Fungal pathogens of plants are routinely classified based upon whether they obtain nutrients from living or dead host tissues (Solomon et al., 2003). Those that feed on living tissues are referred to as biotrophic (or biotrophs), while those that kill plant tissues in order to retrieve nutrients are considered to be necrotrophic (or necrotrophs). In between these extremes are many filamentous fungal pathogens that have an extended period of symptomless host colonization, prior to more rapid invasive growth and asexual reproduction associated with the death of host tissues. These pathogens are referred to as hemibiotrophic (Luttrell, 1974) or alternatively as necrotrophs that have a long symptomless phase of initial host association. The classification also implies that biotrophic pathogens have the ability to continually evade recognition by the host or suppress the activation of plant defense responses, while necrotrophic pathogens may in fact benefit from host cell death associated with the plant defense response. It remains largely unclear, therefore, how hemibiotrophs/necrotrophs with a long symptomless phase might interact with the activation of plant defenses. One hypothesis is that these pathogens might deploy specialized effectors (frequently but not always proteins) during particular phases of plant infection that facilitate their particular nutritional and reproductive lifestyle. These effectors could act either as infection phase-specific suppressors of plant defense responses or alternatively as activators, depending on the lifestyle of the fungus (Friesen et al., 2008;
Considerable progress has recently been made in the characterization of fungal effectors (De Wit et al., 2009; Stergiopoulos and de Wit, 2009; de Jonge et al., 2011). Typically, these effectors are relatively small proteins that are secreted either through classical systems (requiring a signal peptide and transit through endoplasmic reticulum and Golgi) or, alternatively, for some species, may also be secreted through a nonclassical pathway (Ridout et al., 2006). Their putative, and in some cases proven, function is to enable colonization by the pathogen via interfering with a variety of plant-encoded virulence targets, to effectively suppress (or activate) defense responses that might otherwise hinder pathogen colonization. Plant defense responses are normally triggered through the recognition of various pathogen-derived signals collectively described as pathogen-associated molecular patterns (PAMPs; Nürnberger and Brunner, 2002; Nürnberger and Kemmerling, 2009), a process referred to as PAMP-triggered immunity (PTI) that is often targeted by pathogen effectors (van Esse et al., 2007, 2008; Doehlermann et al., 2009). This has led to plants evolving disease resistance proteins able to detect perturbations to virulence targets that then activate a second line of defense described as effector-triggered immunity (ETI; Boller and He, 2009). This subsequent level of immunity can itself be suppressed through pathogen effector functions (Houterman et al., 2008), giving rise to the “zig-zag” model for interactions of plant pathogens with the plant defense machinery (Jones and Dangl, 2006). ETI frequently conforms to a gene-for-gene model (Flor, 1971) whereby single genes/loci in the pathogen interact with single dominant genes/loci in the plant, giving rise to immunity. In contrast, an inverse model of the gene-for-gene system also exists that involves the interaction between individual effector genes from the pathogen and single dominant susceptibility loci in the plant (Friesen et al., 2008). In the latter scenario, the net result is to promote plant infection through the activation of plant cell death signaling pathways often resembling those triggered during ETI (Wolpert et al., 2002). These mechanisms, described as effector-triggered susceptibility (Friesen et al., 2008), have been shown to favor plant infection by various necrotrophic fungi, and in particular two wheat (Triticum aestivum) infecting fungi, Stagonospora nodorum and Pyrenophora tritici repentis (Friesen et al., 2006; Liu et al., 2009), which are the causal agents of leaf blotch and tan spot diseases, respectively.

The most economically important wheat-infecting fungus in western Europe is Mycosphaerella graminicola (Septoria tritici), the causal agent of S. tritici blotch (STB) disease (Eyal, 1999). M. graminicola is host and tissue specific and is only known to parasitize leaves of wheat (Triticum spp.) plants. Leaf penetration occurs most frequently by means of hyphae emerging from germinating, surface-attached spores that enter via stomata (Kema et al., 1996). Subsequently, symptomless colonization typically lasts at least 7 d following inoculation. During this period, the fungus grows slowly as filamentous hyphae extending in the intercellular spaces between wheat mesophyll cells. This slow rate of initial growth has made it difficult to detect significant increases in pathogen biomass via conventional methods (Cohen and Eyal, 1993; Kema et al., 1996; Pnini-Cohen et al., 2000; Rudd et al., 2008). Due to the lack of experimental evidence about how M. graminicola obtains nutrients during this phase, we currently refer to the fungus as necrotrophic but having a long initial period of symptomless host association. Approximately 8 to 10 d after inoculation, disease lesions begin to form on susceptible plants (Kema et al., 1996). This transition is associated with the induction of host defense processes characteristic of hypersensitive response-like programmed cell death and differential regulation of wheat mitogen-activated protein kinase pathways (Keon et al., 2007; Rudd et al., 2008). These events culminate in loss of control of cell permeability, resulting in leakage of nutrients from dying plant cells into the apoplastic spaces where the fungus resides, and coincide with an exponential increase in its growth rate. Asexual sporulation structures (pycnidia) subsequently form in the substomatal cavities of necrotic leaf tissues. The asexual pycnidiospores then extrude through stomatal openings and are dispersed via rain splash throughout the crop canopy, giving rise to polycyclic infections (Kema et al., 1996).

Several features of the M. graminicola infection process are shared by the tomato (Solanum lycopersicum) plant pathogenic fungus Cladosporium fulvum, in particular, host invasion via stomata, intercellular growth in the apoplast, and specific colonization of leaf tissue (Thomma et al., 2005). C. fulvum has proved to be a valuable model for investigating fungal pathogen interactions with host plants (Joosten and de Wit, 1999; Rivas and Thomas, 2005). However, C. fulvum is a biotrophic fungus that infects a dicotyledonous plant and is able to significantly increase its biomass and sporulate in association with living plant cells. In contrast, M. graminicola, which specifically infects only the leaves of monocotyledonous wheat plants, switches to a necrotrophic growth phase to support sporulation. What is currently unclear, therefore, is to what extent M. graminicola might rely on the deployment of effectors to either facilitate the initial symptomless growth phase (evasion or suppression of plant defense) and/or to trigger host cell death underlying the appearance of disease lesions (Deller et al., 2011).

Recent work on C. fulvum has identified a novel effector protein that plays a critical role in virulence through its ability to interfere with the activation of PTI (Bolton et al., 2008; de Jonge and Thomma, 2009; de Jonge et al., 2010). This protein, referred to as Ecp6 (for extracellular protein 6), possesses three Lysin (LysM) domains, which are often involved in the binding of carbohydrate structures. C. fulvum Ecp6 (CFecp6) was secreted at high levels during plant infection and shown to bind chitin (de Jonge et al., 2010).
Moreover, the protein was shown to block the activation of PTI responses through sequestering chitin fragments and preventing their recognition by plant chitin receptors. However, unlike the previously characterized chitin-binding C. fulvum effector Avr4, CfEcp6 did not possess the ability to protect fungal hyphae against plant-derived hydrolytic enzymes (van den Burg et al., 2006; de Jonge et al., 2010). Therefore, the virulence function of Ecp6 in C. fulvum appears to be specifically via pathogen camouflage during leaf colonization by this biotrophic fungus (de Jonge and Thomma, 2009). At the time of writing, no publicly available full genome sequence for C. fulvum exists, and the presence of additional gene family members, termed LysM effector genes, remains unclear. This is of wider relevance, as initial studies often identified multiple LysM effector homologs in the genome sequences of various other plant pathogenic fungi but also in saprophytic species without apparent virulence functions (Bolton et al., 2008).

Our reanalysis of the M. graminicola genome highlighted the presence of potentially three genes encoding relatively small proteins possessing varying numbers of LysM domains, all of which have signal peptides predicting that they might be secreted. This study describes a detailed characterization of these LysM domain-containing proteins, focusing on two that we identified as being highly expressed during the initial phase of symptomless plant infection. Significantly, both MgLysM proteins possess additional putative effector functions that are not observed for CfEcp6, in that they were both able to protect fungal hyphae from hydrolysis by plant-derived hydrolytic enzymes. Evidence is presented, however, that only one of these proteins plays a major role in pathogen virulence on wheat plants. The data provided suggest that this virulence function acts to suppress the activation of chitin-mediated defenses that would otherwise be triggered during symptomless plant infection by the wild-type fungus. These data also suggest that fungal manipulation of chitin-mediated plant defenses in wheat is an important process required for the development of STB disease and therefore might be a conserved virulence function in diverse biotrophic, hemibiotrophic, and necrotrophic pathogens.

RESULTS

Identification of LysM Effector Homologs in the Genome Sequence of M. graminicola

In order to verify the original predictions (Bolton et al., 2008) and to study in more detail the presence and characteristics of putative LysM domain-secreted effector proteins in M. graminicola, we used the amino acid sequence of CfEcp6 (Bolton et al., 2008) in a BLASTP analysis (E value cutoff of 10) against the sequenced genome. This returned five protein models. Only the two most homologous proteins (2e-57 and 2e-05) were Cys rich and had a signal peptide for secretion. The most homologous protein possessed three predicted LysM domains, similar to CfEcp6, and was named Mg3LysM. The second protein possessed only one predicted LysM domain and was referred to as Mg1LysM. Mg3LysM and Mg1LysM protein sequences were then used in BLASTP searches against the current M. graminicola protein model call (version 2; http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html) and also against a previous version (version 1; http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=Mycgr1&id=40100). No further hits were returned from the current version. However, a further candidate-secreted LysM protein was returned from version 1. Intriguingly, this gene also encoded a predicted secreted protein possessing just one LysM domain. The location of this gene, referred to as MgxlLysM, was approximately 5 kb from Mg1LysM on the opposite DNA strand, separated by only one predicted open reading frame. The various sequence properties of all three predicted MgLysM proteins are summarized in Table I. BLASTP analysis versus the National Center for Biotechnology Information protein database returned CfEcp6 as the most homologous protein to all three MgLysM proteins (Table I). It is noteworthy that MgxLysM lacked any previous EST support (Keon et al., 2000, 2005; Kema et al., 2008) and contained a repeat sequence within intron 2 of the predicted gene model, albeit oriented on the opposite DNA strand (Table I). Each MgLysM protein contained variable numbers of Cys residues (ranging from five to nine), and Mg1LysM and MgxLysM were predicted to be significantly smaller proteins than Mg3LysM as a consequence of possessing fewer LysM domains. An alignment of Mg1LysM, MgxLysM, and Mg3LysM with CfEcp6 is shown in Figure 1A, which highlights the overall structural similarity between the latter two proteins.

The approximately 50-residue amino acid sequences predicted to encode each LysM domain found in Mg1LysM, MgxLysM, Mg3LysM, and CfEcp6 were aligned to investigate sequence similarities between each region (Fig. 1B). A phylogram was also generated, indicating their relatedness to one another (Fig. 1C). LysM domains 2 and 3 of Mg3LysM (residing toward the C-terminal region of the predicted protein) were grouped with the corresponding second and third LysM domains of CfEcp6. In contrast, the first LysM domains of both CfEcp6 and Mg3LysM were more similar to the sole LysM domains present in Mg1LysM and MgxLysM, respectively.

All Three MgLysM Genes Are Actively Transcribed, But Only Mg3LysM and Mg1LysM Are Up-Regulated during Plant Infection

We designed PCR primers to further characterize the MgLysM genes and to monitor their expression levels during in vitro growth and throughout wheat
Characteristics of the putative LysM domain secreted effector proteins in M. graminicola

| Name    | Locus Identifier | Genomic Position | Strand | Genomic Sequence | Coding Sequence | No. of Introns | EST Support | Mature Protein Length | Molecular Mass | No. of LysM Domains | No. of Cys Residues | BLASTP Best Hit; Accession No.; E Value | Remarks |
|---------|-----------------|------------------|--------|------------------|-----------------|---------------|--------------|-----------------------|----------------|---------------------|---------------------|------------------------------------------|---------|
| Mg3LysM estExt_fgenesh1_pgc_chr_110126 | Chromosome 11,505,087-506,095 | – | 758 bp | 699 bp | 1 | Yes | 215 | 22,360.05 | 9 | CfECP6; ACF19427; 7e-46 | Second intron contains a repeat sequence on the + strand |
| Mg1LysM estExt_fgenesh1_kg_C_chr_80182 | Chromosome 8, 1,261,560-1,262,312 | + | 478 bp | 294 bp | 3 | Yes | 79 | 8,701.65 | 4 | CfECP6; ACF19427; 3e-08 | |
| MgxLysM fgenesh2_pgc_scaffold_6000321 | Chromosome 8, 1,256,569-1,257,131 | – | 564 bp | 312 bp | 3 | No | 85 | 9,539.99 | 5 | CfECP6; ACF19427; 3e-07 | |

Leaf infection. Selective primer pairs were designed for each of the MgLysM genes (Fig. 2A), and PCR was performed on (1) fungal genomic DNA, (2) cDNA generated from fungus growing in potato dextrose broth (PDB) culture medium, and (3) wheat leaves harvested at 8 d after fungal inoculation. All primers detected transcripts deriving from each of the three genes, and the PCR products generated from cDNA were of the correct size for transcripts that had undergone the anticipated splicing events (Fig. 2B). This suggested that all three predicted MgLysM genes were actively transcribed.

To determine the relative transcript levels for each MgLysM gene, real-time reverse transcription (RT)-PCR was performed on two in vitro growth samples and across five time points of wheat leaf infection, as follows: day 1, where fungal spores are germinating on the leaf surface; days 4 and 9, which span and extend to the limit of the symptomless phase of leaf infection. Selective primer pairs were designed for each of the three predicted MgLysM genes (Fig. 2A), and PCR was performed on two in vitro growth samples

| Name | Locus Identifier | Genomic Position | Strand | Genomic Sequence | Coding Sequence | No. of Introns | EST Support | Mature Protein Length | Molecular Mass | No. of LysM Domains | No. of Cys Residues | BLASTP Best Hit; Accession No.; E Value | Remarks |
|------|-----------------|------------------|--------|------------------|-----------------|---------------|--------------|-----------------------|----------------|---------------------|---------------------|------------------------------------------|---------|
| Mg3LysM estExt_fgenesh1_pgc_chr_110126 | Chromosome 11,505,087-506,095 | – | 758 bp | 699 bp | 1 | Yes | 215 | 22,360.05 | 9 | CfECP6; ACF19427; 7e-46 | Second intron contains a repeat sequence on the + strand |
| Mg1LysM estExt_fgenesh1_kg_C_chr_80182 | Chromosome 8, 1,261,560-1,262,312 | + | 478 bp | 294 bp | 3 | Yes | 79 | 8,701.65 | 4 | CfECP6; ACF19427; 3e-08 | |
| MgxLysM fgenesh2_pgc_scaffold_6000321 | Chromosome 8, 1,256,569-1,257,131 | – | 564 bp | 312 bp | 3 | No | 85 | 9,539.99 | 5 | CfECP6; ACF19427; 3e-07 | |

dropped dramatically to levels comparable to in vitro growth. Expression levels remained low during sporulation in fully necrotic leaf tissue at day 21 (Fig. 2C). These data demonstrate that Mg3LysM and Mg1LysM, but not MgxLysM, are transcriptionally activated during the symptomless phase of leaf infection.

Both Mg3LysM and Mg1LysM Proteins Bind Chitin Fragments, But Only Mg3LysM Blocks the Activation of Chitin-Induced Plant Defense Responses

The CfEcp6 protein exhibits the ability to specifically bind chitin fragments and to block the rapid chitin-induced elicitation of plant cell cultures (de Jonge et al., 2010). Both the Mg3LysM and Mg1LysM proteins produced in Pichia pastoris were tested for comparable activities. Like CfEcp6, both proteins bound chitin and crab shell chitin, but neither bound chitosan, xylan, or cellulose (Fig. 3A). Interestingly, the two proteins could be distinguished by their ability to diminish chitin-induced defense response activation of tomato cell cultures when added simultaneously with the elicitor. The presence of 100 nm Mg3LysM was able to strongly inhibit the rapid (less than 10 min) alkalization of the culture medium following the addition of 10 nm chitin hexamer oligosaccharide (Gn6; Fig. 3B). These data compare favorably with the reported activity of CfEcp6 (de Jonge et al., 2010). In contrast, 100 nm Mg1LysM had no effect on the ability of either 10 nm (Fig. 3B) or 1 nm (data not shown) chitin to induce this defense response, suggesting that Mg1LysM is less able to prevent the recognition of chitin by tomato cells.

In Contrast to CfEcp6, Both Mg3LysM and Mg1LysM Protect Fungal Hyphae against Hydrolytic Plant Enzymes

It was previously demonstrated that the C. fulvum effector Avr4, which binds chitin through an inverte-
brate chitin-binding domain, but not Ecp6, protects *Trichoderma viride* against hydrolysis by plant hydrolytic enzymes, such as chitinases, in vitro (van den Burg et al., 2006; de Jonge et al., 2010). To test whether Mg1LysM and Mg3LysM were able to protect *T. viride* against hydrolytic activity, conidia were germinated, incubated with either of the two proteins, and subsequently treated with a crude extract of tomato leaves containing hydrolytic enzymes (van den Burg et al., 2006). Growth of *T. viride* was clearly inhibited by the hydrolytic enzymes present in this extract, and CfAvr4, but not CfEcp6, was able to protect the fungus against hydrolysis (Fig. 4). Intriguingly, Mg1LysM as well as Mg3LysM protected *T. viride* against hydrolysis. Thus, in contrast to CfEcp6, and similar to CfAvr4, Mg1LysM and Mg3LysM may contribute to fungal self-defense against hydrolytic plant enzymes.

**Allelic Variation in the Mg3LysM and Mg1LysM Genes from a Differential Isolate Set**

Analysis of allelic variation of CfEcp6 across approximately 60 *C. fulvum* isolates identified only five sites exhibiting single nucleotide polymorphisms (SNPs), of which only one led to an amino acid change (Bolton et al., 2008). This change occurred outside the three predicted LysM domains. To test for allelic variation, Mg1LysM and Mg3LysM were sequenced from genomic DNA of nine isolates of *M. graminicola*, each of which has different avirulence specificity toward wheat genotypes (Jing et al., 2008). A number of SNPs were identified in both genes (Fig. 5). Fifteen sites exhibiting SNPs were identified in the genomic sequence of Mg1LysM, of which seven contributed toward a total of five amino acid changes in the protein (Fig. 5A; Supplemental Fig. S1, A and B). Only three SNPs were identified across the three introns. Four SNPs, giving rise to two amino acid changes, were identified in the LysM domain present in Mg1LysM (Fig. 5A; Supplemental Fig. S1, A and B). Twenty-three sites exhibiting SNPs were identified in Mg3LysM, 10 of which contributed toward eight amino acid changes (Fig. 5B; Supplemental Fig. S1, C and D). Five of these SNPs were detected in the intron. Five SNPs giving rise to two amino acid changes were detected in the LysM domain present in Mg1LysM (Fig. 5A; Supplemental Fig. S1, A and B). Twenty-three sites exhibiting SNPs were identified in Mg3LysM, 10 of which contributed toward eight amino acid changes (Fig. 5B; Supplemental Fig. S1, C and D). Five of these SNPs were detected in the intron. Five SNPs giving rise to two amino acid changes were detected in the LysM domain present in Mg1LysM (Fig. 5A), although the amino acids involved differed between the two proteins.
Further analyses of the Mg1LysM and Mg3LysM genes using maximum likelihood methods for detecting adaptive protein evolution (Goldman and Yang, 1994; Bielawski and Yang, 2005; see “Materials and Methods”) showed strongly significant evidence of positive selection for the two proteins (2Δl = 9.4, degrees of freedom = 2, P < 0.01 for Mg1LysM and 2Δl = 9.7, degrees of freedom = 2, P < 0.01 for Mg3LysM). 2Δl is the statistic used in the test of positive selection (see “Materials and Methods”). This confirmed that some sites in Mg1LysM and Mg3LysM have evolved with nonsynonymous/synonymous substitution ratios greater than 1. Maximum likelihood estimates under a positive-selection model indicated that about 5% of the sites in Mg1LysM and about 2% in Mg3LysM are evolving under positive selective pressure, with nonsynonymous/synonymous ratio estimates, ω, of 14.459 and 30.639, respectively. Our estimates further highlighted the large proportion of sites in both proteins under purifying selection: 95% of the sites in Mg1LysM with ω = 0.043 and 98% in Mg3LysM with ω = 0.119. These findings agree with the behavior of protein-coding genes, where the majority of sites are expected to be subjected to strong purifying selection, with only a small fraction of them under positive selective pressure (Bielawski and Yang, 2005, and refs. therein).

The empirical Bayes approach (see “Materials and Methods”) allows us to use the maximum likelihood estimates from above to identify the sites in the coding sequences of each gene that are likely to evolve by positive selection. Figure 5, C and D, shows the posterior probabilities of sites under positive selective pressure in the coding sequences of Mg1LysM and Mg3LysM. Sites with high posterior probabilities are predicted to evolve under strong positive selection. Twelve nucleotides within four codons in Mg1LysM were identified as having very high posterior probabilities at the 90% probability cutoff, while three sites (one codon) in Mg3LysM were predicted to be positively selected at the 90% probability cutoff, and six sites (two codons) in total had high posterior probabilities at the 50% cutoff. Interestingly, sites with high probabilities in Mg1LysM cluster in the central region, and half of these sites match the position of the encoded LysM domain. In Mg3LysM, high posterior probability sites occur toward the 5’ and 3’ regions of the gene, and only the right-most (third) LysM domain seems to contain them.

M. graminicola Mg3LysM Mutants Are Severely Compromised in Virulence toward Wheat Leaves

To test whether Mg3LysM and/or Mg1LysM proteins played a significant role in fungal virulence, we generated gene deletion strains by Agrobacterium tumefaciens-mediated transformation. M. graminicola transformation was performed in a Δku70-modified strain that typically supports homologous recombination frequencies of greater than 70% (Bowler et al., 2010). Three and four independent strains for Mg1LysM and Mg3LysM, respectively, were confirmed to have single
T-DNA integrations within the target locus via Southern analysis (Supplemental Fig. S2) and were subsequently inoculated onto leaves of a susceptible wheat cultivar. All three tested mutants of Mg1LysM (∆Mg1LysM) were fully pathogenic toward wheat leaves of the susceptible hexaploid winter wheat cv Riband at all spore concentrations. We were unable to detect any difference in the timing of lesion formation or the number of sporulation structures (pycnidia) that formed within disease lesions (data not shown). In contrast, all four independent mutant strains of Mg3LysM (∆Mg3LysM) were significantly impaired in their ability to infect wheat leaves at all tested spore concentrations. Figure 6A displays the representative leaf infection phenotypes for all mutant strains. Features of this defect, observed for all four independent ∆Mg3LysM mutant strains, included the following: (1) an early (approximately 6 d post inoculation) appearance of mild leaf chlorosis in the inoculated area that was not seen in the wild-type inoculated leaves, which was less pronounced at lower spore concentrations (Fig. 6B); (2) a complete lack of development of necrotic lesions bearing pycnidia (Fig. 6, A and B) at any time point post inoculation at any tested spore concentration (monitored for up to 31 d; Figure 6, C and D); and (3) a dramatically reduced fungal biomass in leaves infected by the mutant strains, as determined by real-time PCR on fungal genomic DNA. Representative data for two independent mutant strains are shown in Figure 6E. This reduced rate of fungal biomass accumulation became detectable between 6 and 10 d post inoculation, during which lesions began to form on leaves infected with the wild-type fungus. These data suggest that Mg3LysM, but not Mg1LysM, plays a significant role in the virulence of M. graminicola toward wheat leaves.

The Activation of Wheat Defense Genes Is More Rapid and Pronounced during Symptomless Colonization by the ∆Mg3LysM Mutants

It has been suggested that fungi lacking homologs of CfEcp6 have an impaired ability to evade the activation of chitin-induced PTI (Bolton et al., 2008; de Jonge

Figure 3. Both the Mg1LysM and Mg3LysM proteins bind chitin but only Mg3LysM suppresses chitin-induced elicitation of tomato suspension-cultured cells. A, The purified MgLysM proteins were incubated in the presence of the insoluble carbohydrates chitin, crab shell chitin and chitosan, and the plant-derived carbohydrates xylan and cellulose. Following centrifugation, both the pellet (P) and the supernatant (S) were analyzed on protein gels. Both the Mg3LysM and Mg1LysM proteins were pelleted only in the presence of insoluble chitin. B, Tomato cell culture chitin-induced medium alkalinization assays in the presence and absence of Mg3LysM and Mg1LysM proteins. Only Mg3LysM is able to suppress elicitation of the cells by 10 nM chitin (Gn6). Graphs represent an average of two to four measurements in all cases.

Figure 4. Mg1LysM and Mg3LysM protect fungal hyphae from hydrolysis by plant hydrolytic enzymes. Micrographs of T. viride taken 4 to 6 h after addition of water, crude extract of tomato leaves containing intracellular, hydrolytic enzymes including basic chitinases (ChiB), pretreatment with 30 μM CfAvr4 followed by addition of tomato extract (Avr4 + ChiB), pretreatment with 30 μM CfEcp6 followed by addition of tomato extract (Ecp6 + ChiB), pretreatment with 30 μM Mg3LysM followed by addition of tomato extract (Mg3LysM + ChiB), and pretreatment with 30 μM Mg1LysM followed by addition of tomato extract (Mg1LysM + ChiB). A representative figure from three independent experiments is shown. Bars = 10 μm.
The long symptomless phase of wheat leaf colonization by *M. graminicola* is well suited to the analysis of pathogen gene expression but also to temporal analysis of host defense gene expression. We hypothesized that plants inoculated with the ΔMg3LysM mutant strains would accelerate, and perhaps amplify, the expression of *Pathogenesis-Related* (PR) gene homologs, which are normally triggered immediately prior to symptom formation during fully susceptible disease interactions (Adhikari et al., 2007; Motteram et al., 2009). Therefore, we inoculated wheat leaves with either the ΔMg3LysM mutant strains or the wild-type strain and collected leaf material throughout the symptomless phase of colonization for analysis of host defense gene expression by real-time RT-PCR. We used primers to a wheat PR1 homolog (GenBank accession no. AF384143) and to a Chitinase gene (A7437443), both of which are transcriptionally activated between days 8 and 10 post inoculation with wild-type fungus (Motteram et al., 2009). Neither the PR1 nor the Chitinase gene showed any consistent difference in expression levels at day 4 of leaf infection between the wild-type and ΔMg3LysM mutant strains (Fig. 7). However, at day 6, a very strong transcriptional induction of both genes was observed in plants inoculated with the ΔMg3LysM mutants that was not detected in wheat leaves infected by the wild-type fungus. This induction was sustained at day 8 and remained severalfold higher than that subsequently detected during leaf colonization by the wild-type fungus (Fig. 7). These data suggest that susceptible wheat leaves infected by the ΔMg3LysM mutant strains are responding earlier and more strongly to the presence of the fungus compared with leaves inoculated with the wild-type strain.

**DISCUSSION**

The LysM Domain Effector Functions in Biotrophic and Hemibiotrophic/Necrotrophic Fungal Pathogens of Dicotyledonous and Monocotyledonous Plants

To date, to our knowledge this is only the second report concerning the functional analysis of LysM...
effector homologs in fungal plant pathogens and the first to describe functional effector proteins from the wheat pathogen *M. graminicola*. The original study focused upon one protein, CfEcp6, isolated from the biotrophic dicotyledonous (tomato) leaf pathogen *C. fulvum*, which was shown to be produced at high levels during plant infection, to bind chitin fragments, and to interfere with the expression of chitin-triggered immunity (Bolton et al., 2008; de Jonge and Thomma, 2009; Thomma et al., 2005). *M. graminicola* also penetrates wheat leaves through stomata but then only slowly colonizes the intercellular spaces of wheat leaves. However, this fungus appears to benefit from host cell death, which may provide sufficient nutrients to support its asexual sporulation (Keon et al., 2007; Deller et al., 2011). Early symptomless growth is slow, whereas growth rate during host cell death increases very rapidly, suggesting that *M. graminicola* is subject to initial nutrient limitation that is relieved once host cell permeability changes and the necrotrophic phase begins. In fact, the transition to active pathogenesis in this system may depend on initiation of host cell death (Rudd et al., 2008). However, the data we have presented here for the ΔMg3LysM mutant strains support the idea that *M. graminicola* must nevertheless suppress early recognition events and the premature activation of PTI during the symptomless phase in order to be able to transition to the necrotrophic growth stage, accelerate growth, and sporulate. This suggests that fungal pathogens that are predominantly necrotrophic (with regard to the acquisition of nutrients sufficient to support sporulation) may still need to suppress the early PTI systems of the host plant in order to subsequently establish the necrotrophic phase of colonization. This may be achieved by temporal and spatial regulation of the production of suites of effector proteins, the first “battery” of which may function in

**Figure 6.** Gene disruption strains of Mg3LysM (ΔMg3LysM) are severely impaired in their ability to colonize and reproduce in wheat leaves. A, Wheat leaves inoculated with the wild-type strain of *M. graminicola* photographed 21 d after inoculation at three different fungal spore concentrations. B, Wheat leaves inoculated with a ΔMg3LysM mutant (ΔMg3LysM-2) strain photographed 21 d after inoculation at three different spore concentrations. C, Leaf infected with wild-type fungus at 30 d post inoculation highlighting the presence of abundant fungal sporulation structures (black foci). D, Leaf infected with ΔMg3LysM mutant fungus at 30 d post inoculation. E, Quantitative PCR on DNA isolated from fungus-infected leaves to determine the levels of fungal biomass during colonization by the wild-type (WT) and ΔMg3LysM mutant strains. Data are provided for two independent ΔMg3LysM mutants (ΔMg3LysM-2 and ΔMg3LysM-3). The data are shown relative to biomass detected at 6 d after inoculation for the wild-type fungus and are representative of three independent experiments performed on each strain.

**Figure 7.** The ΔMg3LysM mutants trigger more rapid and strong expression of wheat defense-related genes during the early symptomless phase of the interaction. RNA was isolated from wheat leaves inoculated with either the wild type or ΔMg3LysM mutants across the symptomless period of infection. Real-time RT-PCR was performed on the wheat PR1 and Chitinase genes. Gene expression data are normalized to expression of the wheat β-tubulin gene and presented relative to that detected at day 0. The data are representative of duplicate experiments with similar results.
suppressing PTI and establish niche occupancy, which may then pave the way for release of a second battery that might stimulate effector-triggered susceptibility by inducing host cell death responses (Friesen et al., 2008; Solomon and Rathjen, 2010; Deller et al., 2011). This study has also provided clear evidence supporting virulence functions for fungal LysM effectors that are necessary for fungal pathogenesis of both dicotyledonous and monocotyledonous plant species, suggesting an ancient adaptation of these pathogens to the evolution of chitin recognition systems by plants.

Clues to MgLysM Protein Functions Deriving from Gene Expression Profiling

The gene expression profiling experiments provided data supporting biological functions for the Mg3LysM and Mg1LysM proteins during the symptomless phase of leaf infection, as both were very strongly activated only during this phase. The extent to which expression of the Mg3LysM and Mg1LysM genes then decreased upon entry into the necrotrophic growth phase of the fungus is remarkable and suggests that the requirement for the Mg3LysM protein to suppress defense activation had been removed coincident with activation of the host cell death response. This pattern of gene expression has also been described for a M. graminicola homolog of the necrosis- and ethylene-inducing-like protein MgNLPl (Motteram et al., 2009) and for a number of putative secreted proteins containing intragenic repeats identified in the fungal genome sequence (Rudd et al., 2010). Research on other systems has identified similar patterns of effector gene expression in pathogenic fungi. For example, the SIX1 effector gene from the vascular wilt fungus Fusarium oxysporum (f. sp. lycopersici) was strongly expressed only in the presence of living plant cells (van der Does et al., 2008). The gene expression data for Mg1LysM and Mg3LysM suggest that they may also respond to the presence of living wheat cells and are subsequently rapidly down-regulated during the onset of host cell death. How fungal pathogens detect this change in host cell viability needs further research, although it has been shown that selected fungal pathogen effectors can be transcriptionally regulated in response to changes in nutrient availability that might occur as a consequence of host cell death (Thomma et al., 2006).

MgLysM Proteins Have Additional Putative Effector Functions That Distinguish Them from CfEcP6

In contrast to CfEcP6 and Mg3LysM, Mg1LysM possesses only a single LysM domain, and gene expression profiling highlighted that peak expression for Mg3LysM preceded that for Mg1LysM. Moreover, loss of Mg1LysM protein function had no major discernible impact on virulence. This was despite the fact that Mg1LysM was also able to bind chitin fragments. However, Mg1LysM chitin binding did not block the elicitation of tomato defense responses, so presumably it does not effectively conceal the elicitor in the way predicted for Mg3LysM and CfEcP6. Based upon these results, it is likely that the Mg1LysM protein does not function in interfering with the ability of the host plant to detect chitin, which distinguishes it from both CfEcP6 and Mg3LysM. Unexpectedly, both Mg1LysM and Mg3LysM were found to possess a functionality that is not present in CfEcP6, in that they were both able to protect fungal hyphae against hydrolysis by plant enzymes. The redundant self-protection activity, combined with the inability of Mg1LysM to block chitin-triggered defense, may explain why deletion of the Mg1LysM gene did not impact significantly upon fungal virulence.

The protection activity of Mg3LysM and Mg1LysM against plant hydrolytic enzymes distinguishes both proteins from CfEcP6. Intriguingly, C. fulvum has been shown to possess other effectors that possess this activity, foremost among which is the chitin-binding effector Avr4, which lacks LysM domains and binds chitin through an invertebrate chitin-binding domain (van den Burg et al., 2004, 2006). These previous studies and our work here reiterate that CfAvr4 possesses a chitinase protection activity also seen for Mg1LysM and Mg3LysM. Moreover, recent studies on sequenced fungal genomes identified homologs of the CfAvr4 gene in other Mycosphaerellaceae species, namely Mycosphaerella fijiensis and several Cercospora species (Stergiopoulos et al., 2010). However, perhaps significantly, no Avr4 homologs were identified in the M. graminicola genome, which may explain why, in contrast to C. fulvum EcP6, LysM effectors of M. graminicola may have evolved additional functions that serve to protect fungal hyphae against plant hydrolytic enzymes. This also suggests an important potential dual

| Properties                                      | CfEcP6 | Mg1LysM | Mg3LysM |
|------------------------------------------------|--------|---------|---------|
| Expressed during plant infection?              | Yes    | Yes (but only symptomless phase) | Yes (but only symptomless phase) |
| Major role in virulence?                       | Yes    | No (possibly minor or redundant) | Yes |
| Chitin-binding activity?                       | Yes    | Yes     | Yes     |
| Blocks chitin-triggered defense responses?     | Yes    | No      | Yes     |
| Protection against plant-derived chitinases?   | No     | Yes     | Yes     |
| Sequence variation Ka/Ks?                      | Ka < Ks purifying selection | Ka = Ks neutral but strong at particular sites | Ka = Ks neutral but strong at particular sites |

Ka, Nonsynonymous nucleotide substitution rate; Ks, synonymous nucleotide substitution rate.
functionality for the Mg3LysM effector protein during wheat leaf infection. Based upon the experimental evidence to date, loss of Mg3LysM could result in greater access of plant chitinases to the chitin component of the hyphal cell wall. This may result in the release of additional chitin fragments, which will also fail to be sequestered by the deleted Mg3LysM protein. The net result may be a significantly increased amount of chitin fragments available for detection by plant receptors, which would trigger an earlier and stronger defense reaction. This is supported by the gene expression profiling of wheat defense genes following inoculation with the ΔMg3LysM mutant, which identified more rapid and stronger expression of wheat PR-1 and Chitinase genes. The experimentally determined characteristics of CfEcp6, Mg3LysM, and Mg1LysM are summarized in Table II.

**Mg1LysM and Mg3LysM Exhibit More SNPs Giving Rise to Nonsynonymous Amino Acid Substitutions Than Are Found in CfEcp6**

In contrast to what was observed for CfEcp6, many SNPs were identified in the Mg3LysM and Mg1LysM genes, which gave rise to several amino acid substitutions in the protein sequences. Although the overall number of synonymous-to-nonsynonymous substitutions in each gene suggested only neutral levels of selection, certain sites in each gene appear to be under strong selection, particularly in the third LysM domain of Mg3LysM and the LysM domain of Mg1LysM (Fig. 5, C and D). It is currently unclear why more SNPs are detected in the two *M. graminicola* LysM effectors than are found in CfEcp6. Similarly, we do not yet know the contribution of different regions of either protein, including the predicted LysM domains, to the biological functions we have identified. One possibility for an overall increased level of sequence polymorphism is that the added functionality of Mg3LysM and Mg1LysM to protect hyphae against plant chitinases may place additional evolutionary pressure on these two genes, which is not imposed upon CfEcp6. We also cannot rule out the possibility that either gene may function as an avirulence determinant during gene-for-gene resistance interactions with particular wheat cultivars (Brading et al., 2002). However, the precise origins of additional selective pressures on MgLysM sequences and possible consequences of sequence variability for protein function(s) remain to be determined.

An alignment of the LysM domains of CfEcp6 and Mg3LysM suggests that the two domains closest to the C terminus of each protein are most similar to one another. In contrast, the first domain in each protein was more similar to the LysM domains found in the Mg1LysM and MgXlLysM proteins. Previous analyses have also identified LysM effector homologs in many other fungi, and the number of LysM domains can vary considerably (Bolton et al., 2008). As functional studies progress, it might be possible to ascertain precisely how many, and which, LysM domains are required to confer the various effector functions observed for CfEcp6, Mg3LysM, and Mg1LysM. This should be feasible through genetic complementation studies in ΔMgLysM mutants coupled with analyses of protein biochemistry.

**The Impact of Chitin Signaling and Chitinase Protection on Wheat Leaf Infection by *M. graminicola***

Chitin represents only one of a number of PAMPs, which can trigger plant immunity toward fungal pathogens. From studies on model pathosystems, a variety of other fungal and/or oomycete molecules, including glucans, proteins/peptides, and lipids, have been shown to activate PTI-associated defense responses (Nürnberger and Brunner, 2002). Which *M. graminicola* PAMPs might be detected by specific host receptors resident on the surfaces of wheat leaf cells is currently unknown, although it is well established that wheat leaves respond strongly to chitin elicitation with lig- nification and other defense responses (Barber et al., 1989; Vander et al., 1998). However, the considerable reduction in virulence of the Mg3LysM mutants coupled with the magnitude of the host defense gene activation triggered through loss of this effector suggests that chitin recognition and signaling play a major role in controlling STB disease of wheat and, conversely, that evading the activation of chitin-mediated signaling pathways by wheat leaves is of key importance to fungal pathogenesis. Whether cultivated hexaploid wheat possesses functional homologs of the Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*) LysM domain-containing chitin receptors (Miya et al., 2007; Petutschnig et al., 2010; Shimizu et al., 2010) and downstream signaling components, therefore, awaits determination. However, the recent identification of a putative chitin receptor that is homologous to rice CEBIP in barley (*Hordeum vulgare*) suggests that CEBIP chitin receptors are conserved in monocot plant species (Tanaka et al., 2010).

**MATERIALS AND METHODS**

**Plant and Fungal Material and Handling**

A modified version of the fully sequenced *Mycosphaerella graminicola* isolate IPC023 was used in all experiments (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html). This modified strain carries a deletion of the *Ku70* gene, which otherwise facilitates nonhomologous recombination events and increases the rate of generating homologous recombinants via transformation to greater than 70% (Bowler et al., 2010). All strains were stored at −70°C and −80°C in 50% (v/v) glycerol. Fungal spores for plant inoculation were harvested from 7-d-old cultures growing (budding) on yeast extract peptone dextrose plates (*Oxoid*) at 15°C. For estimation of gene expression in vitro, the wild-type fungus was inoculated into cultures of PD8 and CDB and shake flask cultures were harvested during logarithmic growth. *Wheat* (*Triticum aestivum*) leaf infection assays were performed as described previously (Keon et al., 2007).

**Real-Time PCR**

Genomic DNA was isolated from 500 mg of infected leaf tissue (10× 6-cm leaf segments) harvested on various days after inoculation, using a DNeasy Plant Mini Kit (Qiagen), following the supplier’s instructions. Real-Time PCR
was performed in order to monitor levels of fungal biomass in infected leaf tissues using a Cy5-labeled probe to quantify the presence of the Cytochrome b gene of *M. graminicola* (Fraaije et al., 2005). A 50-ng aliquot of DNA was used in a 20-μL PCR. Results were obtained from three replicate-time-course experiments. Real-time RT-PCR was done as described previously with relative expression levels determined in relation to β-tubulin-specific primer sets for both *M. graminicola* and wheat (Morteram et al., 2009; Rudd et al., 2010). A full list of primer sequences is contained in Supplemental Table S1. Primers for the wheat PRTI homolog, Chitinase, and β-tubulin derive from GenBank accession numbers AF384143, A7437443, and U76444, respectively.

### Heterologous Expression of Mg3LysM and Mg1LysM in *Pichia pastoris*

Full-length cDNA clones of Mg3LysM and Mg1LysM were generated by PCR using primers 3LysMcDNAF/3LysMcDNAF and 1LysMcDNAF/1LysMcDNAF, respectively (Supplemental Table S1). The resulting sequences were cloned into pGEM-T Easy (Stratagene). Individual clones were sequenced in both directions using T7 and SP6 primers (MWG Biotech) for sequence verification. Both genes were cloned into *Pichia* expression vector pPIC9 (Invitrogen) after performing PCR using primers to add the N-terminal His and FLAG tags and EcoRI and NdeI restriction sites for directional cloning (Supplemental Table S1). Subsequently, *P. pastoris* strain GS115 was transformed, and a selected clone was cultured in a fermentor (Bioflo 3000; Rooney et al., 2005). After removal of cells and concentration of the culture medium, the His-tagged protein was purified using a nickel-nitriilotriacetic acid agarose column (Qiagen) according to the manufacturer’s protocol. The eluted protein fractions were pooled and dialyzed against Milli-Q water, and the final protein concentration was determined spectrophotometrically at 280 nm and confirmed using the Pierce BCA Protein Assay Kit (Thermo Scientific) with bovine serum albumin as a standard.

### Polysaccharide Affinity Precipitation

These assays were performed as described previously (van den Burg et al., 2006; de Jonge et al., 2010) with 50 μg mL⁻¹ Mg3LysM and with 600 μg mL⁻¹ Mg1LysM. Essentially, the proteins were incubated with 3 mg of chitin beads (New England Biolabs), crab shell chitin, chitosan, xylan, or cellulose (all from Sigma) in 800 μL of water. After gentle rocking at 4°C overnight, the insoluble fraction was pelleted by centrifugation (5 min, 13,000 g) and the supernatant was subsequently boiled in 200 μL of 1% SDS. The presence of protein in supernatant and pellet was examined by Tricine SDS-PAGE followed by Coomassie Brilliant Blue staining.

### Chitin-Induced Medium Alkalinization Assay

These assays were performed as described previously (de Jonge et al., 2010). Suspension cultured tomato (*Solanum lycopersicum*) cell line MsDK was used 5 d after subculture for alkalinization experiments. To this end, 2.5 mL aliquots of the suspension were placed on 12-well culture plates on a rotary shaker at 200 rpm and allowed to equilibrate for a minimum of 2 h. Upon addition of chitin, the pH of the medium was continuously measured for 10 min. Prior to addition, mixtures of effector protein and chitin oligosaccharides were kept at room temperature for at least 1 h.

### In Vitro Fungal Growth Assays

These assays were performed as described previously (van den Burg et al., 2006; de Jonge et al., 2010). Essentially, approximately 10³ conidia of *Trichoderma viride* were incubated overnight at room temperature in 40 μL of PDB on a 96-well microtiter plate. Subsequently, effector proteins were added to the conidal suspensions at a final concentration of 30 μM. After a 2-h incubation period, 5 μL of tomato extract containing hydrolytic enzymes was added. Fungal growth was assayed microscopically after 4 to 6 h of incubation at 22°C.

### Agrobacterium tumefaciens-Mediated Fungal Transformation

For targeting of Mg3LysM, 1,036 bp of fungal genomic DNA was amplified and cloned into vector pCHYG (Morteram et al., 2009) as a SacI-KpnI fragment using primers 3LysMKO-FI1fwd and 3LysMKO-FI1rev (Supplemental Table S1) to generate pCHYG3FL1. A second fragment of 1,382 bp was then amplified and cloned into pCHYG3FL1 using BamHI-XbaI restriction sites to yield the final finished targeting vector pCHYG3LKO. The second fragment was amplified using primers 3LysMKO-FI2fwd and 3LysMKO-FI2rev (Supplemental Table S1). The final plasmid was confirmed to be correct by restriction analysis and then used to transform *A. tumefaciens* strain Agl-1 via the freeze-thaw method (Am et al., 1998). This construct was designed to introduce the T-DNA into the genomic sequence of Mg3LysM within the 5' intron sequence while also deleting approximately 50% of the predicted coding region. Targeting of Mg1LysM followed the same scheme but used primers 1LysMKO-FI1fwd and 1LysMKO-FI1rev for cloning via SacI-KpnI and 1LysMKO-FI2fwd and 1LysMKO-FI2rev for cloning via Xho-HindIII (Supplemental Table S1). The final plasmid, pCHYG1LKO, was designed for the complete deletion of the Mg1LysM gene. Strains harboring the desired plasmids were then used for fungal transformation. *A. tumefaciens* transformation of *M. graminicola* was performed as described previously (Zwiers and De Waard, 2001; Morteram et al., 2009) based upon their ability to grow on hygromycin (100 μg/mL) selective medium. Targeted insertion of the T-DNA to the desired locus for candidate mutants of Mg3LysM and Mg1LysM was determined by Southern-blot analysis using probes generated by PCR using primers SSL fw/SSL rev and SSL fw/SSL rev (respectively, Supplemental Table S1).

### Isolation of Fungal Genomic DNA and Southern-Blot Analysis

Fungal DNA was isolated as described and Southern blot probed as described by Morteram et al. (2009). DNA was digested with *XhoI* for putative ΔMg3LysM transformants or *BamHI* for ΔMg1LysM transformants. Specific probes designed to detect a shift in the size of the cross-hybridizing signal in correctly targeted strains were generated via PCR on genomic fungal DNA.

### Methods for Detecting Adaptive Protein Evolution

We used likelihood-based phylogenetic methods, likelihood ratio tests, and Bayesian statistics to detect adaptive protein evolution, assess variation in selective pressure among sites, and identify the sites within a protein that are likely to evolve under positive selection (Goldman and Yang, 1994; Bielawski and Yang, 2005). We performed the analyses with the software package TOPALi version 2 (Müle et al., 2009), which, in turn, calls an external package called PAML (Yang, 1997).

The theory of hypothesis testing, and in particular the likelihood ratio test, allows us to calculate the support provided by the observed data for or against a null hypothesis relative to an alternative hypothesis. In the context of this study, the observed datum is the protein sequence alignment. The competing hypotheses are, for example, a model that assumes a common nonsynonymous/synonymous substitution ratio, ω, for all sites in the alignment versus an alternative model that includes multiple ω values (thus allowing for variable selective pressures among sites). A second example of this type of test is between a null model that assumes only purifying and neutral evolution versus a more general, alternative model that allows for purifying, neutral, and positive selective pressures. The support of the data for or against the null model (i.e. the “simpler” of the two hypotheses) is measured using the statistic

\[ 2\Delta l = 2 \left( \ln \left( \hat{\theta}_0 \right) - \ln \left( \theta_1 \right) \right) \]

where \( \theta_1 \) is the maximum log likelihood under the null model; \( \theta_0 \) is the maximum log likelihood under the alternative model; \( \hat{\theta}_0 \) and \( \hat{\theta}_1 \) are the corresponding model parameters; and the hat denotes a point estimate of the parameter. In our study, \( \theta_0 \) is a parameter vector that may contain one or multiple ratios \( \omega \) together with the proportion of sites evolving under each \( \omega \). We then compare the \( 2\Delta l \) statistic against a \( \chi^2 \) distribution to find the significance of the test, with degrees of freedom equal to the difference in the number of free parameters between the two models. Some restrictions apply to this type of test, and further details may be found in Bielawski and Yang (2005) or in statistical textbooks.

Once having tested for positive selection and obtained a significant result, Bielawski and Yang (2005) recommend using empirical Bayes to identify the specific sites in the alignment that are likely to evolve by positive selective pressure. This approach uses the maximum-likelihood parameter estimates obtained at the likelihood ratio test stage and computes the posterior probability that a site evolves with the ratio \( \omega \) estimated for the positive class. The
maximum-likelihood estimate of the proportion of sites in the positive class is used as the prior probability.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Alignments of Mg3LysM and Mg1LysM genes and proteins sequenced from a differential isolate set.

**Supplemental Figure S2.** The generation and Southern-blot validation of Mg3LysM and Mg1LysM gene disruption/deletion strains.

**Supplemental Table S1.** Primers used in this study.

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