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Molecular characterization of a Hog1-type MAPK, *MlpHog1*, from *Melampsora larici-populina*

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Abstract The obligate biotrophic fungus *Melampsora larici-populina* (*Mlp*) causes devastating poplar foliar rust disease. In pathogens, the Hog1-type mitogen-activated protein kinase (MAPK) gene is involved in both pathogenesis and the response to various stresses, including osmotic stress. However, there are few reports on its role in *M. larici-populina*. In the present study, we identified and characterized the Hog1-type MAPK gene, *MlpHog1*, in *M. larici-populina*. The MlpHog1 protein consists of 356 amino acids and contains a conserved TGY motif. The function of *MlpHog1* was probed by mutant complementation studies. Expression of *MlpHog1* could partially complement the osmoregulation, sexual reproduction and pathogenicity defects of the *Fusarium graminearum Hog1* mutant and largely rescued the response of the mutant to cytoplasm membrane and oxidative stresses. These observations suggest that *MlpHog1* may play a role in the infectious growth of *M. larici-populina* and the response of the fungus to various environmental stresses.

Keywords Poplar foliar rust · *Melampsora larici-populina* · Hog1-type mitogen-activated protein kinase · Function

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Introduction

Melampsora larici-populina (Basidiomycetes, Pucciniales) is an obligate biotrophic pathogen, similar to many other rust fungi, that has a complex heteroecious macrocyclic lifestyle. This rust completes its life cycle on two different hosts (poplar as the telial host and larch as the aecial host) and successively produces five types of spores, including pycniospores, aeciospores, urediniospores, teliospores and basidiospores (Hacquard et al. 2011; Cao et al. 2000). The poplars that are widely distributed throughout the Northern Hemisphere are commonly used in the plant science community as model trees and recently have received increasing attention as a renewable source of biomass for the energy, lumber and pulping industries (Wan et al. 2013; Duplessis et al. 2009). As the most devastating and widespread pathogen of poplars, *M. larici-populina* restricts the use of these trees for environmental and wood production in many parts of the world (Steenackers et al. 1996). Indeed, the distribution of foliar rust disease caused by *M. larici-populina* is widespread in China, throughout the northeast, northwest and southwest regions. Furthermore, this disease is difficult to control because the new races cause the breakdown of poplar resistance and thus limit the development of plantations (Cao et al. 1998; Tian et al. 2000). Recent advances in the genomics of poplars and *M. larici-populina* have helped to define new strategies to facilitate the study of this tree-rust fungus model pathosystem (Hacquard et al. 2011).

Mitogen-activated protein kinase (MAPK) cascades function as key signal transducers that use protein phosphorylation/dephosphorylation cycles to disseminate information among evolutionarily conserved pathways (Hamel et al. 2012). In general, MAPK cascades in eukaryotes consist of three interlinked protein kinases (PKs) activated sequentially (Widmann et al. 1999) and different conserved MAPK signalling pathways are used by eukaryotic organisms to control gene expression, differentiation, cell survival, and apoptosis in response to diverse extracellular triggers (Herskowitz 1995; Treisman 1996). In budding yeast, five MAPK pathways regulate mating, invasive growth, cell wall integrity, hyperosmolarity responses, and ascospore formation (Chen and Thorner 2007). The high osmolarity glycerol (HOG) pathway is required for the accumulation of osmoprotectant molecules and the maintenance of an osmotic gradient across the stressed plasma membrane when yeast cells are grown under hypertonic conditions (Hamel et al. 2012). Numerous *Hog1* homologues have been identified in plant pathogenic fungi. *Osm1* deletion mutants of *Magnaporthe oryzae*, the rice blast fungus, are highly sensitive to osmotic stress, exhibiting severe morphological defects when grown under hyperosmotic conditions (Dixon et al. 1999). The *Hog1* gene in the wheat scab fungus *F. graminearum* is important for regulating hyphal growth, plant infection, and hyperosmotic and general stress responses (Zheng et al. 2012), and the Hog-homologous stress-activated MAPK *BcSak1* in *Botrytis cinerea*, which causes gray mold disease, has a significant impact on pathogenesis and is involved in the response to oxidative stress (Segmuller et al. 2007). In the human pathogen *Candida albicans*, *CaHog1* is activated by various stress conditions, plays a role in cell wall biosynthesis and integrity, and is important for pathogenesis (Monge et al. 2006).

Given its obligate biotrophic status, *Mlp* is not amenable to genetic transformation, which has hindered the molecular investigations of this rust fungus. In this study, we cloned a Hog1-type MAPK gene named *MlpHog1* and characterized its function by mutant complementation. This study provides important information for understanding the role of *MlpHog1*.

Materials and methods

Strains and culture conditions

The Chinese *Mlp* strain Wh03 was propagated on 1- to 2-year-old potted *Populus purdomii* plants, as described previously (Cao et al. 1998). Fresh urediniospores were harvested from the infected poplar leaves. The *F. graminearum* strains used in this study (Table 1) were maintained at 25 °C, as described previously (Hou et al. 2002). To test sensitivity to various stresses, vegetative growth was assayed on complete medium (CM) plates with 1 M NaCl, 0.01 % SDS or 0.05 % H_2O_2 at 25 °C for 3 days, as described previously (Wang et al. 2011, 2012).

Nucleic acid manipulations

Total RNA of urediniospores was extracted using the RNeasy Plant Mini Kit (QIAGEN, USA) following the recommended protocol. DNaseI treatment was used to remove genomic DNA. First-strand cDNA was synthesized with an Oligo$(dT)_{18}$ primer using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, USA) according to the manufacturer's instructions and used for cloning.

Isolation and sequence analysis of *MlpHog1*

Based on the sequence of the *Mlp87970* gene in the 98AG31 genome deposited at Joint Genome Institute (JGI), *MlpHog1* was cloned from the cDNA of *Mlp* strain Wh03 using the primers H1-F and H1-R (Table 1) and *FastPfu* DNA Polymerase (TransGen Biotech, China). DNA sequencing was performed by Beijing AuGCT Biological Technology. The molecular weight of the deduced protein was predicted using the ProtParam program (http://web.expasy.org/protparam/). Homologues in a set of fungal genomes were determined by Blastp searching at the Broad Institute (http://www.broadinstitute.org/) or NCBI (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments were performed using ClustalX. Phylogenetic analysis was performed using the neighbour-joining (NJ) method in MEGA6. The GenBank accession number of *MlpHog1* is KT183031.

Table 1 Primers and strains used in this study

Primer	Sequence (5′→3′)	
H1-F	ATGGCCGATTTTGCCAAAC	
H1-R	TCAAGCAGCAGGGGCAGC	
H1-F/RP	CAGATCTTGGCTTTCGTAGGAACCCAATCTTCAATGGCCGATTTTGCCAAACTG	
H1-R/RP	CACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACAGCAGCAGGGGCAGCAAGAC	
Strain	Genotype/Comment	Source
PH-1	A wild-type strain of *Fusarium graminearum*	(Cuomo et al. 2007)
ΔFgHog1	The *hog1* deletion mutant of PH-1	(Zheng et al. 2012)
H-4	*ΔFgHog1* complemented with RP27 promoter :: *MlpHog1*	This study
H-6	*ΔFgHog1* complemented with RP27 promoter :: *MlpHog1*	This study
H-8	*ΔFgHog1* complemented with RP27 promoter :: *MlpHog1*	This study
H-11	*ΔFgHog1* complemented with RP27 promoter :: *MlpHog1*	This study

Complementation of the *F. graminearum hog1* mutant with *MlpHog1*

For complementation assays, the PCR product amplified with primers H1-F/RP and H1-R/RP (Table 1) was cloned into pFL2 using the yeast gap repair approach (Bourett et al. 2002). The resulting construct was transformed into protoplasts of the *F. graminearum hog1* mutant, as described previously (Hou et al. 2002). Geneticin-resistant transformants were confirmed by polymerase chain reaction (PCR) analysis as harbouring the plasmid.

Aerial hyphae of 7-day-old carrot agar cultures were pressed down with sterile 0.1 % Tween 20 to induce sexual reproduction, as described previously (Bowden and Leslie 1999; Zheng et al. 2012). Perithecium formation and cirrhi production were examined approximately 2 weeks after induction. Freshly harvested conidia were re-suspended to a final concentration of 2×10^5 conidia/ml in sterile distilled water (Ding et al. 2009). Flowering wheat heads of cultivar XiaoYan 22 were drop-inoculated with 10 μl of conidia suspensions, as described previously (Gale et al. 2002). To maintain humidity, the inoculated wheat heads were capped with a plastic bag for 48 h. After removing the plastic bags, the wheat plants were grown in a greenhouse. Scab symptoms were examined, and the disease index for each strain was estimated by counting the number of diseased spikelets per wheat head at 14 days post-inoculation (dpi).

Results

Cloning and description of *MlpHog1*

The genome of *M. larici-populina* was previously sequenced (Duplessis et al. 2011), facilitating the study of genes involved in pathogen virulence. Based on sequence information of *Hog1* from *M. larici-populina* reference strain 98AG31(Protein ID 87970) (Hamel et al. 2012), an open reading frame of 1071 bp was obtained from the Chinese *Mlp* strain Wh03, which is designated *MlpHog1* in the present study (GenBank accession number KT183031). The deduced MlpHog1 protein consists of 356 amino acids with a predicted molecular mass of 40.48 kDa. *MlpHog1* contains a conserved TGY motif at positions 171 to 173 in the activation loop (Online Resource 1), which is present in the stress-activated protein kinase subgroup of MAPKs (Robinson and Cobb 1997).

MlpHog1 shares greater than 80 % identity with its orthologues from other model fungi, including *S. cerevisiae* Hog1 (83 % identity), *S. pombe* Sty1 (85 % identity), *M. oryzae* Osm1 (88 % identity) and *F. graminearum* Hog1 (85 % identity) (Online Resource 1). A phylogenetic analysis revealed that *MlpHog1* clusters with other Basidiomycete fungi and is most closely related to rust fungi (Fig. 1).

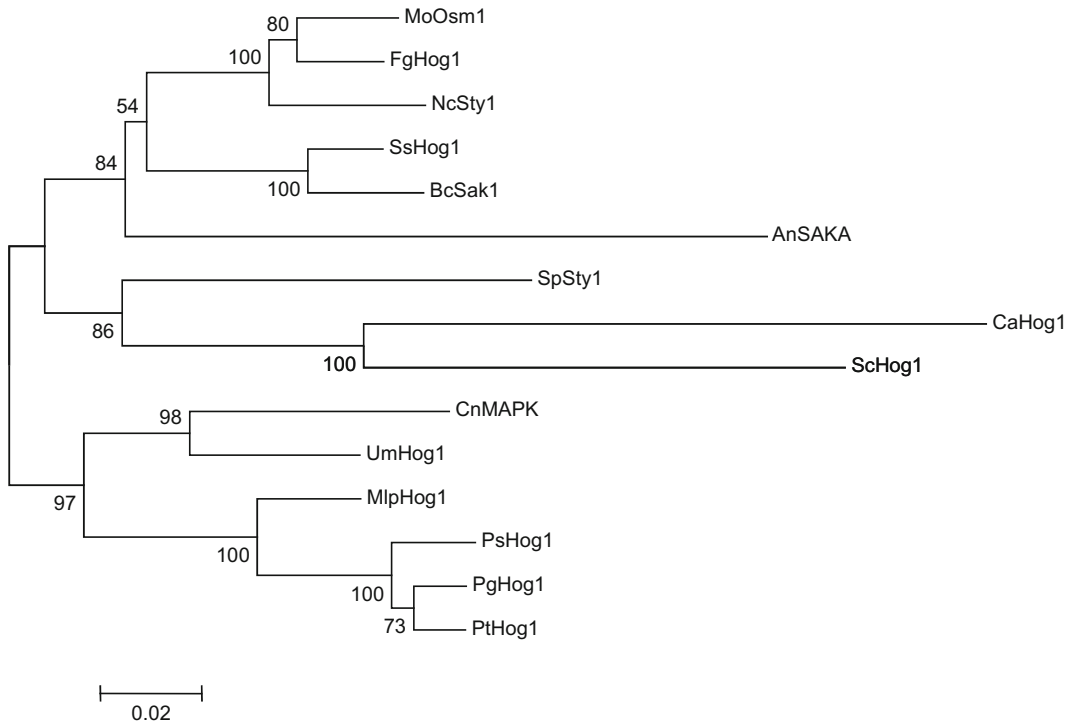

Fig. 1 Phylogenetic analysis of *Hog1* genes. Amino acid sequences encoded by *Hog1* genes from *Aspergillus nidulans* (AAF97243), *B. cinerea* (XP001558337), *C. albicans* (CAWT_04100), *Cryptococcus neoformans var. neoformans* (XP569949), *F. graminearum* (FGSG_09612), *M. larici-populina* (KT183031), *M. oryzae* (AAF09475), *Neurospora crassa* (XP_962163), *P. graminis* f. sp. *tritici* (PGTG_04715), *P. striiformis* f. sp. *tritici* (PSTG_13544), *P. triticina* (PTTG_09272), *S. cerevisiae* (CAA97680), *S. pombe* (NP592843), *Sclerotinia sclerotiorum* (SS1G_07590), and *Ustilago maydis* (XP758504) were examined. The unrooted phylogram was constructed based on NJ analysis. The confidence of groupings was estimated by 1000 bootstrap replicates. The *numbers* next to the branching points indicate the percentage of replicates supporting each branch

Complementation of defects in response to various environmental stresses in the *F. graminearum Hog1* mutant by *MlpHog1*

Given the lack of an available transformation system for *Mlp*, we heterologously expressed *MlpHog1* in the *F. graminearum Hog1* mutant to investigate its function. A construct harbouring *MlpHog1* driven by the RP27 promoter was transformed into protoplasts of the *F. graminearum Hog1* mutant (Zheng et al. 2012). Eleven resulting geneticin-resistant transformants were obtained, four of which carried the *MlpHog1* construct, as confirmed by PCR. The four transformants exhibited identical phenotypes, though only data regarding transformant H-4 are provided here.

On CM plates, the wild-type *F. graminearum* strain PH-1 produced white and aerial colonies, whereas the *F. graminearum Hog1* mutant displayed less aerial hyphal growth and a reduced growth rate (Fig. 2) (Zheng et al. 2012). The colony of transformant H-4 was white and more aerial than the *FgHog1* mutant, and its growth rate was significantly increased compared with that of the *FgHog1* mutant, which were similar to PH-1 (Fig. 2). These results indicated that expression of the *MlpHog1* gene in *F. graminearum* complemented most of the vegetative growth defects of the *Hog1* mutant.

After a 3-day incubation on CM with 1 M NaCl, the *FgHog1* mutant displayed no obvious growth (Fig. 2) (Zheng et al. 2012), whereas the white and aerial hyphae of transformant H-4 were visible, similar to wild-type PH-1. However, growth of the transformant was significantly slower than that of PH-1 ($P = 0.05$) (Fig. 2), indicating partial complementation of the hyperosmotic stress response defect of the *F. graminearum Hog1* mutant by the *MlpHog1* gene.

Because the *FgHog1* mutant is involved in the response to cytoplasm membrane and oxidative stresses in *F. graminearum* (Zheng et al. 2012), we also evaluated

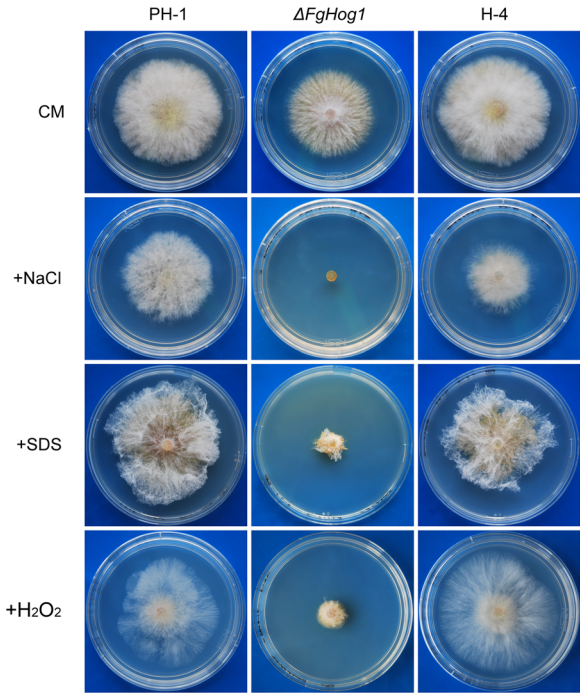

Fig. 2 Colony morphology of *Fusarium graminearum* strains. Colonies of the wild-type strain (PH-1), *FgHog1* deletion mutant, and complemented strain (H-4) grown on CM plates with 1 M NaCl, 0.01 % SDS or 0.05 % H_2O_2 at 25 °C for 3 days

the effect of sodium dodecyl sulfate (SDS) and H_2O_2 treatments. In the presence of 0.01 % SDS or 0.05 % H_2O_2, colony growth of the *FgHog1* mutant was stunted but evident. In contrast, transformant H-4 produced aerial hyphae (Fig. 2), similar to wild-type PH-1. These results indicated that expression of the *MlpHog1* gene in *F. graminearum* largely complemented the impaired response of the *Hog1* mutant to cytoplasm membrane and oxidative stresses.

MlpHog1 partially restores sexual reproduction and plant infection defects in the *F. graminearum Hog1* mutant

Wild-type PH-1 formed numerous perithecia with cirrhi 2 weeks after self-fertilization on self-mating carrot agar, whereas the *FgHog1* mutants were sterile and could not produce perithecia (Fig. 3) (Zheng et al. 2012). Although transformant H-4 did produce perithecia under the same conditions, the number of perithecia with cirrhi was reduced by approximately 40 % (Fig. 3), suggesting that the sexual reproduction defect of the *F. graminearum Hog1* mutant was only partially complemented by the *MlpHog1* gene.

As *FgHog1* is important for plant infection (Zheng et al. 2012), we inoculated flowering wheat heads with wild-type PH-1, *FgHog1* mutant and transformant H-4 conidia suspensions to determine the virulence of the transformant. The wheat spikelets drop-inoculated with wild-type PH-1 developed typical wheat scab symptoms, whereas the *FgHog1* mutant was defective in spreading from the inoculated kernels to other spikelets on the same wheat head (Fig. 4) (Zheng et al. 2012). Conversely, transformant H-4 did spread from the inoculated spikelet to neighbouring spikelets, but to a lesser extent than PH-1 (Fig. 4). At 14 dpi, the average disease index (diseased spikelets per head) was 6.67 for transformant H-4 and 12.33 for PH-1 (Table 2). These results indicated that expression of the *MlpHog1* gene in *F. graminearum* partially complemented the plant infection defects exhibited by the *Hog1* mutant.

Discussion

Although Hog1 plays important roles during numerous biological processes, including osmoregulation, few studies have focused on its role in poplar foliar rust *M. larici-populina*. In this study, we cloned and characterized the Hog1 gene *MlpHog1* from *M. larici-populina*. Based on genomic data provided by the completion of whole-genome sequencing projects, Hamel and colleagues analysed the structure of MAPK protein families in 24 fungal taxa, including both plant pathogens and mycorrhizal symbionts (Hamel et al. 2012). With regard to *M. larici populina*, they predicte that three MAPK systems are present in this fungus (Ste11-Ste7-Kss1 pathway, Bck1-Mkk1-Slt2 pathway, and Ssk2-Pbs2-Hog1 pathway), including the intact Hog1 pathway. *MlpHog1* was cloned and sequenced from Chinese *M. larici-populina* strain Wh03 by homology-based cloning, harbouring a TGY phosphorylation site for the threonine/tyrosine phosphorylation required for kinase activation and a hallmark of stress-activated MAPKs in fungal and mammalian systems (Kultz 1998). Bioinformatic analysis revealed that its sequence shares high identity with *Hog1* othorlogs from other fungi. These results indicate the conservation of *Hog1* across fungi and animals.

Heterologous expression is frequently used for the functional analysis of genes. For example, the yeast transformants expressing *MoMsn2* from *M. oryzae* restored growth to the Δ*Scmsn2* yeast mutant on medium

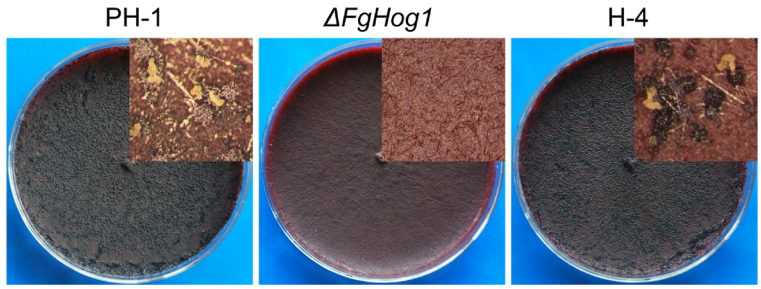

Fig. 3 Sexual reproduction of *Fusarium graminearum* strains. Self-crossing cultures of the wild-type strain (PH-1), *FgHog1* deletion mutant, and complemented strain (H-4) grown on carrot agar plates. The close-up views were obtained using a dissecting microscope

containing 5 mM H_2O_2 (Zhang et al. 2014). *MAPK1* from the wheat stripe rust *Puccinia striiformis* partially complemented the corresponding *kss1* mutants of *F. graminearum* and *M. oryzae* (Guo et al. 2011). For the oomycete *Phytophthora infestans*, *Picdc14* appears to be functionally equivalent to *cdc14* of *S. cerevisiae* based on its complementation of the *cdc14–1*^{ts} mutation (Ah Fong and Judelson 2003). In addition, *Brassica oleracea* ABI5 can rescue the ABA-insensitive phenotype of *abi5-1* during seed germination (Zhou et al. 2013). Overall, the strictly biotrophic parasitism of rust fungi depend on the host's living tissue for their development, proliferation and reproduction (Feau et al. 2007), which has resulted in the lack of a stable transformation system for *M. larici-populina*. Therefore, we utilized a heterologous system to determine the potential function of genes of interest from *M. larici-populina*. Host-induced RNAi system is newly developed to indirectly suppress parasite gene expression by expressing an RNAi construct in vivo in the host (Nunes and Dean 2012). Recent studies show that this technology is also successfully used in obligate biotrophic fungi, such as *Blumeria graminis* and *Puccinia striiformis* f. sp. *tritici*

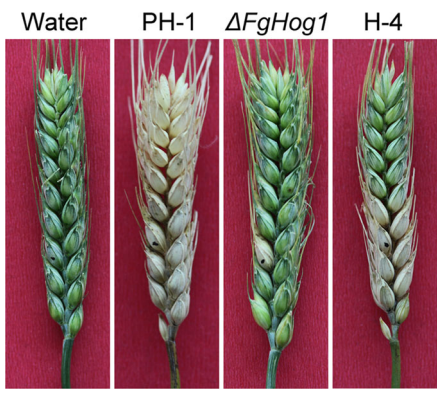

Fig. 4 Infection assays with flowering wheat heads. Wheat heads were drop-inoculated with sterile water or conidia from the wild-type strain (PH-1), *FgHog1* deletion mutant, and complemented strain (H-4). Typical heads were photographed at 14 dpi

(Nowara et al. 2010; Yin et al. 2011; Zhang et al. 2012). It may be available to study the parasite gene funtion for the poplar-*M. larici-populina* pathosystem in future.

The wild type *FgHog1* complements the defects of *the Fghog1* mutant (Zheng et al. 2012). Our observation showed that heterologous expression of *MlpHog1* in *FgHog1* mutant did not fully complement the defects on environmental stresses, sexual reproduction and plant infection, which is similar to the phenomenon that *PstMAPK1* and *PstILC1* from *Puccinia striiformis* partially complemented the defects of corresponding ascomycetous mutants (Guo et al. 2011; Liu et al. 2014). Based on the bioinformatics analysis, the intact Hog1 pathway is present in *M. larici-populina* and *MlpHog1* displays the high degree of conservation. However poplar foliar rust fungi have a distinct life style from *F. graminearum*, which may cause the differences on the regulation of biological processes including signal transduction between them. Thus rust genes including *MlpHog1* are not fully functional in ascomycetous fungi.

Although the functions of Kss1/Fus3-type and Slt2-type MAPKs in fungal pathogenesis appear to be broadly conserved, the role of *Hog1* homologues in pathogenesis differs drastically among plant pathogens (Zhao et al. 2007). *M. oryzae Osm1* is dispensable for plant infection (Dixon et al. 1999), as are *Colletotrichum*

Table 2 Disease index scores of *Fusarium graminearum* strains

	Strain		
	PH-1	Δ*FgHog1*	H-4
Disease index[a]	12.33 ± 2.89^{A}	1.00 ± 0.00^{C}	6.67 ± 0.58^{B}

[a] Diseased spikelets per wheat head were examined at 14 dpi. The mean and standard deviation were calculated from three independent experiments. The data from three independent replicates were analysed with Duncan's multiple range test. Different letters are used to show statistically significant differences ($P = 0.05$)

orbiculare Osc1 and *Bipolaris oryzae Srm1* (Kojima et al. 2004; Moriwaki et al. 2006). In contrast, deletion of *Mycosphaerella graminicola Hog1* or *B. cinerea Sak1* produces nonpathogenic strains (Mehrabi et al. 2006; Segmuller et al. 2007). In the present study, we found that the complementated strains could spread from the inoculated spikelet to neighbouring spikelets, whereas the *FgHog1* mutant was defective in spreading under the same condition. Infection hyphae of *M. larici-populina* ramify extensively and grow throughout the intercellular spaces of poplar leaves, producing haustoria for nutrient acquisition. Therefore, the partial complementation result with regard to plant infection indicates functional conservation between *MlpHog1* and *FgHog1*, and suggests that *MlpHog1* may play a role in regulating infectious growth in *M. larici-populina*.

Although Hog1-type MAPKs play different roles in fungal virulence, their roles are conserved in the response to osmotic stress. *Hog1* deletion mutants of *B. cinerea*, *B. oryzae*, *C. orbiculare*, *C. parasitic* and *M. oryzae* are all sensitive to hyperosmotic stress (Segmuller et al. 2007; Moriwaki et al. 2006; Kojima et al. 2004; Park et al. 2004; Dixon et al. 1999). In our study, *MlpHog1* expression in *F. graminearum* partially complemented the hyperosmotic stress response defect of the *FgHog1* mutant, demonstrating functional conservation between *MlpHog1* and *FgHog1*. Moreover, the *MlpHog1* gene largely complemented the defective response to cytoplasm membrane and oxidative stresses in the *FgHog1* mutant. During the interaction between plants and pathogens, plants exert many kinds of stresses to pathogens, including osmotic stress, oxidative stress or cytoplasm membrane stress. Positive responses to these extracellular stresses may be beneficial for plant infection. These results indicate that *MlpHog1* may play a role in responding to different environmental stresses as well as successful host infection.

Perithecium formation was not observed in the *FgHog1* mutant, however the complemented strains expressing the *MlpHog1* gene could produce perithecia with cirrhi. Because of the decreased cirrhi formation efficiency, *MlpHog1* gene expression in *F. graminearum* partially complemented the defect in sexual reproduction, suggesting that *MlpHog1* may be related to sexual reproduction. The vegetative cycle of *M. larici-populina*, which involves large amounts of dikaryotic urediniospores, occurs on poplar leaves, and successive cycles of uredinia formation throughout summer cause massive epidemics in poplar plantations throughout Europe and worldwide (Barres et al. 2012). Conversely, the sexual stage of this rust fungus occurs on larch needles (*Larix* sp.) in spring (Xhaard et al. 2011), persisting for a long time from the vegetative stage to the sexual stage. Additional work is required to explore the function of the *MlpHog1* gene during the sexual stage.

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