Dissecting the role of histidine kinase and HOG1 mitogen-activated protein kinase signalling in stress tolerance and pathogenicity of *Parastagonospora nodorum* on wheat

Evan John, Francisco Lopez-Ruiz, Kasia Rybak, Carl J. Mousley, Richard P. Oliver and Kar-Chun Tan

1Department of Environment and Agriculture, Centre for Crop and Disease Management, Curtin University, Bentley, WA 6102, Australia
2School of Biomedical Sciences, CHIRI Biosciences Research Precinct and Faculty of Health Sciences, Curtin University, Bentley, WA 6102, Australia

The HOG1 mitogen-activated protein kinase (MAPK) pathway is activated through two-component histidine kinase (HK) signalling. This pathway was first characterized in the budding yeast *Saccharomyces cerevisiae* as a regulator of osmotolerance. The fungus *Parastagonospora nodorum* is the causal agent of septoria nodorum blotch of wheat. This pathogen uses host-specific effectors in tandem with general pathogenicity mechanisms to carry out its infection process. Genes showing strong sequence homology to *S. cerevisiae* HOG1 signalling pathway genes have been identified in the genome of *P. nodorum*. In this study, we examined the role of the pathway in the virulence of *P. nodorum* on wheat by disrupting putative pathway component genes: HOG1 (SNOG_13296) MAPK and NIK1 (SNOG_11631) hybrid HK. Mutants deleted in NIK1 and HOG1 were insensitive to dicarboximide and phenylpyrrole fungicides, but not a fungicide that targets ergosterol biosynthesis. Furthermore, both Δnik1 and Δhog1 mutants showed increased sensitivity to hyperosmotic stress. However, HOG1, but not NIK1, is required for tolerance to elevated temperatures. HOG1 deletion conferred increased tolerance to 6-methoxy-2-benzoxazolinone, a cereal phytoalexin. This suggests that the HOG1 signalling pathway is not exclusively associated with NIK1. Both Δnik1 and Δhog1 mutants retained the ability to infect and cause necrotic lesions on wheat. However, we observed that the Δhog1 mutation resulted in reduced production of pycnidia, asexual fruiting bodies that facilitate spore dispersal during late infection. Our study demonstrated the overlapping and distinct roles of a HOG1 MAPK and two-component HK signalling in *P. nodorum* growth and pathogenicity.

INTRODUCTION

All organisms adapt to environmental changes through the perception of stimuli. Once the stimulus is recognized, a signal is relayed within the cell, which instigates changes in cellular functions that allow the organism to adapt appropriately. Although fungi inhabit a broad range of environmental niches, numerous signalling pathway components are highly conserved across the kingdom (Bahn et al., 2007). A major area of fungal research in recent years has been dedicated to understanding the intricate mechanisms of cellular adaptation to abiotic stress as perceived during infection of animal and plant hosts. The extent of these adaptations may influence the outcome of an infection in an environment where biotic and abiotic stresses are prevalent. Mitogen-activated protein kinase (MAPK) signalling pathways are some of the best-studied signal transduction pathways to date (Xu, 2000; Zhao et al., 2007). The cascade is activated through an upstream receptor responding to a stimulus. Activation results in the phosphorylation of the MAPK kinase kinases (MAPKKKs), which then phosphorylate the MAPK kinases (MAPKKs). Activated MAPKKs
then phosphorylate MAPKs, which regulate gene expression and downstream cellular responses. Five major MAPK pathways have been identified in the budding yeast *Saccharomyces cerevisiae* (Madhani & Fink, 1998). These are the FUS3 (mitaging), KSS1 (pheromone response/filamentous growth), HOG1 (osmoregulation), SLT2 (cell-wall integrity) and SMK1 (sporulation) pathways. MAPK signalling is implicated in growth, differentiation, survival and pathogenesis in many phytopathogenic filamentous fungi (Xu, 2000; Zhao et al., 2007). They typically contain three major MAPK pathways: orthologues of the FUS3/KSS1, SLT2 and HOG1 MAPK signalling pathways (Xu, 2000).

The HOG1 MAPK pathway was first identified in *S. cerevisiae* as a regulator of osmotolerance (Brewster et al., 1993). Activation of this pathway results in the phosphorylation of Ssk2p/Ste11p MAPKKK, which then phosphorylates the Pbs2p MAPKK. Activated Pbs2p then phosphorylates Hog1 (Gustin et al., 1998). Hog1 orthologues in phytopathogenic fungi have a similar role in maintaining osmotolerance (Xu, 2000), but are also involved in other abiotic stress responses, conidiation, pathogenicity, mycotoxin production and susceptibility to dicarboximide/phenylpyrrole fungicides (Table 1). These phenotypes often vary between different fungal pathogens (Table 1). In *S. cerevisiae*, the HOG1 MAPK signalling pathway is regulated by an upstream two-component sensor-signalling system (Maeda et al., 1994). The sensor/histidine kinase (HK) Sln1p is inhibited by osmotic stress, thereby reducing the level of phosphorylation of the phosphotransferase Ypd1 and response regulator (RR) Ssk1. Together, this leads to an Ssk2p-dependent activation of the HOG1 pathway (Posas et al., 1996). Most fungi contain both the HK and RR domains within the same polypeptide (Grebe & Stock, 1999). Hybrid HKs often utilize an additional round of phosphorylation through a histidine phosphotransferase (Hpt) and a secondary RR. Genome sequencing has revealed that filamentous fungi possess a diverse number of genes that encode putative hybrid HKs. Carlett et al. (2003) classified fungal hybrid HKs into 11 major groups. Group III NcNIK1/OS-1 hybrid HKs are involved in osmosensing. Perturbation of group III hybrid HKs in phytopathogens often results in phenotypes that overlap with those of mutants that carry impairment in the HOG1 MAPK cascade (Table 1). However, that is not always the case. Lin & Chung (2010) demonstrated redundant and distinct roles of AaHOG1 and AaHSK1 (group III HK) in the citrus brown spot pathogen *Alternaria alternata*. Mutants disrupted in AaHSK1 and AaHOG1 demonstrate increased sensitivity to hyperosmotic stress. AaHOG1, but not AaHSK1, is required for virulence on citrus and tolerance to oxidative stress. However, deletion of AaHSK1, but not AaHOG1, conferred resistance to dicarboximide/phenylpyrrole fungicides.

*Parastagonospora nodorum* is the causal agent of septoria nodorum blotch on wheat (Solomon et al., 2006a; Quaedvlieg et al., 2013). Septoria nodorum blotch is a major disease in many wheat-growing regions of the world (Oliver et al., 2012). *P. nodorum* is a sophisticated fungal necrotroph that utilizes an array of host-specific necrotrophic effectors to manipulate the plant metabolism to favour infection (Friesen et al., 2008; Tan et al., 2010, 2015). The role of the FUS3/KSS1 MAPK orthologue MAK2 in pathogenicity has been examined in detail in *P. nodorum* (Solomon et al., 2006a; Tan et al., 2009b). Targeted gene deletion of MAK2 resulted in mutants that were non-pathogenic, unable to sporulate and exhibited abnormal vegetative growth (Solomon et al., 2005b). Subsequent proteomic analyses identified a Mak2/Gna1 Go subunit co-regulated putative short-chain dehydrogenase that plays a role in sporulation and mycotoxin production (Tan, 2007; Tan et al., 2008, 2009c). The function of HOG1 signalling during *P. nodorum* pathogenicity and development is poorly understood. Genome sequencing of *P. nodorum* has revealed genes that encode putative components of the HOG1 pathway (Hane et al., 2007). We decided to investigate the role of the pathway by inactivating components in *P. nodorum* that are orthologous to genes of other fungi known for their overlapping involvement in osmotolerance and susceptibility to dicarboximide/phenylpyrrole fungicides. As such, genes that encode putative group III HK and HOG1 MAPK were knocked out using targeted gene deletion. The resulting mutants were examined for their roles in stress tolerance, development and pathogenicity on wheat. This approach allows a simultaneous comparison of phenotypic effects that were incurred from mutations in different parts of the HOG1 MAPK signalling pathway in *P. nodorum* in order to elucidate overlapping and/or distinct roles of both components.

**METHODS**

**Fungal culturing.** All *P. nodorum* strains were maintained on V8-PDA agar (150 ml Campbell’s V8 juice l⁻¹, 3 g CaCO₃ l⁻¹, 30 g sucrose l⁻¹, 10 g Difco PDA l⁻¹ and 10 g agar l⁻¹) at 21°C under a 12 h photoperiod.

**In planta gene expression analysis.** RNA isolation and *in planta* gene expression analysis were performed as previously described with minor modifications (Solomon et al., 2003). Briefly, detached wheat leaves (Halberd) maintained in 0.15 % (w/v) benzimidazole agar were inoculated with 1x10⁶ pycnidiospores in 0.02 % Tween 20 to facilitate infection. Lesions were excised at 3, 6, 8 and 10 days post-infection (dpi), freeze dried and subject to RNA extraction using TRizol reagent (Invitrogen), DNase-treated and reverse transcribed as previously described (Tan et al., 2008). Quantitative real-time PCR (qRT-PCR) was performed using a Quantitect SYBR Green RT-PCR kit (Qiagen) and a Bio-Rad CFX96 system, using SN15 genomic DNA as a quantitative standard. The primer pair Nik1qPCRf and Nik1qPCRr was used to amplify a 129 bp region of NIK1. The primer pair Hog1qPCRf and Hog1qPCRr was used to amplify a 144 bp region of HOG1. The housekeeping gene actin (ACT1) was used to normalize gene expression using the primer pairAct1qPCRf and Act1qPCRr (Tan et al., 2008). Primer sequences are shown in Table S1 (available in the online Supplementary Material).

**Construction of the HOG1 and NIK1 gene knockout vectors.** *P. nodorum* SN15 strains carrying individual deletions in HOG1 (HOG1 MAPK gene) and NIK1 (HK gene) were created through genetic
# Table 1. Characterised functions of putative HK/Hog1 MAPK pathway components in various fungal phytopathogens

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Gene</th>
<th>Family</th>
<th>Gene inactivation phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alternaria alternata</em></td>
<td>AaHSK1</td>
<td>Group III HK</td>
<td>Δaahsk1: non-pathogenic (not Δaahsk1)</td>
<td>Lin &amp; Chung (2010)</td>
</tr>
<tr>
<td></td>
<td>AaHOG1</td>
<td>Hog1 MAPK</td>
<td>Δaah1: resistant to dicarboximide and phenylpyrrole (not Δaahog1) Δaahog1: increased sensitivity to oxidative stress (not Δaahsk1) Δaahsk1/Δaahog1: increased sensitivity to hyperosmotic stress</td>
<td></td>
</tr>
<tr>
<td><em>Alternaria brassicicola</em></td>
<td>AbN1K1</td>
<td>Group III HK</td>
<td>Reduced virulence; increased sensitivity to hyperosmotic stress</td>
<td>Avenot et al. (2005);</td>
</tr>
<tr>
<td><em>Alternaria longipes</em></td>
<td>A1HK1</td>
<td>Group III HK</td>
<td>Increased virulence; increased resistance to dimethachlon and phenylpyrrole; reduced sporulation; increased sensitivity to osmotic stress</td>
<td>Cho et al. (2009)</td>
</tr>
<tr>
<td><em>Bipolaris oryzae</em></td>
<td>SRM1</td>
<td>Hog1 MAPK</td>
<td>Increased sensitivity to oxidative/UV/hyperosmotic stresses; moderate resistance to dicarboximide and phenylpyrrole; positive regulator of melanin biosynthetic genes</td>
<td>Moriwaki et al. (2006)</td>
</tr>
<tr>
<td><em>Botrytis cinerea</em></td>
<td>BcSak1</td>
<td>Hog1 MAPK</td>
<td>Δbcsak1: reduced phytotoxin production</td>
<td>Heller et al. (2012);</td>
</tr>
<tr>
<td></td>
<td>BcOS4</td>
<td>Ssk2 MAPKK</td>
<td>Δbos1: increased resistance to dicarboximide and phenylpyrrole (not Δbos4/Δbcsak1)</td>
<td>Liu et al. (2008);</td>
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<tr>
<td></td>
<td>Bos1</td>
<td>Group III HK</td>
<td>Δbos1: increased sensitivity to oxidative stress; moderate resistance to dicarboximide and phenylpyrrole; positive regulator of melanin biosynthetic genes</td>
<td>Segmuller et al. (2007);</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Δbos1/Δbcsak1/Δbos4: abnormal vegetative growth</td>
<td>Viaud et al. (2006);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Δbos1/Δbcsak1/Δbos4: reduced/abolished conidiation</td>
<td>Yang et al. (2012)</td>
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<tr>
<td><em>Cochliobolus heterostrophus</em></td>
<td>Sk1</td>
<td>RR</td>
<td>Δsk1: increased conidiation <em>in vitro</em> (not Δhog1)</td>
<td>Igbaria et al. (2008);</td>
</tr>
<tr>
<td></td>
<td>Skn7</td>
<td>RR</td>
<td>Δskn1: decreased conidiation <em>in vitro</em> (not Δskn1)</td>
<td>Iizumitsu et al. (2007);</td>
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<td><em>Collotrichium lagenarium</em></td>
<td>OSC1</td>
<td>Hog1 MAPK</td>
<td>Increased sensitivity to hyperosmotic stress; increased resistance to phenylpyrrole</td>
<td>Kojima et al. (2004)</td>
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<tr>
<td><em>Cryphonectria parasitica</em></td>
<td>CpMK1</td>
<td>Hog1 MAPK</td>
<td>Increased sensitivity to hyperosmotic stress; increased resistance to phenylpyrrole</td>
<td>Park et al. (2004)</td>
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<tr>
<td><em>Fusarium graminearum</em></td>
<td>FgOS2</td>
<td>Hog1 MAPK</td>
<td>Δfgos2/Δfghog1/Δfgpbs2/Δfgsk2: increased sensitivity to oxidative and hyperosmotic stresses</td>
<td>Nyugen et al. (2012);</td>
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<td></td>
<td>Fghog1</td>
<td>Hog1 MAPK</td>
<td>Δfghog1/Δfgpbs2/Δfgsk2: cell-wall defect</td>
<td>Zheng et al. (2012)</td>
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<td></td>
<td>Fgbps2</td>
<td>Pbs2 MAPKK</td>
<td>Δfgos2/Δfghog1/Δfgpbs2/Δfgsk2: reduced virulence</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fgssk2</td>
<td>Ssk2 MAPKK</td>
<td>Δfgos2/Δfghog1/Δfgpbs2/Δfgsk2: vegetative growth defect</td>
<td></td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>Fhk1</td>
<td>Group III HK</td>
<td>Increased resistance to dicarboximide and phenylpyrrole; increased sensitivity to hyperosmotic and oxidative stresses; reduced virulence</td>
<td>Rispail &amp; Di Pietro (2010)</td>
</tr>
<tr>
<td><em>Fusarium proliferatum</em></td>
<td>Fphog1</td>
<td>Hog1 MAPK</td>
<td>Increased resistance to phenylpyrrole; increased sensitivity to oxidative, heat, UV and hyperosmotic stresses</td>
<td>Adam et al. (2008a, b);</td>
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<tr>
<td><em>Gibberella zeae</em></td>
<td>Sk1</td>
<td>RR</td>
<td>Increased sensitivity to oxidative stress; increased sensitivity to hyperosmotic stress; decreased conidiation; decreased virulence; hydrophilic; delayed ascospore release</td>
<td>Oide et al. (2010)</td>
</tr>
<tr>
<td><em>Magnaporthe oryzae</em></td>
<td>OSM1</td>
<td>Hog1 MAPK</td>
<td>Δosm1: reduction in arabitol content</td>
<td>Dixon et al. (1999);</td>
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<tr>
<td><em>Magnaporthe oryzae</em></td>
<td>Fghog1</td>
<td>Hog1 MAPK</td>
<td>Increased resistance to phenylpyrrole; increased sensitivity to oxidative, heat, UV and hyperosmotic stresses</td>
<td>Adam et al. (2008a, b);</td>
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<tr>
<td></td>
<td>Fgssk2</td>
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<tr>
<td><em>Magnaporthe oryzae</em></td>
<td>FgOS2</td>
<td>Hog1 MAPK</td>
<td>Δfgos2/Δfghog1/Δfgpbs2/Δfgsk2: reduced virulence</td>
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<tr>
<td><em>Magnaporthe oryzae</em></td>
<td>Fgssk2</td>
<td>Ssk2 MAPKK</td>
<td>Δfgos2/Δfghog1/Δfgpbs2/Δfgsk2: vegetative growth defect</td>
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Table 1. cont.

<table>
<thead>
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<th>Gene family</th>
<th>Genes</th>
<th>Phenotypes</th>
<th>Reference</th>
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<tr>
<td>HIK1</td>
<td>Group III HK</td>
<td>HIK1 increased resistance to dicarboximides, phenylpyrroles (Ahi1)::sktl1) and pentachloronitrobenzene</td>
<td>Jacob et al. (2014); Zhang et al. (2005, 2008); Mehrabi et al. (2006); Zhang et al. (2013)</td>
</tr>
<tr>
<td>MoSIN1</td>
<td>Group V1 HK</td>
<td>HIK1 increased sensitivity to oxidative stress; increased sensitivity to heavy metals; cell defect</td>
<td>Jacob et al. (2014); Zhang et al. (2005, 2008); Mehrabi et al. (2006); Zhang et al. (2013)</td>
</tr>
<tr>
<td>MaSSK1</td>
<td>RR</td>
<td>HIK1 increased sensitivity to heat stress and oxidative stresses; altered vegetative growth and unable to produce sclerotia</td>
<td>Jacob et al. (2014); Zhang et al. (2005, 2008); Mehrabi et al. (2006); Zhang et al. (2013)</td>
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<td>HHI1</td>
<td>Group III HK</td>
<td>HOG1 MAPK</td>
<td>Duan et al. (2013)</td>
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<td>MgHogl</td>
<td>Sn15</td>
<td>Hog1 MAPK</td>
<td>Duan et al. (2013)</td>
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<td>Siki</td>
<td>Group III HK</td>
<td>Hog1 MAPK</td>
<td>Duan et al. (2013)</td>
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<td>Mycosphaerella graminicola</td>
<td>Group III HK</td>
<td>Hog1 MAPK</td>
<td>Duan et al. (2013)</td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>Group III HK</td>
<td>Hog1 MAPK</td>
<td>Duan et al. (2013)</td>
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Table 1. cont.

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Gene</th>
<th>Phenotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>E. coli</td>
<td>hog1</td>
<td>Unable to form filamentous growth; non-pathogenic; impaired pigmentation; increased sensitivity to hyperosmotic stress and oxidative stress</td>
<td>Duan et al. (2013)</td>
</tr>
<tr>
<td>Sclerotinia sclerotiorum</td>
<td>hog1::HOG1 and hog1::C14 in the dark</td>
<td>Increased resistance to dicarboximides, phenylpyrroles (iprodione and procymidone), a phenylpyrrole (fludioxonil) and a triazole (tebuconazole) Sigma-Aldrich.</td>
<td>Thind, 2011; Weber &amp; Hahn, 2011</td>
</tr>
<tr>
<td>P. nodorum</td>
<td>hog1::HOG1 and hog1::C14 in the dark</td>
<td>Increased resistance to dicarboximides, phenylpyrroles (iprodione and procymidone), a phenylpyrrole (fludioxonil) and a triazole (tebuconazole) Sigma-Aldrich.</td>
<td>Thind, 2011; Weber &amp; Hahn, 2011</td>
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</table>

**Genetic complementation.** *P. nodorum* Δhog1.20 and Δnik1.18 were selected for genetic complementation. For *HOG1*, a 3931 bp region containing the gene, native promoter and terminator regions was amplified using Hog1FC and Hog1RC. The resulting DNA fragment was fused to a hygromycin resistance gene (HPH) derived from pAN7-r using fusion PCR (Fig. S2a). For NIK1, a 6317 bp region containing the gene, native promoter and terminator regions was amplified using Nik1FC and Nik1RC. The resulting DNA fragment was fused to HPH (Fig. S2a). All PCR amplifications were performed with Phusion Taq DNA polymerase. Primer sequences for vector construction are shown in Table S1. Genetic complementation vectors were used to transform the respective gene deletion mutants using PEG-mediated transformation (Solomon et al., 2004). Mutants that carried the appropriate gene deletion were identified using PCR (Fig. S1c). A robust quantitative PCR method was used to determine the copy number of HOG1 and NIK1 deletion constructs in all transformants to identify appropriate mutants with single copy integration (Fig. S1c) (Solomon et al., 2008). Gene deletion mutants and an ectopic strain (Ect) with single copy integration were retained for further studies. All fungal strains used in this study are described in Table 2.

**Fungicide tolerance assay.** A 96-well microtitre plate assay was used to assess fungicide resistance (Thind, 2011; Weber & Hahn, 2011). Four fungicides from three classes were tested: two dicarboximides (iprodione and procymidone), a phenylpyrrole (fludioxonil) and a triazole (tebuconazole) Sigma-Aldrich. With each strain, 5 µl spores (10^6 spores ml^-1) for the respective mutants/controls was added to minimal medium (MM) broth for a final volume of 100 µl per well (Solomon et al., 2004). Each well was supplemented with a fungicide pre-dissolved (in ethanol) with concentrations ranging from 0 µg ml^-1 to the maximum workable for the respective fungicides. Growth was measured as the change in optical density at a wavelength of 450 nm from the initial measurement to the final measurement at day 6 post-inoculation using a Synergy HT multi-detection microplate reader (BioTek). Plates were wrapped in parafilm to maintain sterility and incubated at 22°C in the dark. Measurements were taken as the average of nine readings across the area of each well. The experiment was performed with four biological replicates. For each, regression lines were calculated from plots of log(fungicide concentration) versus log(% inhibition). This allowed an estimation of EC₅₀ values (fungicide concentration where growth is inhibited by 50%), which were used to calculate the resistance factor (RF) for Δhog1/nik1 mutants relative to SN15. Where inhibition of growth was not observed for a particular strain, the highest concentration of fungicide tested was used for calculating this value with resulting RF values reported as ‘>x’ where x represents the RF.

**Hyperosmotic and oxidative stress assays.** The ability of the mutants to grow under hyperosmotic and oxidative stress...
conditions was assessed through a microtitre assay set up essentially as described above, but without fungicides. Following Lowe et al. (2008), 0.25, 0.50, 0.75 and 1.00 M NaCl was used for the hyperosmotic stress assay. For the oxidative stress microtitre assay, all strains were inoculated with 0, 1, 3 and 10 mM H₂O₂.

**6-Methoxy-2-benzoxazolinone (MBOA) tolerance assay.** A growth assay was employed to determine the impact of MBOA on the growth of all strains. MM agar plates were supplemented with 0, 0.1, 0.3, 1.0 and 3.0 mM MBOA (Sigma-Aldrich) dissolved in 0.4 % (v/v) ethanol (Du Fall & Solomon, 2013). Following this, plates were inoculated with 1 x 10⁶ spores. The impact of MBOA on fungal growth was determined by measuring colony diameters following 9 days growth at 22 °C under fluorescent light on a 12 h light/dark cycle. The assay was performed in biological triplicates.

**Whole plant spray infection assay.** Virulence was determined using a whole plant spray method as previously described, with minor modifications (Solomon et al., 2005a). Briefly, an inoculum consisting of 1 x 10⁶ spores ml⁻¹ in 0.5 % gelatin (Sigma-Aldrich) was sprayed onto 2-week-old wheat seedlings (Calingiri; InterGrain) via an airbrush system. Disease was allowed to develop in a growth chamber at 22 °C on a 12 h light/dark cycle for 10 days under high humidity prior to scoring. For the whole plant spray assay at an elevated temperature, infection was allowed to develop for 5 days at 22 °C on a 12 h light/dark cycle under high humidity. A temperature cycle of 28 °C for 8 h, followed by 22 °C for 16 h, on a 12 h light/dark cycle under high humidity for 9 days, was used. The first 8 h of the light cycle coincided with the elevated temperature. Following the whole plant spray assessment, five infected first leaves representing each infection were removed and maintained on 0.15 % benzimidazole agar as detached leaves for 14 days for infection at alternating 22 and 28 °C (Solomon et al., 2004). This allowed for an assessment of pycnidiation in planta. Images representing the extent of pycnidia formation were taken with a Nikon SMZ 800 stereoscope coupled to a DS-L3 controller.

**RESULTS**

### Identification of the putative HOG1 MAPK and NcNIK1/OS-1 class HK in *P. nodorum*

The Hog1 MAPK protein sequence from *S. cerevisiae* (Genbank accession no. CAA97680) was used to search the *Phaeosphaeria nodorum* SN15 Genbank nr protein database and identified SNOG_13296 (Genbank accession no. Q0U4L8) as the best BLAST hit. The predicted SNOG_13296 polypeptide sequence consisted of 355 aa encoded by a 1475 bp gene comprising seven exons and six introns. Conserved domain (CD)-BLAST identified a protein kinase domain (pfam00069) from amino acid 20 to 299 (Fig. S3a). Using the filamentous fungal MAPK classification nomenclature described by Xu (2000), phylogenetic analysis classified the SNOG_13296 polypeptide to be a genuine Hog1 MAPK orthologue (Fig. S4). Amino acid identity of the SNOG_13296 polypeptide to characterized Hog1 MAPKs of fungal phytopathogens ranged from 91 % with Osm1 of the rice blast fungus *Magnaporthe oryzae* (Dixon et al., 1999) to 97 % with Hog1 of the southern corn blight pathogen *Cochliobolus heterostrophus* (Yoshimi et al., 2005).

The group III hybrid HK NcNik1/OS-1 from *Neurospora crassa* (Broad Institute accession no. NCU02815.7) identified SNOG_11631 (Genbank accession no. XP_001801869) as the best BLAST hit. The corrected ORF and amino acid sequence of SNOG_11631 were located at: https://github.com/robsyme/Parastagonospora_nodorum_SN15. CD-BLAST analysis identified four pfam domains: a HAMP domain (pfam00672) from amino acid 286 to 630, a phospho-acceptor domain from amino acid 740 to 804 (pfam00512), an HK-like ATPase domain (pfam02518) from amino acid 851 to 965, and a RR domain from amino acid 1117 to 1232 (pfam00072) (Fig. S3b). The domain prediction of SNOG_11631 is consistent with that of the group III hybrid HKs (Catlett et al., 2003). Using the fungal hybrid HK classification nomenclature proposed by Catlett et al. (2003), phylogenetic analysis further classified the SNOG_11631 polypeptide to be a genuine group III hybrid HK (Fig. S5). Amino acid identity of the SNOG_11631 polypeptide to characterized group III hybrid HKs ranged from 66 % for the grey mould fungus *Botrytis cinerea* (Liu et al., 2008) to 88 % for *C. heterostrophus* (Yoshimi et al., 2004). From here, SNOG_13296 and the revised SNOG_11631 genes are referred to as HOG1 and NIK1, respectively.

### Disruption of NIK1 and HOG1 in *P. nodorum*

To determine the role of the HK-Hog1 MAPK pathway in *P. nodorum* development and pathogenicity, we inactivated NIK1 and HOG1 in the wild-type strain SN15 using targeted gene deletion. Consequently, strains deleted in NIK1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td>SN15</td>
<td>Wild-type</td>
<td>Department of Agriculture and Food Western Australia, Western Australia, Australia</td>
</tr>
<tr>
<td>Ect</td>
<td>Ectopic transformant (BLE ectopic integration)</td>
<td>This study</td>
</tr>
<tr>
<td>Anik1.18</td>
<td>SN15 deleted in SNOG_11631 (NIK1)</td>
<td>This study</td>
</tr>
<tr>
<td>Anik1.36</td>
<td>SN15 deleted in SNOG_11631 (NIK1)</td>
<td>This study</td>
</tr>
<tr>
<td>Ahog1.20</td>
<td>SN15 deleted in SNOG_13296 (HOG1)</td>
<td>This study</td>
</tr>
<tr>
<td>Ahog1.29</td>
<td>SN15 deleted in SNOG_13296 (HOG1)</td>
<td>This study</td>
</tr>
<tr>
<td>Anik1 :: NIK1</td>
<td>Δnik1.18 complemented with SNOG_11631 (NIK1)</td>
<td>This study</td>
</tr>
<tr>
<td>Ahog1 :: HOG1</td>
<td>Δhog1.20 complemented with SNOG_13296 (HOG1)</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Table 2. Fungal strains used in this study**

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(Δnik1.18 and Δnik1.36), HOG1 (Δhog1.20 and Δhog1.29) and an ectopic (Ect) mutant were retained for phenotypic characterization (Fig. S1, Table 2). The Ect mutant contained a selectable marker insertion elsewhere in the genome rather than in NIK1 and HOG1; thus, all assayed phenotypes should be similar to SN15.

**HOG1 and NIK1 play a role in osmotic stress tolerance**

Reduction in osmotic stress tolerance is a hallmark of perturbation in the Hog1 MAPK signalling pathway (Table 1). The osmotolerance of *P. nodorum* Hog1 MAPK pathway mutants was assessed by measuring growth in NaCl concentrations ranging from 0.25 to 1 M, as described by Lowe et al. (2008), in a microtitre assay (Fig. 1). To our surprise, SN15 and Ect exhibited a slight increase in growth between 0.25 and 0.75 M NaCl. All strains exhibited similar growth at 0.25 M NaCl. The growth of Δhog1.20 and Δhog1.29 was inhibited at 0.5 M, whereas growth of Δnik1.18 and Δnik1.36 was significantly reduced at 0.75 M. No growth of Δhog1.20, Δhog1.29, Δnik1.18 and Δnik1.36 was seen at 1 M NaCl, while both SN15 and Ect grew to some extent. The results indicate that NIK1 and HOG1 are required for osmotolerance (Fig. 1).

**HOG1 and NIK1 are dispensable for tolerance to oxidative stress**

Inactivation of the fungal Hog1 MAPK signalling pathway often resulted in increased sensitivity to oxidative stress (Table 1). The ability of *P. nodorum* Hog1 pathway mutants to tolerate oxidative stress was assessed by measuring growth in 0 to 1 mM H₂O₂ in a microtitre assay (Fig. S6). Growth of *P. nodorum* SN15 was observed at 0 and 1 mM H₂O₂, but was greatly inhibited at 3 mM and 10 mM H₂O₂. *P. nodorum* Δhog1 and Δnik1 strains did not exhibit increased sensitivity to H₂O₂ compared to strains with intact HOG1 and NIK1 (Fig. S6).

Δnik1 and Δhog1 mutants showed increased resistance to dicarboximide and phenylpyrrole fungicides

Fungal strains perturbed in Hog1 signalling often exhibit resistance to dicarboximide (e.g. iprodione and procymidone) and phenylpyrrole (e.g. fludioxonil) fungicides (Table 1). Microtitre plate growth rates of SN15, Ect, Δhog1.20, Δnik1.18 and complemented strains on various concentrations of iprodione, procymidone, fludioxonil and a control fungicide, tebuconazole, were determined (Table 3, Fig. S7). Both Δhog1.20 and Δnik1.18 displayed high RF values to both dicarboximide and phenylpyrrole fungicides. The EC₅₀ values of the deletion mutants were higher than the maximum concentration of iprodione and procymidone used (10 µg ml⁻¹). Similarly, the EC₂₀ values of the deletion mutants were higher than the maximum concentration of fludioxonil used (5 µg ml⁻¹). We then genetically complemented Δhog1.20 and Δnik1.18. Genetic complementation restored sensitivity to iprodione, procymidone and fludioxonil. No resistance to tebuconazole was observed (Table 3, Fig. S7). This indicates that dicarboximide and phenylpyrrole fungicides inappropriately activate the HOG1 pathway in *P. nodorum*.

**HOG1 deletion limits *P. nodorum* growth and pycnidiation at elevated temperatures in vitro**

During field infection, it is likely that *P. nodorum* experiences changes in the environment, such as temperature fluctuations, and must adapt. The effect of elevated temperatures was investigated to explore further abiotic stress responses associated with NIK1 and HOG1 deletion in *P. nodorum*. Mycelial growth was compared following incubation at 24, 28 and 32 °C for 10 days on V8-PDA agar medium. Vegetative growth of all strains was similar at 24 and 28 °C. However, *P. nodorum* Δhog1.20 and Δhog1.29 were unable to grow at 32 °C (Fig. 2a). Furthermore, hypersensitivity to heat stress in the Δhog1 background was already apparent at 28 °C, as pycnidial development was sparse when compared to SN15 (Fig. 2b). Growth and pycnidiation of the Δnik1 mutants were similar to SN15 (Fig. 2). This indicates that HOG1, but not NIK1, is required for tolerance to heat stress in vitro. Genetic complementation of Δhog1.20 restored the SN15-like vegetative growth and pycnidiation (Fig. S8).
Deletion of HOG1 increases tolerance to the cereal phytoalexin MBOA

The cereal phytoalexin MBOA inhibits the growth of P. nodorum at high concentrations (Du Fall & Solomon, 2013). A petri dish assay was used to determine the radial growth of SN15, Ect, Δhog1.20, Δnik1.18 and genetically complemented Δhog1 :: HOG1 and Δnik1 :: NIK1 in the presence of MBOA at different concentrations (Fig. 3a). Surprisingly, Δhog1.20, but not Δnik1.18, demonstrated increased tolerance to MBOA at 3.0 mM (Fig. 3b).

NIK1 and HOG1 deletion reduces asexual sporulation in vitro

P. nodorum produces asexual fruiting bodies called pycnidia. These fruiting bodies contain pycnidiospores, which are required by the fungus to facilitate secondary host infection (Eyal et al., 1987). We sought to investigate for evidence of fitness reduction of pycnidia in the Δhog1 and Δnik1 mutants by quantifying the level of asexual sporulation in vitro (Fig. 4). All mutants produced significantly fewer pycnidiospores than SN15. P. nodorum Δhog1.20 and Δhog1.29 produced significantly less spores than Δnik1.18 and Δnik1.36.

HOG1 but not NIK1 is required for full pycnidiation in planta

In vitro characterization of Δhog1 and Δnik1 mutants has identified the corresponding genes, HOG1 in particular, to be important in P. nodorum development and stress tolerance. With this in mind, we sought to investigate expression of HOG1 and NIK1 in SN15 during infection using qRT-PCR. Time points reflecting early infection/host penetration (3 dpi), proliferation (6 dpi) and asexual sporulation/pycnidiation (8 and 10 dpi) were chosen (Fig. 5). The level of NIK1 expression was found to remain constant through the sampled infection period. In contrast, HOG1 expression increased approximately threefold during proliferation, leading to the onset of pycnidiation. Since NIK1 and HOG1 are expressed in planta, we then assessed whether these genes play a role in the infection lifecycle of P. nodorum on wheat using a whole plant spray assay at 22 °C. It was observed that P. nodorum Δhog1.20, Δhog1.29, Δnik1.18 and Δnik1.36 caused necrotic lesions on wheat that were comparable to SN15 (Fig. 6a). However, HOG1 deletion abolished pycnidiation and consequently asexual sporulation in planta (Fig. 6b). This suggests that HOG1, but not NIK1, is required to complete asexual development through pycnidiation during wheat infection.

Fig. 2. Growth of all strains at elevated temperatures. (a) Colony morphology and (b) pycnidia development were examined.
As HOG1 is required for tolerance to elevated temperatures in vitro, a whole plant spray assay was undertaken at an elevated temperature to determine whether heat stress affects the virulence of the \( \Delta \text{hog1} \) mutants. A temperature of 28°C was chosen for infection, as \( \Delta \text{hog1} \) mutants first showed a compromised heat-stress-related phenotype at this temperature. \( P. \text{nodorum} \ \Delta \text{hog1.20} \) was able to cause lesions on the host similar to SN15 (Fig. S9a). Surprisingly, \( \Delta \text{hog1.20} \) was able to produce pycnidia in planta at 28°C, unlike the 22°C infection (Fig. S9b), but at a much reduced number (Fig. S9c). Thus, increased temperature partially complements the pycnidiation defect of \( \Delta \text{hog1.20} \) during growth in planta.

**DISCUSSION**

Thus far, the FUS3/KSS1 MAPK class MAK2 is the only MAPK gene that has been characterized in \( P. \text{nodorum} \) for its role in virulence and downstream target regulation (Solomon et al., 2005b; Tan, 2007). Analysis of the \( P. \text{nodorum} \) genome identified two other genes that encode putative MAPKs. One of these is HOG1/\text{SNOG}_13296, which belongs to the HOG1 MAPK class.

### Table 3. EC\(_{50}\) (\( \mu \text{g ml}^{-1} \)) and RF values of the fungicide tolerance assay

<table>
<thead>
<tr>
<th>Strain</th>
<th>Iprodione EC(_{50})</th>
<th>Iprodione RF vs SN15</th>
<th>Procymidine EC(_{50})</th>
<th>Procymidine RF vs SN15</th>
<th>Fludioxonil EC(_{50})</th>
<th>Fludioxonil RF vs SN15</th>
<th>Tebuconazole EC(_{50})</th>
<th>Tebuconazole RF vs SN15</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN15</td>
<td>1.84</td>
<td>1.00</td>
<td>2.06</td>
<td>1.00</td>
<td>0.10</td>
<td>1.00</td>
<td>1.38</td>
<td>1.00</td>
</tr>
<tr>
<td>Ect</td>
<td>1.66</td>
<td>0.90</td>
<td>2.15</td>
<td>1.04</td>
<td>0.20</td>
<td>1.98</td>
<td>1.28</td>
<td>0.93</td>
</tr>
<tr>
<td>( \Delta \text{hog1.20} )</td>
<td>10*</td>
<td>&gt;5.44</td>
<td>10*</td>
<td>&gt;4.86</td>
<td>5*</td>
<td>&gt;48.77</td>
<td>1.66</td>
<td>1.21</td>
</tr>
<tr>
<td>( \Delta \text{nik1.18} )</td>
<td>10*</td>
<td>&gt;5.44</td>
<td>10*</td>
<td>&gt;4.86</td>
<td>5*</td>
<td>&gt;48.77</td>
<td>2.07</td>
<td>1.50</td>
</tr>
<tr>
<td>( \Delta \text{hog1} :: \text{HOG1} )</td>
<td>1.41</td>
<td>0.76</td>
<td>1.55</td>
<td>0.76</td>
<td>0.80</td>
<td>0.74</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>( \Delta \text{nik1} :: \text{NIK1} )</td>
<td>0.63</td>
<td>0.17</td>
<td>0.48</td>
<td>0.23</td>
<td>0.01</td>
<td>0.05</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*EC\(_{50}\) is greater than the measurable concentration used; therefore, the highest concentration of fungicide tested was used for RF calculation. The microtitre growth curves used for calculation.

**Fig. 3.** Growth of all strains on MBOA. (a) Colony diameter was determined from 9 days growth on MM agar supplemented with different MBOA concentrations. The experiment was performed in biological triplicates. ANOVA using the Tukey-Kramer test set at a stringent significance threshold of \( P<0.0005 \) was used to compare the radial growth of all strains. The error bars show SEMs. When indicated by the same number of asterisks above the bars, the mean was not found to be significantly different between strains in each treatment. (b) Colony morphology of all strains grown on 3.0 mM MBOA.
class. Analysis of the *P. nodorum* genome has also uncovered genes that encode amino acid sequence orthologues to yeast Ssk1 MAPKKK (SNOG_09580), Ssk2 MAPKK (SNOG_07196), Ste11 MAPKK (SNOG_07664) and Pbs2 MAPKK (SNOG_02007). We have also identified a putative SLT2 MAPK class gene, SNOG_05764, which encodes a 420 aa polypeptide (Fig. S4). SNOG_05764 is referred to as MPS1.

The role of MPS1 in phytopathogenicity is currently being investigated in our laboratory.

Analysis of the *P. nodorum* SN15 genome identified a large family of potential two-component systems that consisted of 23 HKs, 3 RRs and 1 Hpt (Hane et al., 2007). Analysis of the *C. heterostrophus* genome identified 21 HKs, 3 RRs and 1 Hpt, whereas *N. crassa* possesses 11 HKs, 2 RRs and 1 Hpt (Catlett et al., 2003). Catlett et al. (2003) identified 11 major groups based on phylogenetic analysis of predicted hybrid HKs from *C. heterostrophus, N. crassa, B. cinerea* and *Fusarium verticillioides*. All 11 groups possess predicted phosphoacceptor, ATPase and response-regulator domains. Phylogenetic analysis placed the predicted *P. nodorum* hybrid HKs into the 11 major groups (Fig. S5). NIK1/SNOG_11631 is the only *P. nodorum* gene classified within the group III hybrid HK.

HK signalling is often transduced via the Hog1 MAPK pathway in most eukaryotes. In many fungi, perturbation in group III HK and Hog1 often results in overlapping phenotypes, such as a loss in osmotolerance and increased resistance to dicarboximide and phenylpyrrole fungicides (Table 1). Therefore, we hypothesized that Nik1 function in close association with the Hog1 MAPK pathway in *P. nodorum*. We have generated *P. nodorum* strains that carry gene deletions of two putative components of the HOG1 pathway: a putative group III hybrid HK Nik1 and the Hog1 class MAPK Nik1. Mutants carrying deletions in NIK1 and HOG1 showed increased resistance to both dicarboximide and phenylpyrrole antifungal agents consistent with many other fungal pathogens (Table 1). Phenotypic defects relating to asexual sporulation and hyperosmotic stresses were significantly more pronounced in the Δhog1 than the Δnik1 mutants. Furthermore, Δhog1 mutants, but not Δnik1 mutants, displayed increased sensitivity to heat stress. This study has demonstrated both distinct and overlapping roles of group III HK and HOG1 MAPK in *P. nodorum* that are somewhat similar to the phenomenon described by Lin & Chung (2010) for AaHSK1 and AaHOG1 deletion mutants in *A. alternata* (Table 1).

Since the HOG1 MAPK-MAPKK-MAPKKK cascade is genetically intact in the Δnik1 background, it is highly possible that the pathway is associated with another regulatory mechanism to compensate for NIK1 deletion under different developmental or stress conditions. In yeast, the Sho1p transmembrane osmosensor receptor activates the HOG1 MAPK pathway through an alternate MAPKKK, Ste11p (Maeda et al., 1995). It is not known what the putative regulator(s) is and where it relays its signal along the putative HOG1 MAPK pathway in *P. nodorum*. Yoshimi et al. (2004) demonstrated that the group III HK Dic1 positively regulates Hog1 signalling in *C. heterostrophus*. However, the HOG1 pathway remained active in the absence of Dic1. Furthermore, a group VI HK SLN1 possesses overlapping roles with HK1 (group III HK) as upstream regulators of the HOG1 pathway in *M. oryzae* (Jacob et al., 2014; Zhang et al., 2010). BLAST analysis identified SNOG_11562 as the only group VI HK in *P. nodorum*.

We demonstrated that the Δhog1 mutants possess increased sensitivity to heat stress. The role of the Hog1 pathway in the heat-stress response was first described in *S. cerevisiae* (Winkler et al., 2002). It has been shown that HOG1 is activated during heat stress. In the vascular wilt fungus *Fusarium proliferatum*, conidia of the Δhog1 mutants exposed to heat stress showed reduced viability (Adam et al., 2008b).

![Fig. 4.](image1.png) **Fig. 4.** *In vitro* sporulation assay on V8-PDA. ANOVA using the Tukey–Kramer test set at a significance threshold of *P* ≤ 0.05 was used to compare sporulation of all strains. The error bars show SEMs. When indicated by the same number of asterisks above the bars, the mean was not found to be significantly different between strains.

![Fig. 5.](image2.png) **Fig. 5.** The expression profile of NIK1 and HOG1 in planta. The error bars show SEMs. The experiment was performed in biological triplicates.
through the HOG1 pathway via an alternate unknown regulator or an alternate pathway independent of Nik1 in P. nodorum. Similarly, increased tolerance to MBOA is a phenotype unique to the HOG1 deletion background. The P. nodorum necrotrophic effector ToxA induces MBOA production (Du Fall & Solomon, 2013). Although the biochemical target and mode of action of MBOA are yet to be fully deduced, the phytoalexin alters central carbon metabolism in P. nodorum (Du Fall & Solomon, 2013). Since HOG1 deletion increases tolerance to MBOA, the mutation presents an ideal tool for functional characterization of resistance. In wheat crown rot fungus, Fusarium graminearum, an N-malonyltransferase encoded by the FDB2 gene is required for the detoxification of benzoxazolinones (Kettle et al., 2015). However, reciprocal BLAST analysis indicates that P. nodorum does not possess an FDB2 orthologue (Gardiner et al., 2012). Therefore, P. nodorum must employ another mechanism for detoxifying MBOA. To our knowledge, this study is a first to examine the effect of a phytoalexin on fungal mutants that are defective in HOG1 signalling.

P. nodorum produces fruiting bodies called pycnidia during its asexual state. Pycnidiospores are produced and naturally dispersed via rain splashes to nearby healthy plant tissues to facilitate secondary infection (Eyal et al., 1987; Eyal, 1999). We have demonstrated that Nik1 and HOG1 play a vital role in asexual sporulation. P. nodorum Δhog1 mutants, in particular, produced significantly less spores than SN15 and Δnik1 mutants under axenic conditions. This effect is much more pronounced during infection with the Δhog1 mutants where pycnidial production was either abolished at 32 °C or severely reduced at 28 °C. The Δhog1 mutants are generally more susceptible to environmental stresses than SN15 and Δnik1, as demonstrated in this study. A probable explanation is that the Δhog1 mutants may have encountered a combination of stressors during growth on the host plant that is sufficient to antagonize asexual sporulation through the formation of fruiting bodies in planta as opposed to favourable conditions presented in axenic cultures. This suggests that HOG1 is required by P. nodorum to complete its natural pathogenic lifecycle through asexual sporulation.

The molecular mechanism of asexual sporulation in P. nodorum is a subject of strong investigations. Previous reverse genetic studies have demonstrated the requirement of signalling pathways, mannitol and trehalose metabolism in asexual sporulation (Gummer et al., 2012; IpCho et al., 2010; Li et al., 2008; Lowe et al., 2009; Solomon et al., 2004, 2005a, c, 2006b). Deletion of heterotrimeric G-protein-regulated putative short-chain dehydrogenase genes SCH1 and SCH2 resulted in reduced sporulation (Casey et al., 2010; Tan et al., 2008). In the case of SCH1 deletion, pycnidial maturation was blocked (Tan et al., 2008). Intriguingly, sporulation in vitro is promoted by the supplementation of γ-aminobutyric acid, a metabolite that accumulates in stressed plants (Mead et al., 2013). It is possible that the reduction in asexual sporulation observed for the Δnik1 and Δhog1 mutants is a pleotropic phenotype resulting from the loss of physiological coordination at the biochemical level.

Inactivation of the HOG1 pathway in many phytopathogenic fungi results in an increase in resistance to dicarboximides and phenylpyrroles (Table 1). In this study,
perturbation of HOG1 signalling in *P. nodorum* resulted in increased resistance to a phenylpyrrole and dicarboximide fungicides. Dicarboximides and phenylpyroles are contact non-systemic site-specific fungicides that are used to control a narrow spectrum of fungal diseases caused by *Botrytis* spp. and the closely related genera *Monilinia* and *Sclerotinia* (Dettweiler et al., 1983; Yoshimura et al., 2004). Point mutations in the group III HK accounted for some cases of dicarboximide resistance, observed in field fungal isolates exhibiting low to medium levels of resistance to iprodione. For instance, widespread resistance to iprodione was observed in *B. cinerea* isolated from strawberries (Grabke et al., 2014). Point mutations in the group III HK gene BOS1, efflux pump activity and unknown mechanisms accounted for the different level of resistance observed. Flu-dioxonil resistance in field fungal isolates is commonly associated with up-regulation of multidrug ABC transporters (Hahn & Leroch, 2015), although laboratory-generated fungal mutants clearly demonstrated the role of HOG1 signalling in resistance (Table 1). The primary target site has not been clearly identified; however, it is thought that these fungicides exert their influence on the group III HKs through the HAMP domain (Fujimura et al., 2015).

The role of the osmotolerance pathway in fungal virulence has been extensive investigated. In many cases, perturbation of the pathway resulted in a drastic reduction or loss of fungal virulence (Table 1). Mutants perturbed in the pathway often demonstrated abnormalities in vegetative morphogenesis, such as abnormal appressoria development (Igbaria et al., 2008), altered fungal cell wall (Jacob et al., 2014), or in ability to form invasive filamentous growth (Mehrabi et al., 2006). These pleiotropic effects may contribute to the loss in pathogenic fitness. In this study, deletions in NIK1 and HOG1 did not affect the ability of *P. nodorum* to cause lesions on wheat; thus, indicating a dispensable role of the pathway in virulence. This is similarly observed in *Bipolaris oryzae*, *P. proliferatum* and *Colletotrichum lagenarium* deleted in HOG1 (Adam et al., 2008a; Kojima et al., 2004; Moriwaki et al., 2006), and *Sclerotinia sclerotiorum* deleted in group III HK orthologues (Duan et al., 2013). In one instance, deletion of AlHK1 (a NIK1 group III HK orthologue) in the tobacco pathogen *Alternaria longipes* conferred an increase in virulence (Duan et al., 2013). Thus, the HOG1 pathway plays both overlapping (e.g. osmotolerance) and diverse roles across different pathosystems.

Perturbation of group III HK and components of the Hog1 MAPK pathway often resulted in an increase in sensitivity to oxidative stress (Table 1). However, this was not the case with *P. nodorum* strains deleted in HOG1 and NIK1. Thus, *P. nodorum* uses an alternate pathway to sense and adapt to oxidative stress. It was previously observed that AaHSK1 from *A. alternata* and AbNIK1 from the black spot fungus *Alternaria brassicicola* do not play a role in oxidative stress tolerance (Cho et al., 2009; Lin & Chung, 2010).

Despite the wealth of studies, there is a lack of knowledge of downstream targets associated with the HOG1 pathway in phytopathogenic fungi that could explain phenotypic changes associated with pathway inactivation. In *B. cinerea*, a macroarray approach designed from expressed sequenced tags was used to analyse the gene expression profile of a mutant disrupted in BcSAK1, a HOG1 MAPK gene (Heller et al., 2012). Similar to *P. nodorum* HOG1, BcSAK1 is required for abiotic stress tolerance and conidiation. Interestingly, most genes regulated by BcSAK1 are not associated with stress response. Instead, genes that are associated with the production of phytotoxins secondary metabolites were significantly down-regulated. Furthermore, HOG1 in *F. graminearum* regulates the production of the mycotoxins deoxynivalenol and zearalene (Nyugen et al., 2012). Analysis of the *P. nodorum* genome identified genes encoding 23 polyketide synthases (PKSs), 14 non-ribosomal peptide synthetases (NRPSs), 1 PKS-NRPS hybrid, 3 sesquiterpene synthases and 1 diterpene synthase (Chooi et al., 2014). These genes are often associated in functional gene clusters. This highlights the huge potential for secondary metabolite production in *P. nodorum*. Recent studies have begun to link biologically active secondary metabolites to genes (Chooi et al., 2015a, b; Tan et al., 2009c). It remains to be seen whether the HOG1 pathway regulates secondary metabolism in *P. nodorum* under the appropriate conditions. Therefore, the Δhog1 and Δnik1 mutants generated in this study are ideal tools that can be used to study global regulation. As such, we are currently performing comprehensive analyses of these mutants using RNA sequencing, proteomics, phosphorylation assays and metabolomics to provide a deeper insight to the regulation of Hog1 signalling and its downstream targets. This will allow the dissection of physiological components that contributed to phenotypic impairments, explain differences between both mutants and uncover novel aspects of fungal metabolism in a devastating necrotrophic pathogen of wheat. In the past, these approaches were used to successfully identify downstream (direct and indirect) targets of Fus3/Kss1 MAPK, Stu1 transcription factor and heterotrimeric G-protein signalling in *P. nodorum* (Casey et al., 2010; Guummer et al., 2013; IpCho et al., 2010; Tan, 2007; Tan et al., 2008, 2009a). An establishment of the ‘regulome’ in *P. nodorum* will significantly bolster our understanding of the link between signal transduction and the pathogenic lifecycle.

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