***MgSlt2*, 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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Among expressed sequence tag libraries of *Mycosphaerella graminicola* isolate IPO323, we identified a full-length cDNA clone with high homology to the mitogen-activated protein (MAP) kinase *Slt2* in *Saccharomyces cerevisiae*. This MAP kinase consists of a 1,242-bp open reading frame, and encodes a 414-amino-acid protein. We designated this homolog *MgSlt2*, generated *MgSlt2* knockout strains in *M. graminicola* isolate 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 (2×), bifonazole (>4×), imazalil (5×), and cyproconazole (10×), 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 frequently were unable to branch out and establish invasive growth resulting in highly reduced virulence, and prevented pycnidia formation. Therefore, we conclude that *MgSlt2* is a new pathogenicity factor in *M. graminicola*.

Additional keywords: *A. tumefaciens*-mediated transformation, Septoria tritici leaf blotch, transposon.

Mycosphaerella is the largest genus in the class Dothideomycetes, an extremely large and diverse class of fungi. With over 1,000 named species, plus its associated asexual stages, the genus *Mycosphaerella* contains at least 3,000 species and 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 *Mycosphaerella 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 U.S.\$2.5 billion. *M. citri*, the cause of greasy spot of citrus, is the largest fungicide target in this crop in the United States. 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.

M. graminicola is rapidly developing as a model fungus for the order 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* spp. occurs indirectly through the stomata. In compatible responses, subsequent development is intercellular, in close contact with mesophyll cells and, depending on the pathosystem, symptomless for 14 to 20 days before rapid cell collapse, suggesting an active role of toxic compounds (Duncan and Howard 2000; Kema et al. 1996d). 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 produced continuously throughout the year, which release the airborne 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 *Mycosphaerella graminicola* population genetics the recognized standard for many other plant pathosystems (Hunter et al. 1999; Kema et al. 1996c; Zhan et al. 2003, 2004; Zhan and McDonald 2004). Early evidence for specificity was controversial; hence, the wheat–*M. graminicola* pathosystem traditionally has been thought to be controlled by quantitative characters in host and 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 eventually was falsified and experimental evidence showed that host and pathogen interact according to the gene-for-gene hypothesis (Brading et al. 2002; Kema et al. 1996a,b,c, 2000, 2002; Kema and van Silfhout 1997). Since then, up to eight resistance genes in the host

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were identified and mapped (Adhikari et al. 2003, 2004a,b,c; Arraiano et al. 2001; Brown et al. 2001; 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; Stergiopoulos et al. 2003; Zwiers and De Waard 2001) 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 expressed sequence tags (ESTs) from a range of cultural conditions (Kema et al. 2003). The genomes of *M. graminicola* and *M. fijiensis* currently are being sequenced at the Joint Genome Institute of the United States Department of Energy.

We recently identified the first *M. graminicola* mitogen-activated protein (MAP) kinase, *MgFus3*, a homolog of *Saccharomyces cerevisiae* *Fus3*, which is required for penetration (Mehrabi et al. 2004). The MAP kinase 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 currently is being generated (Kema et al. 2003). MAP kinase pathways, sometimes called extracellular signal-regulated kinase pathways, are well known for their involvement in the transduction of a variety of extra cellular signals. MAP kinases belong to the family of serine/threonine protein kinases and are integral parts of gene cascades generally consisting of three conserved phosphokinases, MAP kinase kinase kinases (MAPKKKs), MAP kinase kinases (MAPKKs), and MAP kinases (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 osmotic stress 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 MAPK genes (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 MAPKs *Fus3* and *Slr2* 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 in *M. graminicola* that we designated as *MgSlr2* because it is a homolog of the *S. cerevisiae* *Slr2* gene (Torres et al. 1991). We isolated and functionally characterized the role of *MgSlr2* in the dimorphic lifestyle of *M. graminicola*, because hardly anything is known

about the relation among pathogenicity, cell growth, and morphogenesis in this important plant-pathogenic fungus.

RESULTS

MgSlr2 is a homolog of the *S. cerevisiae* MAP kinase *Slr2*.

Among EST libraries of *M. graminicola*, we identified a full-length cDNA clone with 62% identity and 75% similarity at protein level to *Slr2*. *MgSlr2* possesses a 1,242-bp open reading frame encoding a 414-amino-acid sequence with a calculated molecular weight of 47.6 kDa. The protein contains all conserved protein kinase subdomains, including the MAP kinase dual phosphorylation sites TEY (residues 185 to 187) (Fig. 1). Phylogenetic studies revealed that *MgSlr2* clusters with *Slr2* homologs in the phytopathogenic fungi *Magnaporthe grisea*, *Colletotrichum lagenarium*, *Claviceps purpurea*, *Fusarium graminearum*, and *Blumeria graminis* as well as the nonphytopathogenic fungus *Emericella nidulans* (Fig. 1).

MgSlr2 is constitutively expressed in planta and in vitro.

The expression of *MgSlr2* was studied, with β -tubulin as control, by reverse-transcription polymerase chain reaction (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).

Isolation of *MgSlr2* disruptants.

Two *Mycosphaerella graminicola* transformants lacking an intact copy of *MgSlr2* (IPO323 Δ *MgSlr2*) were identified among 30 hygromycin-resistant transformants by PCR screening using primers *MgSlr2*F1/*MgSlr2*R1 (Table 1), designed upstream and downstream of the transposon insertion. Replacement of the original copy of *MgSlr2* by the disruption construct was confirmed further by large-size PCR amplification using Herculase DNA polymerase (Fig. 3).

The effect of *MgSlr2* on vegetative properties.

The *M. graminicola* IPO323 Δ *MgSlr2* disruptants and the wild-type IPO323 strain showed identical growth and maintained the same cell shape and growth rate until 5 days postinoculation (dpi) in fresh yeast glucose broth (YGB) medium. From that point onward, the cells of the IPO323 Δ *MgSlr2* strains started to show a defect in polarized growth of the tip cells, causing an enlarged and aberrant shape at 7 dpi. This defect in cytokinesis eventually resulted in the formation of large and swollen cells at 11 dpi (Fig. 4A). A progressive autolyzing process characterized by swollen cells containing up to four nuclei was revealed by 4',6-diamidino-2-phenylindole (DAPI) staining (Fig. 4B), indicating that, despite hampered cell wall formation, the nuclear division continued. This process started gradually at 11 dpi and continued until massive cell collapse at 18 dpi.

The germination frequency of *M. graminicola* IPO323, IPO323 Δ *MgSlr2*, and ectopic control strains was similar on potato dextrose agar (PDA) medium (data not shown), and colony shape remained similar until 3 to 4 days after plating. Subsequently, the IPO323 Δ *MgSlr2* strains grew more slowly, resulting in colonies half the diameter of the wild-type strain IPO323 at 15 dpi. Moreover, IPO323 also produced substantial aerial mycelium that was absent on the IPO323 Δ *MgSlr2* colonies, even after prolonged growth on PDA (Fig. 5). In addition, *M. graminicola* IPO323 started to melanize after 4 to 5 dpi and was completely melanized at 11 dpi, whereas melanization did not occur in the IPO323 Δ *MgSlr2* strains.

On water agar (1%), the growth rate of *M. graminicola* IPO323 and the IPO323 Δ *MgSlr2* strains was similar until 5 dpi, after which the IPO323 Δ *MgSlr2* strains showed a reduced growth rate resulting in colony sizes half the diameter of the

wild-type strain IPO323 at 11 days (Fig. 5). In contrast, the colony size and shape of the IPO323Δ*MgSlt2* strains was similar to IPO323 on *E. nidulans* minimal medium, but the colony shape of the IPO323Δ*MgSlt2* strains appeared less compact, with longer-extending hyphae (Fig. 5).

MgSlt2 is involved in cell wall strengthening and protects the cells from toxic compounds.

We have tested the sensitivity of *M. graminicola* IPO323, IPO323Δ*MgSlt2*, and the ectopic control strains to glucanase, chitinase, and 11 chemical compounds representing several

Fig. 1. A, Phylogenetic comparison of *MgSLT2* mitogen-activated protein kinase (MAPK) homologues based on amino acid sequence alignments. The deduced peptide sequences of *MgSLT2* from *Magnosphaerella 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*, MG1 (AF492766) from *Fusarium graminearum*, MPKA (U59214) from *Emmericella 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* homologues from other related fungi. The conserved dual phosphorylation site TEY in all MAPKs is boxed (residue 185 to 187). Gaps introduced for the alignment are indicated by hyphens.

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 Δ MgSlt2 strains showed (partially) digested cell walls in the majority of cells, leading to significant aggregation after 30

Fig. 2. In vitro and in planta expression analysis of *MgSlt2*. Reverse-transcription polymerase chain reaction (RT-PCR) products were generated using gene-specific primers for the *MgSlt2* gene (upper panel) and β -tubulin gene as control (lower panel). Lane M, 1-kb-plus ladder marker. Lanes 1 to 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 postinoculation, respectively. Lane 7, first-strand cDNAs generated from total RNA isolated from *M. graminicola* IPO323 grown in yeast glucose broth 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>MgSlt2</i> R1	CTCCTCCACAACCTCGAA	<i>MgSlt2</i> gene
<i>MgSlt2</i> F1	TGACATGGACATTCCGAGA	<i>MgSlt2</i> gene
Sp6	TATTTAGGTGACACTATAG	pSport1 border
T7	TAATACGACTCACTATAGGG	pSport1 border
PrimerS	ATAATCCTTAAAACTCCATTTCACCCCT	Left border of transposon (Tn7L)
PrimerN	ACTTTATTGTCATAGTTTAGATCTATTTTG	Right border of transposon (Tn7R)
PrimerE	CAGGAAACAGCTATGACATG	Left side of MCS in pCGN1589
PrimerH	GATTAAGTTGGGTAACGCCAGG	Right side of MCS of pCGN1589

Fig. 3. Diagram of the disruption strategy. **A**, Customized donor construct (pGPS3HygKan) containing a kanamycin and hygromycin cassette was used for transposition into **B**, the target construct pCGN1589*MgSlt2*, resulting in **C**, the disruption construct pCGN1589 Δ *MgSlt2*. 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 Δ *MgSlt2* (primers E and H). **D**, After transposition, the mixture was used to transform *Escherichia coli* DH10 β . By screening 32 *E. coli* colonies in a multiplex colony polymerase chain reaction (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 pCGN1589*MgSlt2* show an amplicon size equal to the size of *MgSlt2* (approximately 2 kb). **E**, PCR identification of homologous recombinants. The two *M. graminicola* IPO323 Δ *MgSlt2* disruptant strains that were selected from the first PCR screening were used for large-size PCR amplification using primers *MgSlt2*F1/*MgSlt2*R1. Lane 1, *M. graminicola* IPO323. Lanes 2 and 3, two homologous recombinants IPO323 Δ *MgSlt2*-1 and IPO323 Δ *MgSlt2*-2. Lane 4, 3, and 5, three ectopic transformants.

min of incubation, suggesting hypersensitivity to this compound (Fig. 6). None of the strains was affected by chitinase, even after prolonged incubation (24 h; not shown).

We determined the minimum inhibitory concentrations (MICs) of the 11 toxic compounds on yeast-like cells of *M. graminicola* IPO323, IPO323 Δ *MgSlt2*, and the ectopic control strains. Compared with IPO323 and the ectopic transformant, the IPO323 Δ *MgSlt2* disruptants showed a significantly increased sensitivity to miconazole (2 $\times$), bifonazole (>4 $\times$), imazalil (5 $\times$), and cyproconazole (10 $\times$) (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 Δ *MgSlt2* strains and both controls (*M. graminicola* IPO323 and the ectopic transformant) by inoculation experiments on fully unfolded first leaves of the susceptible wheat cv. Obelisk. Both controls already showed small chlorotic flecks 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 to 18 dpi (Fig. 7). However, the IPO323 Δ *MgSlt2*-inoculated plants did not develop any symptom until 16 to 18 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 never were 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 IPO323Δ*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 4 dpi. The majority of IPO323Δ*MgSlt2* spores initiated growth and developed germ tubes like IPO323. However, some (1 to 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 or their germ tube initiations had a noncharacteristic shape. However, despite these abnormal phenotypes, these spores eventually were able to develop infectious germ tubes (Figs. 8 and 9A) 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 IPO323Δ*MgSlt2* germ tubes came from infectious hyphae in the substomatal cavities and between mesophyll cells at later stages (Fig. 9D to

Fig. 4. Effect of *MgSlt2* on vegetative reproduction of *Mycosphaerella graminicola* in yeast glucose broth. **A**, Progressive autolysis of IPO323Δ*MgSlt2* cells compared with the regular cell shape of the wild-type isolate IPO323. Cells show defects in polarized growth in the tip cells (arrows) causing enlarged tips at 7 days, eventually resulting in the formation of large and swollen cells at 11 days. **B**, 4',6-diamidino-2-phenylindole staining revealed that the swollen cells of IPO323Δ*MgSlt2* contained two to four nuclei. Scale bar = 10 μm.

F). However, unlike IPO323, in which infectious hyphae branched out into invasive mycelium, the IPO323Δ*MgSlt2* strains failed to do so and never colonized the substomatal cavities and intercellular spaces of the mesophyll (Fig. 9D to F). Further detailed observations revealed that the infectious hyphae of the IPO323Δ*MgSlt2* strains had an aberrant shape in close contact with mesophyll cells. The shape and the thickness of IPO323Δ*MgSlt2* hyphae were similar to IPO323 when growing

Fig. 5. Effect of *MgSlt2* disruption on morphological characteristics of *Mycosphaerella graminicola*. Left and right panels show the wild-type isolate *M. graminicola* IPO323 and the *MgSlt2* disruptant, respectively, growing on potato dextrose agar (PDA), water agar (WA), and *Emicella nidulans* minimal medium (MM) at 11 days at 20°C.

Table 2. Minimal inhibitory concentrations (MICs) and the derived relative increase of sensitivity (Q value)^a

Compounds	MIC (μg/ml)		Q value
	IPO323 ^b	IPO323Δ <i>MgSlt2</i> ^c	
Antibiotics			
Cycloheximide	500	500	1
Fungicides			
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
Plant metabolites			
Berberine	>500	>500	1
Camptothecin	>500	>500	1
Other			
Rhodamine 6G	25	25	1

^a (MICs) of 11 compounds that were used in toxicity assays on *Mycosphaerella graminicola* IPO323 and the disruptant IPO323Δ*MgSlt2* strains as well as the derived relative increase of sensitivity (Q value) of the latter to these compounds

^b *M. graminicola* IPO323 and the ectopic transformant showed similar MIC values.

^c Both independent IPO323Δ*MgSlt2* disruptant strains showed similar MIC values.

in the open space of the apoplast; however, in direct contact with the mesophyll cells, these hyphae were abnormally swollen or collapsed at the contact area (Fig. 9E and F).

DISCUSSION

We have isolated and characterized *MgSlt2*, a homolog of the *Slt2* MAP kinase that regulates cell wall integrity in *S. cerevisiae*. The observed expression pattern was similar to the expression pattern of β -tubulin; therefore, we conclude that *MgSlt2* is constitutively expressed in planta as well as in vitro. In *M. graminicola* and other fungal pathogens, homologs of the *S. cerevisiae* Fus3 MAP kinase are essential for virulence and other functions (Xu 2000). We recently have shown that disruptants of *MgFus3* are nonpathogenic 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 MAP kinase 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 *Magnaporthe grisea*, *Colletotrichum lagenarium*, *Claviceps purpurea*, and *F. graminearum*, but these have significantly different pathogenic lifestyles and represent an entirely different part of the fungal kingdom (Goodwin 2004; Goodwin et al. 2004). In *Colletotrichum lagenarium*, *MAF1* is required for the

early differentiation phase of appressorium formation because mutants failed to form appressoria (Kojima et al. 2002). Contrastingly, *mps1* mutants of *Magnaporthe grisea* could form appressoria but were nonpathogenic because of the inability of these appressoria to penetrate the plant cell surfaces (Xu et al. 1998). In the non-appressorium-forming fungus *Claviceps purpurea*, both *CMPK1* and *CMPK2* are necessary for penetration (Mey et al. 2002). In *Mycosphaerella 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 because 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 IPO323 Δ *MgSlt2* strains were not able to branch out and colonize the mesophyll. A possible explanation for this observation is that plant metabolites, phytoalexins or pathogenesis-related proteins are detrimental to IPO323 Δ *MgSlt2* and inhibit the growth of infectious hyphae. Thus, as in *Magnaporthe grisea*, *Colletotrichum lagenarium*, *Claviceps purpurea*, and *F. graminearum*, *MgSlt2* affects pathogenicity in *Mycosphaerella graminicola*, but the mechanism and time frame significantly differ from these other plant pathogens.

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

Slt2 homologs also have been cloned and characterized in some non-plant-pathogenic fungi. In *S. cerevisiae*, the cell integrity *Slt2* MAP kinase pathway controls cell-wall integrity and promotes cell-wall remodeling under stress conditions. *Slt2* 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 *Slt2* in

Fig. 6. Sensitivity of *MgSlt2* mutants to the cell wall-digesting enzyme glucanase. **A**, Wild-type strain *Mycosphaerella graminicola* IPO323 and **B**, the ectopic transformant did not release many spheroplasts, whereas the cell walls of **C**, the *MgSlt2* disruptant were completely or partially digested under the same conditions. Scale bar = 10 μ m.

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 disruptant strains, IPO323 Δ *MgSlt2*-1 (3) and IPO323 Δ *MgSlt2*-2 (4).

Fig. 8. Scanning electronic microscopy images of *Mycosphaerella graminicola* penetrating leaves of the susceptible wheat cv. Obelisk at 72 h postinoculation. **A**, Penetration of the wild-type strain IPO323. **B**, Penetration of IPO323 Δ *MgSlt2*. 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 μ m.

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 *E. nidulans*, the *Slt2* 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 above-mentioned plant-pathogenic fungi comply partly with our findings. As in *Claviceps purpurea*, *Magnaporthe grisea*, and *F. graminearum* (Hou et al. 2002; Mey et al. 2002; Xu et al. 1998), *MgSlt2* knockouts of *Mycosphaerella graminicola* have an increased sensitivity to cell-wall-degrading enzymes and show auto-sphaeroplasting, but still undergo nuclear division. This latter phenomenon was observed and reported only in *Slt2*-ortholog studies in nonpathogenic fungi. Contrastingly, *MAF1* did not affect cell-wall strength in *Colletotrichum lagenarium* as shown by lytic enzyme studies (Kojima et al. 2002). We extended the information on cell-wall integrity aspects of *Slt2*

orthologs and showed that the *IPO323ΔMgSlt2* strains also are much more sensitive to a number of fungicides and xenobiotic compounds. We conclusively showed that *IPO323ΔMgSlt2* 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 and associates (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 *Cryptococcus 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 by plant defense compounds. In *F. oxysporum*, the overexpression 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 *MgSlt2* is dispensable for penetration but is essential for in-

Fig. 9. Comparative pathogenesis of **A** through **C**, *Mycosphaerella graminicola* IPO323 and **D** through **F**, the disruptant strain *IPO323ΔMgSlt2* on the susceptible wheat cv. Obelisk at 8, 12, and 16 days postinoculation (dpi). **A**, Penetration of stomates. **B**, Early pycnidium development in a heavily colonized substomatal cavity. **C**, Mature pycnidium with exuding cyrrhus, containing the conidia, from the stomatal aperture. **D** through **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 μm.

vasive hyphal growth. We provided direct evidence that *MgSlt2* 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 Laboratories, Detroit, U.S.A.) at 18°C to produce yeast-like spores that were collected by gently scraping cultures incubated for 3 to 4 days and subsequently stored at –80°C (Kema and Van Silfhout 1997). *M. graminicola* IPO323 was grown in YGB (1% yeast extract, 3% glucose) at 18°C in an orbital incubated shaker (Innova 4430; New Brunswick Scientific, Nijmegen, The Netherlands) adjusted to 120 rpm for 5 days to produce inoculum that was collected by centrifugation, washed with water, and adjusted to 10⁷ spores per milliliter for inoculation and 10⁸ spores per milliliter for transformation. *E. nidulans* minimal medium was prepared and used based on Barratt and associates (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). DNA sequences were obtained on an ABI-prism 3100 capillary automated sequencer using the BigDye terminator reaction mix (Applied Biosystems, Amsterdam). Phylogenetic tree generation, DNA and protein sequence alignment, editing, and analysis were performed using MEGALIGN and DNA Star software (Madison, WI, U.S.A.), 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 *MgSlt2* was identified in the cDNA libraries of *M. graminicola* IPO323 (Kema et al. 2003). The cDNA insert was excised from the pSport1-*MgSlt2* plasmid using *KpnI/XbaI* and cloned into pCGN1589. We used the GPS-Mutagenesis system (New England Biolabs, Leusden, The Netherlands) to make a disruption construct of *MgSlt2*. A new, customized donor construct was made by insertion of the kanamycin resistance gene into pGPS3Hyg, which previously was 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 *EcoRV* 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 T7-based transposon borders (T7L and T7R), 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 instructions. The transposition mixture then was cloned to *E. coli* DH10 β that was subsequently grown on selective gentamicin and kanamycin media. A colony PCR was performed by using primerH and primerE (located on the right and left side of the multiple cloning site of pCGN1589, respec-

tively) together with PrimerN (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 s, and 72°C for 90 s; 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 pCGN1589 Δ *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 PrimerN and PrimerS. 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 *Agrobacterium tumefaciens*-mediated transformation according to Zwiers and de Waard (2001).

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

Construct pCGN*MgSlt2* was cloned into *A. tumefaciens* LBA1100 by electroporation and *A. tumefaciens*-mediated transformation was carried out according to Zwiers and de Waard (2001). After 3 weeks, individual *M. graminicola* transformant colonies were collected and transferred to PDA containing hygromycin at 100 μ g per ml and cefatoxime at 200 μ g per ml. The stability of the putative transformants was verified by transferring them three times to fresh PDA medium containing hygromycin at 100 μ g per ml 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 *MgSlt2*F1 and *MgSlt2*R1 (Table 1), which were designed in the flanking regions of the inserted transposon that disrupts the *MgSlt2* gene and did not render an amplicon in case homologous recombination took place. Such clones were selected and analyzed using additional large amplification PCR with the same primers (*MgSlt2*F1 and *MgSlt2*R1) and Herculanase 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 μ l, 10⁷ spores per milliliter) were centrifuged and spores were resuspended in either 500 μ l (1 mg per ml) of 1,3(1,3:1,4)- β -D-glucan 3(4) glucanohydrolase (Sigma, Zwijndrecht, The Netherlands) or chitinase (250 μ g per ml) (Sigma, Zwijndrecht, The Netherlands) and incubated at 37 and 25°C, respectively. The activity of the cell wall-degrading enzyme was monitored microscopically every 30 min.

The 11 compounds comprising fungicides, plant metabolites, an antibiotic, and rhodamine 6G that we used for toxicity assays are listed in Table 2. Spore suspensions (5 μ l; 4 $\times$ 10⁴ spores per milliliter) harvested 5 days after inoculating YGB were spotted on 9-cm PDA (39 g liter⁻¹) plates amended with different concentration of these compounds. MICs were assessed for yeast-like cells of *M. graminicola* IPO323, IPO323 Δ *MgSlt2*, and the ectopic control strains after 10 days of incubation at 18°C in the dark. The relative increase of sensitivity (Q value) of the IPO323 Δ *MgSlt2* strains was calculated by dividing the MIC value of IPO323 by the MIC value of IPO323 Δ *MgSlt2*.

Virulence assays.

Fifteen seeds of the susceptible wheat cv. Obelisk were planted in 5-cm² 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 (10⁷ spores per milliliter) supplemented with 0.15% Tween 20 as a surfactant. After inoculation, pots were placed in transparent polyethylene boxes (6 by 22 by 34 cm), incubated at 20°C in the dark for 48 h, and subsequently incubated at 16 h of light (166 µE s⁻¹ m⁻²) and 8 h of 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 fresh YGB at a concentration of 4 to 5 × 10⁵ spores per milliliter. Approximately 200 µl of 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, vol/vol) for 30 min and flame dried. Samples were stained with DAPI 1 mg per ml dissolved in anti-fading mounting solution (Johnson and Araujo 1981). Observations were made using an epifluorescence microscope (Zeiss Axioscop, Oberkochen, Germany) equipped with a Nikon CoolSNAP digital camera (RS Photometrics, Roper Scientific Inc., Tucson, AZ, U.S.A.).

Four cytological analysis leaves from inoculated plantlets were harvested and cut into 2- to 3-cm segments and immediately immersed in 20 ml of 0.05% trypan blue dissolved in lactophenol-ethanol (1:2, vol/vol) and boiled for 15 min. Samples were destained overnight in a saturated chloral hydrate solution (5:2, wt/vol), 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 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, Münster, Germany) and subsequently frozen in liquid nitrogen. The sample was transferred under vacuum to a dedicated cryopreparation chamber (CT 1500 HF; Oxford Cryo-System, Eynsham, U.K.) onto a sample stage at -90°C. Cryofracture was conducted at approximately -90°C using a cold (-196°C) scalpel blade. The fractured sample was freeze dried for 3 min at -90°C in vacuum at 3 × 10⁻⁷ Pa to remove water vapor contamination. After the sample surface was sputter coated with 10 nm of 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; Tokyo) and subsequently analyzed with an accelerating voltage of 5 kV. Images were digitally recorded (Orion, Brussels).

Expression analysis.

Expression analysis of the *MgSlt2* gene was performed in vitro and in planta at different time points using RT-PCR. We collected 10 leaves per sample from inoculated plants that subsequently were 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) and potential DNA contamination was removed using the DNA-free kit (Ambion, Cambridgeshire, U.K.), 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 manu-

facturer's instructions. PCR reactions were subsequently carried out with 5-µl aliquots of this reaction in a 5-µl PCR reaction volume, using the same primers and conditions as described above for the detection of homologous recombinants, and amplicons finally were analyzed on 1% agarose gels using 10-µl aliquots of the PCR products.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

United States Department of Energy-Joint Genome Institute:
www.jgi.doe.gov/sequencing/why/CSP2005/mycosphaerella.html
 Centraal Bureau voor Schimmelcultures, an institute of the Royal
 Netherlands Academy of Arts and Sciences: www.cbs.knaw.nl