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Functional characterization of *Melampsora larici-populina* Hog1-type MAPK in response to osmotic stress

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Abstract

The obligate biotrophic pathogen *Melampsora larici-populina*, responsible for poplar foliar rust disease, causes annual epidemics and severe damages to poplar plantations worldwide. Flexible responses to external osmotic pressure changes are important for the growth and survival of pathogens. In a previous study, we suggested that the Hog1-type MAPK *MlpHog1* in *M. larici-populina* may play a role in infectious growth and responses to various environmental stresses. In the present study, we analysed its biological characteristics. *MlpHog1* displayed a conserved coding sequence pattern among five strains from different regions of China. *MlpHog1* restored the *Hog1*-orthologue mutant defects responding to hyperosmotic stress in both *Saccharomyces cerevisiae* and *Magnaporthe oryzae*. Transient expression in wheat protoplasts revealed that MlpHog1 is localized in the cytoplasm and nucleus. These results indicate that *MlpHog1* plays a positive role in responding to osmotic stress in the poplar rust *M. larici-populina*.

KEYWORDS
Melampsora larici-populina, *MlpHog1*, osmotic stress, poplar foliar rust

1 | INTRODUCTION

Yeast growth is limited under high external osmolarity, and the high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway from *Saccharomyces cerevisiae* is a comprehensively understood osmoresponsive system (Hohmann, 2002). In this pathway, two independent upstream branches converge on the MAPK kinase Pbs2. Activated Pbs2 phosphorylates the core MAPK Hog1, which is the homologue of mammalian p38 stress-activated protein kinase (SAPK). *S. cerevisiae* Hog1 is responsible mainly for the accumulation of glycerol in the presence of high osmolarity (Albertyn et al., 1994). In response to osmostress, *S. cerevisiae* Hog1 is rapidly activated and then imported into the nucleus to conduct multiple types of transcription regulation (Alepuz et al., 2001; Capaldi et al., 2008; Nadal & Posas, 2010; Ni et al., 2009; Proft & Struhl, 2002; Zapater et al., 2007). Moreover, Hog1 affects mRNA stability for osmostress-upregulated genes (Saito & Posas, 2012). Activated by osmostress, *S. cerevisiae* Hog1 modulates a rapid and transient delay at various stages of the cell cycle to enable cells to adapt before cell-cycle progression (Adrover et al., 2011; Alexander et al., 2001; Clotet et al., 2006; Escote et al., 2004; Yaakov et al., 2009; Zapater et al., 2005).

The importance of the budding yeast *Hog1* homologues in the high osmolarity response is well established in many plant pathogenic fungi. *Hog1* homologue deletion mutants display growth defects in response to high osmotic stress in many phytopathogenic fungi (Dixon et al., 1999; Kojima et al., 2004; Mehrabi et al., 2006; Segmuller et al., 2007; Wang et al., 2016; Zheng et al., 2012, 2016). Among *Magnaporthe oryzae*, *Fusarium graminearum* and *Ustilaginoidea virens*, the accumulation of the main compatible solute or compatible solutes under hyperosmotic conditions is controlled by the *Hog1* MAP kinase pathway (Dixon et al., 1999; Zheng et al., 2012, 2016). ClOsc1 from *Colletotrichum lagenarium* is phosphorylated and accumulates in the nucleus under high osmotic stress (Kojima

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et al., 2004), and similarly, increased phosphorylation of a stress-activated MAPK from *Botrytis cinerea* (BcSak1) is detected in mycelia treated with 0.8 M NaCl (Segmuller et al., 2007).

Poplar leaf rust disease caused by *Melampsora larici-populina* results in threats to poplar plantations worldwide, including plantations in many regions of China (Pinon & Frey, 2005; Tian et al., 2000). Using resistant poplar cultivars is the most effective and environmentally friendly approach to control this disease. However, similar to other rust diseases, it is difficult to control because rapidly evolved races lead to the breakdown of poplar resistance (Tian et al., 2000). Our ability to control poplar leaf rust disease will benefit from understanding the molecular mechanism of development and pathogenesis. In recent years, accompanied by genome release and transcriptome analyses, researches on the function of interested genes from *M. larici-populina*, especially effectors, have yielded much progress (Duplessis, Cuomo, et al., 2011; Duplessis, Hacquard, et al., 2011; Hacquard et al., 2012, 2013; Lorrain et al., 2018, 2019). 11 *M. larici-populina* candidate effectors are emphasized because of specific localization and/or plant protein interactors (Germain et al., 2017; Petre et al., 2015; Petre, Lorrain, et al., 2016; Petre, Saunders, et al., 2016). A set of candidate effectors in *M. larici-populina* has been characterized. Mlp124478 has a virulence activity and remodels transcription by binding DNA to suppress normal transcriptional responses to pathogens (Ahmed et al., 2018). Mlp124357 increases plant susceptibility to bacterial and oomycete pathogens and associates with AtPDI-11 likely acting as a helper protein recruited by Mlp124357 to enhance plant susceptibility (Madina et al., 2020). Mlp37347 accumulates exclusively at plasmodesmata, increases plasmodesmatal flux and reduces plasmodesmatal callose deposition, also could promote *Hyalonoperospora arabidopsidis* growth in infection assays (Rahman et al., 2021).

Positive responses to extracellular stresses are important for normal growth of the pathogen and further plant infection. There were few reports on how the HOG pathway responds to hyperosmotic stress in *M. larici-populina*. Previously, we conducted the molecular characterization of the Hog1-type MAPK gene, *MlpHog1* (Yu et al., 2016), and here we explored its potential function in response to osmotic stress.

2 | MATERIALS AND METHODS

2.1 | Polymorphism analysis of *MlpHog1* among different *M. larici-populina* strains

Four Chinese *M. larici-populina* (*Mlp*) strains from different regions (Cao et al., 2012), including Zst (Heilongjiang Province, Northeast China), Bq (Beijing, North China), Nm (Inner Mongolia Autonomous Region, Northwest China) and Sb052 (Sichuan Province, Sorthwest China), were selected to assay coding region polymorphisms of *MlpHog1*. *MlpHog1* fragments were amplified from urediospore cDNA of the four strains with *TransStart FastPfu* DNA polymerase (TransGen, Beijing) and primers H1-F/R (Yu et al., 2016). PCR fragments were inserted into the pMD19-T vector (TaKaRa, Japan) and sequenced at AuGCT Biotech Company (Beijing, China) to obtain the coding sequences of *MlpHog1* from the four *Mlp* strains.

2.2 | Complementation of the *S. cerevisiae Hog1* mutant

The *S. cerevisiae* wild-type strain BY4741 and *Hog1* deletion mutant strain Δ*ScHog1* (BY4741 background) were purchased from the EUROpean *S. cerevisiae* Archive (EUROSCARF). The coding sequence of *MlpHog1* was amplified using the primers MlpH1-YE/F and MlpH1-YE/R1 (Table S1). The purified PCR product and pYES2 vector (Invitrogen Co., CA, USA) were digested with *Eco*RI and *Xho*I and connected with T4 ligase. The Δ*ScHog1* strain was transformed with the construct pYES2::*MlpHog1* and with the empty vector pYES2, according to the lithium acetate method (Schiestl & Gietz, 1989). Yeast transformants were selected on synthetic medium lacking uracil and screened by PCR using the corresponding primers (H1-pYseq/F1 and Hog1-pYseq/R for the construct pYES2::*MlpHog1*, and H1-pYseq/F2 and Hog1-pYseq/R for the empty vector pYES2, Table S1). Yeast complementation assays were performed as described previously (Yu et al., 2014). Serial dilutions of the cell suspension of each yeast strain were spotted on yeast peptone galactose medium (YPG; 1% yeast extract, 2% bactopeptone, 2% galactose) and YPG medium supplemented with 0.4 M NaCl and incubated at 30°C for 3 days. The experiments were repeated three times.

2.3 | Complementation of the *M. oryzae Hog1* mutant

The vector pFL2::*MlpHog1* (Yu et al., 2016) was transformed into protoplasts of the *M. oryzae Hog1* mutant Δ*OSM1* as described previously (Sweigard et al., 1992). Geneticin-resistant transformants were confirmed by PCR analysis with primers MlpHog1-F/RP27 and pFL2 insert/R (Table S1). The diameters of all strains on complete medium (CM) plates (the control) and CM plates with 0.4 M NaCl (the treatment) were measured after growth at 25°C for 10 days and used to calculate the inhibition rate (in%), which was estimated as (control diameter-treatment diameter)/(control diameter-plug diameter) × 100.

2.4 | Subcellular localization analysis

Triticum aestivum Suwon 11 plants were used for protoplast transformation and grown in a glasshouse for approximately 2–3 weeks. The coding sequence of *MlpHog1* was amplified with the primers H1-163F and H1-163R1 (Table S1), and the purified PCR product and pCaMV35S::GFP vector were digested with *Pst* I and *Xba* I and finally connected with T4 ligase. The fusion expression construct pCaMV35S::*MlpHog1*-GFP and empty vector pCaMV35S::GFP were

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separately transformed into *T. aestivum* protoplasts through the polyethyleneglycol(PEG)-calcium method, as described previously (Zhu et al., 2018). After incubation overnight, transformed protoplasts were examined with an FV1000 confocal laser microscope (Olympus, Tokyo, Japan). The experiments were repeated three times.

3 | RESULTS

3.1 | *MlpHog1* shows a conserved coding sequence pattern

To identify the intraspecific sequence polymorphism in *MlpHog1*, we gained the coding sequences of other four Chinese *Mlp* strains from different regions, including Zst, Bq, Nm and Sb052, and compared them with that of the Chinese *Mlp* strain Wh03 (Shaanxi Province, Northwest China) obtained previously (Yu et al., 2016). Interestingly, no sequence variations in the coding sequences were present among these five *Mlp* strains, suggesting that *MlpHog1* may be highly conserved in strains from different regions of China.

3.2 | Complementation of *S. cerevisiae Hog1* mutant with *MlpHog1* restores adaptation to hyperosmotic stress

We transformed the *MlpHog1* gene into the yeast *Hog1* mutant Δ*ScHog1* to test whether *MlpHog1* could complement this mutant. All strains displayed similar growth under normal conditions (Figure 1). After adding exogenous 0.4 M NaCl, the growth of the mutant Δ*ScHog1* and the mutant carrying the empty vector pYES2 were significantly inhibited (Figure 1), which was in accordance with other studies (Dixon et al., 1999). However, transformants with pYES2::*MlpHog1* displayed obvious growth, similar to wild-type BY4741 (Figure 1). These results indicated that the phenotypic defect in response to high osmotic pressure was well rescued by genetic complementation of the yeast mutant Δ*ScHog1* with *M. larici-populina MlpHog1*.

3.3 | *MlpHog1* partially complements the *M. oryzae OSM1* mutant

The *MlpHog1* gene was transformed into the *M. oryzae Hog1* mutant Δ*OSM1* to analyse the function of *MlpHog1*. The resultant transformants showed identical phenotypes and only data regarding H1-11 are provided here. When exposed to hyperosmotic stress (0.4 M NaCl), the growth of the mutant Δ*OSM1* was more sensitive than that of the wild-type Guy11; however, vegetative growth inhibition of the transformant H1-11 was significantly decreased compared with Δ*OSM1* (p =.05) (Figure 2). This observation shows that *MlpHog1* could partially complement *M. oryzae OSM1* mutant defects in response to hyperosmotic stress.

3.4 | Subcellular localization of MlpHog1 in plant protoplasts

The pCaMV35S::*MlpHog1*-GFP fusion construct was transformed into *T. aestivum* protoplasts to conduct the subcellular localization assay. When the MlpHog1-GFP fusion protein was transiently expressed in *T. aestivum* protoplasts, fluorescence was distributed in the cytoplasm and nucleus of protoplasts, while GFP protein as a control displayed fluorescence in the cytomembrane, cytoplasm and nucleus of protoplasts (Figure 3), suggesting that MlpHog1 is localized in the cytoplasm and nucleus in wheat plants.

4 | DISCUSSIONS

In the current study, we found no coding single nucleotide polymorphism (cSNP) of *MlpHog1* among five *Mlp* strains from different regions of China. In comparing the coding sequence of *MlpHog1* between the Chinese strain Wh03 and the French reference strain 98AG31, only three cSNPs, including nucleotide loci 222,465 and 468, are present, and all three are synonymous cSNPs. Similarly, Krantz et al. identified the HOG pathway components in 20 fungal species by a comparative genomics approach and found that

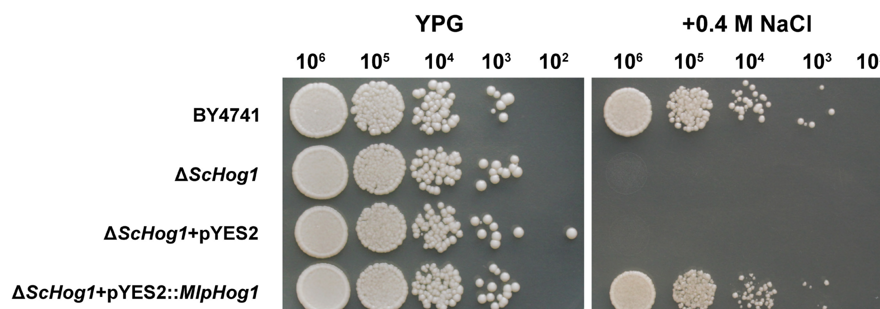

FIGURE 1 Complementation of the *Saccharomyces cerevisiae Hog1* mutant with *Melampsora larici-populina MlpHog1*. Serial dilutions of cell suspensions from all yeast strains, including wild-type BY4741, Δ*ScHog1*, Δ*ScHog1* (pYES2) and Δ*ScHog1* (pYES2::*MlpHog1*) were spotted onto yeast peptone galactose (YPG) plates containing no additions (the control) and 0.4 M NaCl (the treatment). Yeast cells were incubated at 30°C for 3 days

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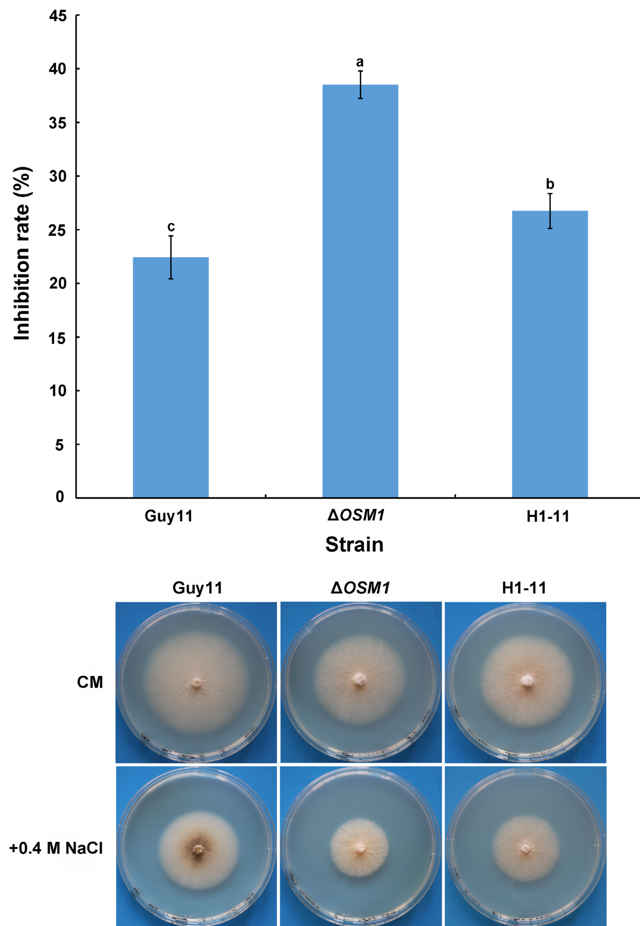

FIGURE 2 Complementation of the *Magnaporthe oryzae OSM1* mutant with *Melampsora larici-populina MlpHog1*. The inhibition rates grown on CM (the control) and CM with 0.4 M NaCl (the treatment) were shown from the wild-type (Guy11), the *Hog1* deletion mutant (Δ*OSM1*) and the transformant H1-11. The mean and standard deviation were calculated from three independent replicates and analysed with Tukey's HSD test. Different letters show statistically significant differences ($p = .05$). Colonies of all the strains were also shown

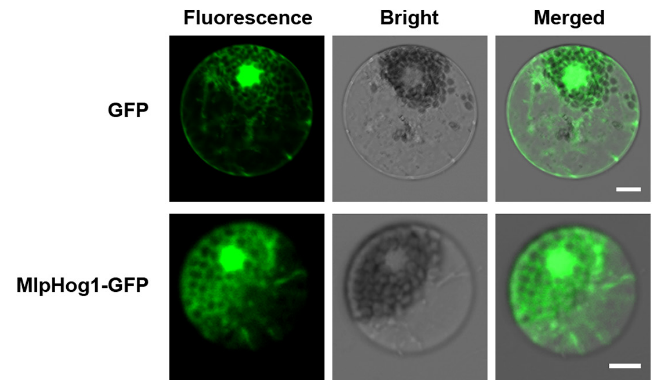

FIGURE 3 Subcellular localization of the MlpHog1 protein. Green fluorescent protein (GFP) (the control) and MlpHog1-GFP fusion proteins were expressed in the wheat protoplasts following PEG-mediated transformation. Bar = 10 μm

Cdc42 and Hog1 were the most highly conserved among 40 proteins linked to this pathway (Krantz et al., 2006). However, for one Fus3/Kss1-type MAPK from *Puccinia striiformis* f. sp. *tritic*, 32 cSNPs with abundant types exist among six different Chinese *Pst* races (Guo et al., 2011).

In our previous study, partial complementation of the hyperosmotic stress response defect of the *F. graminearum Hog1* mutant by the *MlpHog1* gene demonstrated the functional conservation between *MlpHog1* and *FgHog1* (Yu et al., 2016). Unfortunately, at present, this poplar rust fungus still cannot be genetically manipulated to understand the function of genes of interest in *M. larici-populina*, even in a host-induced RNAi system (Qi et al., 2018). We continued to analyse the function of *MlpHog1* during osmotic stress by means of the other two fungal systems. In response to hyperosmotic stress, *MlpHog1* expression restores the growth defect in the *S. cerevisiae Hog1* mutant and partially complements the *M.oryzae OSM1* mutant defect, indicating the functional conservation between *MlpHog1* and *ScHog1* and also between *MlpHog1* and *OSM1*. Thus, we demonstrate that *MlpHog1* could rescue the *Hog1*-orthologue mutant defects responding to hyperosmotic stress in the three fungal systems.

Following osmostress the budding yeast Hog1 is rapidly activated and then imported into the nucleus to phosphorylate its nuclear substrates (Saito & Posas, 2012). By observing GFP signals of the *FgHog1*-GFP transformant, it was suggested that FgHog1 is activated and localized to the nucleus by a 0.3 M NaCl treatment (Zheng et al., 2012). Similar observation was made for the *C. lagenarium* Hog1-related MAPK ClOsc1 (Kojima et al., 2004). In the current study, *T. aestivum* protoplasts with the *MlpHog1*-GFP fusion construct were incubated in the W5 solution overnight to maintain the homeostasis of protoplasts and induce transient expression. W5 solution contains 0.15 M NaCl, so we inferred that under NaCl treatment, MlpHog1-GFP signals could be detected in the nucleus.

A higher intracellular osmotic pressure has to be maintained in microbial cells relative to the growth medium to generate cell turgor pressure, by which cell extension, growth and division are driven (Welsh, 2000). Microorganisms are exposed to highly variable environments with diverse stresses, including osmotic stress. Thus, reasonable adjustments to fluctuations in the osmolarity of the growth medium are crucial for growth and survival, and microorganisms have developed many strategies in response to such fluctuations (Welsh, 2000). Overall, analyses of heterologous expression in three fungal systems and subcellular localization in wheat protoplasts suggest that *MlpHog1* from the poplar rust *M. larici-populina* plays a positive role in responding to osmotic stress, helping this rust fungus adapt to its environment and exert its pathogenicity.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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