The *pmk1*-like mitogen-activated protein kinase from *Lecanicillium* (*Verticillium*) *fungicola* is not required for virulence on *Agaricus bisporus*

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In plant-pathogenic fungi, the *pmk1* mitogen-activated protein kinase (MAPK) signalling pathway plays an essential role in regulating the development of penetration structures and the sensing of host-derived cues, but its role in other pathosystems such as fungal–fungal interactions is less clear. We report the use of a gene disruption strategy to investigate the *pmk1*-like MAPK, *Lf pmk1* in the development of *Lecanicillium fungicola* (formerly *Verticillium fungicola*) infection on the cultivated mushroom *Agaricus bisporus*. *Lf pmk1* was isolated using a degenerate PCR-based approach and was shown to be present in a single copy by Southern blot analysis. Quantitative RT-PCR showed the transcript to be fivefold upregulated in cap lesions compared with pure culture. *Agrobacterium*-mediated targeted disruption was used to delete a central portion of the *Lf pmk1* gene. The resulting mutants showed normal symptom development as assessed by *A. bisporus* mushroom cap assays, sporulation patterns were normal and there were no apparent changes in overall growth rates. Our results indicate that, unlike the situation in fungal–plant pathogens, the *pmk1*-like MAPK pathway is not required for virulence in the fungal–fungal interaction between the *L. fungicola* pathogen and *A. bisporus* host. This observation may be of wider significance in other fungal–fungal and/or fungal–invertebrate interactions.

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INTRODUCTION

Lecanicillium fungicola (Pruess) Hassebrauk is the causal agent of dry bubble disease of the cultivated button mushroom *Agaricus bisporus* (Lange) Imbach. It is one of the most serious pathogens of this crop and is worldwide in distribution. The disease manifests itself in three different symptoms: dry bubble, stipe blow-out and cap-spotting diseases (Mills *et al.*, 2008). Disease outbreaks remain a serious threat to the mushroom industry and can devastate crops where successful management of this disease mainly relies on good cultural practices and strict sanitation. The broad-spectrum azole fungicide prochloraz-manganese has sometimes been used to control infections; however, resistance is becoming more common within the pathogen population, which limits the usefulness of this product (Bonnen and Hopkins, 1997). Other fungicides are less effective or impact on the host fungus (Chrysai-Tokousbalides *et al.*, 2007) and may give concerns regard-

ing residues in the crop or in the spent mushroom compost. To date, no other acceptable means of chemical control for *L. fungicola* have been marketed, probably due to prohibitive development and licensing costs, whilst difficulties involved with breeding of *A. bisporus* have prevented disease-resistant strains from being developed. Although formerly known as *Verticillium fungicola*, the taxonomy of these fungi has recently been revised, with the group renamed *Lecanicillium* (Zare & Gams, 2001, 2008; Amey *et al.*, 2007), showing it to be more closely related to the insect pathogenic fungi, such as *L. longisporum* (formerly *V. lecanii*), and nematode pathogens, such as those within the genus *Haptocillium* (e.g. *V. balanoides*). Indeed, there are reports of *L. fungicola* being isolated from other invertebrates such as nematodes (Gené *et al.*, 2005).

Many fungal pathogens are sensitive to chemical and physical cues from their host surfaces and these cues often promote the formation of penetration structures, such as appressoria. Mitogen activated protein kinases (MAPKs) are a family of eukaryotic serine/threonine protein kinases (Kültz, 1998) involved in the transduction of extracellular signals, growth regulation and differentiation processes (Nishida & Gotoh, 1993; Dickman & Yarden, 1999; Schaeffer & Weber, 1999). MAPK kinases (MAPKKs)

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Abbreviations: DI, deionized (water); MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MM, minimal medium; PDA, potato dextrose agar; Q-PCR, quantitative RT-PCR.

activate MAPKs, with MAPKK kinases (MAPKKKs) activating MAPKKs. These MAPKKK–MAPKK–MAPK cascades are conserved in many organisms (Xu, 2000), suggesting the probable presence of similar MAPK homologues in *L. fungicola*. The pathways have been intensively studied in model fungi such as *Aspergillus nidulans* or *Neurospora crassa*, with genome sequences revealing the presences of three or occasionally four MAPK genes for most ascomycete fungi.

Pathogenesis-related functions of MAPKs have been demonstrated in a number of plant-pathogenic fungi, including *Magnaporthe grisea* (Xu & Hamer, 1996; Xu *et al.*, 1998), *Ustilago maydis* (Muller *et al.*, 1999; Mayorga & Gold, 2001), *Cochliobolus heterostrophus* (Lev *et al.*, 1999), *Botrytis cinerea* (Zheng *et al.*, 2000), *Colletotrichum lagenarium* (Takano *et al.*, 2000), *Fusarium oxysporum* (Di Pietro *et al.*, 2001), *Claviceps purpurea* (Mey *et al.*, 2002) and *Cryphonectria parasitica* (Park *et al.*, 2004). *M. grisea* is the most intensively studied of the plant pathogenic ascomycetes, with three MAPK homologues being characterized (Xu, 2000; Zhao *et al.*, 2007).

It has been suggested that the MAPK family of genes has probably co-evolved with their targets, scaffold proteins and kinases that activate them (Lev *et al.*, 1999; Park *et al.*, 2003), so the function of a MAPK gene in one species may be slightly different from one with a similar sequence in another species. For example, the *pmk1* gene in *M. grisea* has a specific role in forming large, melanized appressoria, whereas in *C. heterostrophus*, the equivalent gene homologue, *chk1*, has a wider set of developmental roles and a smaller role in appressoria formation (Lev & Horwitz, 2003). Despite these small differences in effectors, there is a general phenomenon that, amongst fungal plant pathogens, *pmk1* homologues are essential for virulence and that gene disruption compromises pathogenicity. Whether the same is true for pathogens of other hosts is less clear. In the animal pathogen *Cryptococcus neoformans*, impairment of this signalling pathway has only a small impact on virulence (Clarke *et al.*, 2001). Infection of immune-depressed mice with the *pmk1*-deficient *F. oxysporum* showed normal infection in these mammals, despite eliminating virulence against their normal plant hosts (Ortoneda *et al.*, 2004). The role of a MAPK signal transduction pathway in disease development of fungal–fungal interactions is largely unresolved. In this study, a *pmk1*-like MAPK homologue from *L. fungicola* was cloned, characterized and used to determine expression in *L. fungicola* infections of *A. bisporus*.

METHODS

Fungal culture. All experiments were performed with *L. fungicola* isolate 150-1, which was originally isolated from a diseased mushroom (Mills *et al.*, 2008). Cultures for experimental purposes were grown as described previously (Amey *et al.*, 2002) on Czapek-Dox agar (CDA) potato dextrose agar (PDA) supplemented, when appropriate, with 650 µg hygromycin B ml⁻¹, or using *L. fungicola*

minimal medium (MM; Calonje *et al.*, 1997) with the addition of 0.1% *A. bisporus* cell wall material (ACWM). *Agaricus* cell wall extracts were prepared by using the method described by Novaes-Ledieu & Mendoza (1981): button mushrooms were homogenized in a Waring blender, the homogenate was centrifuged to harvest the cell wall material and the pellet was then washed twice in deionized (DI) water, twice in 10% (w/v) sucrose and twice in 1 M NaCl. Two further washes with DI water were performed before the precipitate was freeze-dried and ground to a fine powder.

Nucleic acid manipulations. *L. fungicola* genomic DNA was isolated essentially as described by Keon & Hargreaves (1998) and used for Southern analysis. Genomic DNA (5 µg) of wild-type, disruptants and a control transformant was digested with *Bgl*II or with *Sac*I and separated by gel electrophoresis. Gels were denatured and DNA was blotted onto Hybond-N⁺ membranes. These were probed with a ³²P-labelled dCTP fragment of the right flanking sequence that was generated by PCR using primers 5'-CGTGAAGTGGGTTCAGGAAAA-3' and 5'-AACACCCCTACCTC-GAGCCAT-3'. Hybridizations were carried out according to the method described by Amey *et al.* (2002). Plasmid DNA was purified using the Promega wizard SV mini-prep kit.

PCR was performed on a PTC-100 thermocycler (MJ Research) in 20 µl reaction volumes. Cycling parameters for degenerate primers were: 1 cycle of 94 °C 2 min; 30 cycles of 94 °C 30 s, 50 °C 30 s, 72 °C 30 s; 1 cycle of 72 °C 4 min. To permit amplification of longer products, the annealing temperature (55–60 °C), extension time (2 min) and number of cycles (*n*=35) were modified.

RNA extraction and quantitative RT-PCR (Q-PCR). Total RNA was extracted from *L. fungicola* agar cultures and from lesions formed on *A. bisporus* sporophores using Tri reagent (Invitrogen). RNA (1 µg) was digested in 10 µl volumes with 1 µl DNase Q and 1 µl 10× DNase Q buffer (Promega) for 30 min at 37 °C. To stop the reaction, 1 µl DNase Q stop solution (Promega) was added to the mixture and heated to 65 °C for 10 min. DNase-treated RNA (1 µg) was converted to cDNA using the ThermoScript RT-PCR System (Invitrogen) with random hexamers (Amersham), following the manufacturer's instructions, except for the following modifications: random hexamers together with RNA were heated (65 °C, 10 min) and cooled on ice before the 50 °C incubation step, which was increased to 1 h 30 min. At the end of the reaction, cDNA was diluted to 10 ng µl⁻¹ by the addition of 80 µl H₂O (assumed 100% conversion of RNA to cDNA). Primer pairs for the *Lf pmk1* gene (5'-GATGTCGTCGGCGAAGGT-3'/5'-TGGCCTGATGGTTTGTGGAT-3') and 18S (5'-CAACGGGTAACGGAGGGTTA-3'/5'-TGCTGCC-TTCCTTGGATGTAG-3') were designed using ABI Primer Express (v 2.0). The 18S primers were tested against *A. bisporus* cDNA and did not yield any significant values. Q-PCR was carried out using the ABI TaqMan 9700HT with standard cycling conditions (40 cycles of 95 °C for 15 s followed by 1 min at 60 °C). Reaction mixtures (15 µl) comprised 7.5 µl SYBR Green master mix (Eurogentech), 1 µM each primer and 2 µl (20 ng) cDNA. Because of the large amount of 18S RNA present, only 5 ng cDNA was used. Three independent biological replicates each from *L. fungicola* agar cultures and *A. bisporus* lesions were analysed. Two (technical replicate) reactions were carried out for each sample and also for each standard, with the mean of two replicates being used in subsequent calculations. Standard curves were generated for each gene (primer combination) using fourfold dilutions of a mixture of appropriate cDNA samples. TaqMan SDS software (v 2.1) was used to obtain the cycle threshold (C_t) for each sample and to calculate relative values based on appropriate standard curves, which were imported into Microsoft Excel for subsequent analysis. Relative values were normalized against 18S values to indicate relative gene expression.

Isolation of the *Lf pmk1* gene. To obtain the full-length genomic clone, the lambda-BlueSTAR genomic library described by Amey *et al.* (2003) was screened in duplicate by probing with the degenerate PCR-generated gene fragment obtained using primers Map3 (5'-GAYGAYCAYTGYCARTAYTTYAT-3') and Map7 (5'-TTDATNC-CRTARTARTCYTCCAT-3'), designed using conserved regions of *pmk1*-like MAPKs from various filamentous fungi. Library screening under high-stringency conditions and routine procedures was carried out using standard protocols. Sequence data were analysed using Sequencher software (Gene Codes). The BLASTnr algorithm was used to search DNA and protein databases at the National Center for Biotechnology Information. Multiple sequence alignments were prepared using CLUSTAL_X and neighbour-joining, bootstrapped trees were produced using MEGA3.1 (Kumar *et al.*, 2004).

Knockout plasmid construction. The disruption cassette was constructed in pZEJ (Johnson, 2006), a mini-binary vector based on pGREENII (Hellens *et al.*, 2000), containing a hygromycin resistance cassette within the T-DNA. The 1141 bp upstream targeting region (left arm) was amplified by PCR from a plasmid template and restriction sites for *Clal* and *EcoRI* were incorporated into the primers for directional cloning upstream of the Hygromycin cassette in pZEJ. In the same manner, the 1089 bp downstream targeting region (right arm) was amplified but included restriction sites for *SpeI* and *NotI* to yield the transformation plasmid pZMK37. Concurrently, an 826 bp downstream targeting region (right arm) with the same included restriction sites was constructed to yield transformation plasmid pZMK38.

Agrobacterium (*Ag.*) *tumefaciens*-mediated transformation. Transformation of *L. fungicola* 150-1 with either pZMK37 or pZMK38 was performed essentially as described by Amey *et al.* (2002) using the pGreen/pSoup system (Hellens *et al.*, 2000). Briefly, 100 µl of an *L. fungicola* 150-1 spore suspension (10^6 spores ml⁻¹) was mixed with 100 µl *Ag. tumefaciens* and plated onto cellophane disks on induction medium (Hooykaas *et al.*, 1979). Following 2 days co-cultivation, cellophane disks were transferred to PDA supplemented with 200 µg cefotaxime ml⁻¹ and 650 µg hygromycin B ml⁻¹. After a further 4 days, transformants were subcultured onto PDA with 650 µg hygromycin B ml⁻¹ alone and incubated at 20 °C.

Phenotypic assays. Plate-based clearing assays were performed to determine the abilities of *L. fungicola* mutants to degrade various substrates. Agar culture plugs (5 mm diameter) from transformants and wild-type strains were grown for 7 days on MM and ACWM plates supplemented with starch (0.5% w/v), gelatin (0.5% w/v), xylan (0.5% w/v) or skimmed-milk powder (1.5% w/v). Clearing zones were visualized by use of an appropriate stain: starch, iodine; gelatin, amido black; xylan, congo red. Dehydrated milk cultures did not require staining to visualize clearing zones.

Gel diffusion assays (Zou *et al.*, 2002) for degradation of glycol chitin, a soluble, modified form of chitin, were performed using 14-day-old ACWM cultures, grown at 22 °C with gentle shaking. Mycelia were removed by centrifugation (27 000 g, 60 min, 4 °C) and supernatants were filtered to ensure removal of cells and cell wall material. Sodium azide was added to a final concentration of 0.01% and the filtrate was concentrated using a Centricon Plus 20 (Millipore) with a 5 kDa cut-off. Protein concentration of the retentate was determined as described by Bradford (1976) and equal protein concentrations for the retentate were used for this assay.

Infection assays were performed as previously described by Collopy *et al.* (2001). Harvested *A. bisporus* sporophores with the veil still intact had their stipes removed and were then inoculated with *L. fungicola* conidia to induce infection. Caps were inoculated with 5 µl of a conidial suspension (10^6 ml⁻¹) and incubated at 22 °C for 5 days, after which, the lesion diameter was measured and recorded.

RESULTS

Isolation of *Lf pmk1*

PCR amplification using *L. fungicola* genomic DNA with degenerate primers designed against *pmk1*-like proteins yielded an approximately 420 bp product, which was cloned using pGEMT; the sequences of three independent recombinants proved identical and database comparisons showed strong similarity to fungal *pmk1* homologues. Due to this high homology to other fungal MAPK genes, and particularly the *pmk1*-like MAPKs, the isolated *L. fungicola* gene was termed *Lf pmk1*. The cloned PCR fragment was used to probe a Southern blot and the presence of a single band in each lane was indicative of a single-copy gene within the *L. fungicola* genome (data not shown). This fragment was then used to screen a lambda genomic library and two independent plaques were purified. Inserts were subsequently excised to yield plasmids pMapKCC and pMapKFC and used as templates for sequencing by a primer-walking strategy. In total, a region of 3908 bp was sequenced, including 1.3 kb of predicted promoter region, the 1.3 kb encoding the *Lf pmk1* open reading frame and an additional 1.3 kb downstream region (GenBank accession no. AY184496).

Three introns were predicted on the basis of the expected amino acid sequence and conserved splice sequences. RT-PCR was used to confirm the presence of these introns within the gene. This yielded the predicted size product of 765 bp compared with the 964 bp derived from genomic DNA, and the locations were subsequently confirmed by sequencing of this product. The predicted protein of 356 aa exhibits more than 90% aa identity to other fungal *pmk1* homologues, as illustrated by neighbour-joining analysis (Fig. 1).

Q-PCR

Comparative transcription of *Lf pmk1* in vegetative mycelium and infection lesions was determined using Q-PCR. Expression was detected from cultures grown in both conditions. In vegetative mycelium, the amount of *Lf pmk1* transcript when normalized to 18S gave a relative value of 3.5 (± 0.48 SE) compared with 18.8 (± 3.40 SE) from cap lesions. These data are indicative of a more than fivefold increase in the relative expression of *Lf pmk1* during infection.

Transformation for targeted gene disruption

The *Lf pmk1* gene-specific disruption cassette was constructed in pZEJ, a pGREEN derivative containing a hygromycin resistance cassette. The two final vectors were each composed of a hygromycin B resistance cassette flanked by left and right arms of the *Lf pmk1* gene. Both constructs contained a left arm of 1141 bp, but two different right arms of 1089 and 826 bp were used for pZMK37 and pZMK38, respectively. In addition to the disruption of the *Lf pmk1* gene by the hygromycin resistance

Fig. 1. Neighbour-joining phylogenetic tree of selected fungal MAPK proteins, showing the relationship of *Lf pmk1* to the other members of the *pmk1*-like group and to other MAPK families. LF PMK1, *L. fungicola* AY184496; Fomk1, *F. oxysporum* AAG01162; TVK1, *T. virens* AAO46014; MgPmk1, *M. grisea* ACC49521; CIMK1, *Colletotrichum lagenarium* AAD50496; NcMak2, *Neurospora crassa* AAK25816; BcMK1, *B. cinereus* AAG231320; Chmk1, *C. heterostrophus* AAF05913; UmUbc3, *Ustilago maydis* AAF09452; ScKss1, *Saccharomyces cerevisiae* AAA34882; ScFus3, *S. cerevisiae* AAA34613; ScSlt2, *S. cerevisiae* CAA41954; EnMPKA, *Emericella nidulans* AAD24428; MgMps1, *M. grisea* AAC63682; NcMps1, *N. crassa* XP_958040; EnMpkC, *E. nidulans* AAG28463; ScHog, *S. cerevisiae* AAA34680; EnHogA, *E. nidulans* AAF97243; MgOs1, *M. grisea* AAF09475; NcSty1, *N. crassa* EAA32927. Bar, 0.1 base substitutions per site.

gene cassette, a region of approximately 500 bp of the ORF was deleted between the left and right arm flanks to ensure inactivation of the *Lf pmk1* reading frame. Transformation of the construct was performed using *Ag. tumefaciens* LBA1126 containing pSoup and either the pZMK37 or pZMK38 vectors. Hygromycin-resistant lines were selected at random and screened by PCR to confirm the presence of the hygromycin transgene (Fig. 2a and b). A PCR was performed using a primer pair amplifying from within the hygromycin gene to an area outside the targeting region to confirm that homologous recombination had occurred. Three transformants 37MK1, 37MK3 (derived from pZMK37) and 38MK1 (derived from pZMK38) yielded the expected product of 1.6 kb (Fig. 2b). Southern analysis of these transformants showed the expected loss of wild-type band and gain of recombinant band in 37MK1 and 38MK1 (Fig. 2c), indicative of gene disruption. Strains 37MK1 and 38MK1 were therefore selected as representatives of the two knockout types for further analysis.

Phenotypic effect of *Lf pmk1* disruption on *L. fungicola*

In other fungi, disruption of the *pmk1*-like MAPK has been reported to have a number of different phenotypic effects, including changes in virulence, growth patterns and sporulation; these phenotypes were investigated in these disrupted strains.

Growth rate comparisons between Δ LFMK1 knockouts and wild-type (150-1) *L. fungicola* grown on CDA, PDA or ACWM showed no significant differences, additionally

there were no obvious differences in the colour of the mycelia or the colony morphology (e.g. amount of aerial hyphae). Growth rates were measured on CDA solidified with a range of agar concentrations from 0.5 to 8 %, but again there were no differences between the wild-type and disrupted strains, showing that the mutants were not impaired in their ability to penetrate the media. Growth rates were also assessed on a range of osmotic stabilizers including sucrose (1 M), KCl (0.6 M), NaCl (0.9 M) and sorbitol (1.2 M) but again there was no difference between wild-type and disrupted strains.

Spores were harvested from centrally inoculated plates after 7 days growth and were counted and measured. Conidiation rates and spore sizes were similar, indicating no apparent alteration in sporulation rates or morphology between the disruptants and the wild-type (data not shown). Although the growth of 37MK1 was slightly reduced on chitin- or xylan-modified media (Fig. 3a), this was isolate-specific and did not occur in the disruptant 38MK1, again indicating no obvious change in growth rates between wild-type and Δ LFMK1 mutants.

There have been reports of altered levels of secreted enzymes associated with disruption of *pmk1*-like MAPKs (e.g. Lev & Horwitz, 2003). We used plate-based assays to monitor production of amylase and protease. In our clearing zone experiments, there were no significant differences in the rate of growth or size of clearing zone on starch plates, indicating that amylase activity was unaffected (Fig. 3b). Likewise, there was no difference in protease secretion as indicated by clearing zones on either

Fig. 2. (a) Schematic showing primers used for confirmation of transformation and disruption of *Lf pmk1*. Primers hygA and hygB were used to confirm the presence of the hygromycin resistance cassette among potential *L. fungicola* transformants. Primers LAFKO and hygoutC2 were used to confirm homologous integration of the gene disruption cassette into *LFMK1*. (b) PCR products amplified using LAFKO with hygoutC2 (to confirm homologous integration) and hygA with hygB (to confirm genuine hygromycin resistance). WT lanes are the results of PCR from 150-1 (wild-type) DNA. 37MK1, 37MK3 and 38MK1 are PCR results from probable deletion strains. The left lane contains a molecular marker (Hyperladder I, Bioline). (c) Southern blot analysis. Lanes 1–5 and 6–10 contain digested DNA of 150-1 (wild-type), 37MK1, 37MK3 and 38MK1, and a control transformant, cut with *Bgl*II and *Sac*I, respectively, and probed with the right flank sequence. 37MK1 and 38MK1 show the loss of the wild-type band (1.1 kb and 1.4 kb for *Bgl*II and *Sac*I) and gain of recombinant bands (2.1 kb and 2.2 kb for *Bgl*II and *Sac*I) indicative of gene disruption. 37MK3 shows both the wild-type and recombinant bands.

skimmed-milk- or gelatin-modified media (Fig. 3b). There was also no significant difference between wild-type and disruption transformants in the diffusible chitinase activity assays performed on liquid media in which *L. fungicola* had been grown (data not shown).

Virulence of *Lf pmk1* mutants

Detached cap bioassays, which we have previously used to assess virulence of *L. fungicola* transgenic lines (Amey *et al.*, 2003), were deployed to compare *Lf pmk1* disruption transformants with the wild-type. No significant differences were observed during pathogenicity assays using conidia suspensions on *A. bisporus* detached mushrooms. In control inoculations, wild-type *L. fungicola* conidia resulted in necrotic lesions, and sterile DI water produced no lesions. All lesions appeared the same as the wild-type, with a sunken appearance and brown coloration. There was no difference in lesion diameters (Fig. 4) or the onset of development of lesions formed by wild-type and *Lf pmk1* disruption transformants.

DISCUSSION

The development of cap lesions and other dry bubble symptoms caused by the mushroom pathogen *L. fungicola*,

are considered to be the results of both physical and chemical damage to host tissue (Dragt *et al.*, 1996; Calonje *et al.*, 1997). It could be expected that the fungal–fungal interaction requires mechanisms to detect suitable host material and that a signal transduction process triggers the expression of appropriate virulence factors, as has been well characterized in numerous fungal–plant pathogens. The recent development of a gene disruption strategy (Amey *et al.*, 2003) for *L. fungicola* affords an opportunity to study the mechanisms of mycoparasitism in this intriguing pathosystem (Amey *et al.*, 2003) and to elucidate the roles of signal transduction pathways. Degenerate PCR was successfully employed to isolate a portion of *Lf pmk1* and subsequent library screening and analysis led to the isolation of the entire gene. This is predicted to encode a MAPK with a molecular mass of 41.2 kDa which displays high levels of similarity to the *pmk1*-like group of MAPKs. As would be expected from a gene that might potentially play a role in pathogenicity, *Lf pmk1* transcripts were substantially upregulated during infection of *A. bisporus*.

Agrobacterium-mediated transformation permitted recovery of transformants with disrupted *Lf pmk1* sequences. Disruption transformants did not exhibit altered growth rates or secreted enzyme profiles, which is in contrast with observations in other fungi, in which sporulation can be impaired or secreted enzyme profiles can be altered (e.g. Di

Fig. 3. (a) Colony diameter of wild-type and *Lf pmk1* disrupted strains 37MK1 and 38MK1 after 5 days growth on various media, showing no changes in growth rate. (b) Clearing zone (empty bars) and colony diameters (solid bars) after 5 days growth on starch, milk or gelatin plates for wild-type and *Lf pmk1* disrupted strains 37MK1 and 38MK1, showing no difference in amylase or protease secretion.

Pietro *et al.*, 2001; Lev & Horwitz, 2003; Cho *et al.*, 2007). The pathogenicity of disruption transformants was also indistinguishable from the wild-type in terms of phenotype or virulence, which is in stark contrast with the situation in plant-pathogenic fungi where all previous reports of *pmk1*-like MAPK disruptions have resulted in either reduction or elimination of virulence.

Signal transduction pathways are thought to play integral roles in pathogenesis and the establishment of infection in a number of host-pathogen interactions (Ruiz-Roldan *et al.*, 2001). Numerous studies have demonstrated that disruption of MAPK homologues in various plant pathogens interferes with signal transduction pathways that are

integral to pathogenesis and typically result in decreased virulence (Xu & Hamer, 1996; Xu *et al.*, 1998; Lev *et al.*, 1999; Muller *et al.*, 1999; Takano *et al.*, 2000; Zheng *et al.*, 2000; Mayorga & Gold, 2001; Ruiz-Roldan *et al.*, 2001). Such pathogenicity determinants are not always specific to the interaction of a plant pathogen with one particular host. Disruption of *BMK1* in *B. cinerea* or *VMK1* in *V. dahliae* resulted in severely reduced virulence on a number of different host plants (Zheng *et al.*, 2000; Rauiyaree *et al.*, 2005). Such results suggest that the MAPK signalling pathway may have a conserved role in plant-pathogenic fungi despite the absence of an intimate host-pathogen interaction. Although MAPK signalling pathways have a universal role in displaying full virulence in fungal

Fig. 4. Mean diameter ($n=20$) of cap lesions on *A. bisporus* 5 days post-inoculation with wild-type (150-1) or an *Lf pmk1* disruption transformant (37MK1 and 38MK1). There was no significant difference in virulence between the disruption transformants and the wild-type.

pathogenesis against plant hosts, *fmk1* mutants of *F. oxysporum* displayed normal virulence in immunorepressed mice (Ortoneda *et al.*, 2004), whilst showing reduced virulence *in planta*. Whilst this is not a natural situation, it does show that *fmk1* was not required to support growth on such a host, although such animals are also subject to infection by other microbes not usually regarded as pathogens.

Trichoderma species, which are commonly used as biocontrol agents to reduce the effects of fungal plant pathogens, exhibit various effects when specific *pmk1*-like MAPKs are disrupted; reduced biological control was reported against *Rhizoctonia solani* and *Sclerotium rolfsii* for *tmkA* mutants (Mukherjee *et al.*, 2003), whereas increased control was seen for *Trichoderma virens* *tvk1* mutants against *R. solani* and *Pythium ultimum*, but with more variable behaviour reported against other target fungi (Mendoza-Mendoza *et al.*, 2003). Additional, indirect, effects on apparent biocontrol activity, such as the induction of plant resistance pathways (Viterbo *et al.*, 2005; Reithner *et al.*, 2007), have further complicated the interpretation of gene disruptions in other systems. Collectively, these observations indicate that the roles of *pmk1*-like MAPKs in other pathogenic interactions can be more diverse than those found in fungal-plant interactions.

The observation that *Lf pmk1* disruptants do not exhibit reduced virulence suggests that the signalling pathway does not play a direct role in pathogenicity or sensing of environmental cues from its fungal host. Alternatively, it is possible that a different MAPK pathway has evolved to regulate virulence in *L. fungicola* or to act as a substitute for it in *Lf pmk1* mutants, but we saw no evidence for a similar gene following Southern blot analysis. *L. fungicola* does not obviously parasitize vegetative hyphae of *Agaricus* (*in vitro*) but does attack developing mushrooms, indicating that there must be a detection mechanism that can differentiate between these two host physiologies. Recent investigations into the recognition and binding of *L. fungicola* on *A. bisporus* mushrooms have identified a lectin present in

fruit bodies that is not present in *A. bisporus* mycelium (Bernardo *et al.*, 2004). A glucogalactomannan on the surface of *L. fungicola* specifically complements this lectin and may explain the inability of *L. fungicola* to infect *A. bisporus* mycelium. In this research, we investigated the ability of *L. fungicola* to cause cap lesions on maturing mushrooms, as these are the symptoms of most economic significance due to loss of visual quality of the resulting crop. Different *L. fungicola* isolates vary in their abilities to cause the three symptoms of disease and it is possible that different regulatory pathways are activated with other symptoms.

Recent publications have reported the isolation of *L. fungicola* from nematodes (Gené *et al.*, 2005) along with numerous reports that *L. longisporum* (*V. lecanii*) can be used for the biological control of not only insects but also mildews and rusts (e.g. Jun-Kim *et al.*, 2007; Spencer & Atkey, 1981). It would be interesting to investigate whether disrupting *pmk1* homologues in *L. longisporum* results in altered pathogenicity and to determine whether the gene plays a significant role in other fungal-invertebrate and/or mycopathogen pathosystems.

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