Definition of Tissue-Specific and General Requirements for Plant Infection in a Phytopathogenic Fungus

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Although plant diseases are usually characterized by the part of the plant that is affected (e.g., leaf spots, root rots, wilts), surprisingly little is known about the factors that condition the ability of pathogens to colonize different plant tissues. Here we demonstrate that the leaf blast pathogen *Magnaporthe grisea* also can infect plant roots, and we exploit this finding to distinguish tissue-specific and general requirements for plant infection. Tests of a *M. grisea* mutant collection identified some mutants that were defective specifically in infection of either leaves or roots, and others such as the map kinase mutant *pmk1* that were generally defective in pathogenicity. Conservation of a functional PMK1-related MAP kinase in the root pathogen *Gaeumannomyces graminis* was also demonstrated. Exploitation of the ability of *M. grisea* to infect distinct plant tissues thus represents a powerful tool for the comprehensive dissection of genetic determinants of tissue specificity and global requirements for plant infection.

Additional keywords: root infection.

Plant diseases are characterized by the tissue types that they affect. For example, colonization of the vascular tissue leads to wilt diseases by alteration of transpirational flow, whereas proliferation of a pathogen in leaf tissue reduces photosynthetic output and leads to leaf spot or leaf mould diseases (Agrios 1997). The genetic components that condition the ability of a pathogen to colonize different plant tissues are, however, unknown, even though they are probably among the most important determinants for successful establishment of disease. In addition to these tissue-specificity determinants, there is likely to be a core set of “basic” pathogenicity factors that are generally required for plant colonization.

Phytopathogenic fungi can be divided broadly into two types: those infecting leaves and stems of plants and those proliferating in root tissue (Agrios 1997). Infection of these tissues and survival within them require distinct strategies. Leaf-infecting fungi often have to breach the tough waxy cuticle that coats aerial plant structures (Mendgen and Deising

1993). The primary mechanical barriers confronting root-infecting fungi are likely to be less substantial. Soilborne pathogens, however, must be able to compete effectively with the extensive community of rhizosphere microorganisms and contend with antimicrobial plant metabolites that are exuded by roots (Carter et al. 1999; Deacon 1996).

Here we examined a foliar pathogen that has been extensively characterized genetically to determine whether it is able to colonize root tissue and to establish whether the genetic components governing its ability to grow in a living plant could be differentiated into tissue-specific and general determinants, respectively. The pathogen of choice was *Magnaporthe grisea*, which causes blast disease on leaves of cereals and grasses (Ou 1985). This fungus has emerged as a paradigm for molecular genetic dissection of factors determining fungal pathogenicity to leaves, and mutational analyses have identified a number of genes that are required by *M. grisea* for pathogenic differentiation and colonization of leaf tissue (Balhadère et al. 1999; Hamer and Talbot 1998; Howard and Valent 1996). In contrast, very little is known about factors required by soilborne fungal pathogens for successful colonization of root tissues, although root-infecting fungi are also extremely important as disease-causing agents (Deacon 1996). This lack of knowledge is in part a result of the difficulty of studying such processes below ground, and also because of the genetic intractability of many root-infecting organisms.

In this paper, we demonstrate that *M. grisea* can infect the roots of cereals and cause lesion development and exploit this to define tissue-specific and general requirements for disease.

RESULTS

Magnaporthe grisea causes disease symptoms on the roots of cereals.

Barley is often used as an experimental host for *M. grisea* when testing strains for pathogenicity to leaves (Sweigard et al. 1998). The *M. grisea* strain Guy11 is pathogenic to barley leaves but is unable to infect wheat leaves (Leung et al. 1988). This was confirmed in our leaf infection assays (data not shown). When Guy11 was assessed for the ability to infect cereal roots, lesions were clearly visible on the roots of barley and wheat seedlings after 2 weeks incubation, whereas there were no symptoms on roots of mock-inoculated plants (Fig. 1A and B). The lesions were more extensive and darker on wheat roots (Fig. 1B) and were very similar to typical “take-

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Gaeumannomyces graminis GMK1 gene GenBank accession no. AF258529.

all" disease symptoms caused by the cereal root pathogen *Gaeumannomyces graminis*, although their development was slower. Cytological observations of cross sections of infected roots taken within and around lesions confirmed that fungal growth was present in the cortex and stele (Fig. 1C). The fact that the leaves of the wheat cultivar used in these experiments were immune to infection by *M. grisea* while the roots were susceptible implies that determinism of host range is tissue specific.

Infection of leaves by *M. grisea* is initiated by adhesion of conidia to the leaf surface. The germ tubes arising from these conidia differentiate melanized structures, known as appressoria, that are essential for host penetration (Hamer and Talbot 1998; Howard and Valent 1996). In our experiments, appressoria were not observed when roots infected by Guy11 were examined, although brown, rounded, swollen cells similar to the vesicle-like structures previously described for root-infecting pathogens such as *G. graminis*, *Phialophora* spp. (Deacon 1981), and *Magnaporthe rhizophila* (Scott and Deacon 1983) were seen within the root cortex. Vegetative mycelium of *M. grisea* is not known to differentiate appressoria and since we used mycelium as the inoculum in our root-infection assays, the absence of these infection structures is, perhaps, not surprising. However, appressoria also failed to form when suspensions of Guy11 conidia were applied to wheat and barley roots even after prolonged (72 h) incubation (data not shown). Penetration of cereal roots by *M. grisea* therefore does not appear to involve appressoria.

Analysis of a *M. grisea* mutant collection identifies tissue-specific and general requirements for disease.

To establish whether *M. grisea* could be used to investigate the genetic requirements for infection of different plant tissues, the collection of mutants listed in Table 1 was tested for the ability to cause disease on barley and wheat roots. Mutants also were inoculated onto barley leaves to confirm their phenotypes on this tissue. The mutants could be subdivided into four classes on the basis of their ability to infect leaves and roots (Fig. 2). Mutants belonging to the first class were unable to infect leaves, but were still able to form lesions on roots. These included the melanin-deficient mutants *alb1* and *buf1* (Chumley and Valent 1990; Romao and Hamer 1992) and the mutant strain *npr1*, which is defective in the regulation of nitrogen assimilation and pathogenicity (Lau and Hamer 1996). The *nut1* mutant was the only representative of the second class. This mutant was fully pathogenic to leaves but

failed to give clear lesions on roots. *nut1* is defective in a gene that is homologous to the *Aspergillus nidulans* *AREA* gene (Caddick et al. 1986) and is involved in global regulation of nitrogen utilization (Froeliger and Carpenter 1996). The third class consists of mutants *pmk1*, *abc1*, *igd1*, and *npr2*, which gave reduced disease symptoms on leaves and roots and are therefore deficient in factors required for successful infection of both tissues. Mutants *gde1* and *met1* (previously reported to be defective in leaf infection) (Balhadere et al. 1999) were able to cause disease on leaves and roots in our assays. The discrepancies between our results and those of Balhadere et al. (1999) are likely the result of differences in inoculation procedures and/or experimental conditions. For all mutant strains used in this study, including *nut1*, fungal hyphae were clearly visible on the surface of trypan blue-stained roots of barley and wheat, indicating that failure to infect is not a result of the inability to survive in the assay conditions prior to making contact with the roots.

The PMK1 signal transduction pathway: a global regulator of pathogenesis.

The MAP kinase PMK1 plays a key role in the regulation of pathogenesis in *M. grisea* (Xu and Hamer 1996). *pmk1* mutants of *M. grisea* are unable to infect leaves of rice and barley and are defective in both appressorium formation and invasive growth within the leaf. The *M. grisea* *pmk1* mutant also was unable to cause lesions on the roots of barley or wheat (Fig. 2), indicating that *PMK1* is required for infection of both leaves and roots. Cytological analysis confirmed the absence of fungal growth within the root tissues (data not shown).

To determine whether there is functional conservation of MAP kinase signaling mechanisms in root- and leaf-infecting pathogens, we isolated a *PMK1* homolog from the root-infecting pathogen *G. graminis*, which is unable to infect aerial plant tissues (Walker 1981). Using degenerate polymerase chain reaction (PCR) primers designed for the amplification of genes encoding FUS3-related MAP kinases (Xu and Hamer 1996), two products (400 and 360 bp) were obtained following PCR amplification with *G. graminis* genomic DNA. The 400-bp PCR product contained a partial open reading frame that shared more than 97% amino acid identity with the corresponding region of the PMK1 product, whereas the predicted amino acid sequence of the 360-bp PCR shared 80% identity with *CDC2*-related kinases (data not shown).

The 400-bp PCR product was used as a probe to isolate the *G. graminis* gene *GMK1*. *GMK1* is predicted to encode a 356

Table 1. Characteristics of *Magnaporthe grisea* strains used in this study

Strain	Defect	Pathogenicity to barley leaves	Reference
Wild-type Guy11	None	Wild type	Leung et al. 1988
Mutants ^a			
<i>pmk1</i>	MAP-kinase deficient mutant	Reduced	Xu and Hamer 1996
<i>npr1</i> , <i>npr2</i>	Nitrogen regulation and pathogenicity	Reduced	Lau and Hamer 1996
<i>nut1</i>	Global regulator of nitrogen utilization	Wild type	Froeliger and Carpenter 1996
<i>met1</i>	Methionine biosynthesis	Wild type	Balhadere et al. 1999
<i>igd1</i>	Invasive growth	Reduced	Balhadere et al. 1999
<i>gde1</i>	Conidial germination	Wild type	Balhadere et al. 1999
<i>abc1</i>	Putative ABC transporter	Reduced	Urban et al. 1999
<i>buf1</i> , <i>alb1</i>	Melanin biosynthesis	Reduced	Romao and Hamer 1992

^a All mutant strains were generated from the wild-type strain Guy11, except for the melanin-deficient mutants, which were generated by crossing strain 4136-4 to original mutant strains obtained spontaneously (*alb1*) or by UV mutagenesis (*buf1*) from strain 4091-5-8 (Chumley and Valent 1990).

amino acid protein with approximately 97% amino acid sequence identity to *M. grisea* PMK1. Two independently isolated full-length cDNA clones also were sequenced, allowing the presence of the three introns to be confirmed and a putative transcription start to be located 118 bp upstream of the initiation codon. The deduced GMK1 protein contains the 11 conserved serine–threonine protein kinase subdomains (Hanks et al. 1988) and the characteristic MAP kinase phosphorylation sites (TEY, residues 184–186) (Nishida and Gotoh 1993) (Fig. 3). Southern blot analysis indicated that *GMK1* is a single-copy gene (data not shown). This gene is expressed in culture and during infection of wheat roots (Fig. 4). A comparison of the DNA sequences of approximately 0.8 kb of the promoter regions of *GMK1* and *PMK1* revealed that the sequence similarity in this region was low (38% identity following alignment with the CLUSTAL V algorithm) (Higgins et al. 1992) and conserved regions with homology to known regulatory motifs were not identified.

A *pmk1*-deleted mutant of *M. grisea* (nn 78) was cotransformed with the pAN8-1 vector (Mattern et al. 1988) and a plasmid carrying the 2.7 kb *KpnI*–*PstI* fragment containing the whole *GMK1* gene to test whether the *G. graminis* *GMK1* gene was able to complement the *pmk1* mutation. Phleomycin-resistant transformants were recovered, purified by single-spore isolation, and screened for the presence of the *KpnI*–*PstI* fragment by Southern blot analysis. Appressorium formation and pathogenicity to barley leaves was restored in

cotransformants containing the *GMK1* gene (Table 2 and Fig. 5A), indicating that *GMK1* can complement both the appressorial development and invasive growth defects of *pmk1* mutants. Importantly, the *GMK1* cotransformants also regained the ability to cause lesions on roots of barley and wheat (Fig. 5B).

DISCUSSION

These experiments demonstrate that the foliar blast pathogen *M. grisea* can infect cereal roots and cause disease symptoms. Clearly, *M. grisea* is not regarded as an economically important root pathogen, although it does have close relatives that cause root diseases, including *Magnaporthe poae* and *M. rhizophila*, which are pathogens of Kentucky bluegrass (*Poa pratensis*) and millet (*Setaria* spp.), respectively (Landschoot and Jackson 1989; Scott and Deacon 1983), and the take-all pathogen *G. graminis* (Bryan et al. 1995; Cannon 1994). When the *M. grisea* mutants listed in Table 1 were assessed in root-infection assays, mutants with leaf-specific (*alb1*, *bud1*, *npr1*), root-specific (*nut1*), and general (*pmk1*, *abc1*, *igd1*, and *npr2*) pathogenicity defects could be identified. The resolution of genetic determinants of tissue specificity and global requirements for the infection of different plant tissues represents an important development in our understanding of pathogenesis and the manifestation of disease.

The differentiation of melanized appressoria is a key step in the process of leaf infection by *M. grisea* (Howard and Valent 1996). Mutants such as *bud1* and *alb1*, which are unable to synthesize DHN melanin, fail to generate the appressorial turgor pressure required for successful penetration of the leaf surface (Chumley and Valent 1990; De Jong et al. 1997; Howard and Ferrari 1989). Our experiments indicate that appressoria are not required for infection of cereal roots by *M. grisea*. Furthermore, the *alb1* and *bud1* mutants were able to produce lesions on cereal roots, confirming that DHN melanin is not required for root infection by *M. grisea*. Some root pathogens, including different *G. graminis* varieties, form melanized appressoria-like structures, known as hyphopodia, on the root surface (Landschoot and Jackson 1989; Scott and Deacon 1983; Walker 1981). Both melanin and hyphopodia are dispensable for root infection by *G. graminis* var. *graminis* (Epstein et al. 1994; Frederick et al. 1999). The related fungus *G. graminis* cv. *tritici* does require melanin for pathogenicity, but this may be for the production of ectotrophic macrohyphae on roots rather than for host penetration (Henson et al. 1999).

Fig. 1. Infection of cereal roots by *Magnaporthe grisea*. **A**, Barley; **B**, wheat. 1) Mock-inoculated control; 2) wild-type *M. grisea* strain Guy11. Seedlings were assessed for disease symptoms after 3 weeks incubation. **C**, Growth of the *M. grisea* strain Guy11 in wheat root tissues. Arrows indicate trypan blue-stained fungal hyphae in the cortex and stele (scale bar = 25 μ m).

Table 2. Complementation of appressorial formation of the *Magnaporthe grisea pmk1* mutant by the *Gaeumannomyces graminis GMK1* gene

Strain	% Conidia germination ^a	% Appressorium formation ^a
Wild type (Guy11)	98.2 $\pm$ 1.3	92.5 $\pm$ 1.9
<i>pmk1</i> (nn78)	96.8 $\pm$ 1.9	0
<i>pmk1::GMK1</i> (nn 78–T2)	98.6 $\pm$ 0.8	87.9 $\pm$ 5.4

^a Germination frequencies and appressorium formation were assessed following microscopic examination of at least 200 conidia per strain 24 h after application of 10 μ l of each spore suspension onto plastic coverslips. Mean and standard deviations were calculated from five independent replicates.

Nitrogen is generally deficient in cultivated soils, and the lack of this nutrient element is associated with increased severity of take-all disease in cereals (Huber 1981). Regulation of nitrogen assimilation is important for disease development by *M. grisea* on leaves and roots, although there are subtle differences in the requirements for colonization of the two different tissues. The *M. grisea* mutants *npr1* and *npr2* are

substantially reduced in pathogenicity to leaves (Lau and Hamer 1996), whereas the *nut1* mutant is pathogenic on this tissue (Froeliger and Carpenter 1996). *npr2* is unable to cause lesion formation on roots and thus is affected in pathogenesis on both tissues, whereas *npr1* does give symptoms on roots. *nut1* fails to give clear lesions on roots and so appears to be defective specifically in root infection. The *npr1* and *npr2*

Fig. 2. Lesion formation caused by *Magnaporthe grisea* mutants on leaves and roots. **A**, Barley leaf sections. **B**, Wheat roots (similar results were obtained with barley but the lesions were not as well defined, so wheat is shown for clarity). Arrows give an indication of lesions that were clearly visible on the roots.

Fig. 3. Characterization of the *Gaeumannomyces graminis* *GMK1* gene. Alignment of the predicted amino acid sequences of *GMK1*, *PMK1*, and other related MAP kinases. Accession nos.: *G. graminis* *GMK1*, AF258529; *M. grisea* *PMK1*, U70134; *Colletotrichum lagenarium* *CMK1* gene, AF174649; *Fusarium solani* *FsMAPK*, U52963; *Cochliobolus heterostrophus* *CHX1* gene, AF178977; *Candida albicans* *CEK1* gene, A47211; *Saccharomyces cerevisiae* *FUS3*, M31132; *S. cerevisiae* *KSS1*, P14681; Black boxes represent identical residues. Roman numerals indicate conserved subdomains among the serine-threonine protein kinase family (Hanks et al. 1988). Sequence alignment was performed with the CLUSTALV algorithm (Higgins et al. 1992).

mutants were originally isolated on the basis of chlorate resistance, a selection that is used routinely for the isolation of mutants that are defective in nitrogen assimilation. These mutants have complex phenotypes, and their genetic defects have not been fully characterized (Lau and Hamer 1996). Under nitrogen-starvation conditions the *NPR1* gene is required for the expression of the hydrophobin-encoding gene *MPG1*, which is essential for pathogenicity to leaves (Talbot et al. 1993) but not for the induction of the nitrate reductase gene (Lau and Hamer 1996). Under the same conditions, *NPR2* is required for the induction of both *MPG1* and nitrate reductase genes (Lau and Hamer 1996). The *NUT1* gene, which is a homolog of the *A. nidulans* *AREA* gene, is a global regulator of nitrogen utilization (Froeliger and Carpenter 1996) and is also involved in the induction of the nitrate-reductase structural gene under nitrogen-starvation conditions. However, *NUT1* does not regulate the expression of *MPG1* (Lau and Hamer 1996).

While some *M. grisea* mutants were specifically defective in the ability to give symptoms on either leaves or roots of cereals, others were generally deficient in disease development on both tissues. We have shown that the MAP kinase *PMK1* is required for lesion development on both leaves and roots. *PMK1*-related MAP kinases are essential for pathogenicity of other leaf-infecting fungi, including *Cochliobolus heterostrophus* (Lev et al. 1999), *Colletotrichum lagenarium* (Takano et al. 2000), and *Botrytis cinerea* (Zheng et al. 2000). With the exception of this study, however, their role in infection of other plant tissues has not been addressed. The conservation of a functional *PMK1*-related MAP kinase in the take-all fungus *G. graminis* suggests that these MAP kinase signal transduction pathways are global regulators of fungal pathogenesis on different plant tissues. Furthermore, the *G. graminis* *GMK1* gene was able to complement the *pmk1* mutant when expressed under the control of its own promoter, indicating that the regulation of expression of *GMK1* in *M. grisea* is also conserved. Although transformation of *G. graminis* has been achieved on occasion in our laboratory and elsewhere (Bowyer et al. 1995; Henson et al. 1988; Pilgeram and Henson 1990), success is sporadic and gene-disruption experiments are extremely problematic. Attempts are under way to develop reliable transformation systems for *G. graminis* and root-infecting *Magnaporthe* species to enable direct tests of the function of *PMK1*-related MAP kinases, and also of other genes of interest that emerge from studies with the more amenable *M. grisea* pathosystem.

The identification of additional *M. grisea* mutants unable to give lesions on roots will enable us to identify more genes that are required for infection of this tissue. Random insertional mutagenesis by restriction enzyme-mediated integration (REMI) has proved to be a powerful tool for the identification of pathogenicity determinants in a number of foliar pathogens (Maier and Schäfer 1999). To date, we have screened 175 REMI mutants of *M. grisea* in our root-infection assay and identified two that fail to give lesions on wheat and barley roots. Interestingly, both mutants are fully pathogenic to leaves. The characterization of the genetic defects in these and other mutants isolated in further screening experiments will allow the identification of novel tissue-specific and general factors that are required for plant colonization by *M. grisea*.

MATERIALS AND METHODS

Fungal strains and growth conditions.

The *M. grisea* fungal strains used are listed in Table 1. *G. graminis* var. *tritici* strain R1 (Bryan et al. 1995) was also used. Fungi were grown in potato dextrose broth at 22°C for 5 or 7 days for RNA and DNA extraction, respectively (Bryan et al. 1995).

Transformation of *M. grisea*.

M. grisea transformation was performed as described by Kershaw et al. (1998), except that 20% sucrose was used as the osmoticum. The plasmid containing the *G. graminis* *GMK1* gene was introduced by cotransformation with the plasmid pAN8-1, which carries a selectable marker for phleomycin resistance (Mattern et al. 1988). Transformants were selected for resistance to 35 µg phleomycin per ml (Cayla, Toulouse, France) and purified by two successive rounds of monoconidial isolation on complete medium agar.

Appressorium formation.

Appressorium formation of *M. grisea* strains was assessed as described by Hamer et al. (1988). Aliquots (100 µl) of spore suspensions (10^5 spores per ml) were deposited on plastic cover slips (PGS Scientific, Frederick, MD, U.S.A.) and incubated under humid conditions for 24 h at 28°C in the dark. At least 200 conidia per strain were examined microscopically for germination and appressoria formation.

Infection of leaves and roots.

The cereals used were barley cultivar Golden Promise and wheat cultivar Riband. Leaf-infection assays were as described by Hamer et al. (1988). *M. grisea* strains were sporulated on complete medium agar (Talbot et al. 1993) at 28°C for 7 days, and an inoculum concentration of 10^5 spores per ml in 0.25% gelatin was used for brush inoculation of leaf segments. Symptoms were assessed visually after 4 to 8 days. Root infection was carried out following a method used routinely with *G. graminis* (Bowyer et al. 1995). Seeds were surface sterilized by soaking in several volumes of bleach (1.2% available chlorine) for 10 min before washing ten times in several volumes of sterile water. *M. grisea* cultures for root-infection assays were grown on potato dextrose agar for 8 days at 22°C (*M. grisea* forms few or no spores under these conditions), and mycelial plugs were taken from the colony

margin with a sterile No. 3 cork borer. Fifty-milliliter plastic centrifuge tubes (Corning, Corning, NY, U.S.A.) were filled with 35 ml of sterile moist vermiculite. Four mycelial plugs were placed on top of this and covered with a further 5 ml of vermiculite. Mock-inoculated tubes received sterile agar plugs. Two surface-sterilized seeds were placed on top of the second vermiculite layer, followed by an additional 5 ml of vermiculite. Four replicate tubes were set up for each treatment. Tubes were sealed with Parafilm (American National Can, Greenwich, CT, U.S.A.) and incubated at 22°C (1,800 lux, photoperiod of 16 h light–8 h dark) for 2 to 3 weeks. The seedling roots were then washed in water and examined for lesions. Fungal colonization of roots was evaluated by staining with a 0.1% trypan blue solution in lactophenol (23% [wt/vol] phenol, 18.4% [vol/vol] lactic acid, and 35.6% [vol/vol] glycerol). Stained samples were mounted in 60% glycerol on glass slides and observed at 100 or 200 times magnification with a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY, U.S.A.) under bright-field illumination. All leaf and root infection experiments were carried out at least three times to confirm the reproducibility of the results.

DNA isolation and manipulation.

Plasmid DNA for fungal transformation experiments was isolated by the alkaline lysis procedure and purified by ultracentrifugation on a cesium chloride density gradient (Sambrook et al. 1989). *M. grisea* and *G. graminis* genomic DNA was isolated following the method of Daboussi et al. (1989). DNA restriction, agarose gel fractionation, and transfer to nylon membranes (Hybond NX, Amersham Pharmacia Biotech, St. Albans, U.K.) were performed according to the manufacturer's instructions and standard procedures (Sambrook et al. 1989). Hybridizations for Southern blot analysis and library screening were carried out at 65°C according to standard procedures. Probes were 32 P-labeled with the random priming method with the Oligolabelling kit (Amersham Pharmacia Biotech).

PCR cloning of the *GMK1* gene.

The primers (MAK2, MAK4, and MEK3) and PCR conditions that were used to isolate the *PMK1* gene from *M. grisea* (Xu and Hamer 1996) were applied to *G. graminis*. PCR products were cloned into the pCR2.1 vector (Invitrogen, Groningen, Netherlands) and sequenced with the ABI Prism Big Dye Terminator reaction mix (Perkin-Elmer, Norwalk, CT, U.S.A.).

Screening of *G. graminis* genomic DNA and cDNA libraries.

The *G. graminis* genomic DNA library had been previously constructed in the λZAPII vector (Stratagene, La Jolla, CA, U.S.A.) (P. Garosi and A. Osbourn, *unpublished results*). To construct the cDNA library, total RNA of *G. graminis* strain R1 grown in potato dextrose broth was isolated as described (Vallélian-Bindschedler et al. 1998). Messenger RNA was purified with magnetic oligo(dT)₂₅ Dynabeads (Dyna, Broomborough, U.K.) following the manufacturer's instructions, and a cDNA library was constructed in the vector λZAP Express with the ZAP-cDNA synthesis kit (Stratagene). After in vitro packaging with the GigapackII Gold Packaging Extract Kit (Stratagene), recombinant phages were transfected into *Es-*

Fig. 4. Analysis of *GMK1* expression. Ethidium bromide-stained polymerase chain reaction (PCR) products generated by reverse transcription (RT)-PCR with primers for *GMK1* (upper panel) and for the *Gaeumannomyces graminis* actin gene (lower panel). Lane 1, fungal genomic DNA control. Lane 2, total RNA from *G. graminis* grown in liquid culture, from mock-inoculated wheat roots (lane 3), and from wheat roots inoculated with *G. graminis* 4, 5, 6, 7 and 8 days after inoculation (lanes 4–8 respectively). The presence of an intron between the annealing locations of the two primers allows differentiation of PCR products derived from genomic DNA and mRNA for *GMK1*.

Fig. 5. *GMK1* restores pathogenicity of the *Magnaporthe grisea pmk1* mutant to both leaves and roots. **A**, Barley leaf sections. **B**, Wheat roots (similar results were obtained with barley but the lesions were not as well defined, so wheat is shown for clarity). 1) Mock-inoculated control; 2) Guy11 wild type; 3) *pmk1* mutant nn78; 4, an nn 78 transformant containing the *Gaeumannomyces graminis GMK1* gene (nn 78/T2).

cherichia coli strain XL1Blue-MRF' (Sambrook et al. 1989). *E. coli* strain Q358 (Sambrook et al. 1989) was used for subsequent amplification steps and screening.

Northern blot analysis and reverse transcription (RT)-PCR.

Northern blot experiments were conducted according to standard procedures (Sambrook et al. 1989). Hybridizations were carried out at 65°C in a sodium phosphate hybridization buffer (Church and Gilbert 1984). Membranes were washed at high stringency (Sambrook et al. 1989). For RT-PCR, 5 µg of total RNA from each sample was treated with RNase-free DNase (Amersham Pharmacia Biotech) and reactions were performed with hexanucleotides and the Expand Reverse Transcriptase (Roche Diagnostics, Lewes, U.K.). PCR reactions were then carried out with 5 µl aliquots of this reaction for each set of primers. Two primers for specific amplification of the *G. graminis* actin gene transcript were designed based on the gene sequence (P. Garosi and A. Osbourn, *unpublished results*). These were ACTF (forward) 5'-ACACCTTCTACA ACGAGCTGC-3' and ACTR (reverse) 5'-AGCGACAGG ACGGCCTGGATC-3'. Annealing was conducted at 51°C. For specific amplification of *GMK1* transcripts, primers GMK1.1 (forward) 5'-GGTCGCCATCAAGAAGATCACC CCC-3' and GMK1.2 (reverse) 5'-GGTCATCCGAAAGGT CCTGGGTGC-3' were used. Primer annealing was conducted at 60°C.

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