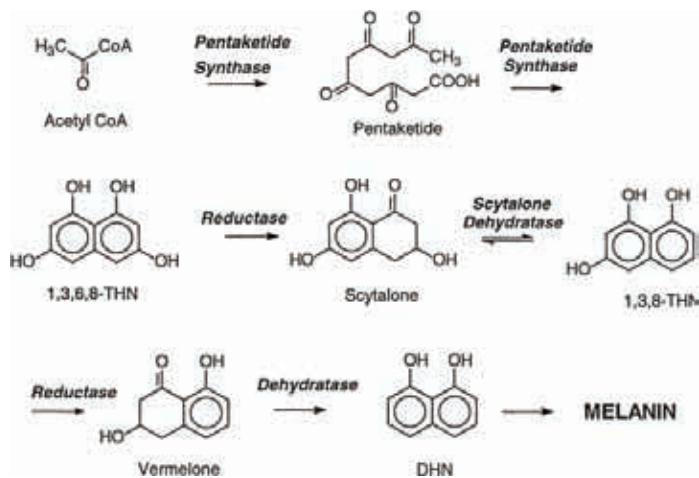


Fig. 3. Melanin biosynthesis pathway in fungi. Adapted from Wang et al. (2002).

developmental regulation of the melanin biosynthesis genes by the *Fus3* ortholog in this fungus (Takano et al. 2000).

As mentioned before mutation in *MgGpa1*, *MgGpb1*, *MgFus3*, *MgSlt2* and *MgHog1* also strongly affected melanization in *M. graminicola*. Therefore, it might be interesting to monitor the expression profile of melanin biosynthesis genes in melanized and non-melanized *M. graminicola* mutants. This might reveal whether there is post-transcriptional or post-translational regulation of the melanin biosynthesis pathway. Strikingly, the non-melanized phenotype of *MgGpa1*, *MgGpb1* and *MgHog1* mutants could be restored when the mutants were grown at an elevated temperature. In addition, hyperosmotic conditions suppressed melanization of all strains including the wild-type strain, suggesting that the melanin biosynthesis in *M. graminicola* likely is a complex process involving cross-talk between the signaling and biosynthesis pathways that can also be affected by environmental conditions.

The necessity of melanin for pathogenicity of several plant pathogenic fungi has been well documented (Kawamura et al. 1997; Kubo et al. 1991). For example, in appressorium-forming pathogens such as *M. grisea* and *C. lagenarium*, melanization is extremely important for the generation of turgor pressure and mechanical penetration of epidermis cells (Money and Howard 1996; Perpetua et al. 1996; Talbot 2003). Unlike appressorium-forming pathogens, *M. graminicola* enters its host through stomata and, hence, impaired melanization does not affect penetration. Indeed, non-melanized mutants of *MgSlt2* and the wild-type strain

were equally effective in entry of the host (Mehrabi et al. 2006), which suggest that melanization is a not crucial factor for the early stage of infection. However, it has been shown that pycnidia of *M. graminicola* are heavily melanized (Duncan and Howard 2000; Kema et al. 1996; Mehrabi et al. 2006), indicating that melanization is required for stroma formation and/or (a)sexual fructification. The requirement of *MgFus3* for pycnidia formation *in vitro* corroborates this hypothesis (This thesis, chapter 2).

### Concluding remarks

The fungal kingdom includes a range of extremely diverse organisms, from saprophytes to animal and plant pathogens that have completely different lifestyles. Several phytopathogenic fungi such as *M. grisea* and *U. maydis* have been extensively studied to identify the role of signaling pathways in virulence and development. Here we have characterized highly conserved genes involved in the major signaling pathways and have shown that these genes have distinct biological functions in development and pathogenic growth of *M. graminicola*. We conclude that *M. graminicola* is rapidly developing as a new model among plant pathogenic fungi.

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# **APPENDIX**

**Summary in English**

**Summary in Dutch**

**Summary in Farsi**

**Acknowledgments**

**About the author**

**List of publications**

**Education certificate of the EPS graduate school**

**Title page in Farsi**

**Color figures**

## SUMMARY

*Mycosphaerella graminicola* (Fuckel) J. Schröt is the causal agent of septoria tritici leaf blotch, which is the major foliar wheat disease in most temperate areas. Direct losses and the cost of control strategies contribute to the huge economical importance of this disease. *M. graminicola* is a dimorphic fungal pathogen that grows in yeast-like or filamentous fashion depending on the environmental conditions. Filamentation is required for pathogenicity of this non-appressorium forming pathogen, which has a hemibiotrophic lifestyle characterized by an initial biotrophic phase (about 10 days) that is followed by a necrotrophic phase during which numerous asexual and eventually sexual fructifications are produced.

This thesis is part of an ongoing research program aimed at understanding the genetic and molecular control of pathogenicity. The research was initiated with the generation of EST and genomic sequence data of the pathogen. This thesis describes the functional analyses of 10 genes encoding proteins involved in signal transduction pathways operating in *M. graminicola* when grown *in vitro* and *in planta*. **Chapter one** gives an introduction to the research topic. In **chapter two**, the role of the mitogen-activated protein kinase (MAPK)-encoding gene, *MgFus3*, the ortholog of *Fus3* controlling mating in *Saccharomyces cerevisiae*, is described. Disruption of this gene prevented melanization of mycelia and formation of pycnidia *in vitro*. *MgFus3* mutants are non-pathogenic. This is ascribed to impaired penetration of stomata, possibly due to inability of the mutants to recognize stomata.

**Chapter three** describes the MAPK-encoding gene, *MgSlt2*. This is the ortholog of *Slt2* in *S. cerevisiae* that regulates cell wall biosynthesis. In *M. graminicola*, *MgSlt2* plays a role in cell wall integrity since *MgSlt2* mutants were affected in polarized growth and showed progressive autolysis during aging. They were also hypersensitive to glucanase and several fungicides and did not produce aerial mycelium or melanin on potato dextrose agar (PDA). Pathogenicity assays revealed that virulence of *MgSlt2* mutants was severely reduced. Electron microscopy and histopathological analyses showed that *MgSlt2* mutants penetrated wheat stomata regularly, but were unable to establish invasive growth and did not produce asexual fructifications. Because *MgSlt2* is involved in cell wall integrity, *MgSlt2* mutants are probably more sensitive to hitherto unknown plant defense compounds, which might explain the compromised colonization of mesophyll tissue.

**Chapter four** describes characterization of MAPK-encoding gene, *MgHog1*, and the p21-activated kinase (PAK)-encoding gene, *MgSte20*. *MgHog1* mutants were osmosensitive, highly resistant to the fungicides fludioxonil, fenpiclonil and iprodione did not melanize and

were unable to switch from yeast-like to filamentous growth. As a result of the impaired dimorphic switch, *MgHog1* mutants were unable to establish infectious germ tubes and therefore failed to penetrate wheat leaves. This demonstrates that dimorphic transition is a key factor in pathogenicity of *M. graminicola*. Phenotypes of *MgSte20* mutants were identical to the wild-type isolate in all tested conditions.

Fructification of *M. graminicola* is a complex process requiring differentiation of the infectious hyphae in the substomatal cavities during the latter stages of infection. In **Chapter five**, functional analyses of genes encoding the catalytic (*MgTpk2*) and the regulatory (*MgBcy1*) subunits of PKA showed that these genes are essential for asexual fructification. *MgTpk2* and *MgBcy1* mutants were able to germinate, penetrate and colonize mesophyll tissue, but were unable to differentiate pycnidia. Our data provide evidence that the cAMP pathway regulates filamentation through *MgTpk2* and *MgBcy1*. Disruption of *MgTpk2* impaired filamentation. In addition, the *MgTpk2* mutants became melanized faster and secreted a dark-brown pigment into yeast glucose broth medium (YGB), whereas *MgBcy1* mutants showed delayed melanization on PDA and were osmosensitive. Overall, the divergent functions of the regulatory and the catalytic subunits of PKA indicate that proper regulation of PKA activity is required for various physiological processes including differentiation, filamentation, osmoregulation and melanization.

**Chapter six** describes the characterization of three G $\alpha$  protein-encoding genes (*MgGpa1*, *MgGpa2* and *MgGpa3*) and one G $\beta$  protein-encoding gene (*MgGpb1*) in *M. graminicola*. Phylogenetic comparisons and sequence analyses of G $\alpha$  proteins of *M. graminicola* revealed that MgGPA1 and MgGPA3 can be categorized as homologs of the mammalian G $\alpha_i$  and G $\alpha_s$  families, respectively, whereas MgGPA2 is unrelated to mammalian G $\alpha$  proteins. *MgGpa1*, *MgGpa3* and *MgGpb1* mutants exhibited different phenotypes when grown on PDA at 20 °C that were not observed when the temperature was raised to 28 °C. Melanization did not occur in the *MgGpa1* and *MgGpb1* mutants, and the former formed fluffy mycelia in YGB and hardly produced spores. *MgGpb1* mutants showed a nested type of growth on PDA that resulted from hampered filamentation, numerous cell fusions and increased anastomosis. Therefore, we concluded that *MgGpa1* negatively regulates filamentation, which is positively regulated by *MgGpa3* and *MgGpb1*. Interestingly, unlike the response of the *MgGpa1* mutants, exogenous cAMP restored the phenotype of the wild-type in the *MgGpb1* and *MgGpa3* mutants, indicating a stimulating function for MgGPB1 and MgGPA3 and an inhibitory function for MgGPA1 in the regulation of the cAMP pathway.

Pathogenicity assays revealed that *MgGpa1*, *MgGpa3* and *MgGpb1* are required for virulence of *M. graminicola* whereas *MgGpa2* is dispensable.

Finally, in **chapter seven** the results described in this thesis are discussed in a broader perspective. The roles of the various kinases as well as G proteins during development *in vitro* and *in planta* for fungal pathogenesis in general and for *M. graminicola* in particular are highlighted. Also, the potential of *M. graminicola* as a new model fungus is addressed. The power of comparative genomics can be fully exploited once the genomic sequence of the fungus is available.

## SAMENVATTING

*Mycosphaerella graminicola* (Fuckel) . Schröt is de verwekker van septoria tritici bladvlekkenziekte, de belangrijkste schimmelziekte van tarwe in gematigde gebieden. Opbrengstverliezen en de kosten van gewasbeschermingsmaatregelen dragen bij aan de enorme economische betekenis van deze ziekte. *M. graminicola* is een pathogene schimmel die, afhankelijk van de omstandigheden, dimorfisme vertoont en als een gist of een filamentuze schimmel kan groeien. Filamentuze groei is noodzakelijk voor pathogeniteit van dit niet appressorium-vormende pathogeen, die een hemibiotrofe levensvorm heeft die zich kenmerkt door een initiële biotrofe fase (één of twee weken), en gevolgd wordt door een necrotische fase waarin zich talloze aseksuele en uiteindelijk geslachtelijke vruchtlichamen vormen.

Dit proefschrift maakt deel uit van een doorlopende strategie die gericht is op het begrijpen van de genetische en moleculaire regulering van pathogeniteit. Het onderzoek werd geïnitieerd door EST en genomische sequentiegegevens van dit pathogeen. Dit proefschrift beschrijft de functionele analyse van 10 genen die coderen voor eiwitten die betrokken zijn bij de signaaltransductie tijdens *in vitro* en *in planta* ontwikkeling van *M. graminicola*. **Hoofdstuk 1** is een introductie van het onderzoeksonderwerp. In **hoofdstuk 2** wordt de rol beschreven van het mitogen geactiveerde proteïne kinase (MAPK)-coderende gen *MgFus3*, een ortholoog van het *Fus3* gen, dat de paring in de gist *Saccharomyces cerevisiae* reguleert. Uitschakeling van dit gen voorkomt melanisering van het mycelium en de sporen en de *in vitro* vorming van pycnidiën. *MgFus3* mutanten zijn niet pathogeen. Dit wordt toegeschreven aan de geblokkeerde penetratie van de huidmondjes, die waarschijnlijk samenhangt met de verstoorde herkenning van deze structuren.

**Hoofdstuk 3** beschrijft het MAPK-coderende gen *MgSlt2*. Dit is een ortholoog van het *Slt* gen in *S. cerevisiae* dat de synthese van de celwand reguleert. *MgSlt2* speelt een rol in de celwandintegriteit van *M. graminicola* hetgeen blijkt uit het feit dat *MgSlt2* mutanten tijdens veroudering geen polaire groei en een toenemende celwandafbraak vertoonden. Deze mutanten waren ook overgevoelig voor glucanase en verschillende fungiciden en produceerden geen luchtmycelium of melanine op aardappel dextrose agar (PDA). Pathogeniteitsproeven toonden de sterk gereduceerde virulentie van de *MgSlt* mutanten aan. Elektronenmicroscopische en histologische analyses lieten zien dat de *MgSlt* mutanten de huidmondjes wel penetreerden, maar niet in staat waren om verder te koloniseren en derhalve geen aseksuele vruchtlichamen vormden. Omdat *MgSlt2* betrokken is bij de celwandintegriteit

zijn *MgSlt2* mutanten waarschijnlijk gevoeliger voor tot nu toe onbekende afweerstoffen van de plant, die de verminderde kolonisering van het mesophyl weefsel kunnen verklaren.

**Hoofdstuk vier** beschrijft de karakterisering van het MAPK-coderende gen *MgHog1* en *MgSte20*, het gen dat codeert voor het door p21 geactiveerde kinase (PAK). *MgHog1* mutanten zijn gevoelig voor osmose en resistent tegen de fungiciden fludioxonil, fenpiclonil en iprodione. Verder melaniseren zij niet en zijn zij niet in staat om van de gistvorm naar filamenteuze groei over te schakelen. Dit resulteert in een geblokkeerd dimorfisme die de infectieuze groei van kiembuizen van *MgHog1* mutanten verhinderd waardoor zij tarwebladeren niet kunnen penetreren. Dit demonstreert dat de omschakeling van de gistvorm naar filamenteuze groei een cruciale rol speelt in de pathogeniteit van *M. graminicola*. Fenotypen van *MgSte20* mutanten waren in alle testen identiek aan de wild type isolaten.

De vorming van vruchtlichamen door *M. graminicola* is een complex proces dat differentiatie van de infectieuze hyfen in de substomatale kamers gedurende de latere stadia van infectie vereist. In **hoofdstuk vijf** werd aangetoond dat de coderende genen voor de katalytische (*MgTpk2*) en de regulerende (*MgBcy1*) subunit van het PKA gen een essentiële rol spelen bij de vorming van vruchtlichamen. *MgTpk2* en *MgBcy1* mutanten kunnen kiemen, penetreren en het mesofyl weefsel koloniseren, maar zijn niet in staat om pycnidiën te vormen. Onze gegevens verschaffen aanwijzingen dat de cAMP route de filamenteuze groei via *MgTpk2* en *MgBcy1* reguleert. Uitschakeling van *MgTpk2* leidt tot een verstoorde filamenteuze groei. Tevens vertoonden *MgTpk2* mutanten een versnelde melanisering en scheidden zij een donkerbruin pigment af in gist glucose medium (YGB), terwijl *MgBcy1* mutanten juist een vertraagde melanisering vertoonden op PDA en osmosegevoelig waren. Samenvattend kan worden gesteld dat de uiteenlopende functies van de regulerende en de katalyserende subunit van het PKA gen aantonen dat de juiste regulering van PKA activiteit vereist is voor verschillende fysiologische processen zoals differentiatie, filamenteuze groei, gevoelheid voor osmose en melanisering.

**Hoofdstuk zes** beschrijft de karakterisering van drie genen die G $\alpha$  eiwitten in *M. graminicola* coderen (*MgGpa1*, *MgGpa2* en *MgGpa3*), en een gen dat een G $\beta$  eiwit (*MgGpb1*) codeert. Fylogenetische en sequentievergelijkingen van G $\alpha$  eiwitten in *M. graminicola* tonen aan dat MgGPA1 en MgGPA3, respectievelijk als homologen van G $\alpha_i$  en G $\alpha_s$  families in zoogdieren kunnen worden ingedeeld, terwijl MgGPA2 daar geen verwantschap mee vertoont. *MgGpa1*, *MgGpa3* en *MgGpb1* mutanten vertoonden verschillende fenotypen in een kweek op PDA bij 20°C, die niet werden waargenomen bij een

verhoging van de temperatuur tot 28 °C. In *MgGpa1* en *MgGpb1* mutanten trad geen melanisering op en *MgGpa1* mutanten produceerden nauwelijks sporen, maar vormden luchtmycelium in YGB. *MgGpb1* mutanten vertoonden een verstengeld groeipatroon op PDA als gevolg van verstoerde filamentatie, talloze celfusies en toegenomen anastomose. Wij hebben daarom geconcludeerd dat *MgGpa1* filamentatie negatief reguleert, terwijl *MgGpa3* en *MgGpb1* dit positief reguleren. In tegenstelling tot de reactie van de *MgGpa1* mutanten, herstelde exogeen toegediend cAMP het fenotype van het wild-type isolaat in de *MgGpb1* en *MgGpa3* mutanten, wat aangeeft dat MgGPB1 en MgGPA3 de cAMP route stimuleren en dat MgGPA1 deze route blokkeert. Pathogeniteitstoetsen toonden aan dat *MgGpa1*, *MgGpa3* en *MgGpb1* noodzakelijk zijn voor de virulentie van *M. graminicola* terwijl *MgGpa2* gemist kan worden.

Tenslotte worden in **hoofdstuk zeven** de resultaten die in dit proefschrift beschreven worden in een breder perspectief geplaatst. De rol van de verschillende kinasen en de G proteïnen tijdens de *in vitro* en *in planta* ontwikkeling en de pathogeniteit van schimmels wordt zowel in algemene zin als in het bijzonder voor *M. graminicola* besproken. Tevens wordt de potentie van *M. graminicola* als nieuwe model schimmel naar voren gebracht. De kracht van vergelijkende genomica kan volledig uitgebuit worden zodra de genoomsequentie van deze schimmel beschikbaar is.

## خلاصه:

بیماری سپتومیکوز برگی گندم که عامل آن قارچ *Mycosphaerella graminicola* است مهمترین بیماری گندم در بسیاری از مناطق با آب و هوای معتدل می‌باشد. هزینه‌های کنترل و خسارت مستقیم این بیماری بسیار قابل توجه می‌باشد. قارچ عامل بیماری بسته به شرایط محیطی به صورت مخمری<sup>۱</sup> و یا میسلیومی<sup>۲</sup> رشد می‌کند. چرخه بیماری با نفوذ لوله تندش<sup>۳</sup> قارچ به بافت‌های گیاه میزبان از طریق منافذ طبیعی مانند روزنه‌ها شروع می‌گردد. این قارچ برای ایجاد آلودگی اندامهای اختصاصی مانند اپرسوریوم<sup>۴</sup> تولید نمی‌نماید. قارچ عامل بیماری از نظر زیستی نیمه بیوتروفیک بوده و دوره بیماری شامل یک مرحله بیوتروفی است که در حدود ده روز طول می‌کشد. پس از این دوره، مرحله نکروتروفی آغاز که طی آن سلولهای گیاه متلاشی و در نهایت تعداد بسیار زیادی اندامهای غیر جنسی قارچ به نام پیکنید تولید می‌شود. تولید پیکنید در این قارچ که در مراحل آخر بیماریزایی اتفاق می‌افتد یک مرحله پیچیده بوده که تیاز به تمایز و تکامل سلولی و میسلیومی قارچ در اتاق زیر روزنه‌ها در بافت گیاه دارد.

در این تحقیق ده ژن دخیل در مسیرهای انتقال سیگنال<sup>۵</sup> مورد شناسایی و کلون سازی قرار گرفته و با ایجاد موتاسیون نقش آنها در بیماریزایی و رشد و نمو و تمایز قارچ بررسی گردید. در فصل اول این رساله مسیرهای انتقال سیگنال در قارچها و اهداف این تحقیق به طور خلاصه تشریح شده است.

در فصل دوم ژن *MgFus3* مورد مطالعه قرار گرفته است. سویه‌های تغییر یافته ژنتیکی که با موتاسیون در این ژن ایجاد شدند کاملاً غیر بیماریزا بوده و در محیط آزمایشگاهی قادر به تولید پیکنید و ملانین نبودند. عدم ایجاد بیماری بوسیله این موتانت‌ها بدلیل ناتوانی آنها در نفوذ به داخل بافت‌های گیاه بوده و بنابراین احتمالاً این ژن در شناسایی بافت و یا روزنه‌های گیاه دخالت دارد.

در فصل سوم نقش ژن *MgSlc2* مورد مطالعه قرار گرفته است. موتانت‌ها ایجاد شده در محیط آزمایشگاهی قادر به تولید ملانین نبوده و در مقایسه با سویه وحشی<sup>۶</sup> به آنزیم هضم کننده دیواره سلولی (glucanase) و تعدادی از قارچکش‌ها حساسیت بیشتری نشان دادند. نتایج آزمایشات نشان داد که این ژن در تنظیم ساختن دیواره سلولی نقش داشته و همچنین بیماریزایی موتانت‌های ایجاد شده به شدت کاهش پیدا کرد. آزمایشات میکروسکوپی نشان داد که این موتانت‌ها مانند سویه وحشی قادر به نفوذ به درون میزبان از طریق روزنه‌ها بوده اما در مرحله بعد قادر به کلونیزه کردن بافت گیاه نمی‌باشند. شواهد غیر مستقیم دال بر حساسیت بیش از حد این موتانت‌ها به ترکیبات دفاعی ناشناخته ای که احتمالاً از طرف سلولهای گیاه علیه عوامل بیماریزا تولید می‌شود نشان دهنده نقش دیواره سلولی قارچها در حفاظت سلولی می‌باشد.

در فصل چهارم نقش دو ژن *MgHog1* و *MgSte20* در بیماریزایی، رشد و تمایز سلولی مورد بررسی قرار گرفته است. موتانت‌های *MgHog1* در شرایط بافشار اسمزی بالا قادر به رشد نبوده، به تعدادی قارچکش مقاوم بوده، ملانین تولید نکرده و همچنین قادر به تمایز از مرحله مخمری به مرحله میسلیومی نبودند. آزمایشات میکروسکوپی نشان

<sup>1</sup> Yeast-like<sup>2</sup> Filamentation<sup>3</sup> Germ tube<sup>4</sup> Appressorium<sup>5</sup> Signal transduction pathways<sup>6</sup> Wild-type strain

داد که این موتانت ها توانایی تمایز لوله تندش و رخنه به درون بافت های گیاهی را نداشتند. بین موتانت های ژن *MgSte20* با سویه وحشی هیچگونه تفاوتی مشاهده نگردید.

در **فصل پنجم** دو ژن *Mg2* و *MgBcy1* و *MgTpk2* مورد بررسی قرار گرفته است. بررسی های انجام شده بیانگر نقش این دو ژن در تولید پیکنید می باشد. آزمایش های بیماریزابی و میکروسکوپی نشان داد که موتانت های این دو ژن مانند سویه وحشی توانایی جوانه زنی، نفوذ به درون بافت گیاه و کلونیزه کردن بافت گیاه را داشته اما درنهایت قادر به تولید و تمایز پیکنید نبوده و بنابراین بیماریزابی این موتانت ها کاهش پیدا کرد. علاوه بر این نقش این ژنها درکنترل تولید ملانین، رشد در شرایط با فشار اسمزی بالا و تمایز از مرحله مخرمی به مرحله میسلیومی کشف گردید.

در **فصل ششم چهار ژن** *MgGpa1*, *MgGpa2*, *MgGpa3*, *MgGpb1* مورد بررسی قرار گرفته است. نتایج آزمایشات نشان داد که ژن *MgGpa1* به طور منفی میسلیوم زایی را کنترل می کند و بنابراین موتانت های این ژن بندرت تولید اسپور های مخرمی می کردند. بر عکس، ژنهای *MgGpb1* و *MgGpa3* میسلیوم زایی را تقویت کرده و بنابراین موتانت های این ژنها بیشتر از طریق مخرمی رشد کردند. همچنین موتانت های ژن *MgGpb1* بشدت تمایل به همد瀚ی<sup>7</sup> داشتند. علاوه بیماریزابی موتانت های *MgGpa1*, *MgGpa3*, *MgGpb1* به شدت کاهش پیدا کرد. موتانت های ژن *MgGpa2* هیچگونه تفاوتی با سویه وحشی نداشتند. همچنین بر اساس نتایج بدست آمده در این فصل و فصل پنجم یک مدل برای مسیر انتقال سیگناال که در آن پروتئین های تولید شده توسط این ژنها دخالت دارند پیشنهاد گردیده است.

در **فصل هفتم** نتایج بدست آمده از این تحقیق در بعد از وسیع تر و مقایسه ای مورد بحث قرار گرفته و همچنین اهمیت این قارچ به عنوان یک مدل قارچی جدید تشریح گردیده است.

با تشکر از نظرات ارزنده دوستان گرامی آقای حسین جعفری و آقای رامین روح پروردر ویراستاری چکیده فارسی

<sup>7</sup> Anastomosis

## ACKNOWLEDGMENTS

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## **ABOUT THE AUTHOR**

Rahim Mehrabi was born in 1973 in Kooshk, Isfahan, Iran. After completing high school in biological sciences in 1991, he began his studies at the University of Orumiyeh, (West Azarbayan) and obtained a BSc degree (with distinguished degree) in the field of Plant Protection in 1995. He continued his studies at Tarbiat Modarres University (Tehran, Iran) and completed his MSc in the field of Plant Pathology in 1997. After finishing his Master's studies, for completion the required military service, he worked at the Sadooghi Hospital in Isfahan for two years, and then in 2000 he was employed as the academic staff at the Seed and Plant Improvement Institute in Karaj. In 2002, he was awarded a scholarship from the Ministry of Science, Research and Technology (MSRT) financed by the Agricultural Research and Education Organization (AREO) of Iran, allowing him to do a PhD abroad. In April 2002, he started his PhD program at Wageningen University and Research Center, Plant Research International and Laboratory of Phytopathology and initially started to clone an avirulence gene. Eventually, after one and half year he started to work on signal transduction pathways in *Mycosphaerella graminicola*. This dissertation presents the results of his PhD study on the role of signal transduction pathways in *M. graminicola*.

## LIST OF PUBLICATIONS:

**Full papers:**

- Mehrabi, R.**, van der Lee, T., Waalwijk, C., and Kema, G. H. J. 2006. *MgSl2*, a cellular integrity MAP kinase gene of the fungal wheat pathogen *Mycosphaerella graminicola*, is dispensable for penetration but essential for invasive growth. *Mol. Plant-Microbe Interact.* 19:389-398.
- Mehrabi, R.**, and Kema, G. H. J. 2006. Protein Kinase A subunits of the ascomycete pathogen *Mycosphaerella graminicola* regulate asexual fructification, filamentation, melanization and osmosensing. Submitted.
- Mehrabi, R.**, van der Lee, T., and Kema, G. H. J. 2006. G $\alpha$  and G $\beta$  proteins regulate the cAMP pathway required for development and pathogenicity of the fungal wheat pathogen *Mycosphaerella graminicola*. Submitted.
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- Cousin, A., **R. Mehrabi**, M. Guilleroux, M. Dufresne, T. van der Lee, C. Waalwijk, T. Langin and G. H. J. Kema. 2006. The MAP kinase-encoding gene *MgFus3* of the non-appressorium phytopathogen *Mycosphaerella graminicola* is required for penetration and *in vitro* pycnidia formation. Accepted by *Mol. Plant Pathol.*

**Abstracts:**

- Mehrabi, R.**, Mendes, O., Waalwijk, C., van der Lee, T. A. J., and Kema, G. H. J. 2003. A fine-mapping strategy to clone an Avr gene from *Mycosphaerella graminicola*. Pages 165-168 in: Global Insight into the Septoria and Stagonospora Diseases of Cereals. G. H. J. Kema, M. Van Ginkel and M. Harrabi eds. Proc. 6th Int. Symp. Septoria Stagonospora Dis. Cereal. Tunis, Tunisia.
- Mehrabi, R.**, Taga, M., Waalwijk, C., and Kema, G. H. J. 2003. Electrophoretic karyotyping and cytological analysis of mitotic chromosomes of *Mycosphaerella graminicola*, the causal agent of septoria tritici leaf blotch of wheat. Pages 25-28 in: Global Insight into the Septoria and Stagonospora Diseases of Cereals. G. H. J. Kema, M. Van Ginkel and M. Harrabi eds. Proc. 6th Int. Symp. Septoria Stagonospora Dis. Cereal. Tunis, Tunisia.
- Ben M'Barak, R., S. Ware, C. Waalweijk, T. van der Lee, **R. Mehrabi** and G.H.J. Kema. 2003. A TaqMan approach to monitor and quantify *Mycosphaerella graminicola*. Page 161 in: Global Insight into the Septoria and Stagonospora Diseases of Cereals. G. H. J. Kema, M. Van Ginkel and M. Harrabi eds. Proc. 6th Int. Symp. Septoria Stagonospora Dis. Cereal. Tunis, Tunisia.
- Mehrabi, R.**, Waalwijk, C., van der Lee, T. A. J., Ware, S., and Kema, G. H. J. 2004. A MAP kinase gene, *MgMK1*, is essential for pathogenicity in *Mycosphaerella graminicola*. Page 75 in: Proc. 7th Eur. Conf. Fungal Genet. Copenhagen, Denmark.
- Mehrabi, R.**, Waalwijk, C., van der Lee, T., and Kema, G. H. J. 2005. HTP Functional genomics of signal transduction pathways using transposon mutagenesis system in the fungal phytopathogen *Mycosphaerella graminicola*. *Clin. Biochem.* 38:832.
- Mehrabi, R.**, Waalwijk, C., van der Lee, T., and Kema, G. H. J. 2005. *Mlt2*, a MAP kinase of *Mycosphaerella graminicola* involved in cell wall integrity, is dispensable for penetration but is essential for invasive growth. Page 129 in: Proc. 23rd Fungal Genet. Conf. Asilomar, California, USA.
- Mehrabi, R.** and G.H.J. Kema. 2005. Signaling in *Mycosphaerella graminicola*: Insight into the role of signaling pathways in virulence and stress responses. Page 29 in: Proc. 13<sup>th</sup> multi-disciplinary Iranian Researchers Conference in Europe. Leeds University, Leeds, UK.
- Mehrabi, R.**, Waalwijk, C., van der Lee, T. A. J., and Kema, G. H. J. 2005. Genetic and functional analyses of a 96 kb region harboring a putative avirulence locus from the fungal wheat pathogen *Mycosphaerella graminicola*. Page 129 in: Proc. 23rd Fungal Genet. Conf. Asilomar, California, USA.
- Mehrabi, R.**, Waalwijk, C., van der Lee, T. A. J., and Kema, G. H. J. 2006. Divergent role of signaling pathways in development and pathogenicity of *Mycosphaerella graminicola*. Page 307 in: Proc. 8th Eur. Conf. Fungal Genet. Vienna, Austria.

**Education Statement of the Graduate School**  
**Experimental Plant Sciences**

The Graduate School  
  
**EXPERIMENTAL  
PLANT  
SCIENCES**

Issued to: Rahim Mehrabi  
 Date: 03 May 2006  
 Group: Laboratory of Phytopathology, Wageningen University

<b>1) Start-up phase</b>	<u>date</u>
► First presentation of your project Progress in cloning of a putative Avr gene in <i>Mycosphaerella graminicola</i>	24 Jan 2003
► Writing or rewriting a project proposal Functional analysis of virulence genes in <i>Mycosphaerella graminicola</i>	Apr - Oct 2002
► Writing a review or book chapter MSc courses	
► Laboratory use of isotopes Safe handling with radioactive materials and sources	07-09 Jan 2003
	<i>Subtotal Start-up Phase</i>
	9.0 credits*

<b>2) Scientific Exposure</b>	<u>date</u>
► EPS PhD student days Utrecht University Free University of Amsterdam Radboud University, Nijmegen	27 Mar 2003 03 Jun 2004 02 Jun 2005
► EPS theme symposia Theme 2 'Interactions between Plants and Biotic Agents', Wageningen University Theme 2 'Interactions between Plants and Biotic Agents', Utrecht University	12 Dec 2003 17 Sep 2004
► NWO Lunteren days and other National Platforms ALW Platform Molecular Genetics Annual Meeting, Lunteren ALW Platform Molecular Genetics Annual Meeting, Lunteren	26-27 Oct 2003 28-29 Oct 2004
► Seminars (series), workshops and symposia WCS Day WCS Day	30 Jan 2003 22 Jan 2004
► Seminar plus	
► International symposia and congresses 6th International Symposium on Septoria and Stagonospora Diseases of Cereals, Tunisia 7th European Conference on Fungal Genetics, Copenhagen, Denmark 23rd Fungal Genetics Conference, Asilomar, California, U.S.A. 13th multi-disciplinary Iranian Researchers Conference in Europe, Leeds University, England First International Conference of Biochemistry and Molecular Biology, Tehran, Iran 8th European Conference on Fungal Genetics, Vienna, Austria	08-12 Dec 2003 17-20 Apr 2004 15-20 Mar 2005 02 Jul 2005 11-15 Sep 2005 08-11 Apr 2006
► Presentations Poster presentation, ALW Platform Molecular Genetics Annual Meeting, Lunteren, The Netherlands Poster presentation, 6th International Symposium on Septoria and Stagonospora Diseases of Cereals, Tunisia Oral presentation, 6th International Symposium on Septoria and Stagonospora Diseases of Cereals, Tunisia Oral presentation, WCS day, Utrecht University, The Netherlands Poster presentation, 7th European Conference on Fungal Genetics, Copenhagen, Denmark Oral presentation, Molecular Genetics Annual Meeting, Lunteren, The Netherlands Poster presentation (1), 23rd Fungal Genetics Conference, Asilomar, California, U.S.A. Poster presentation (2), 23rd Fungal Genetics Conference, Asilomar, California, U.S.A. Oral presentation, Purdue University, West Lafayette, U.S.A. Oral presentation, 13th Multi-disciplinary Iranian Researchers Conference in Europe, Leeds, England Oral presentation, First International Conference of Biochemistry and Molecular Biology, Tehran, Iran Oral presentation, 8th European Conference on Fungal Genetics, Vienna, Austria	26-27 Oct 2003 08-12 Dec 2003 08 Dec 2003 22 Jan 2004 17-20 Apr 2004 29 Oct 2004 15-20 Mar 2005 15-20 Mar 2005 22 Mar 2005 02 Jul 2005 15 Sep 2005 08-11 Apr 2006 02 Jun 2005
► IAB interview	
► Excursions Field visit, breeding for resistance to <i>Septoria</i> and <i>Fusarium</i> , Lille, France Control of cereal diseases by new fungicides, Syngenta, Germany Summer excursion for cereal diseases in Belgium and France	25 Jun 2003 04 Jul 2003 28 Jun 2004
	<i>Subtotal Scientific Exposure</i>
	21.9 credits*

<b>3) In-Depth Studies</b>	<u>date</u>
► EPS courses or other PhD courses Disease resistance in plants, Wageningen University Bioinformation Technology -1, Wageningen University Molecular basis of Microbe-Plant Interactions, Leiden University Functional Genomics: theory and hands-on data analysis, Utrecht University	14-16 Oct 2002 12-21 May 2003 05-07 Jun 2003 25-28 Aug 2003
► Journal club Weekly phytopathology lab meeting Biweekly pathogen genetics cluster discussion Monthly Dutch <i>Mycosphaerella</i> group discussion	2002-2006 2002-2006 2002-2006
► Individual research training	
	<i>Subtotal In-Depth Studies</i>
	8.4 credits*

<b>4) Personal development</b>	<u>date</u>
► Skill training courses Techniques for writing and presenting a scientific paper, Wageningen University Scientific publishing, Wageningen University	26-28 Mar 2003 13 Oct 2005
► Organisation of PhD students day, course or conference	
► Membership of Board, Committee or PhD council	
	<i>Subtotal Personal Development</i>
	1.2 credits*

<b>TOTAL NUMBER OF CREDIT POINTS*</b>	<b>40.5</b>
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Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

\* A credit represents a normative study load of 28 hours of study

مسیرهای انتقال سیگنال موثر در بیماریزایی و  
رشد و نمو پاتوژن قارچی گندم  
*Mycosphaerella graminicola*

رحیم مهرابی

۱۳۸۵ بهار

رساله دکترای تخصصی

دانشگاه واخینینگن

هلند

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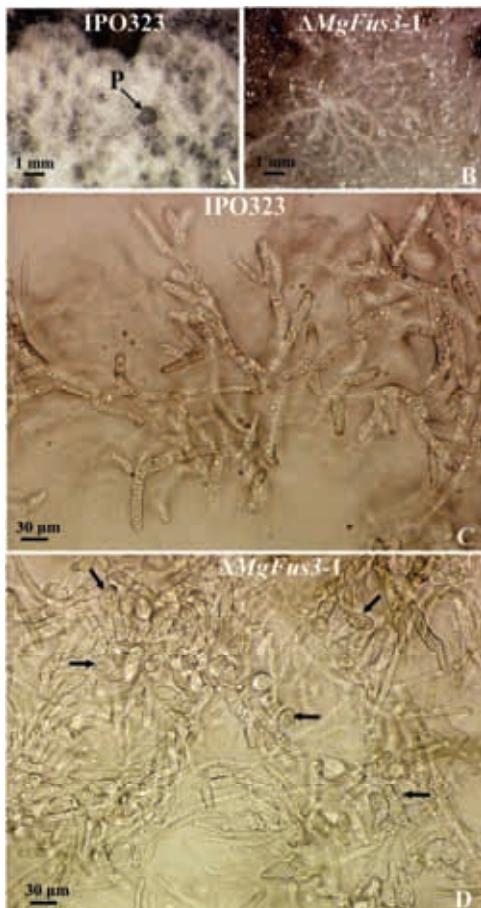
Layout and design: by the author.

**Front page:** Macroscopic symptom of septoria tritici leaf blotch of wheat caused by *Mycosphaerella graminicola* strain IPO323 on cv. Obelisk

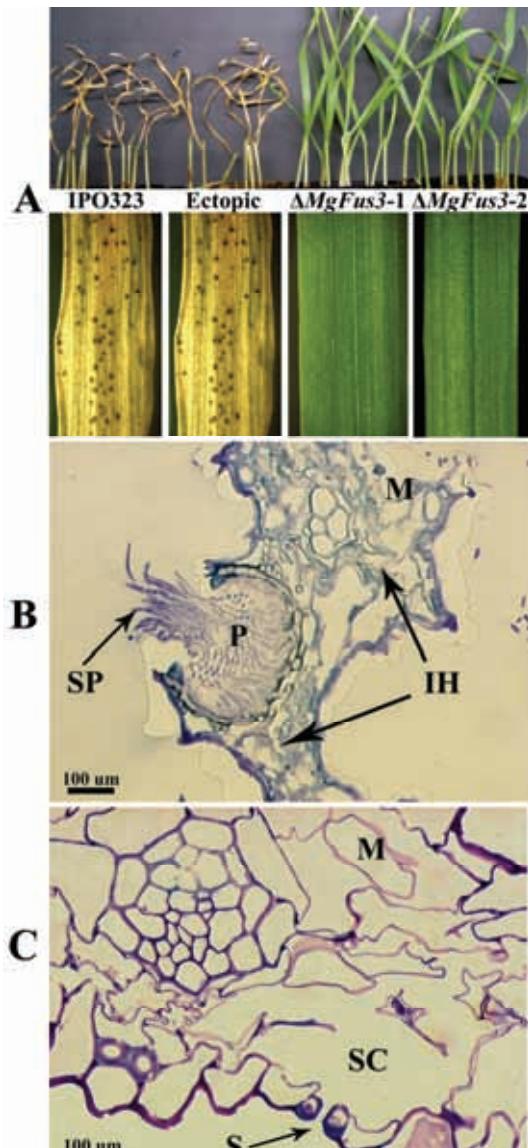
**Back page:** The simplified MAP kinase and cAMP signaling pathways of *Saccharomyces cerevisiae* on the background of the front page.

The Training and Supervision Plan was completed at a Graduate School Experimental Plant Sciences

## **Color figures, chapter two**



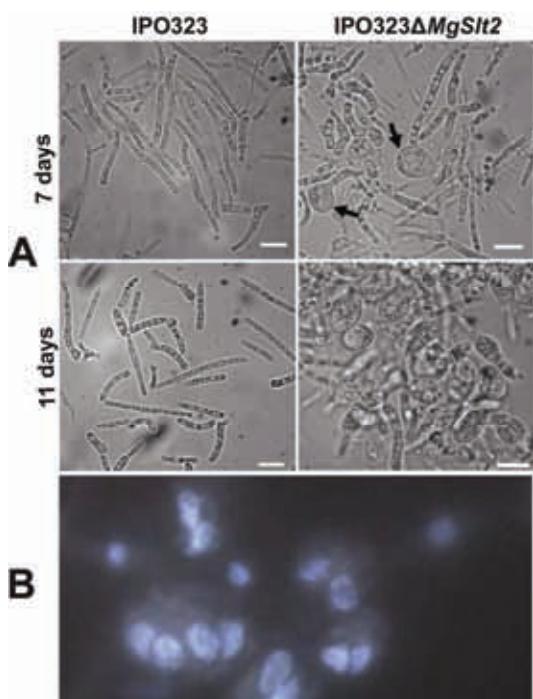
Chapter 2, Page 25, Fig. 3.



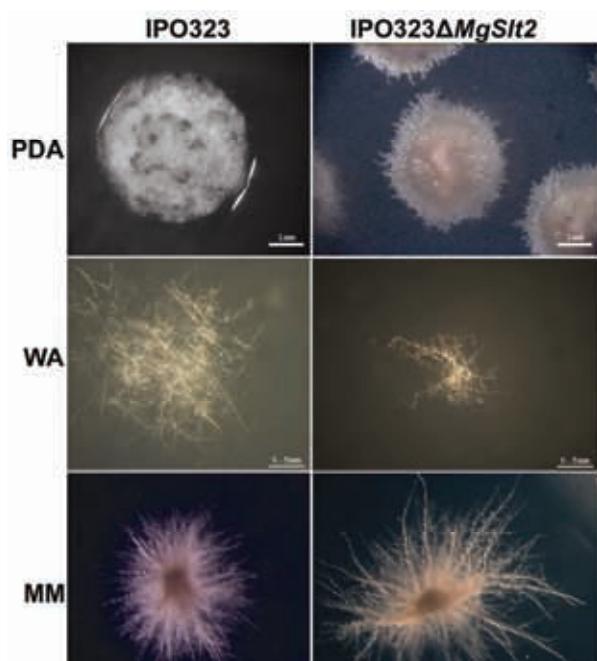
Chapter 2, Page 25, Fig. 4.



**Color figures, chapter three**



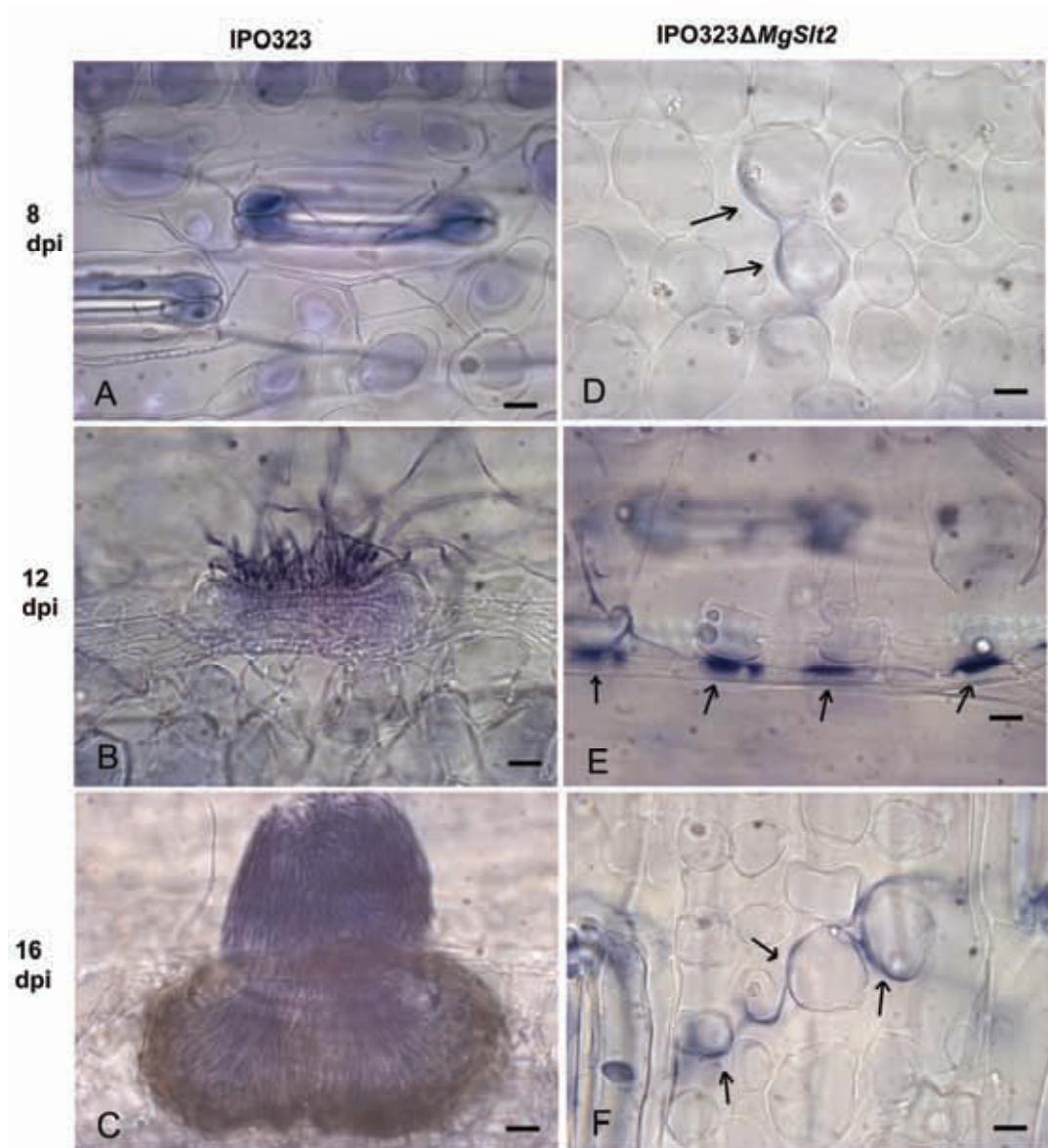
Chapter 3, Page 45, Fig. 4.



Chapter 3, Page 45, Fig. 5.



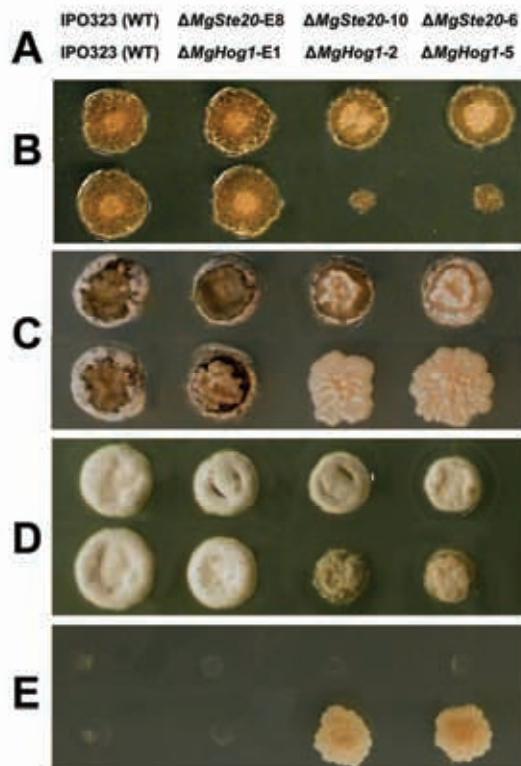
Chapter 3, Page 47, Fig. 7.



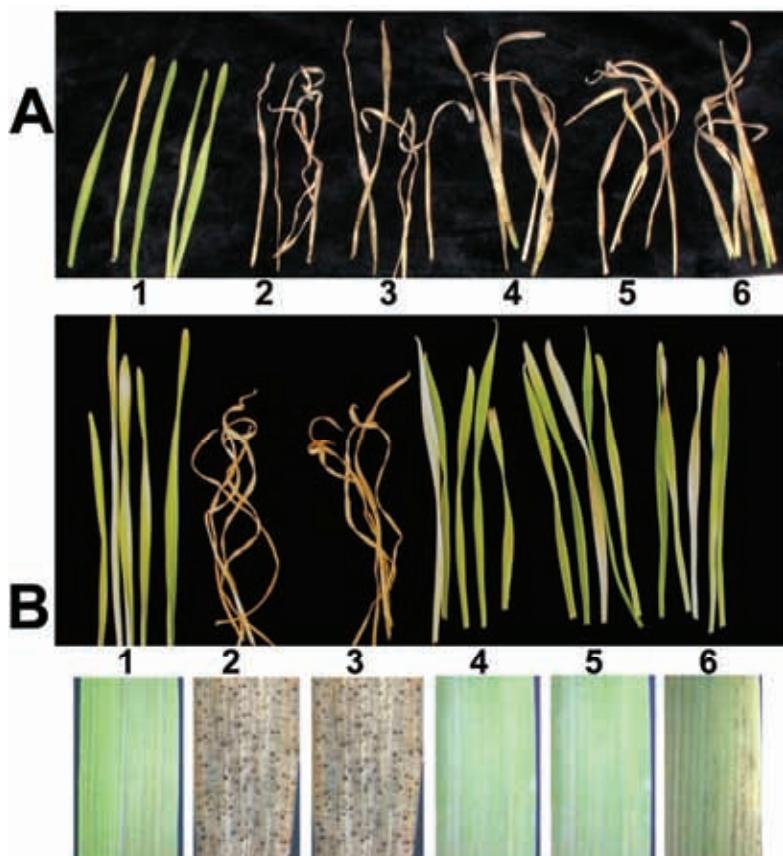
Chapter 3, Page 49, Fig. 9.



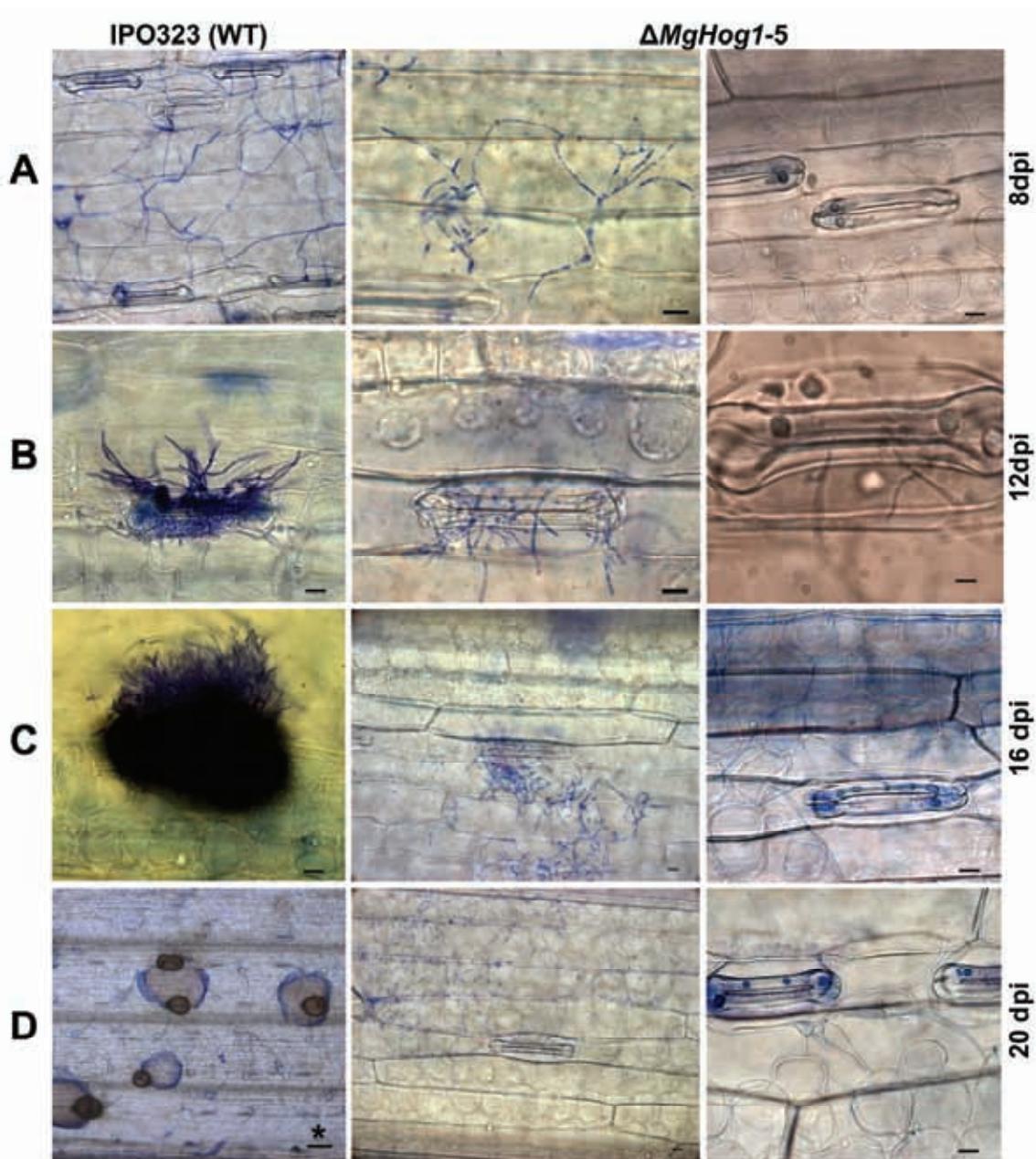
## **Color figures, chapter four**



Chapter 4, Page 68, Fig. 3.



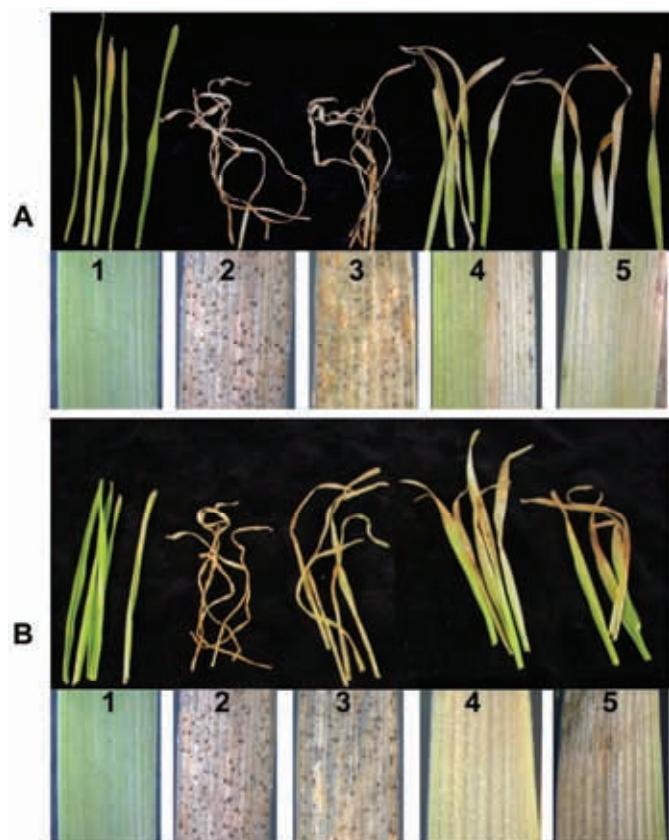
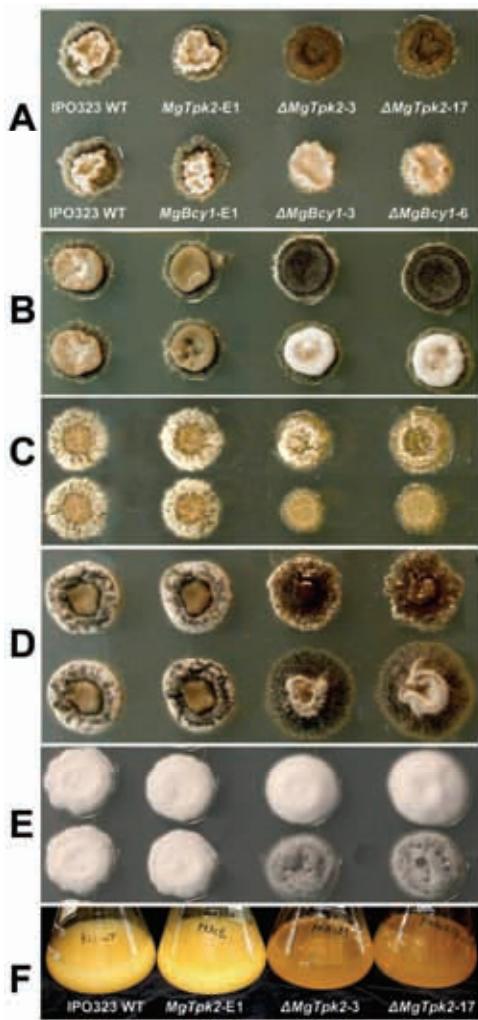
Chapter 4, Page 72, Fig. 5.



Chapter 4, Page 73, Fig. 6.

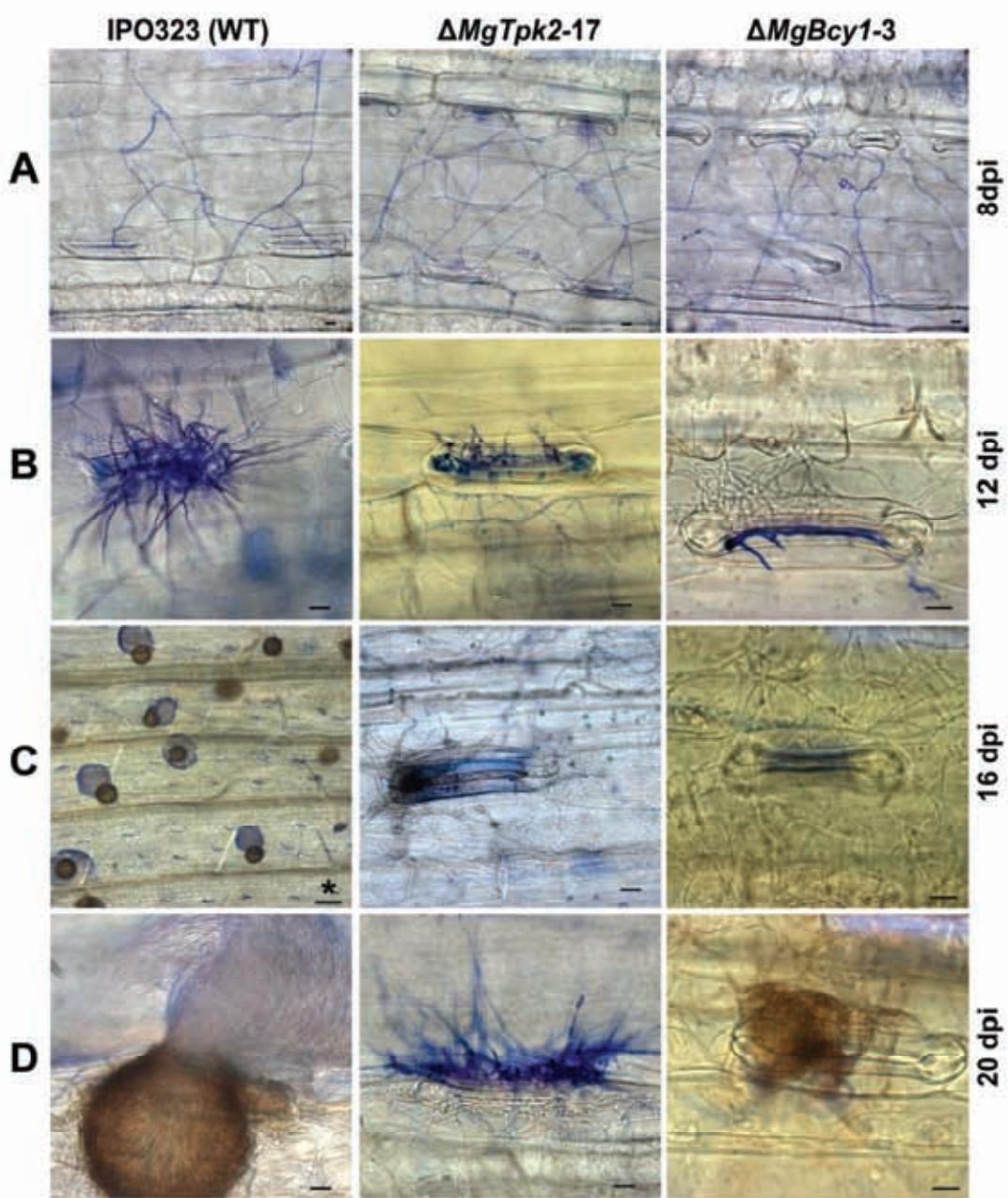


## **Color figures, chapter five**



Chapter 5, Page 95, Fig. 6.

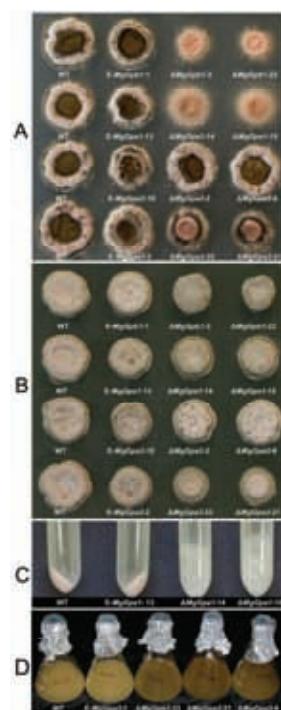
Chapter 5, Page 92, Fig. 4.



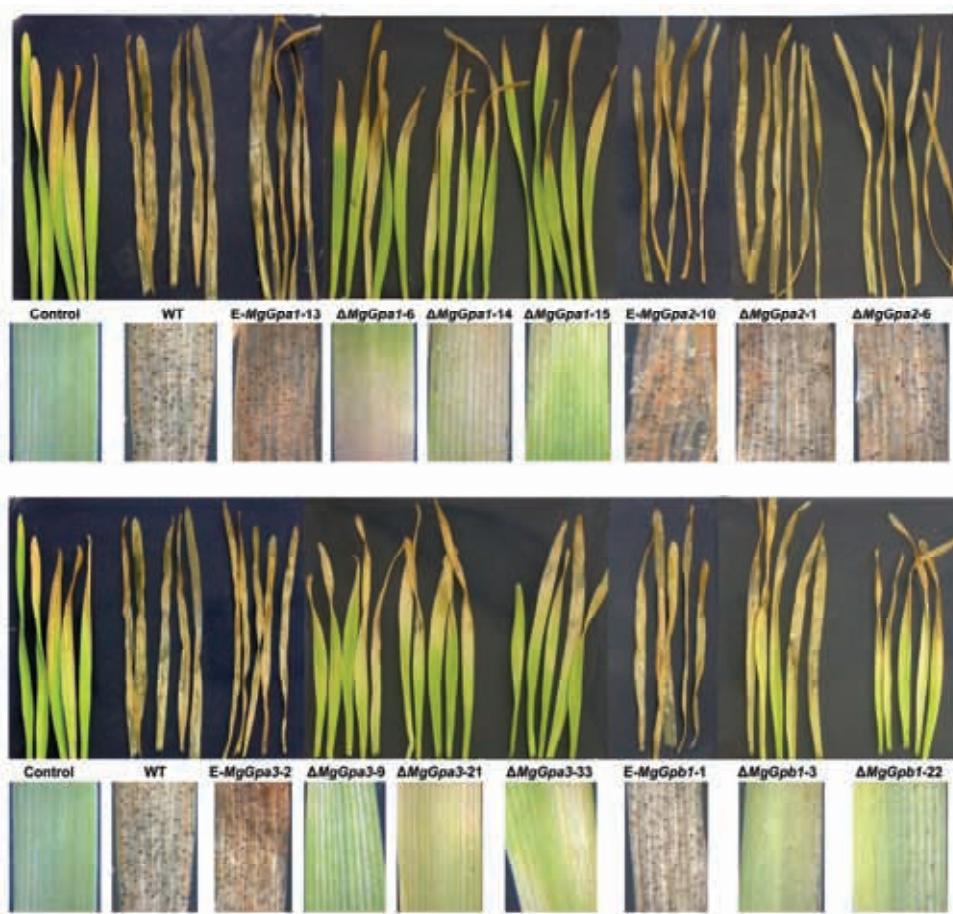
Chapter 5, Page 97, Fig. 7.



## **Color figures, chapter six**



Chapter 6, Page 113, Fig. 3.



Chapter 6, Page 118, Fig. 7.