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Molecular Characterization and Functional Analysis of *MgNLP*, the Sole NPP1 Domain–Containing Protein, from the Fungal Wheat Leaf Pathogen *Mycosphaerella graminicola*

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Analysis of the fully sequenced genome of the wheat leaf-specific fungal pathogen *Mycosphaerella graminicola* identified only a single gene encoding a member of the necrosis- and ethylene-inducing peptide 1 (Nep1)-like protein family (NLP). NLP proteins have frequently been shown to trigger cell death and the activation of defense signaling reactions in dicotyledonous plants. However, complete loss-of-function reverse genetics analyses for their importance in the virulence of eukaryotic plant pathogens are generally lacking. Real-time quantitative polymerase chain reaction on *MgNLP* demonstrated the gene to be specifically expressed in planta. Peak expression was observed during the immediate presymptomatic phase of colonization of a susceptible host genotype. This was followed by a dramatic decrease during disease lesion formation which, in this system, exhibits characteristics of host programmed cell death (PCD). No comparable peak in transcript levels was seen during an incompatible interaction with a host genotype exhibiting gene-for-gene–based disease resistance. Heterologously expressed MgNLP protein induced necrotic cell death and the activation of defense-related genes when infiltrated into *Arabidopsis* leaves but not in leaves of a susceptible wheat genotype. MgNLP infiltration also failed to stimulate wheat mitogen-activated protein kinase activities. Finally, targeted deletion of *M. graminicola MgNLP* caused no detectable reduction in plant pathogenicity or virulence, suggesting that this protein is not a major virulence determinant during fungal infection of its host plant. To our knowledge, this represents the first complete loss-of-function analysis of NLP in a eukaryotic plant pathogen and we discuss our findings in the context of possible functions for NLP in pathogens which only infect monocotyledonous plants.

Plant pathogens have been shown to deploy secreted or injected proteins and metabolites in order to influence the outcome of interactions with host plants in their favor (Birch et al. 2008; Block et al. 2008; Kamoun 2006). Numerous proteins have been identified that either function directly as toxins to induce plant cell death or in suppressing or evading plant defense responses, thereby favoring early pathogen colonization (Espinosa and Alfano 2004; Friesen et al. 2008). These molecules have been collectively referred to as effectors (Jones and Dangl 2006). Among the host cell-death-inducing secreted proteins of plant pathogens are the necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLP), first detected in culture filtrates of the vascular wilt fungus *Fusarium oxysporum* (Bailey 1995). NLP are widespread among bacteria, fungi, and oomycetes and typically possess a conserved domain, referred to as the NPP1 domain (PFAM domain PF05630), deriving from studies describing the plant necrosis-inducing activity of *Phytophthora parasitica* NLP (Fellbrich et al. 2000, 2002). Although exceptions have been noted, the majority of experimentally analyzed NLP have been shown to induce necrotic cell death when infiltrated into plant leaves or used to treat cultured cells (Gijzen and Nürnberger 2006; Qutob et al. 2002). Intriguingly, this biological activity appears to impact only upon dicotyledonous plants (dicots), because several reports have already described the failure of NLP to induce the same effect upon monocotyledonous plants (monocots) (Bailey 1995; Fellbrich et al. 2002; Qutob et al. 2006; Schouten et al. 2008; Veit et al. 2001).

NLP proteins have also been shown to trigger many characteristic defense responses in dicots, now frequently associated with plant perception of pathogen-associated molecular patterns (PAMPs). These include, for example, an oxidative burst and activation of mitogen-activated protein kinases (MAPK) (Fellbrich et al. 2000, 2002; Qutob et al. 2006). Profound plant transcriptional changes are also commonly associated with these responses (Keates et al. 2003; Qutob et al. 2006). The observation that necrotic cell death is often triggered in dicot plants but not in monocots has prompted the suggestion that specific sensitivity factors are present in the plasma membranes of the former that are lacking in the latter. However, these sensitivity factors do not appear to be proteinaceous receptors and, to date, there is no evidence for specific host sensitivity genes or loci existing toward these proteins (Qutob et al. 2006). In addition to plasma membrane targets, association of NLP proteins with nuclei of susceptible plant cells has also been reported (Schouten et al. 2008).

The recent dramatic increase in the availability of fully sequenced genomes for oomycete and fungal plant pathogens

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*The *e*-Xtra logo stands for "electronic extra" and indicates that Figure 3 appears in color and five supplemental figures are published online.

enables, among many other things, a comparative analysis of the distribution and copy number of *NLP*-encoding genes containing the NPP1 domain. Oomycetes (for example, *P. infestans*, the causal agent of potato late blight disease) can typically contain multiples of NLP-encoding genes which can range to up to 60 independent loci in a given sequenced genome (Gijzen and Nürnberger 2006). Sequenced filamentous fungal genomes, on the other hand, do not appear to have such large numbers of NLP-encoding genes. However, even in these species, it is relatively rare for only a single NLP-encoding gene to be present (Gijzen and Nürnberger 2006). For instance, analysis of the sequenced genome of the rice blast fungus *Magnaporthe grisea* suggests that it contains four NLP homologues, and at least three are present in *F. graminearum.* Both these fungi are pathogens of monocot plants. It should also be noted that NLP-encoding genes have also been identified in non-plant-pathogenic fungi such as *Neurospora crassa*. For these reasons, definitive evidence (or otherwise) for NLP proteins functioning in plant pathogenesis is still generally lacking. Possible contributory reasons for this may include, for example, the lack of an experimental system for testing gene function via reverse genetics or the potential for functional redundancy in organisms where NLP-encoding genes are present in multiple copies.

The full genome sequence has recently become available for the fungus *Mycosphaerella graminicola*, which is the causal agent of Septoria tritici blotch disease of wheat. *M. graminicola* is a wheat leaf-specific fungal pathogen of significant agricultural importance (Eyal 1999). The *M. graminicola*–wheat interaction as an experimental model has several interesting features. Prominent among these is a relatively long period of symptomless leaf association (>7 days after inoculation) before the sudden appearance of leaf lesions within which the fungus asexually sporulates. Plant pathogenesis is achieved without any form of host cell penetration and the fungus does not form either appressoria or haustoria during infection. Thus, leaf entry and exit is exclusively through stomatal apertures and colonization throughout is strictly extracellular with respect to host cells. For these reasons, it has been suggested that communication between pathogen and host may rely heavily on the exchange and recognition of secreted molecules (Kema et al. 1996; Rudd et al. 2008). Recent data has shown that the sudden appearance of strictly localized leaf lesions following a period of symptomless association bears hallmarks of host programmed cell death (PCD) (Keon et al. 2007). Various levels of control are also imposed upon the wheat MAPK TaMPK3 and TaMPK6 during this process (Rudd et al. 2008). In addition, gene-for-gene–based disease resistance, which occurs in the absence of hypersensitive cell death (Kema et al. 1996), has been demonstrated for interactions between individual *M. graminicola* isolates and wheat genotypes (Brading et al. 2002).

A number of questions remain to be addressed for this system, including i) to what extent does the fungus produce effectors that might contribute to the lack of aggressive host responses during the symptomless phase of host colonization and ii) does the fungus trigger wheat leaf MAPK signaling, PCD responses, and disease lesion formation through the concerted production of proteinaceous or metabolite toxins? We have begun to initiate a functional genomics analysis of candidate effector or toxin-encoding genes identified in the genome sequence of *M. graminicola.* An examination of this sequence for genes encoding putative *NLP* homologues identified only a single gene encoding a protein possessing the characteristic NPP1 domain. We have named this gene *MgNLP*. Here, we report several aspects relating to *MgNLP* gene expression, protein activity, and plant responses toward the protein. Significantly, by targeted gene deletion of *MgNLP*, we were able to render *M. graminicola* completely devoid of the NPP1 domain and thereby test its importance for disease-causing ability on wheat plants. We discuss these results in the context of possible roles for NLP proteins produced by microbial pathogens during colonization of monocot plants.

RESULTS

Identification of only a single NPP1 domain–containing gene (*MgNLP*) in the fully sequenced genome of *M. graminicola*.

In order to identify putative *M. graminicola* genes encoding proteins containing NPP1 domains, we performed a blastp analysis on the fully sequenced (finished) fungal genome using the original NPP1 amino acid sequence from *P. parasitica* (Fellbrich et al. 2002). This analysis returned only one hit (3e-46) on chromosome 13 with the locus identification (ID) fgenesh1_pm.C_chr_13000083, which we refer to as MgNLP. A tblastn search with the NPP1 protein sequence also returned the same locus ID and no additional homologous regions. The *MgNLP*-predicted gene model suggested an open reading frame of 714 bp encoding a 238-amino-acid protein with an N-terminal signal peptide for extracellular secretion. Both blastp and tblastn analyses performed on the *M. graminicola* genome using the MgNLP amino acid sequence returned no further hits, whereas blastp performed against GenBank returned many highly significant sequence homologues from both fungal and oomycete plant pathogens in addition to nonplant pathogens. MgNLP shared most amino acid sequence similarity to NLP proteins of fungal origin, including both pathogenic and nonpathogenic species.

Using blastp, we sought to determine how many putative NLP proteins were encoded in a variety of other sequenced fungal genomes hosted at the Joint Genome Institute (JGI) and Broad websites. Of the eight additional genomes interrogated, including six plant pathogens and two nonpathogens, only three possessed a single gene encoding a putative NLP homologue. These were *N. crassa*, *Pyrenophora tritici-repentis*, and *M. fijiensis*. Three genomes possessed two NLP homologues (*Stagonospora nodorum*, *Botrytis cinerea*, and *Aspergillus nidulans*) while *F. graminearum* and *Magnaporthe grisea* had three and four putative *NLP*-encoding genes, respectively. The amino acid sequence relationships as determined by Neighbor analysis with 1,000 bootstrap replications between each of these NLP is shown in Figure 1. The original *Phytophthora parasitica* NPP1 sequence was also included for reference. This analysis provided moderate bootstrap support (67%) for MgNLP residing within a group comprising NLP from six independent fungal species, including both plant pathogens and nonplant pathogens (Fig. 1).

MgNLP is highly expressed specifically toward the end of the symptomless phase of colonization of a susceptible wheat leaf.

Use of a *Mycosphaerella graminicola*-attached wheat leaf infection bioassay has previously enabled us to study fungal transcriptional changes at distinct phases of the interaction with a susceptible wheat genotype. Typically, this interaction occurs without disease symptoms until approximately 9 or 10 days after leaf surface inoculation. Thereafter, disease lesions form in these inoculated areas and the fungus sporulates asexually within these regions. Lesion formation itself bears hallmarks of a host PCD response (Keon et al. 2007; Rudd et al. 2008). The previous studies using fungal cDNA microarrays identified clear transcriptional reprogramming events occurring within the fungus as disease symptoms appeared and progressed between 9 and 14 days after inoculation (Keon et al. 2007).

Real-time quantitative polymerase chain reaction (qPCR) analysis on the expression of *MgNLP* in *M. graminicola* growing in vitro and during infection of susceptible wheat leaves demonstrated this gene to be specifically expressed in planta (Fig. 2A). Peak expression was seen during the symptomless phase of the interaction at 6 and 9 days after inoculation on the leaf surface. A dramatic decrease in *MgNLP* expression was subsequently seen as disease lesions developed by day 14 and asexual sporulation structures matured by day 21. This result was consistent regardless of the internal expression control used for data normalization (Supplementary Fig. 1). Only very low levels of *MgNLP* expression were seen during fungal growth in either rich medium (potato dextrose broth) or minimal media (Czapek-Dox and *Aspergillus* minimal medium), suggesting that the expression of *MgNLP* is induced specifically during the symptomless phase of wheat leaf infection.

Gene-for-gene specific resistance occurs between the experimental fungal isolate IPO323 and wheat cultivars containing the *Stb6* resistance gene (Brading et al. 2002). This occurs in the absence of a hypersensitive response (HR) or any other form of host cell death (Kema et al. 1996). Investigation of *MgNLP* expression during the early phases (days 2, 4, and 8) of an incompatible interaction identified only a very modest transcriptional activation of *MgNLP* (Fig. 2B). This was in sharp contrast to the compatible interaction which, although also exhibiting only modest induction by day 4, was followed by significantly increased expression by day 8 (Fig. 2B). The lack of a similar induction of *MgNLP* transcript in the resistant host was not attributable to a decrease in expression of the gene used for normalization, *β-tubulin* (Supplementary Fig. 2). Collectively, these data indicate that *MgNLP* is a gene specifically expressed in planta which displays peak expression during the immediate presymptomatic phase of infection of a fully susceptible host plant.

MgNLP has cell-death-inducing and elicitor activity on *Arabidopsis* but not on wheat leaves.

NLP gene family members from various microorganisms have been described to differ in their ability to induce necrotic

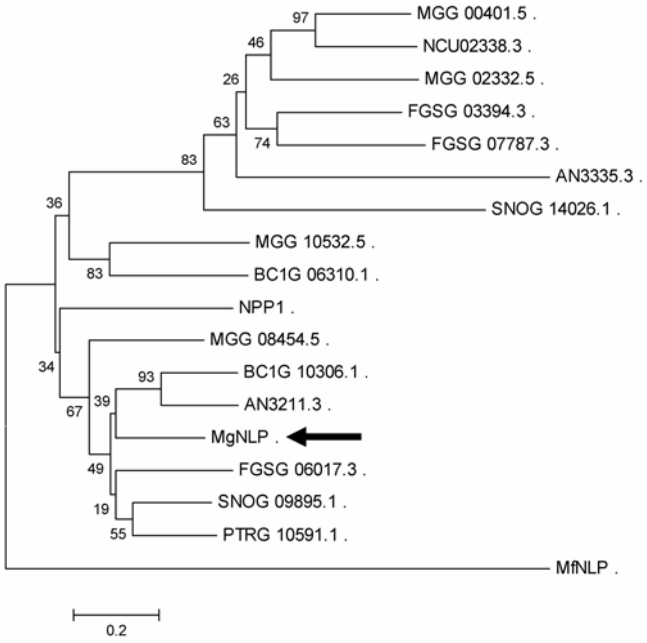

Fig. 1. Phylogenetic tree of *Mycosphaerella graminicola* MgNLP with necrosis- and ethylene-inducing peptide 1-like protein family (NLP) proteins encoded in other sequenced fungal genomes. The figure presents the result of Neighbor analysis of the predicted amino acid sequences of NLP homologues in a range of sequenced fungal genomes in addition to the original NPP1 protein from *Phytophthora parasitica*. The analysis was performed in the package MEGA4 (Tamura et al. 2007). Percent bootstrap values (1,000 replicates) are shown above the forks. Key to species abbreviations: MGG = *Magnaporthe grisea*, BC1G = *Botrytis cinerea*, An = *Aspergillus nidulans*, FGSG = *Fusarium graminearum*, SNOG = *Stagonospora nodorum*, PTRG = *Pyrenophora tritici-repentis*, NCU = *Neurospora crassa*. The numbers following these abbreviations form part of the gene model identifiers retrievable from the Broad genome website. The gene model identifiers for *MgNLP* and *MfNLP* (*Mycosphaerella fijiensis*) are fgenesh1_pm.C_chr_13000083 and fgenesh1_pg.C_scaffold_21000141, retrievable from the Joint Genome Institute website.

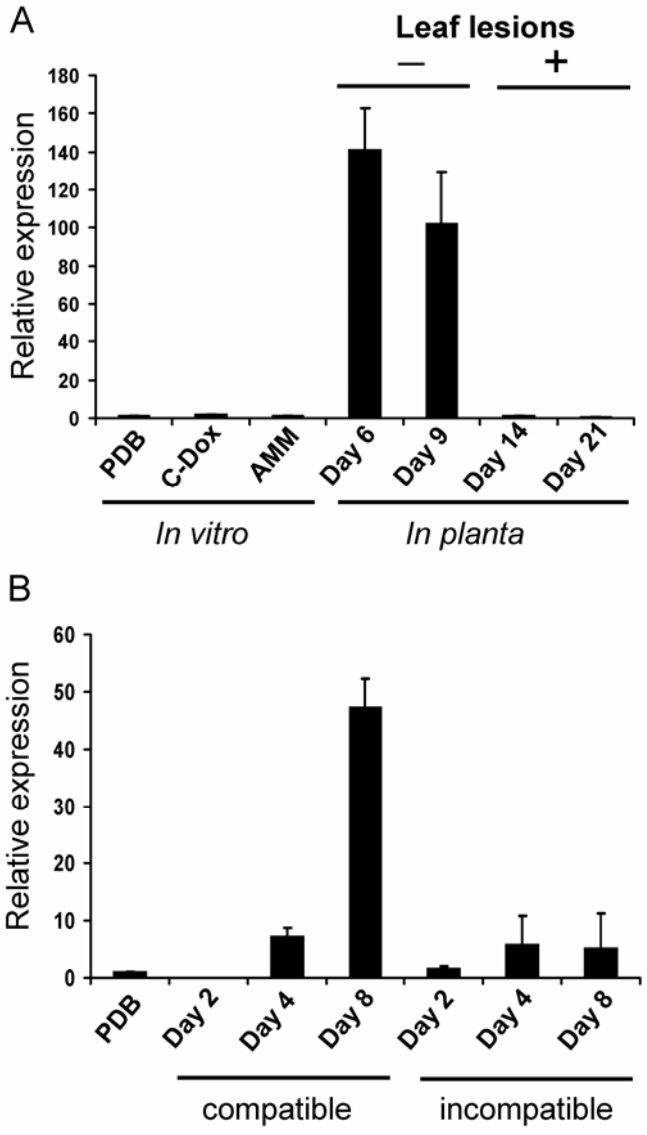

Fig. 2. MgNLP is highly expressed in planta toward the end of the symptomless phase of infection of susceptible wheat leaves. **A,** Real-time quantitative polymerase chain reaction analysis of *MgNLP* transcript levels during in vitro growth and during both asymptomatic growth and within disease lesions of infected wheat leaves. Leaf material collected 6 days after fungal inoculation is completely devoid of visible disease symptoms. First disease symptoms appear 9 days after inoculation. Key to in vitro growth conditions: PDB = potato dextrose broth, C-Dox = Czapek Dox, AMM = *Aspergillus* minimal medium. The figure identifies *MgNLP* as an in planta-expressed gene with peak expression during the symptomless phase of host colonization. **B,** *MgNLP* transcript levels during symptomless interactions with both susceptible and resistant (gene-for-gene) plants. The figure represents the result of two independent experiments and highlights the fact that peak *MgNLP* expression was observed during the immediate presymptomatic phase of colonization of susceptible wheat leaves.

cell death in dicot plants (Gijzen and Nürnberger 2006; Qutob et al. 2002). Where it occurs, cell-death -inducing activity is typically seen within 48 h after leaf infiltration with up to 1 to 2 μM of the purified proteins. In order to test whether MgNLP had cell-death-inducing activity, the protein was heterologously expressed in *Pichia pastoris*, purified, and used in leaf infiltration experiments. Both 1- and 2-μM concentrations of the purified MgNLP protein induced cell death within 48 h following infiltration into leaves of *Arabidopsis* ecotype Col-0 (Fig. 3A) and tobacco leaves (data not shown). Infiltration with either water or heat-denatured MgNLP failed to induce this response (Fig. 3A). These data demonstrate that MgNLP is a cell-death-inducing NLP. In contrast, the same concentrations of protein failed to induce cell death following infiltration into leaves of wheat cultivars extremely susceptible to the MgNLP parent isolate IPO323 (Fig. 3C). Nor was wheat leaf necrosis observed following leaf infiltration of a cultivar which exhibits gene-for-gene–based resistance to the same isolate (data not shown).

NLP proteins have been shown to trigger a variety of plant defense responses in dicot plants in addition to necrosis or cell death. Included among these responses are the expression of defense-related genes and the post-translational activation of MAPK (Fellbrich et al. 2002; Keates et al. 2003; Qutob et al.

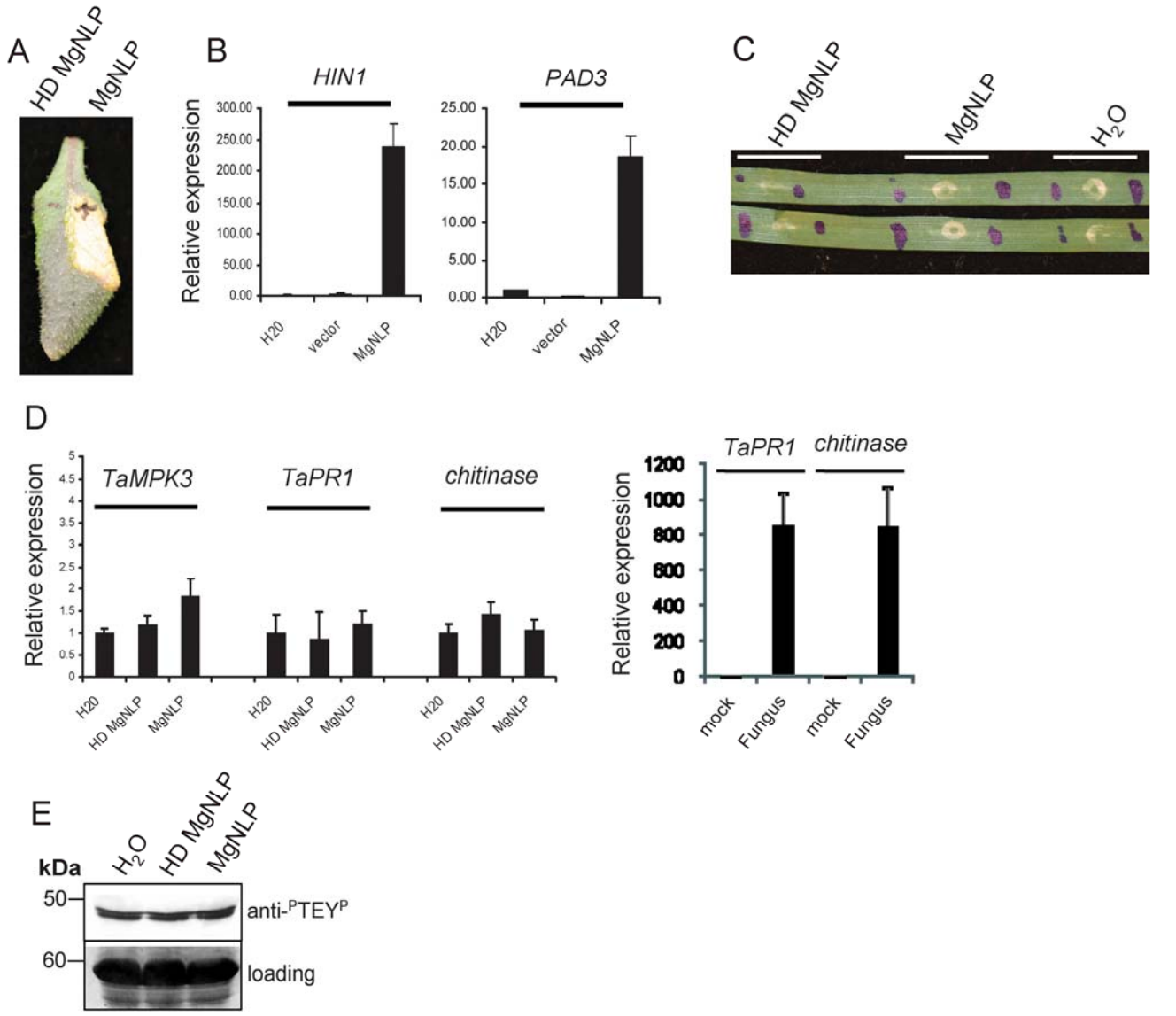

Fig. 3. MgNLP triggers necrotic cell death and defense responses in *Arabidopsis* leaves but not in wheat leaves. **A,** Heterologously expressed MgNLP (2 μM) triggers necrosis in infiltrated *Arabidopsis* leaves. Photograph taken 3 days after leaf infiltration with either active or heat-denatured (HD) MgNLP. **B,** Infiltration of *Arabidopsis* leaves with 2 μM MgNLP stimulates the expression of the plant defense-response-associated genes *Hin1* and *PAD3* detected 4 h after treatment. **C,** MgNLP (2 μM) fails to induce necrotic lesions when infiltrated into wheat leaves of a susceptible wheat cultivar. Tick marks upon the leaves and the horizontal white bars above indicate the leaf areas successfully infiltrated (areas that appeared dark green and water soaked during or following infiltration). Photographs were taken 5 days after infiltration and the small necrotic regions reflect physical damage during infiltration. HD refers to infiltration with heat-denatured MgNLP as an additional control. **D,** Wheat leaf infiltration with MgNLP fails to induce the expression of defense-related genes. Real-time quantitative polymerase chain reaction analysis of wheat mitogen-activated protein kinase (MAPK) *TaMPK3, TaPR1*, and *chitinase* transcripts was performed 4 h after infiltration with either H_2O (control) HD MgNLP (control) or the active MgNLP protein. The expression levels of the *TaPR1* and *chitinase* genes in wheat leaves 8 days after fungal inoculation are shown for comparison. **E,** MgNLP (2 μM) failed to stimulate post-translational activation of MAPK in infiltrated wheat leaves as determined by Western blotting with an anti-active MAPK ($^{P}TEY^{P}$) antibody. Leaf material was analyzed 20 min after leaf infiltration. All analyses were performed at least twice with similar results. These data collectively demonstrate that MgNLP has characteristic necrosis- and ethylene-inducing peptide 1-like protein family (NLP) activity toward the dicotyledonous *Arabidopsis* but not against the monocotyledonous wheat host plant.

2006). During this study, we were unable to conclusively determine whether MgNLP infiltration into *Arabidopsis* leaves stimulated plant MAPK. However, 2 μM MgNLP had a strong effect upon the expression of specific plant defense-related genes. For example, genes coding for harpin-induced protein 1 *(HIN1*) and phytoalexin-deficient 3 (*PAD3*) were strongly induced 4 h following infiltration (Fig. 3B). These data support the conclusion that MgNLP has elicitor activity toward dicot plant species.

Despite the lack of cell-death-inducing activity of MgNLP on wheat, we sought to determine whether any defense responses were activated following MgNLP infiltration of leaves. Therefore, we tested the ability of 2 μM protein to induce the expression of defense-related genes 4 h after infiltration or stimulate activation of MAPK 20 min after leaf infiltration. These time points are typically used to investigate similar responses in dicots (Qutob et al. 2006). Real-time qPCR analysis was performed on three independent plant defense-related genes, *TaMPK3*, *TaPR1* (pathogenesis-related protein 1), and *chitinase*, which were selected based on their strong expression immediately preceding disease symptom development during successful fungal infection of susceptible wheat leaves (Adhikari et al. 2007; Ray et al. 2003; Rudd et al. 2008). Despite the fact that these defense genes responded strongly to inoculation by the fungus, in two independent repeat experiments no significant increase in their expression was detected above control levels as a consequence of MgNLP infiltration (Fig. 3D). Similarly, no stimulation of MAPK above basal

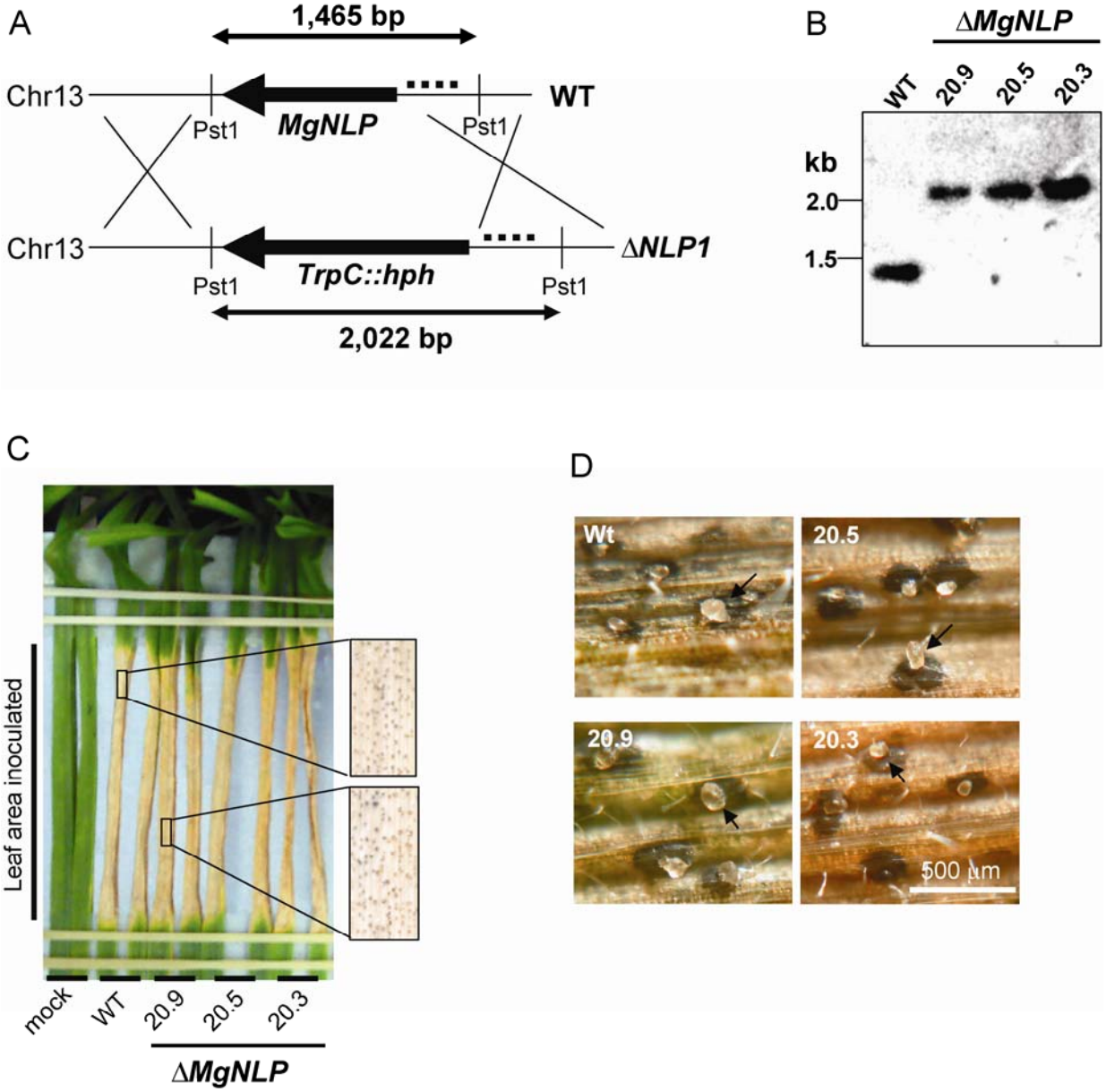

Fig. 4. Plant infection assays with gene deletion mutants of *MgNLP* demonstrate that this gene is fully dispensable for fungal plant pathogenesis. **A,** Schematic representation of the homologous recombination event on chromosome 13 involved in the targeted replacement of *MgNLP* with the *TrpC promoter::hygromycin phosphotransferase* resistance cassette. The diagram depicts the presence of *Pst*1 restriction enzyme sites and indicated the expected shift in size of the hybridizing band in the case of successful gene replacement. The dotted line depicts the hybridization site of the radio-labeled probe. **B,** Southern blot demonstrating the successful gene replacement for three independent transformants 20.9, 20.5, and 20.3. **C,** Wheat leaf infection assays with the wild-type strain and the three independent *MgNLP* deletion strains at inoculum levels of 1×10^7 spores/ml. The photograph was taken 18 days after plant inoculation and demonstrates that no differences were detected between the cell-death-inducing and asexual sporulation characteristics of the wild-type and *MgNLP* gene deletion strains. **D,** The *MgNLP* deletion strains produce viable pycnidia (asexual sporulation structures) which exude pycnidiospores. Arrows indicate cirrhi bearing spores emerging from melanized pycnidia.

control levels (infiltration with either water or heat-denatured MgNLP) was seen when the native protein was infiltrated into wheat leaves (Fig. 3E). This is in stark contrast to the strong MAPK activation detected by the same approach during the formation of disease symptoms (lesions) induced by the fungus (Rudd et al. 2008) Therefore, these data suggest that, in addition to the lack of host necrosis-inducing activity, the MgNLP protein fails to strongly elicit characteristic host defense responses in infiltrated wheat leaves.

MgNLP is dispensable for fungal infection of susceptible wheat cultivars.

In order to investigate a functional role for MgNLP during plant pathogenesis, fungal gene deletion strains were generated by *Agrobacterium*-mediated transformation and homologous recombination. Following fungal transformation, many recombinants were identified through a PCR-based screen on fungal genomic DNA. Three homologous recombinants were selected for further analysis (referred to as 20.9, 20.5, and 20.3). All three strains displayed normal growth in vitro, including the characteristic budding growth on yeast extract peptone dextrose (YEPD) agar (Supplementary Fig. 3). Successful targeted gene deletion of *MgNLP* in each independent transformant was confirmed by visualization of an anticipated shift in size of a *Pst*1-digested genomic DNA fragment in a Southern blot hybridization. This is due to the targeted replacement of the *MgNLP* open reading frame with the *A. nidulans TrpC* promoter-driven hygromycin phosphotransferase (*hph*) gene (Fig. 4A and B).

The three independent single-gene deletion strains were then tested for their disease-causing ability on wheat leaves compared with the wild-type strain. Several features were monitored, including i) the ability to induce disease lesion formation, ii) the time taken for the appearance of first lesions, iii) the time taken for the formation of asexual fruiting bodies within these lesions, and iv) extrusion of viable asexual spores from these fruiting bodies. No differences were seen between the three *MgNLP* gene-deletion strains and the wild-type strain and all were fully able to induce the formation of disease lesions bearing viable fungal sporulation structures within an identical time frame. This is demonstrated by Figure 4C and D for wheat leaves photographed 18 days after fungal inoculation. This result was also confirmed at a range of inoculum levels via a serial dilution experiment (Supplementary Fig. 4). The *MgNLP* deletion strains also remained avirulent on barley leaves and on resistant (*Stb6*) wheat leaves, suggesting that MgNLP is not a major avirulence determinant during either nonhost or resistant host interactions (data not shown). Collectively, these data demonstrate that the *MgNLP* gene and, therefore, the NPP1 domain in general, is functionally dispensable for wheat leaf infection by *M. graminicola.*

DISCUSSION

In recent years, the advances made in sequencing the genomes of fungal and oomycete plant pathogens has greatly impacted upon gene discovery and functional genomics analyses. The finished genome of the wheat leaf-infecting fungal pathogen *M. graminicola* was publicly released in September 2008. Recent work has suggested that this pathogen may co-opt various plant defense responses to initiate disease lesion formation during successful infection. These include the transcriptional and post-translational activation of a wheat MAPK, referred to as TaMPK3, and an "apoptotic-like" form of host PCD reminiscent of a macroscopic HR (Keon et al. 2007; Rudd et al. 2008). The trigger or triggers for these responses remain unknown. One hypothesis is that intercellular fungal hyphae may produce effector-type secreted proteins which might function as toxins recognized by the plant, which hyperactivates host defenses. A number of different secreted proteins have been identified from various plant pathogens that elicit host defense reactions (Friesen et al. 2008; Thomma et al. 2005; Wolpert et al. 2002). One family of such proteins which are found in various microbes which either do or do not infect plants are the NPP1 domain-containing proteins, referred to as NLP. Characteristically, these proteins, when used in isolation, induce a multitude of defense responses in dicot plants, including the post-translational activation of MAPK, defense-related genes, and host cell death (Gijzen and Nürnberger 2006). Intriguingly, numerous previous studies have demonstrated a lack of cell-death-inducing activity toward monocot plants, and subsequent studies have led to the suggestion that particular elements of host cell membranes that confer susceptibility to NLP protein activities are restricted to dicot plants (Qutob et al. 2006).

Attempts to address the function of *NLP* genes of pathogens during plant infection have been hampered in many cases by the presence of multiple copies of these genes in pathogen genomes. For example, an earlier gene deletion approach performed on the vascular wilt pathogen *F. oxysporum* had previously demonstrated an *NLP*-encoding gene to be functionally dispensable for plant infection (Bailey et al. 2002). However, recent sequencing of the genome of this fungus has revealed that at least four and potentially as many as seven secreted proteins possessing the NPP1 domain are present in this organism, which still implies a potential for functional redundancy. Although gain-of-function–type studies have suggested roles for NLP during plant infection (Amsellem et al. 2002), to date, only a single example exists whereby inactivation of an *NLP* gene compromised pathogen infection. This was for the soft-rot-causing bacterium *Erwinia carotovora* on potato (Mattinen et al. 2004; Pemberton et al. 2005).

For many of the available sequenced fungal and oomycete plant pathogen genomes, the copy number of putative *NLP*-encoding genes varies dramatically. This is particularly the case for oomycetes where, in the most extreme cases, *NLP*-encoding genes can be present at up to 60 copies in a genome (Gijzen and Nürnberger 2006). Fungal pathogens, on the other hand, have significantly less although, even here, it is relatively rare for *NLP* genes to exist as a single copy in a genome. The current study was initiated following the realization that the fully sequenced genome of *M. graminicola* isolate IPO323 possessed only a single putative *NLP*-encoding gene. Somewhat intriguingly, our phylogenetic analysis did not suggest a strong amino acid similarity between MgNLP and the only NLP we identified in the genome sequence of the banana pathogen *M. fijiensis*. The reasons for this are currently unclear; however, recent data suggest that, in some respects, this latter fungus may have more evolutionary similarity to the tomato leaf mold fungus *Cladosporium fulvum* than to *M. graminicola* (Bolton et al. 2008). More significantly, *MgNLP* was shown to be highly expressed specifically during the end of the symptomless phase of wheat leaf infection. This observation is reminiscent of what has been reported for a *Phytophthora sojae NLP* during infection of soybean, where peak expression was associated with the appearance of disease symptoms on infected plants as the pathogen transitioned from a biotrophic to a necrotrophic growth state (Qutob et al. 2002). The strong in planta expression of *MgNLP* and its single-copy status in the genome enabled us to answer a question for this pathosystem that has proven difficult for systems where *NLP* genes are multicopy, namely; are they major virulence factors for the pathogenic lifestyle of the microorganism? Targeted gene deletion of *MgNLP* had no apparent effect upon any of the parameters monitored for plant infection, implying that, in this system, the protein is not a major pathogenicity or virulence factor. This is

in agreement with data describing the deletion of two independent NLP genes from *B. elliptica*, neither of which tested in isolation had any effect upon fungal pathogenicity against lily plants (Staats et al. 2007). Furthermore, loss of *MgNLP* had no effect upon any of the in vitro growth characteristics studied, which was anticipated because the gene appears to be expressed at high levels only during plant infection.

The availability of heterologously expressed MgNLP protein allowed us to investigate other characteristics of the protein. First, we were able to demonstrate that MgNLP encoded a true necrosis-inducing protein due to its ability to trigger cell death in infiltrated *Arabidopsis* leaves and to stimulate the expression of defense-related genes in these tissues. Conversely, neither of these responses was seen following infiltration of wheat, the natural host plant. We were also unable to detect either increased expression of the wheat MAPK homologue *TaMPK3* or any effect on the post-translational activation state of wheat MAPK in general. These latter points are noteworthy because plant infection experiments with the parent fungal isolate have previously demonstrated that transcriptional activation of *TaMPK3* also precedes the development of disease symptoms (Rudd et al. 2008) and, thus, parallels the expression profile of the fungal *MgNLP*. In these studies, the TaMPK3 protein was also then shown to be subsequently post-translationally activated during the appearance of disease lesions (Rudd et al. 2008). Based upon these observations, we conclude that MgNLP fails to elicit any of the tested defense responses in wheat leaves normally seen during *M. graminicola* infection, despite the fact that the protein has the typical NLP activities toward leaves of dicot plants.

Many additional questions arise from this study. Foremost among these is why the fungus might waste energy in expressing this gene to high levels only when in contact with its only known host plant, which happens to be a monocot. What is the function of MgNLP in this system? To consider these questions, it is perhaps worth restating that NLP homologues are also found in the genomes of nonpathogenic fungi and other microorganisms. Extensive studies have been performed with NLP proteins from *P. parasitica*, *Pythium aphanidermatum*, *F. oxysporum*, and *B. cinerea*, which have demonstrated an ability to trigger cell death in dicot plants (Bailey 1995; Fellbrich et al. 2002; Qutob et al. 2002, 2006; Schouten et al. 2008). Qutob and associates (2006) also tested various other cell and tissue types, including mammalian cell lines, lower plants, and *Pichia pastoris* (a yeast) spheroplasts, for their sensitivity to NLP. The survival rate of each of these cell types was not significantly compromised by incubation with up to 1 μM NLP. The gene expression profile of *MgNLP* suggests that its potential function is primarily during the period immediately preceding the activation of host cell death signaling in a susceptible wheat leaf. A potentially key aspect of this subsequent host reaction is an associated dramatic loss of mesophyll cell membrane integrity and large-scale release of nutrients into the apoplastic space where the fungus resides (Keon et al. 2007). These events are not seen during gene-for-gene–based resistance and, therefore, it is interesting to note that no significant elevation of *MgNLP* transcript levels occurred at the corresponding time point of the incompatible interaction.

Based upon the features described above, we might therefore speculate over an alternative target (should one exist) for MgNLP function during the immediate phase preceding host cell death during wheat leaf infection. It is likely that *M. graminicola* will be occupying an environmental niche which may be shared with various other microorganisms in the period before the activation of host cell death. These cohabiting microbes might also potentially benefit from the nutrient release that will subsequently occur during this process. Therefore, it would be interesting to test whether MgNLP had any inhibitory activity against other filamentous fungi or other microbes that might normally occupy the same ecological niche during this immediate presymptomatic period. This line of speculation surrounding niche occupancy, if substantiated, may also go some way toward explaining why nonplant pathogens, including saprophytic fungi, also possess NLP-encoding genes.

In summary, to our knowledge, this represents the first example where a fungal plant pathogen has been rendered completely devoid of the NPP1 domain via a reverse genetic approach and subsequently tested for alterations in plant-infecting capabilities. This was greatly facilitated by the presence of only a single gene encoding an NPP1 domain-containing protein in the fully sequenced *M. graminicola* genome. Our results suggest that MgNLP and, therefore, the NPP1 domain is fully dispensable for the ability of the fungus to infect and reproduce asexually in wheat leaves, although we cannot discount a role in sexual reproduction. Given the presence of *NLP*-encoding genes in the genomes of many other monocot-infecting fungal pathogens, it will be of interest to determine whether they function as pathogenicity or virulence factors in these systems. Perhaps the definitive test for determining significant roles for NLP during plant infection will be to render a dicot-infecting filamentous plant pathogen completely devoid of genes encoding proteins with an NPP1 domain. This will no doubt be addressed soon and will begin to form the basis of useful comparisons between different pathosystems.

MATERIALS AND METHODS

Plant and fungal material and handling.

The fully sequenced *M. graminicola* isolate IPO323 was used in all experiments. The isolates were stored at –80°C in 50% (vol/vol) glycerol. Fungal spores for plant inoculation were harvested from 7-day-old cultures growing (budding) on YEPD plates (Oxoid Ltd., Hampshire, U.K.) at 15°C.

For plant infection, the second leaf of 17-day-old wheat seedlings of cvs. Riband (susceptible), Avalon (susceptible), or Cadenza (resistant) were attached, adaxial side up, to Perspex sheets using double-sided tape. The inoculation procedure was as described previously (Keon et al. 2007). The leaves were inoculated evenly with fungal spores at a density of 1×10^7 cells/ml (or serial dilutions) in water containing 0.1% (vol/vol) Tween 20. Following 72 h of incubation at 100% relative humidity, inoculated plants were incubated at 16°C with a 16-h light period at 88% relative humidity for up to 21 days. For fungus-induced expression of plant defense genes, materials were harvested 8 days after leaf inoculation. MgNLP leaf infiltration experiments were performed using blunt-ended syringe pressure infiltration with 2 μM protein on leaves of wheat cvs. Riband or Avalon (17 days old) or *Arabidopsis thaliana* ecotype Col-0 (14 days old). Leaf tissues were excised at various time points after inoculation or infiltration and stored at –80°C for RNA isolation, or was immediately used to generate protein extracts.

Generation of an amino acid phylogenetic tree for NLP.

All amino acid sequences detailed in the legend of Figure 1 were retrieved from either the JGI or Broad websites. The sequences were aligned using ClustalW and the alignment was edited in Genedoc and then analyzed using the phylogenetics package MEGA4 (Tamura et al. 2007). The tree was obtained using Neighbor analysis with 1,000 bootstrap replications.

Western blot detection of activated MAPK.

For the MAPK activity assays, three infiltrated leaves of wheat were collected 20 min after treatment and homogenized

in kinase extraction buffer as described previously (Ahlfors et al. 2004; Kroj et al. 2003). Following centrifugation (13,200 rpm for 15 min at 4°C), the supernatant was collected and immediately analyzed by Western blotting with anti-active ERK antibody. Approximately 100 µg of protein was separated on 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis gels and blotted onto Hybond ECL nitrocellulose (Amersham Pharmacia, , Uppsala, Sweden). The blots were probed with Phospho-p44/42 Map Kinase (Thr202/Tyr204) antibody according to the suppliers' guidelines (Cell Signaling Technology, Ltd., Danvers, CT, U.S.A,) and subsequently with 1:10,000 dilution of anti-rabbit immunoglobulin G horseradish peroxidase conjugate (Sigma Aldrich, Poole, U.K.). Blots were developed using Chemiluminescence (Amersham ECL-plus).

RNA isolation and real-time reverse-transcription PCR with SYBR-Green detection.

Total RNA was isolated from freeze-dried, filtered fungal material collected during log phase growth in liquid cultures or from leaf tissues infected by *M. graminicola* or infiltrated with MgNLP protein using the TRIZOL procedure (Invitrogen, San Diego, CA, U.S.A.). Total RNA was used for all real-time reverse-transcription (RT)-PCR analyses. For RT-PCR analysis, first-strand cDNA was synthesized from total RNA using the SuperScript III First_Strand Synthesis System for RT-PCR (Invitrogen). A 5-µg aliquot of total RNA primed with oligo$(dT)_{20}$ was used in a 20-µl reaction, following the supplier's instructions. The resulting cDNA was analyzed using a QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, U.S.A.), following the supplier's instruction. A 0.5-µl aliquot of cDNA was used in a 20-µl PCR reaction, with an annealing temperature of 56°C. Primers were added at a final concentration of 0.25 µM. The PCR reactions were run and analyzed using an ABI 7500 Real-Time PCR System. For expression normalization, the respective *β-tubulin* genes were used for *M. graminicola* and wheat, in addition to a second normalization gene for the fungus, *elongation factor 1-α.* Gene expression studies in *A. thaliana* employed the *elongation factor 1-α* gene for normalization.

Heterologous expression of MgNLP in *P. pastoris.*

A full-length cDNA clone of *MgNLP* was isolated by PCR with primers MgNLP-c fwd and MgNLP-c rev and was cloned into pGEM-Teasy (Stratagene, La Jolla, CA, U.S.A.). Clones were sequenced in both directions using T7 and SP6 primers (MWG Biotech, Ebersberg, Germany) to ensure that no erroneous base changes had resulted from PCR amplification. Secretory expression of MgNLP was performed in *P. pastoris* GS115 according to the Multi-Copy Pichia expression kit instructions (Invitrogen). The cDNA encoding for MgNLP without signal peptide was amplified via PCR using the primers MgNLPforEcoRI and MgNLPrevNotI and cloned into the secretory expression plasmid pPIC9K.

Purification of the protein from the culture medium of *P. pastoris* was achieved by ion exchange chromatography followed by gel filtration. Culture medium containing MgNLP was dialyzed against 20 mM Tris-HCl, pH 8.5 (buffer A), and applied to HiTrap Q FF 1-ml columns (GE Healthcare, Buckinghamshire, U.K.) equilibrated in buffer A. After extensive washing, bound proteins were eluted with a linear gradient of 0 to 500 mM KCl in buffer A. Following gel electrophoretic analysis, MgNLP-containing fractions were pooled and subjected to a HiLoadTM 16/60 Superdex 75 column (GE Healthcare) equilibrated in 150 mM KCl in buffer A. Upon visual inspection on an SDS gel, MgNLP-containing fractions were pooled and dialyzed against deionized sterile distilled water.

***Agrobacterium*-mediated targeted gene deletion of *MgNLP*.**

Approximately 2-kb of flanking fungal genomic DNA was amplified either side of the predicted *MgNLP* open reading

Table 1. Primers used in this work

Forward		Reverse		
Primer name	**Primer sequence 5' to 3'**	**Primer name**	**Primer sequence 5' to 3'**	**Purpose**
TaPR1 fwd	ACGTACGCCAACCAGAG GATCA	TaPR1 rev	GCATGCGATTAGGGACGA AAGAC	Expression (Wheat)
chitinase fwd	CACACAACTACAACTACG GGCCG	Chitinase rev	CGAGCTCTATCGAAACGC CATT	Expression (Wheat)
TaMPK3 fwd	TACATGAGGCACCTGCCG CAGT	TaMPK3 rev	GGTTCAACTCCAGGGCTT CGTTG	Expression (Wheat)
wheat beta-tubulin fwd	GCTCACATCTCGTGGGTC ACAGA	Wheat beta tubulin rev	CGCCAGTGTACCAATGCA AGAAA	Expression (Wheat) normalize
MgNLP-e fwd	CTTCAGCCCACTGGCGGA TCAA	MgNLP-e rev	ATCAGAGGATGAGTGCCA CCGC	Expression (*Mycosphaerella graminicola*)
Mg beta-tubulin fwd	ATCACCAGCCCGCAAAGC TT	Mg beta-tubulin rev	ACGATCTTGTGTCCGAGT ACCAGC	Expression (*M. graminicola*) normalize
Mg EF1alpha fwd	TGCGCTGTGGCATTACTT CTGG	Mg EF1alpha rev	GGCGTATCTTTAAACCAG TCCCGA	Expression (*M. graminicola*) normalize
HIN1_fwd	ACGTTAACTCCTTTCTATC AAGG	HIN1_rev	TCTTTAGAAGATAAGTCG TAGG	Expression (*Arabidopsis thaliana*)
PAD3_fwd	AGGAGACATTAAGGTTAC ATCC	PAD3_rev	ACCAAACCATATATTCAG TGG	Expression (*A. thaliana*)
EF1alpha_fwd	TCACATCAACATTGTGGT CATTGG	EF1alpha_rev	TTGATCTGGTCAAGAGCC TACAG	Expression (*A. thaliana*) normalize
MgNLP-c fwd	ATGAGGCTCGCGCTATTG CT	MgNLP-c rev	TCAGTCAAGCTGAGCCTC GT	Full length clone
MgNLP fwd EcoRI	TTTGAATTCTCTAGCATC CATCAGAAACGCGCTG	MgNLP rev NotI	TTTGCGGCCGCTCAGTCA AGCTGAGCCTCGTCC	Protein expression
P1	AAGAGCTCTCTTGGACAC GAAGGATGAT	P2	AAGAATTCAGATTCTCGG CCATCGACAC	Gene deletion
P3	AATCTAGACTAATTGGTT TGGTTGGAGT	P4	AAGGATCCAGTGGTTGTC ACATGCAAGT	Gene deletion
P5	TGAGAAGATTCTCATTCG CATGGCG	P6	CGACATCAACTAGCCGTC ATAGCCA	Hybridization probe

frame by PCR on genomic DNA with primers P1 and P2 for flank1 and P3 and P4 for flank2. Flank1 was then cloned into vector pCHYG (Supplementary Fig. 5), which was generated as follows: the *hph* resistance cassette conferring resistance to the antibiotic hygromycin was excised from pCB1004 (Carroll et al. 1994) via a pUC18 derivative called pUCHYG (obtained from Andy Bailey, University of Bristol, U.K.). The resistance cassette was excised from pUCHYG as a *Hin*dIII-*Eco*RI fragment and ligated into pCAMBIA0380 (Hajdukiewicz et al. 1994). Flank1 was introduced as a *Sac*1/*Eco*R1 fragment into pCHYG. The resulting construct was then used for ligation of Flank2 following *Xba*1 and *Bam*H1 restriction digest. The final plasmid (pCHYG-MgNLP) was confirmed to be correct by restriction analysis. The plasmid was then used to transform *Agrobacterium* strain Agl-1 via the freeze-thaw method (An et al. 1988). Strains harboring the plasmid were then used for fungal transformation.

Agrobacterium transformation of *M. graminicola* was performed as previously described (Zwiers and de Waard 2001) but with the following modifications. *M. graminicola* sporidia exhibiting yeast-like growth were suspended in induction medium (IM) and *Agrobacterium* spp. growing at exponential phase in IM were used for the transformation. Fungal suspension and *Agrobacterium* culture were mixed in equal parts (vol/vol) with 200 μM acetosyringone (Sigma) and plated on a cellophane membrane on top of an IM plate also containing 200 μM acetosyringone. After 48 h at 16°C, the cellophane membranes were transferred onto *Aspergillus nidulans* minimal medium plates containing hygromycin B (Calbiochem, San Diego, CA, U.S.A.) at 100 μg/ml and Timentin (ticarcillin disodium salt/potassium clavanulate) (Melford, Ipswich, U.K.) at 100 μg/ml. After approximately 7 days of incubation at 16°C, the plates were screened for putative hygromycin-resistant transformants. Independent fungal colonies of putative transformants were picked onto YEPD plates (Oxoid Ltd.) containing hygromycin and timentin as before and subsequently subcultured twice to obtain material from a single yeast-like fungal colony and to kill off *Agrobacterium* cells. Targeted insertion of the T-DNA was initially confirmed by PCR on genomic DNA directed against *Hph* and *MgNLP*.

Isolation of fungal genomic DNA.

DNA for use in PCR screening was extracted from 5 to 10 mg of freeze-dried and ground fungal mycelium using Fastprep FP120 with 350 μl of TEN buffer (500 mM NaCl, 400 mM Tris-HCL, 50 mM EDTA, pH 8.0), 1% β-mercaptoethanol, 5 mM 1,10 phenanthroline, and 2% (wt/vol) polyvinylpyrrolidone K30. The resulting suspension was then mixed thoroughly with 350 μl of 2% (wt/vol) SDS and incubated for 30 min at 65°C. Following the addition of 300 μl of ice-cold ammonium acetate (7.5 M), the sample was kept on ice for 20 min and then centrifuged at 13,000 rpm for 10 min. An equal volume of cold isopropanol (–20°C) was added to the supernatant and centrifuged for 5 min. The DNA pellet was washed in ice-cold 70% (vol/vol) ethanol and dissolved in sterile distilled water. All DNA samples were quantified using a Thermo Scientific NanoDrop 1000 spectrophotometer and diluted to 10ng/μl for use in PCR.

Genomic DNA for use in Southern blotting was extracted from 40 mg of freeze-dried and ground (Fastprep FP120) mycelium. Nucleic acid extraction was achieved by disruption in 800 μl of Lee and Taylor lysis buffer (Lee and Taylor 1990) and incubation at 65°C for 60 min. An equal volume of phenol/chloroform was added to the heat-treated homogenate and centrifuged at 13,000 rpm for 15 min. The aqueous phase was precipitated with 40 μl of ice-cold 6 M ammonium acetate and 600 μl of isopropanol and centrifuged for 2 min. The pellet was dissolved in 100 μl of 600-μg/ml RNase in Tris-EDTA buffer and incubated at 37°C for 15 min. A 400-μl aliquot of phenol solution (Sigma-Aldrich) was added to the RNase-treated sample and centrifuged for 10 min. Genomic DNA was precipitated from the aqueous phase following addition of 800 μl of ice-cold isopropanol and 20 μl of 6 M ammonium acetate. The DNA pellet was washed in ice-cold 70% (vol/vol) ethanol and resuspended in sterile distilled water. DNA was quantified using a Thermo Scientific NanoDrop 1000 spectrophotometer and diluted. For Southern hybridization, 7 μg of DNA was digested with *Pst*1 prior to electrophoresis.

Southern blot analysis.

Fungal genomic DNA (7 μg) was digested with *Pst*1 and subjected to alkaline Southern blotting via capillary transfer onto nylon membranes (Hybond N+) (Amersham Pharmacia). An 185-bp DNA probe was amplified by PCR using primers P5 and P6. Probe labeling was achieved with ^{32}Pα-dCTP (3,000 Ci/mmol) (NEN Ltd., Boston) using the Amersham Pharmacia Rediprime random prime labeling kit. Prehybridization and probe hybridization was performed at 65°C according to the method of Church and Gilbert (1984). Blots were washed at 65°C twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)/0.1% SDS and once with 0.5× SSC/0.1% SDS. Filters were subsequently developed by phosphorimaging (Typhoon 8600) (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Primers.

Primers used in this work are shown in Table 1.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Broad Institute: www.broad.mit.edu
GeneDoc: A tool for editing and annotating multiple sequence alignments: www.psc.edu/biomed/genedoc
Joint Genome Initiaive (JGI) Genome Portal *Mycosphaerella graminicola* page: genome.jgi-psf.org/Mycgr3/Mycgr3.home.html
U.S. Department of Energy Joint Genome Institute: www.jgi.doe.gov