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# The *Fusarium graminearum MAP1* gene is essential for pathogenicity and development of perithecia

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## SUMMARY

*Fusarium graminearum* is the causal agent of ear blight disease of cereals. Infection occurs at anthesis when ascospores and/or conidia directly penetrate exposed anther and ovary tissue. The hemibiotrophic hyphae colonize floral tissues and developing grains to cause premature ear senescence. During infection, *Fusarium* hyphae can also produce hazardous trichothecene mycotoxins, thereby posing a threat to human and animal health and safety. The *Fusarium MAP1* gene was identified using a PCR approach by its homology to a known pathogenicity gene of *Magnaporthe grisea*, the mitogen-activated protein kinase gene *PMK1*. Gene replacement *F. graminearum map1* mutants were non-pathogenic on wheat flowers and roots, and also could not infect wounded wheat floral tissue or tomato fruits. Unlike the wild-type strain, *map1* mutant inoculations did not compromise grain yield. *Map1* mutants lost their ability to form perithecia *in vitro*, but their rate of asexual conidiation was unaffected. DON mycotoxin production *in planta* was still detected. Collectively, the observed phenotypes suggest that the Map1 signalling protein controls multiple events in disease establishment and propagation. Novel approaches to control *Fusarium* ear blight disease by blocking perithecial development are discussed.

## INTRODUCTION

*Fusarium* ear blight disease of wheat, barley and rye crops, also commonly known as head scab, is predominantly caused by the homothallic fungal species *Fusarium graminearum* (teleomorph *Gibberella zeae*) and *F. culmorum* (Pugh and Johann, 1933; http://www.scabusa.org). Annually, this disease causes considerable losses to grain quality and safety in numerous geographical regions (Ward *et al.*, 2002). Grain safety is compromised because both *Fusarium* species can synthesize a range of mycotoxins during plant infection including the sesquiterpenoid epoxide trichothecenes, deoxynivalenol (DON), 3-acetyl DON, 15-acetyl DON and nivalenol (NIV) (O'Donnell *et al.*, 2000). These toxins are potent inhibitors of protein synthesis in eukaryotic cells (Sharma and Kim, 1991).

Perithecial development is considered to play an important role in the disease cycle of *F. graminearum*. The sexual ascopores formed in perithecia are proposed to be the primary inoculum for ear blight disease in the USA (Fernando *et al.*, 1997; Paulitz, 1996). Cereal ear infection only occurs during a short period of time at flowering (anthesis) (Pugh and Johann, 1933).

Molecular genetic analysis of *Fusarium* ear infection is highly fragmentary. Earlier studies focused on the regulation and biosynthesis of the DON mycotoxin (Hohn *et al.*, 1998; Proctor *et al.*, 1995; Tag *et al.*, 2000). However, the role of trichothecene mycotoxins in plant disease is unclear (Proctor *et al.*, 2002). DON production has been shown to contribute to the virulence of *F. graminearum* towards wheat, but is not an essential pathogenicity factor (Proctor *et al.*, 1995). Recently, the first targeted deletion of a homologue of a previously known fungal pathogenicity gene was reported for *F. graminearum. MVG1* encodes a mitogen-activated protein kinase and is homologous to the *MPS1* gene of the rice blast pathogen *Magnaporthe grisea* (Hou *et al.*, 2002). In *M. grisea*, *MPS1* is essential for appressorium penetration. *F. graminearum* does not form an appressorium prior to plant tissue penetration. In contrast to the *M. grisea mps1* mutant, the *F. graminearum mgv1* mutant is still able to infect wheat ears, although virulence is reduced. *Mgv1* mutants produce DON mycotoxin and conidiate; however, vegetative growth rates are reduced *in vitro* and perithecia fail to form. Therefore the *F. graminearum MGV1* gene can be considered to be contributing to virulence but is not essential for pathogenicity.

Signal transduction has recently been shown to play a crucial role during infection in diverse plant and animal pathogens (Xu, 2000). Fungi must perceive chemical and physical signals from the host and respond with the appropriate metabolic and morphogenetic changes required for fungal development. Key intracellular signalling components identified for *Magnaporthe grisea*

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Nucleotide sequence data can be found at *GenBank* database as accession no. AF448230.

 

and other plant pathogenic fungi include G proteins, adenylate cyclase for cAMP signalling and 10 mitogen-activated protein kinases (reviewed by Idnurm and Howlett, 2001; Xu, 2000). These proteins were shown to be involved in conidiation, appressorium formation, penetration and/or invasive growth in host tissue.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases which activate transcription factors in response to extracellular signals in a variety of eukaryotic organisms including yeast and human (Herskowitz, 1995; Schaeffer and Webber, 1999). In the plant pathogen *M. grisea*, three different and functionally important MAP kinase pathways exist that contain either the MAP kinase protein *PMK1* (pathogenicity MAPK), *MPS1* (MAPK for penetration and sporulation) or *OSM1* (osmoregulation MAPK) (see Xu, 2000 for review). These genes are homologues of *S. cerevisiae* genes with known regulatory functions: *FUS3/KSS1* (pheromone response), *SLT2* (cell integrity) and *HOG1* (growth under high osmolarity conditions (see Gustin *et al.*, 1998 for review). The *M. grisea PMK1* gene is essential for both infection structure formation and *in planta* growth (Xu and Hamer, 1996), whilst *MPS1* is required for appressorial penetration but not *in planta* growth (Xu *et al.*, 1998). *OSM1* is dispensable for pathogenicity (Dixon *et al.*, 1999). It is an emerging pattern that homologues of *M. grisea PMK1* and *MPS1* are conserved in phytopathogenic fungi, where they regulate similar but distinct pathways (Xu, 2000).

A molecular understanding of the signalling events in *F. graminearum* controlling fungal penetration, cereal ear colonization and spore formation would be extremely valuable. Mycotoxin contamination would no longer be an issue if plant infection could be prevented. Homologues of the *M. grisea PMK1* gene, in particular, appear to play an important role in the pathogenicity of several fungal species with highly diverse infection biology (Di Pietro *et al.*, 2001; Mey *et al.*, 2002b; Ruiz-Roldan *et al.*, 2001; Xu, 2000).

In this paper we describe the isolation and disruption of the *F. graminearum FUS3/KSS1/PMK1* related *MAPK* gene, designated *MAP1*. The diverse roles of *MAP1* in the fungal life cycle and during plant disease are examined.

## RESULTS

### Isolation and features of the *MAP1* gene

A ligation-mediated inverse PCR experiment was undertaken to recover the complete genomic gene sequence of an *F. graminearum* MAP kinase with a high degree of identity to *M. grisea PMK1* (see Experimental procedures). Sequence analysis revealed an open reading frame which was predicted to encode a 355-amino acid protein with features of typical MAP kinases, including 11 protein kinase domains and the double phosphorylation sites TEY (Fig. 1a). Three introns of 58, 55 and 62 bp were identified on the basis of alignment with other MAP kinases and the presence of conserved splice-site consensus sequences characteristic of filamentous fungi (Bruchez *et al.*, 1993). No obvious promoter elements could be identified, with the exception of a putative CAAT consensus sequence at position −110.

A comparison of the deduced protein sequence from the isolated *F. graminearum* gene with MAPK Pmk1 from the closely related Ascomycete *Magnaporthe grisea* and Ubc3 from the more distantly related Heterobasidiomycete *Ustilago maydis* gave identity scores of 97% and 76%, respectively, using the BLAST algorithms (Altschul *et al.*, 1997). We designated the *F. graminearum* gene as *MAP1*. The deduced Map1 protein is also highly homologous to two other protein kinases isolated from different phytopathogenic *Fusarium* species, namely Fmk1 from *F. oxysporum* (98%) (Di Pietro *et al.*, 2001) and FsMAPK from *Nectria haematococca* (98%) (Li *et al.*, 1997). A phylogenetic analysis of related proteins indicate that Map1 is more closely related to *M. grisea* Pmk1 than to MAP kinases belonging to the slt2 and Hog1 clades (Fig. 1b). Map1 is only 47% identical to *F. graminearum* Mgv1 (Hou *et al.*, 2002). We conclude that *MAP1* is the *F. graminearum* homologue of *PMK1*.

Low stringency gel blot hybridization analysis of *F. graminearum* genomic DNA digested with *Bgl*II and *Eco*RV using the gene probe U-1 (shown in Fig. 2a) produced a hybridization pattern with single bands (data not shown). This data suggests that the identified sequence is a single-copy gene.

### Disruption of the *MAP1* gene

We analysed the function of the *MAP1* gene in *F. graminearum* by gene replacement (Fig. 2a). The *MAP1* gene replacement vector, pΔMAP1, was constructed by removing 563 bp of the C-terminal region containing the kinase domains VIII to XI (Nishida and Gotoh, 1993) and inserting the gene encoding hygromycin phosphotransferase (*HPH*) (Carroll *et al.*, 1994). The vector contained a total of 1.8 kb of flanking and *MAP1* gene *F. graminearum* DNA to facilitate homologous recombination (Fig. 2a). Transformants were selected on hygromycin-containing medium. Twenty-two out of 29 hygromycin-resistant transformants that had the *MAP1* gene replaced with the *Δmap1* allele were identified in a preliminary PCR screen (data not shown). This indicates a 76% efficiency in gene replacement. Six of the isolated *MAP1* deletion transformants and two ectopic *Δmap1* allele gene insertions were confirmed by DNA gel blot analysis (Fig. 2b and data not shown). Hybridization analysis of selected transformants and the progenitor strain 16A using probe U-1 (Fig. 2a) revealed that the strain 16A and transformant CAM8 contain the wild-type *MAP1* 1.2 kb *Eco*RV fragment (Fig. 2b). In addition CAM8 contained a 2.0 kb *EcoRV* DNA fragment characteristic of the *Δmap1* allele, indicating an integration of the gene replacement vector elsewhere in the genome. Transformants CAM1 and CAM2 show

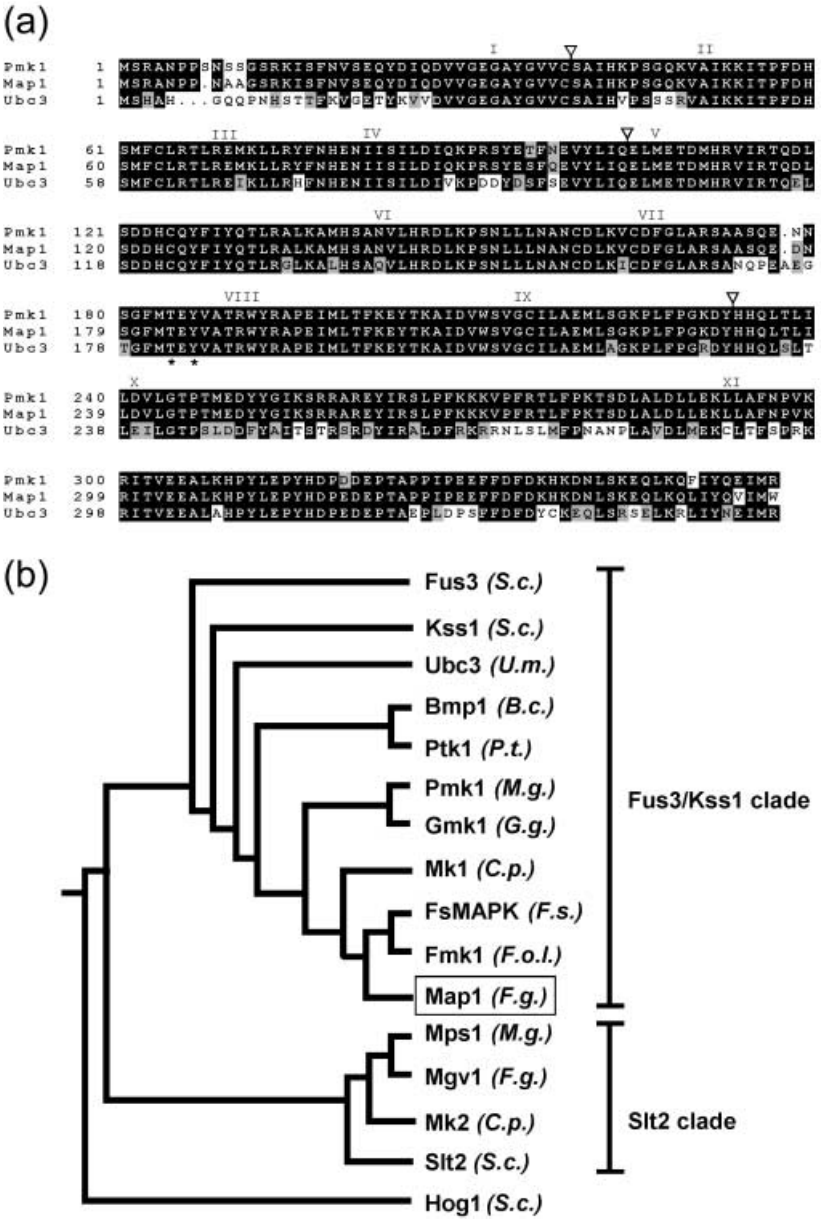


**Fig. 1** Alignment showing the homology of Map1 to other MAP kinases from phytopathogenic and non-phytopathogenic Ascomycete fungi and one Heterobasidiomycete fungus. (a) Amino acid sequence alignment of Map1 with Pmk1 from the Ascomycete fungus *M. grisea* and Ubc3 from the Heterobasidiomycete fungus *U. maydis*. Identical amino acids are highlighted on a black background, while similar amino acids are shown on a grey background. Gaps in the alignment are indicated by dots. The inverted triangles above the three sequences indicate the locations of the three introns in the *MAP1* sequence. The 11 protein kinase domains are labelled at the top in Roman numerals (Hanks *et al.*, 1988). The GenBank accession no. for the *MAP1* sequence is AF448230. (b) Phylogenetic tree based on amino acid sequence alignments of selected fungal MAP kinase homologues. The dendrogram was prepared with the ClustalW alignment program <http://clustalw.genome.ad.jp>. The DNA GenBank accession numbers are: M31132 for *FUS3*, A33297 for *KSS1*, AF170532 for *UBC3*, AF205375 for *BMP1*, AF272831 for *PTK1*, U70134 for *PMK1*, AF258529 for *GMK1*, AJ318517 for *MK1*, U52963 for *FsMAPK*, AF286533 for *FMK1*, AF020316 for *MPS1*, AF492766 for *MGV1*, AJ320496 for *MK2*, S43737 for *SLT2*, and L06279 for *HOG1*. Fungal species abbreviations are: *B.c.* for *Botrytis cinerea*, *C. p.* for *Claviceps purpurea*, *F.g.* for *Fusarium graminearum*, *F.s.* for *Fusarium solani*, *F.o.l.* for *Fusarium oxysporum f.* sp. *lycopersici*, *G.g.* for *Gaeumannomyces graminis*, *M.g.* for *Magnaporthe grisea*, *P.t.* for *Pyrenophora teres*, *S.c.* for *Saccharomyces cerevisiae*, *U.m.* for *Ustilago maydis*.

only the 2.0 kb *EcoRV* fragment as expected for a double homologous recombination event to create the *Δmap1* allele.

### *MAP1* is essential for root colonization

*F. graminearum* causes a blight disease at the stem/root interface on young cereal seedlings (Cook, 1968; Nicholson *et al.*, 2002; Proctor *et al.*, 1995). Root invasion was examined using a container assay in which the sown wheat seeds germinate into vermiculite inoculated with a mixture of fungal mycelium and conidia (see Experimental procedures). The wild-type strain 16A and the ectopic strain CAM8 caused brown lesions to form on the wheat roots within 2 weeks of inoculation. The infected seedlings either failed to emerge above the surface of the growing media, or they exhibited a severely stunted phenotype compared to the uninoculated seedlings. When the entire root system was examined at 21 days post-inoculation, all seedlings inoculated with either the wild-type strain 16A or the ectopic strain CAM8 had a stunted primary root and lacked secondary root branching (Fig. 3a). This phenotype resulted in a severe reduction in total above ground wheat seedling biomass, 5.5-fold for strain 16A and 3-fold for CAM8 compared to the control SNA only inoculated seedlings (Table 1). In contrast, *Δmap1* mutant strains CAM1 and CAM2 did not inhibit wheat seed germination, root

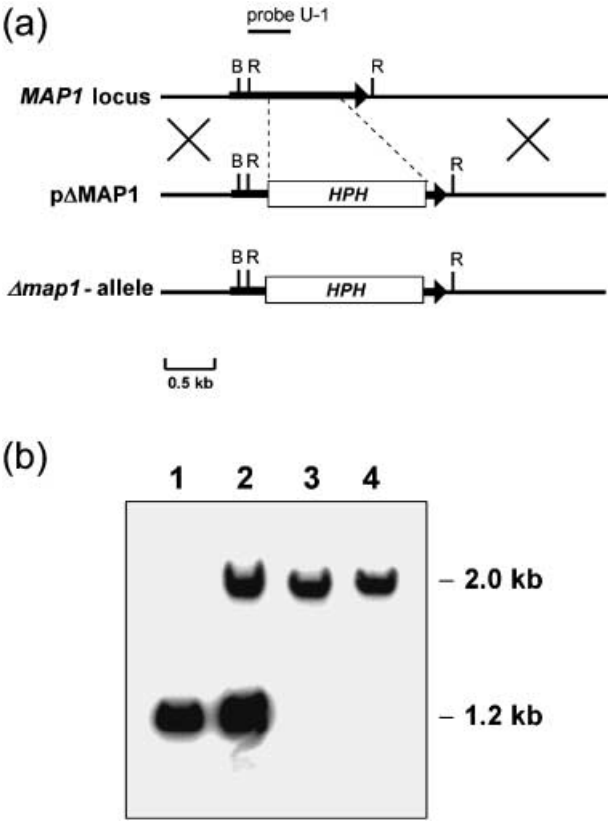


**Fig. 2** Strategy used and evidence for the *F. graminearum MAP1* gene replacement with a disrupted allele. (a) *MAP1* locus and gene-replacement vector pΔMAP1. U-1-indicates a 540 bp PCR fragment (nucleotides 771–1310 in AF448230) used as the hybridization probe. Recombination events between the *MAP1* locus (top row) and gene replacement vector pΔMAP1 (middle row) are indicated by large crosses and result in the creation of a *Δmap1* allele (bottom row). Restriction enzyme sites: B, *Bgl*II, R, *Eco*RV. (b) DNA gel blot analysis of *Eco*RV digested genomic DNA from wild-type strain 16A (lane 1), ectopic integration transformant CAM8 (lane 2) and *Δmap1* mutants CAM1, CAM2 (lanes 3 and 4, respectively). The blot was hybridized with probe U-1 (Fig. 2a). The integration of the deletion vector pΔMAP1 causes a band shift from 1.2 to 2.0 kb.

formation and above ground biomass development (Fig. 3a and Table 1).

Epifluorescence microscopy of stained wheat roots 21 days after inoculation revealed fungal growth in all root tissues for wild-type strain 16A, but not for the mutant strains (data not shown). We conclude that the *Δmap1* mutants cannot colonize root tissue.

### *MAP1* is essential for wheat ear colonization

To determine whether the wild-type *MAP1* gene is essential for pathogenicity on wheat ears, wheat plants of the susceptible cultivar Bobwhite were point-inoculated at anthesis with agar plugs containing *F. graminearum* hyphae (Fig. 3b). The six independently obtained *Δmap1* mutant strains were each non-pathogenic and did not show any disease spread 20 days after inoculation (Fig. 3b and data not shown). In contrast, wild-type strain 16A and the ectopic mutants CAM7 and CAM8 invaded the whole wheat ear and caused the typical bleached disease phenotype. Point-inoculations with each isolate were repeated on at least three separate occasions. A closer inspection of the CAM1 strain during the disease assay revealed that the *Δmap1* mutants were still able to form small amounts of aerial mycelium on dehiscing anther and anther filament tissue (Fig. 3c). However, disease symptoms did not spread into the adjacent lemma, palea and glume tissues. To test the role of *MAP1* in invasive growth *in planta*, wheat ears were wound-inoculated. A spikelet row and rachis close to the top of the ear were cut, and wound sites were then overlaid with a conidial suspension. While the mutant strains did not produce disease symptoms after 20 days, the wild-type strain 16A colonized the whole wheat ear (data not shown).

When wheat plants at anthesis were spray inoculated using a high-density conidial inoculum ($5 \times 10^5$ conidia/mL), again the *Δmap1* mutant strains only colonized anthers (data not shown). In contrast, strain 16A and the ectopic strain CAM7 and CAM8 invaded the whole wheat ear within 8 days and caused the typical bleached disease phenotype.

To elucidate the biological events occurring post-inoculation of the *Δmap1* and wild-type 16A strains, we used bright field and epifluorescent microscopy to study the infection process. Wheat flowers consist of a range of diverse tissues. Lemma and palea tissue can be infected within 36 h by using a detached tissue assay (Pritsch *et al.*, 2000, 2001). The infection process in palea tissue was investigated after 2, 4 and 6 days post-inoculation. Infected samples were processed by autoclaving tissue samples in potassium hydroxide and staining with Aniline Blue prior to microscope observation (Hood and Shew, 1996). The wild-type strain 16A had successfully invaded and colonized all the tissue layers after 4 and 6 days (Fig. 3d). In contrast, the *Δmap1* mutant CAM1 was unable to invade the palea tissue and could not be detected within plant cells (Fig. 3e). All experiments were done with five replicates and repeated once.

We conclude that hyphae of the *Δmap1* mutants were unable to penetrate and spread into the various tissues comprising the spikelet to cause the typical ear bleached symptoms. However, the ability of the *Δmap1* mutants to colonize anther tissue was not affected.

### *MAP1* mutants do not compromise final grain yield

The influence of *F. graminearum* infections on final grain yield was explored, because wheat ear regions not invaded by fungal hyphae can also exhibit compromised grain set, through various postulated mechanisms (Mesterhazy, 2002). Following point-inoculation at anthesis, the *Δmap1* strains did not reduce the total grain weight

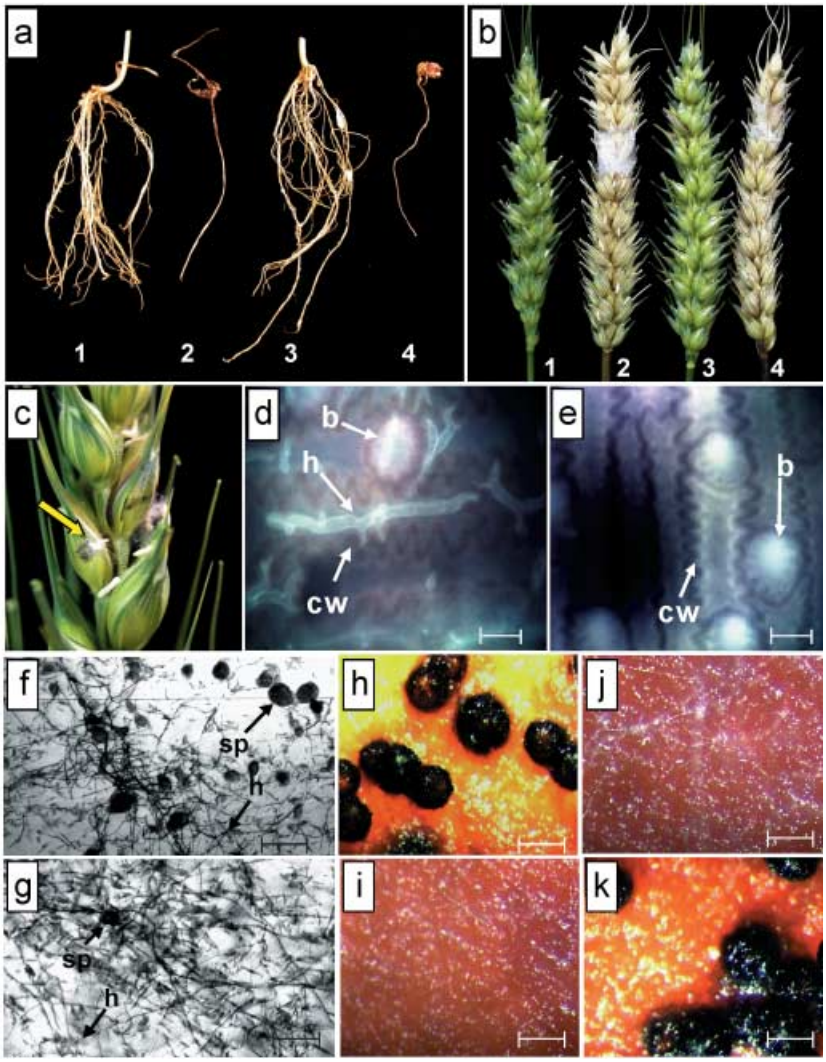


**Fig. 3** The various wild-type and *map1* mutant *F. graminearum* phenotypes associated with plant infections and *in vitro* cultures. (a) Wheat roots of cv. Bobwhite, 21 days after inoculations. 1. Control, SNA only inoculated; 2, wild-type strain 16A; 3, *Δmap1* mutant, CAM1 and 4, ectopic strain CAM8. Root infected by both the wild-type (2) and ectopic (4) strains are severely stunted and have no lateral roots. In contrast, control SNA only inoculated (1) and CAM1 (3) inoculated seeds have produced very extensive root systems of comparable size. Typical roots from inoculated plants are shown. (b) Wheat ears of cv. Bobwhite, 20 days after point-inoculations. Numbers 1–4 indicate the same *F. graminearum* strains as described for panel (a). Both the wild-type and ectopic CAM8 inoculated ears are completely infected and have senesced prematurely. (c) Close up of a wheat ear after inoculation with *Δmap1* mutant strain CAM1. A small amount of aerial mycelium is associated with the extruded anthers (arrow). Inoculated spikelets were marked using a black fibre tip pen. Photograph taken 20 days after inoculation. (d, e) Photomicrographs of *F. graminearum* inoculated wheat palea tissue stained with aniline blue and viewed by epifluorescence microscopy. (d) Hyphae (h) of the wild-type strain 16A growing both inter- and intracellular. cw, plant cell wall. b, basal hair cell. (e) No hyphae of the mutant strain *Δmap1* are visible in the silicanized palea cells. The tissue samples were taken 4 days after inoculation. (f, g) Asexual conidia developing on SNA agar plates. (f) a wild-type colony containing numerous sporodochia (conidial aggregates) (sp.). (g) a CAM1 mutant colony with an occasional sporodochia (sp.). h, hyphae. (h, i, j, k) Sexual perithecia formation on carrot agar. The black circular perithecia have only formed in the cultures of the (h) wild-type and (k) ectopic strains. No perithecia are formed in cultures of *Δmap1* mutants (i) CAM1 and (j) CAM2. Bars = 120 μm in (d), (e); 250 μm in (f)–(k).

recovered from the inoculated ear compared to the control treatment (Table 2). In contrast, infection by wild-type strains 16A and CAM8 resulted in significant reduction in the weight of wheat grain recovered (≈ 73%). However, the ovaries directly in contact with the agar plugs containing the *Δmap1* strain failed to develop and turned brown. The ovary tissue did not appear to have been penetrated by fungal hyphae and we therefore concluded that grain abortion was caused by compounds/molecules released from either the hyphae saprophytically colonizing the surface or the ovary, or from the continuous presence of the inoculum.

**Table 1** The effect of F. graminearum infection by various *Δmap1* mutant and wild-type strains on wheat seedling emergence and aerial biomass of the emerged wheat seedlings.

| | *F. graminearum* strains | | | | |
|---|---|---|---|---|---|
| Parameter measured | CAM1* | CAM2 | CAM8 | 16A | Control† |
| Per cent seed emergence‡ | 100.0 | 100.0 | 38.1 | 14.3 | 100.0 |
| Biomass§ (g) | 0.33 (100%)¶ | 0.30 (91%) | 0.11 (33%) | 0.06 (18%) | 0.33 (100%) |

*CAM1 and CAM2 (2 independent *Δmap1* mutants), CAM8 (ectopic mutant), 16A (wild-type).
†Control inoculated only with plugs of SNA.
‡Per cent seed emergence: assessment 14 days after inoculation, where 100% = 21 seeds.
§Biomass (g): mean above ground biomass for 21 germinated seedlings assessed 21 days after inoculation.
¶Per cent (%) of the control seedling biomass. The experiment was repeated twice with the same results.

**Table 2** Comparison of various *in vitro* and *in planta* parameters for the two *Δmap1* mutant and two wild-type *F. graminearum* strains.

| | *F. graminearum* strains | | | | |
|---|---|---|---|---|---|
| Parameter measured | CAM1* | CAM2 | CAM8 | 16A | Control |
| Spore production† ($\times 10^4$ conidia/mL) | 13 ± 9 | 16 ± 13 | nt‡ | 20 ± 7 | na§ |
| Grain weight¶ (g) | 1.3 ± 0.6 | 1.7 ± 0.6 | 0.4 ± 0.2 | 0.5 ± 0.2 | 1.7 ± 0.4 |
| DON production in wheat ears†† (p.p.m) | 0.6 ± 0.2 | 0.3 ± 0.2 | nt | 1.0 ± 0.1 | < 0.25** |

*See legend to Table 1 for strain identity.
†Number ± standard deviation ($n = 5$) of conidia produced *in vitro* after 17 growth days on SNA media. Numbers are not significantly different ($P \leq 0.479$).
‡Not tested.
§Not applicable.
¶Threshed weight of dry grain recovered from each mature wheat ear ± standard deviation ($n = 5$). Grain weights are significantly higher in CAM1, CAM2 and mock control than in 16A or CAM8 inoculations ($P \leq 0.001$).
**Samples below detection limit of 0.25 p.p.m.
††DON content of whole wheat ears (grain and chaff) assessed 40 days after point-inoculation of wheat cv. Bobwhite by competitive ELISA are significantly reduced in CAM1 and CAM2 compared to 16A ($P \leq 0.01$).

## Colony morphology, growth rates and other *in vitro* phenotypes

Colonies of the two independent *Δmap1* mutants grown *in vitro* showed various morphological phenotypes. Close inspection revealed that the *Δmap1* strains CAM1 and CAM2 both have poorly developed aerial hyphae, in contrast to strains 16A and CAM8 after 7 days incubation on SNA minimal agar plates (data not shown). No obvious reduction or delay in conidial germination was observed when vegetative conidia were collected from two independent *Δmap1* mutants, resuspended in medium, and examined quantitatively for conidial germination on glass slides for 48 h (data not shown). However, both CAM1 and CAM2 were 20–30% reduced in growth rates compared to wild-type 16A and the ectopic mutant CAM8 on SNA agar plates (Table 3). Further growth tests on V8 and oatmeal agar medium confirmed these results (data not shown) indicating that this reduction in fungal growth was medium independent.

On SNA agar plates, wild-type strain 16A forms the vast majority of its asexual conidia together in discrete locations called sporodochia (Fig. 3f). However, the two *Δmap1* mutants rarely formed sporodochia. Instead, their conidia were spread evenly across the surface of the agar plate (Fig. 3g). The failure to form sporodochia, however, was not accompanied by a quantitative reduction in asexual sporulation. The spore production of CAM1 and CAM2 was found to be equivalent to wild-type strain 16A in five independent experiments when harvested from fully developed fungal mycelium (Table 2).

**Table 3** *In vitro* growth of the two *Δmap1* mutant and two wild-type *F. graminearum* strains on SNA media

| | *F. graminearum* strains | | | |
|---|---|---|---|---|
| Incubation time | CAM1* | CAM2 | CAM8 | 16A |
| 48 h | 13.7 ± 0.6† | 11.3 ± 1.5 | 18.3 ± 1.5 | 18.0 ± 1.7 |
| 72 h | 23.3 ± 1.2 | 20.0 ± 1.7 | 30.0 ± 1.7 | 30.0 ± 1.7 |
| 120 h | 58.7 ± 1.5 | 53.3 ± 2.5 | 81.0 ± 2.0 | 78.7 ± 1.2 |

*See legend to Table 1 for strain identity.
†Values are mean colony diameters in mm ± standard deviation. The mean and standard deviation were calculated from two independent trials with three replicates each. At all time points growth rates in CAM1 and CAM2 are significantly reduced compared to CAM8 and 16A ($P \leq 0.001$).

These data suggest that, while *MAP1* is not essential for conidial germination or conidiation, it is involved in the formation of sporodochia, the establishment of aerial mycelium *in vitro* and in the maintenance of optimal mycelial growth rates.

### *MAP1* and DON mycotoxin synthesis

The production of trichothecene mycotoxins is an important aspect of *Fusarium* ear blight infections because of its impact on grain safety (Scholten *et al.*, 2001). It has also been reported that *F. graminearum* strains can spread within infected wheat tissue without causing visible disease symptoms (Jones and Clifford, 1978). The effect of the *Δmap1* allele on mycotoxin production in mature wheat ears was therefore quantified by competitive ELISA. Total 3-acetyl DON and DON mycotoxin content in whole ears following *F. graminearum* inoculation were found to be significantly reduced in CAM1 (1.7-fold) and CAM2 (3.3-fold), compared to wild-type strain 16A (Table 2). We conclude that *Δmap1* strains, although unable to cause typical wheat ear blight symptoms, were still capable of producing DON mycotoxin at the site of initial inoculation.

### *MAP1* is required for perithecia formation

*F. graminearum* is a homothallic Ascomycete fungus. Ascospores are produced within perithecia without the prerequisite for a compatible mating partner under conducive conditions (Pugh and Johann, 1933; Trail and Common, 2000). To determine whether the two *Δmap1* mutants were altered in their ability to form perithecia and/or ascospores, the various *F. graminearum* strains were grown on carrot agar plates (Proctor *et al.*, 1997). The wild-type strain 16A and the ectopic strain CAM8 produced abundant black perithecia containing viable ascospores within 2 weeks. In contrast, the two *Δmap1* mutants CAM1 and CAM2 did not produce any perithecia, even after a 2-month incubation period (Fig. 3h–k). Instead, both mutant strains produced white aerial hyphae, which frequently collapsed on to the surface of the carrot agar plates. There was no evidence of ascospore formation within the mycelium on the surface of the carrot agar, or within the aerial hyphae. The perithecial/ascospore formation assay was performed for four other independently derived *Δmap1* strains and gave identical results to those described above (data not shown). *MAP1* is therefore essential for perithecia and hence ascospore formation in *F. graminearum.*

### *MAP1* is essential to cause tomato fruit rot

Although *F. graminearum* is primarily known to cause disease symptoms on cereal plant species, earlier studies indicated that this fungus causes a rot when introduced into green and red tomato fruits (Crozier and Boothroyd, 1959). To test whether the two *Δmap1* strains were able to cause disease on other hosts, each was inoculated on to superficially wounded ripe tomato fruits. Both mutants did not colonize the tomato fruits (Fig. 4). Microscope analysis revealed that the inoculated CAM1 and CAM2 conidia had germinated and formed a small mycelial network exclusively at the wound surface. In contrast, strains 16A and CAM8 colonized the pericarp tissue and formed abundant masses of white aerial mycelium on its surface (Fig. 4). This experiment was repeated in triplicate.

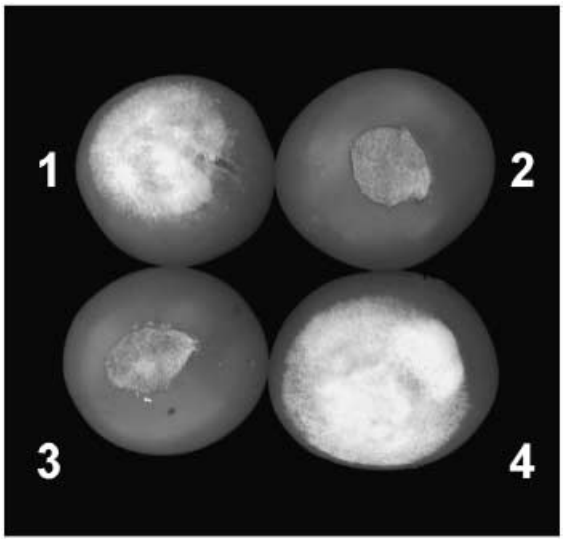


**Fig. 4** Superficially wounded tomato fruits, 6 days after inoculation with various *F. graminearum* strains. Both the (1) wild-type and (4) ectopic strain have formed abundant mycelium both inside and on the surface of the tomato fruit. The two *Δmap1* mutant strains (2) CAM1 and (3) CAM2 only form a small mycelial colony on the fruit's surface.

## DISCUSSION

Molecular genetic analyses of mitogen activated protein kinases (MAPK) have recently identified two signal transduction cascades which are important for pathogenicity in a range of phytopathogenic fungi. The two MAPK mutants originally shown to be impaired in plant pathogenicity were generated for the rice blast fungus *M. grisea.* In *M. grisea,* the *PMK1* gene, which is homologous to the *S. cerevisiae FUS3/KSS1* genes, is essential for appressoria formation, penetration and invasive growth (Xu and Hamer, 1996). In contrast, the *M. grisea MPS1* gene, homologous to the *S. cerevisiae SLT2* gene, is essential for penetration but is not required for invasive growth (Xu *et al.*, 1998). Both pathways are thought to relay external signals and to modulate pathogenicity gene expression. In other plant pathogenic fungi, mutants generated in either the Fus3/Kss1 or Slt2 pathways are frequently impaired in pathogenicity (reviewed by Idnurm and Howlett, 2001; Mey *et al.*, 2002a,b). These mutants also display

a range of distinct phenotypes which are likely to be important for the specific infection strategies of each species.

In this study we used a PCR and bioinformatic strategy to identify the *F. graminearum MAP1* gene as the homologue of the *M. grisea PMK1* gene. The phylogenetic analysis shows that the *MAP1* gene, together with the *M. grisea PMK1* gene, is predicted to encode a serine/threonine related MAP kinase belonging to the clade of FUS3/KSS1 related kinases (Fig. 1).

## Pathogenicity phenotypes of *MAP1* mutants

Six independent *map1* deletion mutant strains were obtained using a gene replacement strategy (Fig. 2). Inactivation of the Map1 dependent signalling pathway inhibited pathogenicity in *F. graminearum*. The *map1* mutants were not able to infect either point- or spray-inoculated wheat florets. Mycotoxin production in wheat ears was twofold reduced. In addition, *map1* mutant inoculations did not cause a reduction in grain yield, seed germination or seedling growth rates. Interestingly, however, the *map1* mutants were still able to colonize senescing anther and anther filaments and to form aerial hyphae on these tissues (Fig. 3c). The pathogenicity defect did not seem to be host or tissue specific, because the *map1* mutants also did not cause tomato fruit rot. The ability of the *map1* mutants to infect other cereal and non-cereal host species has not yet been determined.

Post-penetration, *F. graminearum map1* mutants have severely impaired hyphal growth. Like *M. grisea pmk1* mutants, *map1* hyphae lack the ability to colonize host tissue from a wound site. The finding that drop-inoculated *map1* mutants are unable to penetrate intact palea tissue *in vitro* (Fig. 3e) confirms the result that Map1 protein is required during the early stages of the infection process. The palea and lemma tissues are the least silicanized of all the other plant tissues surrounding the developing grain, i.e. glume and rachis tissues. There are at least three possible explanations why *in planta F. graminearum* colonization rates are reduced. Firstly, an alteration in the expression profile of cell wall-degrading enzymes could compromise both the inter- and intracellular colonization routes. Secondly, the *map1* mutants may lack an ability to overcome both pre-formed and induced plant defence responses. Thirdly, the *map1* mutation may cause gross changes in fungal metabolism and this now enables plant cells to more effectively recognize new signals coming from the invading *Fusarium* hyphae and thereby to activate a stronger defence response.

The *MAP1* sequence is highly related to *MAPK* genes isolated from two other *Fusarium* species, namely *FsMAPK* from *Nectria haematococca* (*Fusarium solani*) (Li *et al.*, 1997) and *Fmk1* from *F. oxysporum* f.sp. *lycopersici* (Di Pietro *et al.*, 2001). The generation and phenotype of an *FsMAPK* mutant has not been reported. The *fmk1* mutant is non-pathogenic on tomato roots and wounded tomato fruits. Although the root pathogen *F. oxysporum* and ear invading *F. graminearum* have contrasting modes of infection, the Fus3/Kss1 signalling pathway is required for penetration in both species.

The phenotype reported for the *F. graminearum mgv1* mutant (Hou *et al.*, 2002), affected in the Slt2 signalling pathway, appears to be less severe than that of the *F. graminearum map1* mutant analysed in this study. However, this comparison should be treated cautiously. The *map1* and *mgv1* mutations were made in two related but different strains of USA origin, 16A and PH-1, respectively. Point-inoculations with *mgv1* mutants caused typical disease symptoms and frequently spread into the adjacent spikelets. A bleaching phenotype, i.e. the typical field symptom, was never observed following *map1* mutant inoculations. This suggests that *mgv1* mutants are attenuated in their ability to spread through developed wheat tissue, while *map1* mutants are inhibited at an earlier infection stage.

Mutants in phytopathogenic Ascomycetes for both the Fus3/Kss1 and Slt2 MAPK pathways exist only in *M. grisea, F. graminearum* and *C. purpurea*. Similar to *M. grisea,* in the cereal floral pathogen *Claviceps purpurea*, a *cpmk1* mutant (*FUS3/KSS1* homologue) appears to be completely non-pathogenic on rye ears (Mey *et al.*, 2002b), while a *cpmk2* mutant (*SLT2* homologue) retains a limited ability to colonize rye pistils (Mey *et al.*, 2002a). These interspecies comparisons suggest that the lack of the yeast Fus3/Kss1 MAPK pathway compromises plant pathogenicity more severely than a lack of the homologous Slt2 MAPK pathway.

## Trichothecene production

Various trichothecene mycotoxins are produced by *F. graminearum* hyphae (Ueno *et al.*, 1975; Ward *et al.*, 2002) and are hypothesized to interfere with plant defence induction (Kang and Buchenauer, 1999). DON has been shown to enhance virulence towards some wheat cultivars but not in others (Proctor *et al.*, 1995). *Map1* mutants are still able to produce DON mycotoxin on superficially infected wheat ears. However, the combined amount of DON and 3-acetyl DON mycotoxin was twofold lower than the amounts recovered from fully diseased wheat ears inoculated with the wild-type strain 16A (Table 2). Thus, the inability of *map1* mutants to spread within wheat ear tissue is unlikely to be caused by a deficiency to produce DON mycotoxin.

## *In vitro* changes to mycelial morphology and asexual spore formation

Several subtle phenotype changes were identified for the *map1* mutants CAM1 and CAM2 when grown *in vitro*, even though spore germination rates and the ability to form conidia were the same as the wild-type (Table 2). These changes included a small delay in radial mycelial growth rate (25%) (Table 3), reduced

aerial hyphae formation on various artificial substrates, and a clear reduction in the development of sporodochia (conidial aggregations) on minimal medium (Fig. 3). The observed growth rate reduction clearly cannot explain the pathogenicity defect. Growth rate reductions have also been reported for three of the nine fungal species harbouring disruptions of the homologous *PMK1* gene. Deletion of the *FMK1* gene in *Fusarium oxysporum* (Di Pietro *et al*., 2001) and the *CHK1* gene in *Cochliobulus heterostrophus* (Lev *et al*., 1999) causes a slight growth reduction, while growth rates in the *Botrytis cinerea bmp1* mutant were more severely reduced (≈ 65%) (Zheng *et al*., 2000). Reduced aerial hyphae formation on artificial substrates was observed for the *F. oxysporum fmk1* mutant (Di Pietro *et al*., 2001). However, the reduction of sporodochia development on minimal agar plates is likely to be specific for *F. graminearum map1* mutants. Sporodochia are cushion-shaped compact masses of hyphae (stroma) containing conidiophores formed under inducive conditions, which only form in Ascomycete *Fusarium* species (Agrios, 1997).

## Perithecia formation

*F. graminearum* produces ascospores enclosed within dark morphological structures, called perithecia. Plant infections by ascospores are known to contribute significantly to the severity of *F. graminearum* ear blight epidemics (Fernando *et al*., 1997; Parry *et al*., 1995). Wild-type strains of this homothallic fungus readily form perithecia and viable ascospores within 2–3 weeks *in vitro*. On infected stubble, the same process takes several months (Pugh and Johann, 1933). Six independent *map1* mutants each did not produce perithecia *in vitro*. Studies on perithecial development in *Fusarium* species indicate that perithecia are frequently formed in stroma (Hawksworth *et al*., 1995; Trail and Common, 2000). It is possible that the *map1* mutation blocks an early phase in hyphal stroma development, causing the defects in both perithecia and sporodochia formation. Alternatively, the Map1 MAP kinase may also regulate mating and cell fusion events, similar to the Fus3/Kss1 proteins in *S. cerevisiae* (Sprague and Thorner, 1992).

Like *MAP1*, the *SLT2* related *F. graminearum* MAPK, *MGV1* (Hou *et al*., 2002), is essential for perithecial formation. Therefore, at least two different MAPK pathway functions exist in *F. graminearum* to control perithecial development. In two other phytopathogenic Ascomycetes, ascospore formation is reported to be controlled by the Fus3/Kss1 signalling cascade, namely the fungal species *M. grisea* and *Cochliobulus heterostrophus* causing Southern corn leaf blight (Lev *et al*., 1999; Xu and Hamer, 1996). The Slt2 MAPK signalling cascade has only been shown to be required for perithecial formation in *M. grisea* and *F. graminearum*. In *C. purpurea* the effect of the *PMK1* and *SLT2* homologue, *CPMK1* and *CPMK2*, on sexual structure formation has not been determined.

## A comparison of Map1 function between different *F. graminearum* strains

*MAP1* gene function was recently analysed in two other *F. graminearum* strains, the American isolate PH-1 and the German isolate 8/1. In both strains the loss of wheat pathogencity and perithecia development was confirmed. In strain PH-1, a complete gene deletion was generated (Xu and Kistler, pers. com.). In strain 8/1, a gene insertion mutation was phenotypically characterized (Jenczmionka *et al*., 2003). However, no sequence data or data on the impact of the *map1* mutation on DON mycotoxin production, root infection, infection of another plant species and quantitative data on wheat grain yield were reported. Jenczmionka *et al*. (2003) observed reduced conidiation on *map1* mutant mycelium grown for 7 days on minimal medium. In our study, conidiation assessed at 17 days was not significantly different to wild-type conidiation. This discrepancy may be explained by the differences in genetic background of the two strains, the experimental conditions used, or subtle temporal differences in spore formation. For example in *M. grisea*, gene deletion of the *CPKA* gene leads to a delay in infection structure formation (Xu *et al*., 1997). Side-by-side comparisons of the three different *F. graminearum map1* mutant and their respective wild-type strains may resolve whether other genetic differences between the three strains influence Map1 protein function.

## Prospects

The elimination of perithecial formation is particularly attractive for the control of *Fusarium graminearum* (*Gibberella zeae*) on cereals. The disease epidemic is primarily monocyclic, with infections by ascospores timed to crop anthesis and silk emergence for maize (Fernando *et al*., 1997; Paulitz, 1996). The growing of highly susceptible maize crops into traditional wheat growing areas is predicted to increase the incidence of wheat ear blight disease (Fernando *et al*., 1997). Perithecia formation only occurs on exposed crop residues in previously infected fields. Therefore, specific disease control measures can be targeted to these high-risk fields.

The assumption that MAP kinase pathways can be suitable drug targets was recently confirmed by the finding that phenylpyrrole fungicides in *Neurospora crassa* target the os-2 MAP kinase (*S. cerevisiae* Hog1 homologue) signalling cascade (Zhang *et al*., 2002). Phenylpyrrole fungicides are proposed to hyper activate the os-2 MAP kinase pathway and cause *N. crassa* conidia and hyphal cells to swell and burst.

*Map1* and wild-type strain gene expression can be compared in microarray experiments. Recently, large collections of ESTs were prepared from developing perithecia and from conditions that mimic *in planta* growth (Trail *et al*., 2003; http://cogeme.ex.ac.uk). In addition, 10X sequence coverage of the *F. graminearum* genome is now available (http://www-genome.wi.mit.edu/annotation/fungi/

fusarium/index.html). The availability of three different *map1* mutant and wild-type strain pair combinations will permit a comprehensive comparative transcriptome analysis to be undertaken. This approach will identify *MAP1* responsive gene sets that are likely to encode signal receptors, regulatory proteins and enzymes/structural proteins. We speculate that several of the *MAP1*-regulated proteins may be novel fungicide targets that are suitable for the control of *F. graminearum* perithecial development, ear infection and mycotoxin contamination of cereal grains.

## EXPERIMENTAL PROCEDURES

### Origin and maintenance of *F. graminearum* strains

The trichothecene-producing wild-type strain 16A (Urban *et al.*, 2002) has been deposited at the Fungal Genetics Stock Center as FGSC#8733. All *Fusarium* strains were routinely cultured as described (Urban *et al.*, 2002). Experiments involving the *F. graminearum* strain 16A were conducted in a biological containment facility under DEFRA licences PHL 39B/3819 (5/2001) and PHL 39 A/3493 (01/2001).

### Nucleic acid manipulations

Fungal genomic DNA was extracted using the CTAB protocol (Xu and Leslie, 1996). Standard molecular biology procedures were performed as previously described (Sambrook *et al.*, 1989). Homology searches of DNA/protein sequence databases were performed with the BLAST algorithm (Altschul *et al.*, 1997). Amino acid sequence comparison and alignments were made using the BESTFIT, PILEUP, and BOXSHADE programs in GCG Wisconsin software package (Accelrys).

### Cloning and sequence analysis of MAP1

A polymerase chain reaction (PCR) was conducted to amplify MAP kinase homologues using degenerate primers as previously described (Xu and Hamer, 1996) using genomic DNA of *F. graminearum* strain 16A. A major band of 0.5 kb amplified with the degenerate primers GT(A/C/G/T)GC(A/C/G/T)AT(A/G)AA(A/G)AA(A/G)AT and CCA(A/C/G/T)C(G/T)(A/C/G/T)GT(A/C/G/T)GC(A/C/G/T)AC(A/G)TA(C/T)TC. PCR fragments were cloned into the Invitrogen pCR2.1 Topo/TA vector. One PCR clone, pCA14, was found to be 97% identical to the *M. grisea PMK1* gene. From the recovered sequence, primers were designed for use in ligation-mediated inverse PCR (Thomas *et al.*, 1994), namely U20, GCAGCTTCATCTCTCGCAGAGT and U21, CCTCAACGCCAACTGTGATCTC. 1 μg of genomic *F. graminearum* DNA was cut with different restriction enzymes and the products re-ligated. The subsequent PCR was performed using the Expand Long PCR kit (Roche). Cycling conditions were 93 °C for 2 min for pre-denaturation, cycles 2–11 at 93 °C for 15 s, 60 °C for 15 s, and 68 °C for 3 min, cycles 12–36 at 93 °C for 15 s, 60 °C for 15 s and 68 °C for 3 min with a 5 s extension per cycle. Reactions were held at 4 °C after a final extension at 68 °C for 3 min. The recovered PCR fragment from *Eco*RI digested and ligated *Fusarium* DNA (size 10 kb) was cloned into the Invitrogen pCR2.1 TOPO/TA vector and sequenced on both strands by primer walking.

To verify the genomic sequence containing the *MAP1* gene, two additional sets of primers situated either just 5′ of the start codon or just 3′ of the stop codon were used to amplify a PCR product of 1.25 kb that was directly sequenced on both strands by primer walking. All sequencing was performed using a Big Dye Terminator Cycle Sequencing Ready Reaction kit (Prism), on a modified ABI 373 DNA sequencer (Applied Biosystems, USA). DNA sequencing data were analysed using the GCG Wisconsin Sequence Analysis Package. Genetic nomenclature in this article follows the guidelines for plant pathogenic fungi (Yoder *et al.*, 1986). The wild-type gene is *MAP1*, which encodes the protein Map1. The recessive mutant allele is *map1*.

### Disruption of the *MAP1* gene

To generate the *MAP1* gene replacement vector pΔMAP1, a 884 bp 5′ fragment encompassing positions +42 to +926 and a 2 kb 3′ fragment encompassing positions +1459 to +3585 in AF448230 were amplified by PCR using the oligonucleotides U17, GGGGTACCGACTGGTCCTCTATTGAC, U18, GGGGTACCTCAACATGATCTCAGGCG with flanking *Kpn*I sites and U13, GTAAACGTGGTGCCTCTGTA, U14, TAGCCAGAGTCCATAGTGTC, respectively. Both fragments were cloned into pCR2.1-TOPO™ (Invitrogen) and sequenced. The 884 bp 5′ and 2 kb 3′ fragments were then inserted as *Eco*RI and *Kpn*I fragments, respectively, into pBS-Hyg to generate pΔMAP1. The orientations of subcloned fragments in pΔMAP1 were confirmed by sequencing. To construct pBS-Hyg, the modified *HPH* gene from pCB1004 (Carroll *et al.*, 1994) was introduced as a 1.4 kb *Hpa*I fragment into the *EcoR*V site of pBluescriptII SK+ (Stratagene). For the *F. graminearum* transformation, pΔMAP1 was linearized with *Not*I and transformed into *F. graminearum* strain 16A as previously described (Hohn and Desjardins, 1992; Proctor *et al.*, 1997). Single hygromycin resistant transformants were pre-screened by PCR analysis of crude DNA extracts (Wu *et al.*, 1997) with *MAP1* primers U9, CGCCTGAGATCATGTTGACT, and U8, GAGCTTCCTCCACAGTA, to determine the presence or absence of a 401 bp *MAP1* fragment.

### Phenotypic analysis of *Δmap1* mutant and wild-type strains

To assay germination rates, $1 \times 10^6$ conidia were used to inoculate 20 mL of GYEP medium (Hohn *et al.*, 1995). Conidial GYEP suspensions were incubated for 2 days in a shaking incubator at

26 °C at 100 r.p.m. Aliquots were taken after 14, 24 and 48 h and germination rates determined using a haemocytometer. To measure fungal growth rates on different solid media, mycelium plugs were transferred on to fresh agar plates of SNA agar, V8 juice agar (36% V8 juice, 0.5% $CaCO_3$, 1.4% Bacto-agar) and Oatmeal agar (Crawford *et al.*, 1986) using a 5 mm cork borer. Fungal growth was recorded as colony diameter in daily intervals during 5 days. To quantify spore production, fungal strains were grown for 17 days on SNA agar plates and quantified as previously described (Urban *et al.*, 2002). Perithecia formation was assayed on carrot agar (Proctor *et al.*, 1997).

### Wheat infection assays

For the wheat inoculation experiments, plants with ears at anthesis BBCH growth scale 65 (Stauss, 1994) were grown as previously described (Urban *et al.*, 2002). For point-inoculations, fungal strains were grown on SNA agar plates for 10 days. Agar plugs with fungal mycelium were then transferred with a Pasteur pipette tip into a single wheat floret. Each wheat ear was inoculated with agar plugs placed in four individual florets of two adjacent spikelets close to the top of the wheat ear. Spray inoculations were conducted as described (Urban *et al.*, 2002). The point- and spray-inoculated plants were kept at 100% relative humidity for the next 4 days. For the first day the chambers were shaded to exclude light. Individual plants were scored for disease symptoms from day 4 onwards in 2–4 day intervals with the final disease score taken at day 20 post-inoculation. Disease progress was recorded as the number of visibly diseased spikelets per wheat ear. All experiments were performed at least twice and at different times. For wound inoculation, wheat ears at anthesis were cut with scissors through the middle of a spikelet row close to the top of the ear. The spikelet and rachis wound sites were droplet inoculated with conidial suspension ($5 \times 10^5$ spore/mL) until run off. Experiments were performed in triplicate.

For the *in vitro* agar plate assays, spikelets were taken from the middle of a wheat ear at anthesis using forceps. The palea tissues of the two external flowers were separated at the spikelet base and mounted on water agar plates (1.5% Bacto agar). The inner surface of the boat shaped palea tissue was drop inoculated using 20 μL conidial suspensions ($5 \times 10^4$ spores/mL) and kept at high humidity in the dark at 20 °C. Disease development was monitored by aniline blue fluorescence (Hood and Shew, 1996) of the samples over 6 days.

For the wheat root infection assays, a plant container test developed for the takeall fungus *Gaeumanomyces graminis* was employed (Dufresne and Osbourn, 2001). Seedling emergence and aerial growth was recorded after 2 weeks. Plants were destructively harvested after 3 weeks. Fresh weight measurements were taken of the shoot biomass, and root disease symptoms assessed.

For the mycotoxin analysis, whole wheat ears were harvested 40 days after inoculation and ground to a fine powder. One gram of each sample was resuspended in 5 g of water and thoroughly mixed using a polytron (PT 3100 Kinematica AG, Switzerland). The mixture was then incubated for 30 min at 30 °C in a water bath and all solid parts were removed by centrifugation. The supernatants were analysed quantitatively for DON and 3-acetyl DON content using a Veratox 5/5 kit competitive ELISA kit (Adgen, Ayr, Scotland) according to the manufacturer's instructions. Each biological sample was quantified twice. Statistical analyses were conducted as described previously (Urban *et al.*, 2002).

### Infection assay on tomato fruits

To assay the colonization of *F. graminearum* strains on wounded tomato fruits, ripe tomatoes of cultivar Moneymaker (Cf-0) were surface sterilized by wiping with 95% ethanol. A 5 $mm^2$ region of the epidermis was carefully removed and the wound site was overlaid with a 15 μL spore solution containing $2 \times 10^5$ conidia/mL. Fruits were incubated at room temperature under conditions of 100% relative humidity.

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## REFERENCES

**Agrios, G.N.** (1997) *Plant Pathology*. San Diego, CA: Academic Press.

**Altschul, S.F., Madden, T.L., Shaffer, A.A., Zhang, Z., Miller, W. and Lipman, D.J.** (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res.* **25**, 3389–3402.

**Bruchez, J.J.P., Eberle, J. and Russo, V.E.A.** (1993) Regulatory sequences in the transcription of *Neurospora crassa* genes: CAAT box, TATA box, Introns, Poly(A) tail formation sequences. *Fungal Genet. Newsl.* **40**, 89–96.

**Carroll, A.N., Sweigard, J.A. and Valent, B.** (1994) Improved vectors for selecting resistance to hygromycin. *Fungal Genet. Newsl.* **41**, 22.

**Cook, R.J.** (1968) Fusarium root and foot rot of cereals in the Pacific Northwest. *Phytopathol.* **58**, 127–131.

**Crawford, M.S., Chumley, F.G., Weaver, C.G. and Valent, B.** (1986) Characterization of the heterokaryotic and vegetative diploid phases of *Magnaporthe grisea*. *Genetics*, **114**, 1111–1129.

**Crozier, J.A. and Boothroyd, C.W.** (1959) Tomato a new suscept of *Gibberella zeae* (Schw.) Petch. *Plant Dis. Reporter*, **43**, 446–447.

**Di Pietro, A., Garcia-Maceira, F.I., Meglecz, E. and Roncero, M.I.G.** (2001) A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is

essential for root penetration and pathogenesis. *Mol. Microbiol.* **39**, 1140–1152.

**Dixon, K.P., Xu, J.R., Smirnoff, N. and Talbot, N.J.** (1999) Independent signaling pathways regulate cellular turgor during hyperosmotic stress and appressorium-mediated plant infection by *Magnaporthe grisea*. *Plant Cell*, **11**, 2045–2058.

**Dufresne, M. and Osbourn, A.E.** (2001) Definition of tissue-specific and general requirements for plant infection in a phytopathogenic fungus. *Mol. Plant-Microbe Interact.* **14**, 300–307.

**Fernando, W.G.D., Paulitz, T.C., Seaman, W.L., Dutilleul, P. and Miller, J.D.** (1997) Head blight gradients caused by *Gibberella zeae* from area sources of inoculum in wheat field plots. *Phytopathol.* **87**, 414–421.

**Gustin, M.C., Albertyn, J., Alexander, M. and Davenport, K.** (1998) MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **62**, 1264–1300.

**Hanks, S.K., Quinn, A.M. and Hunter, T.** (1988) The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. *Science*, **241**, 42–52.

**Hawksworth, D.L., Kirth, P.M., Sutton, B.C. and Pegler, D.N.** (1995) *Ainsworth and Bisby's Dictionary of the Fungi.* Wallingford, UK: CAB Interational.

**Herskowitz, I.** (1995) MAP kinase pathways in yeast: For mating and more. *Cell*, **80**, 187–197.

**Hohn, T.M. and Desjardins, A.E.** (1992) Isolation and gene disruption of the *Tox5* gene encoding trichodiene synthase in *Gibberella pulicaris*. *Mol. Plant-Microbe Interact.* **5**, 249–256.

**Hohn, T.M., Desjardins, A.E. and McCormick, S.P.** (1995) The *Tri4* gene of *Fusarium sporotrichioides* encodes a cytochrome P450 monooxygenase involved in trichothecene biosynthesis. *Mol. Gen. Genet.* **248**, 95–102.

**Hohn, T.M., McCormick, S.P., Alexander, N.J., Desjardins, A.E. and Proctor, R.H.** (1998) Function and biosynthesis of trichothecenes produced by *Fusarium* species. *Mol Genet. Host-Specific Toxins. Plant Dis.* **13**, 17–24.

**Hood, M.E. and Shew, H.D.** (1996) Applications of KOH-aniline blue fluorescence in the study of plant–fungal interactions. *Phytopathol.* **86**, 704–708.

**Hou, Z., Xue, C., Peng, Y., Katan, T., Kistler, H.C. and Xu, J.-R.** (2002) A mitogen-activated protein kinase gene (*MGV1*) in *Fusarium graminearum* is required for female fertility, heterokaryon formation, and plant infection. *Mol. Plant-Microbe Interact.* **15**, 1119–1127.

**Idnurm, A. and Howlett, B.J.** (2001) Pathogenicity genes of phytopathogenic fungi. *Mol. Plant Pathol.* **2**, 241–255.

**Jenczmionka, N.J., Maier, F.J., Losch, A.P. and Schafer, W.** (2003) Mating, conidiation and pathogenicity of *Fusarium graminearum*, the main causal agent of the head-blight disease of wheat, are regulated by the MAP kinase *gpmk1*. *Curr. Genet.* **43**, 87–95.

**Jones, G.D. and Clifford, B.C.** (1978) *Cereal Diseases—Their Pathology and Control*. Hadleigh, Ipswich, Suffolk, UK: BASF United Kingdom Ltd, Agrochemical Division.

**Kang, Z. and Buchenauer, H.** (1999) Immunocytochemical localization of *Fusarium* toxins in infected wheat spikes by *Fusarium culmorum*. *Physiol. Mol. Plant Pathol.* **55**, 275–288.

**Lev, S., Sharon, A., Hadar, R., Ma, H. and Horwitz, B.A.** (1999) A mitogen-activated protein kinase of the corn leaf pathogen *Cochliobolus heterostrophus* is involved in conidiation, appressorium formation, and pathogenicity: Divers roles for mitogen-activated protein kinase homologs in foliar pathogens. *Proc. Natl Acad. Sci. USA*, **96**, 13542–13547.

**Li, D., Rogers, L. and Kolattukudy, P.E.** (1997) Cloning and expression of cDNA encoding a mitogen-activated protein kinase from a phytopathogenic filamentous fungus. *Gene*, **195**, 161–166.

**Mesterhazy, A.** (2002) Role of deoxynivalenol in agressiveness of *Fusarium graminearum* and *F. culmorum* and in resistance to Fusarium head blight. *Eur. J. Plant. Pathol.* **108**, 675–684.

**Mey, G., Held, K., Scheffer, J., Tenberge, K.B. and Tudzynski, P.** (2002a) CPMK2, an SLT2-homologous mitogen-activated protein (MAP) kinase, is essential for pathogenesis of *Claviceps purpurea* on rye: Evidence for a second conserved pathogenesis-related MAP kinase cascade in phytopathogenic fungi. *Mol. Microbiol.* **46**, 305–318.

**Mey, G., Oeser, B., Lebrun, M.H. and Tudzynski, P.** (2002b) The biotrophic, non-appressoria forming grass pathogen *Claviceps purpurea* needs a Fus3/Pmk1 homologous MAP kinase for colonization of rye ovarian tissue. *Mol. Plant-Microbe Interact.* **15**, 303–312.

**Nicholson, P., Turner, A.S., Edwards, S.G., Bateman, G.L., Morgan, L.W., Parry, D.W., Marshall, J. and Nuttall, M.** (2002) Development of stem-base pathogens on different cultivars of winter wheat determined by quantitative PCR. *Eur. J. Plant Pathol.* **108**, 163–177.

**Nishida, E. and Gotoh, Y.** (1993) The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.* **18**, 128–131.

**O'Donnell, K., Kistler, H.C., Tacke, B.K. and Casper, H.H.** (2000) Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. *Proc. Natl Acad. Sci. USA*, **97**, 7905–7910.

**Parry, D.W., Jenkinson, P. and McLeod, L.** (1995) *Fusarium* ear blight (scab) in small grain cereals—a review. *Plant Pathol.* **44**, 207–238.

**Paulitz, T.C.** (1996) Diurnal release of ascospores by *Gibberella zeae* in inoculated wheat plots. *Plant Dis.* **80**, 674–678.

**Pritsch, C., Muehlbauer, G.J., Bushnell, W.R., Somers, D.A. and Vance, C.P.** (2000) Fungal development and induction of defense response genes during early infection of wheat spikes by *Fusarium graminearum*. *Mol. Plant-Microbe Interact.* **13**, 159–169.

**Pritsch, C., Vance, C.P., Bushnell, W.R., Somers, D.A., Hohn, T.M. and Muehlbauer, G.J.** (2001) Systemic expression of defense response genes in wheat spikes as a response to *Fusarium graminearum* infection. *Physiol. Mol. Plant Pathol.* **58**, 1–12.

**Proctor, R.H., Desjardins, A.E., McCormick, S.P., Plattner, R.D., Alexander, N.J. and Brown, D.W.** (2002) Genetic analysis of the role of trichothecene and fumonisin mycotoxins in the virulence of *Fusarium*. *Eur. J. Plant Pathol.* **108**, 691–698.

**Proctor, R.H., Hohn, T.M. and McCormick, S.P.** (1995) Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. *Mol. Plant-Microbe Interact.* **8**, 593–601.

**Proctor, R.H., Hohn, T.M. and McCormick, S.P.** (1997) Restoration of wild-type virulence to *Tri5* disruption mutants of *Gibberella zeae* via gene reversion and mutant complementation. *Microbiology*, **143**, 2583–2591.

**Pugh, W.G. and Johann, H.** (1933) Factors affecting infection of wheat heads by *Gibberella saubinetti*. *J. Agri. Res.* **46**, 771–797.

**Ruiz-Roldan, M.C., Maier, F.J. and Schafer, W.** (2001) *PTK1*, a mitogen-activated-protein kinase gene, is required for conidiation, appressorium formation, and pathogenicity of *Pyrenophora teres* on barley. *Mol. Plant-Microbe Interact.* **14**, 116–125.

**Sambrook, J., Fritsch, E.F. and Maniatis, T.** (1989) *Molecular cloning: A Laboratory Manual*. Plainview, NY: Cold Spring Harbor Laboratory Press.

**Schaeffer, H.J. and Webber, M.J.** (1999) Mitogen-activated protein kinases: Specific messages from ubiquitous messengers. *Mol. Cell. Biol.* **19**, 2435–2444.

**Scholten, O.E., Ruckenbauer, P., Visconti, A., van Osenbruggen, W.A. and den Nijs, A.P.M.** (2001) *Food safety of cereals: a chain-wide approach to reduce Fusarium mycotoxins*. EU FAIR-Ct98-4094. Commission of the European Communities.

**Sharma, R.P. and Kim, Y.W.** (1991) Trichothecenes. In *Mycotoxins and Phytoalexins* (Slunkhe, D.K., ed.). Boca Raton, FL: CRC Press.

**Sprague, G.F. and Thorner, J.W.** (1992) Pheromone response and signal transduction during the mating process of *Saccharomyces cerevisiae*. In *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression*, pp. 657–743. New York: Cold Spring Harbor Laboratory Press.

**Stauss, R.** (1994) *Compendium of growth stage identification keys of mono- and dicotyledonous plants, extended BBCH scale*. BBA, BSA, IGZ, IVA, AgrEvo, BASF, Bayer, CibaBasel.

**Tag, A., Hicks, J., Garifullina, G., Ake Jr, C., Phillips, T.D., Beremand, M. and Keller, N.** (2000) G-protein signalling mediates differential production of toxic secondary metabolites. *Mol. Microbiol.* **38**, 658–665.

**Thomas, C.M., Jones, D.A., English, J.J., Carroll, B.J., Bennetzen, J.L., Harrison, K., Burbidge, A., Bishop, G.J. and Jones, J.D.G.** (1994) Analysis of the chromosomal distribution of transposon-carrying T-DNAs in tomato using the inverse polymerase chain-reaction. *Mol. Gen. Genet.* **242**, 573–585.

**Trail, F. and Common, R.** (2000) Perithecial development by *Gibberella zeae*: a light microscopy study. *Mycologia*, **92**, 130–138.

**Trail, F., Xu, J.-R., San Miguel, P., Halgren, R.G. and Kistler, H.C.** (2003) Analysis of expressed sequence tags from *Gibberella zeae* (anamorph *Fusarium graminearum*). *Fungal Genet. Biol.* **38**, 187–197.

**Ueno, Y., Sawano, M. and Ishii, K.** (1975) Production of trichothecene mycotoxins by *Fusarium* species in shake culture. *Appl. Microbiol.* **30**, 4–9.

**Urban, M., Daniels, S., Mott, E. and Hammond-Kosack, K.** (2002) *Arabidopsis* is susceptible to the cereal ear blight fungal pathogens *Fusarium graminearum* and *Fusarium culmorum*. *Plant J.* **32**, 961–973.

**Ward, T.J., Bielawski, J.P., Kistler, H.C., Sullivan, E. and O'Donnell, K.** (2002) Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. *Proc. Natl Acad. Sci. USA*, **99**, 9278–9283.

**Wu, S.C., Ham, K.S., Darvill, A.G. and Albersheim, P.** (1997) Deletion of two endo-β-1,4-xylanase genes reveals additional isozymes secreted by the rice blast fungus. *Mol. Plant-Microbe Interact.* **10**, 700–708.

**Xu, J.R.** (2000) MAP kinases in fungal pathogens (review). *Fungal Genet. Biol.* **31**, 137–152.

**Xu, J.-R. and Hamer, J.E.** (1996) MAP-kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes Dev.* **10**, 2696–2706.

**Xu, J.R. and Leslie, J.F.** (1996) A genetic map of *Gibberella fujikuroi* mating population A (*Fusarium moniliforme*). *Genetics*, **143**, 175–189.

**Xu, J.R., Staiger, C.J. and Hamer, J.E.** (1998) Inactivation of the mitogen-activated protein kinase Mps1 from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses. *Proc. Natl Acad. Sci. USA*, **95**, 12713–12718.

**Xu, J.-R., Urban, M., Sweigard, J. and Hamer, J.E.** (1997) The *CPKA* gene of *Magnaporthe grisea* is essential for appressorial function. *Mol. Plant-Microbe Interact.* **10**, 187–194.

**Yoder, O.C., Valent, B. and Chumley, F.** (1986) Genetic nomenclature and practice for plant pathogenic fungi. *Phytopathol.* **76**, 383–385.

**Zhang, Y., Lamm, R., Pillonel, C., Lam, S. and Xu, J.-R.** (2002) Osmoregulation and fungicide resistance: the *Neurospora crassa os-2* gene encodes a HOG1 mitogen-activated protein kinase homologue. *Appl. Environ. Microbiol.* **68**, 532–538.

**Zheng, L., Campbell, M., Murphy, J., Lam, S. and Xu, J.R.** (2000) The *BMP1* gene is essential for pathogenicity in the gray mold fungus *Botrytis cinerea*. *Mol. Plant-Microbe Interact.* **13**, 724–732.