The Sclerotinia sclerotiorum Slt2 mitogen-activated protein kinase ortholog, SMK3, is required for infection initiation, but not lesion expansion

Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2016-0091.R2
Manuscript Type:	Article
Date Submitted by the Author:	24-May-2016
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Keyword:	Sclerotinia sclerotiorum, mitogen activated protein kinase, infection, cell wall integrity

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1 **The *Sclerotinia sclerotiorum* Slt2 mitogen-activated protein kinase ortholog, *SMK3*, is required**
2 **for infection initiation, but not lesion expansion**

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Abstract

Mitogen activated protein kinases (MAPKs) play a central role in transferring signals and regulating gene expression in response to extracellular stimuli. An ortholog of the *Saccharomyces cerevisiae* cell wall integrity MAPK was identified in the phytopathogenic fungus *Sclerotinia sclerotiorum*. Disruption of the *S. sclerotiorum Smk3* gene severely reduced virulence on intact host plant leaves, but not on leaves stripped of cuticle wax. This was attributed to alterations in hyphal apical dominance leading to the inability to aggregate and form infection cushions. The mutation also caused loss of the ability to produce sclerotia, increased aerial hyphae formation, altered hyphal hydrophobicity and cell wall integrity. Mutants had slower radial expansion rates on solid media, but more tolerant to elevated temperatures. Loss of the SMK3 cell wall integrity MAPK appears to have impaired the ability of *S. sclerotiorum* to sense its surrounding environment leading to misregulation of a variety of functions. Many of the phenotypes were similar to those observed in *S. sclerotiorum* adenylate cyclase and SMK1 MAPK mutants suggesting that these signalling pathways interact to co-regulate aspects of fungal growth, physiology and pathogenicity.

Key Words

Sclerotinia sclerotiorum, mitogen activated protein kinase, infection, cell wall integrity.

46 Introduction

47 *Sclerotinia sclerotiorum* is an aggressive, cosmopolitan plant pathogen that infects over 400
48 different species (Boland and Hall 1994). Though able to germinate on the host cuticle surface, *S.*
49 *sclerotiorum* ascospores are generally unable to directly infect many hosts (Lumsden 1979). Often,
50 senescent materials, such as shed petals lodged in the axis between the main stem and branch as
51 in *Brassica napus*, provide a source of water and nutrients to generate sufficient mycelial biomass
52 to initiate infection (Abawi et al. 1975; Lumsden and Dow 1973). Upon contact with the host,
53 mycelia form finger-like appendages that develop into multi-celled, dome-shaped structures
54 referred to as infection cushions (Abawi et al. 1975). Following cuticle penetration, a short
55 biotrophic phase allows the pathogen to establish within the host (Kabbage et al. 2015). Mycelia
56 then ramify throughout host tissue, assisted by the release of hydrolytic enzymes (Martel et al.
57 1996; Poussereau et al. 2001a, b; Kasza et al. 2004; Li et al. 2004; Bashir et al. 2012; Zhang et al.
58 2014), oxalic acid (Godoy et al. 1990) and necrosis-inducing proteins (Bashir et al. 2010a; Bashir et
59 al. 2013). Consumption of necrotic host tissue provides the resources needed for sclerotial
60 development and completion of the life cycle. The transition between these phases is determined
61 by numerous signals, including contact with hard surfaces (Bashir et al. 2012), glucose levels
62 (Vautard-Mey et al. 1999), cAMP levels (Rollins and Dickman 1998; Girard et al. 2004; Jurick and
63 Rollins 2007), ambient pH (Rollins and Dickman 2001; Cotton et al. 2003; Rollins 2003; Favoron et
64 al. 2004), and oxidative stress (Kim et al. 2011; Papapostolou et al. 2014). Furthermore,
65 coordination of these signaling pathways may involve various protein kinases (Vautard-Mey and
66 Fevre 2000; Vacher et al. 2003; Chen et al. 2004; 2005; Jurick et al. 2004; Harel et al. 2005;
67 Erental et al. 2008; Bashir et al. 2010b; Duan et al. 2013) and phosphatases (Harel et al. 2006;
68 Erental et al. 2007). An increasing number of extra-cellular effectors have also been shown to
69 affect host-pathogen interactions and disease progression (Liang et al. 2013; Zhu et al. 2013;
70 Guyon et al. 2014; Xiao et al. 2014; Zhang et al. 2014; Lyu et al. 2015, 2016; Pan et al. 2015).
71
72 Extracellular-signal-regulated kinases (ERKs), also known as classical mitogen-activated protein
73 kinases (MAPK), are components of pathways that transfer signals from the external environment
74 to the nucleus. Such signals initiate a cascade of phosphorylation events that eventually impact
75 the transcription control apparatus. MAPK are activated by phosphorylation by upstream MAPK
76 kinases. Five main MAPK pathways have been characterized in *Saccharomyces cerevisiae*; these
77 are described as the mating pheromone response pathway, the filamentation-invasion pathway,

the spore wall assembly pathway, the high osmotic growth pathway and the cell wall integrity pathway (Gustin et al. 1998). MAPK pathways with orthologous elements also exist in many fungal pathogens, though in some cases they have evolved to regulate different biological processes (Xu 2000). For example, MAPKs encoded by the orthologs of *S. cerevisiae* *Fus3* (pheromone) and *Kss1* (filamentation-invasion) genes, including *Botrytis cinerea* BMP1 (Zheng et al. 2000), *Cochliobolus heterostrophus* CHK1 (Lev et al. 1999), *Colletotrichum lagenarium* CMK1 (Takano et al. 2000), *Fusarium oxysporum* FMK1 (Di Pietro et al. 2001), *Fusarium verticillioides* FvMK1 (Zhang et al., 2011), and *Ustilago maydis* UBC3 (Mayorga and Gold 1999), are essential for pathogenesis and are referred to as pathogenicity MAPKs. In *S. sclerotiorum*, the KSS1-related MAPK, SMK1, was also found to play a central role in coordinating signals from pH, cAMP and oxalic acid during the formation of sclerotia (Chen et al. 2004).

The *S. sclerotiorum* orthologs of the *S. cerevisiae* MAPK associated with the cell wall integrity pathway, SLT2, have also been identified in several pathogenic yeasts and filamentous fungi, including *S. sclerotiorum* (Hegedus et al. 2015). *Slr2* ortholog MAPK mutants frequently have an osmotic-remediable lytic phenotype caused by impaired cell wall biogenesis (De Nobel 2000; Levin 2005). In *Candida albicans*, MKC1 has been shown to be essential in sensing contact with surfaces and transitioning to invasive growth (Kumamoto 2005). The *Mkc1* mutant was unable to form filamentous invasive hyphae and was non-pathogenic. In addition, the mutant had increased sensitivity to cell wall degrading enzymes suggesting that cell wall architecture was altered (Navarro-Garcia et al. 1995). Mutation of *Mps1* in *Magnaporthe grisea* caused a range of changes in physiological functions including reduced aerial hyphae growth and conidiation. Colonies also exhibited central progressive autolysis that was recoverable with the addition of 1 M sorbitol to the medium, indicating a deficiency in osmotolerance. Similar to the *C. albicans mkc1* mutant, the *M. grisea Mps1* mutant was also more susceptible to treatment with cell wall degrading enzymes and was avirulent (Xu et al. 1998). In *Fusarium graminearum*, mutation of *MGV1* caused reduced growth on solid medium as well as reduced aerial hyphae formation and conidiation. Mutant colonies also had reduced pigmentation indicating that secondary metabolism was also affected. Similar to those above, this mutant was more susceptible to cell wall degrading enzymes and high temperatures and was less virulent. Interestingly, the mutant was found to be only male fertile with reduced hyphal fusion and heterokaryon formation (Hou et al. 2002).

110 In *B. cinerea*, a close relative of *S. sclerotiorum*, mutation of the *Slt2* ortholog *Bmp3*, caused a
111 severe reduction in aerial hyphae formation and conidiation (Rui and Hahn 2007) similar to the *F.*
112 *graminearum* *MGV1* mutant. Generally, radial expansion of mutant colonies was slower than the
113 wild type and supplementation with nutrients failed to recover the phenotype. Unlike previously
114 characterized mutants in other filamentous fungi, the *B. cinerea* mutant did not exhibit heat
115 sensitivity or susceptibility to cell wall degrading enzymes. Conidial germination was unaffected;
116 however, germ tubes elongated excessively and were unable to penetrate the host. Hyphae also
117 exhibited reduced epidermal cell penetration and fewer lesions formed on intact leaves, though
118 pathogenicity was impacted to a lesser extent on wounded leaves where the cuticle had been
119 damaged (Rui and Hahn 2007). These observations clearly demonstrate that the cell wall integrity
120 MAPK pathway controls diverse physiological functions and is important for pathogenicity;
121 however, different aspects of the infection process are involved dependent upon the pathogen,
122 its lifestyle and the host.
123
124 In this study, the *S. sclerotiorum* *Slt2* ortholog, *Smk3*, was characterized and a wide range of
125 primary and secondary effects were caused by its disruption. Most importantly, *SMK3* was found
126 to be required for sclerotia formation as well as initiation of infection, but not subsequent
127 proliferation within host tissues.
128

129 **Materials and Methods**

130 ***Fungal strains***

131 *Sclerotinia sclerotiorum* strain 1980 (ATCC18683) was used throughout this study. The generation
132 and selection of homokaryotic *Smk3* ν disruption mutant lines derived from 1980 has been
133 described previously (Bashi et al. 2010b) and were cultured under the same conditions.

134

135 A line was also generated in which the threonine and tyrosine residues in the TEY motif of SMK3
136 that are phosphorylated during MAPK activation were substituted for aspartic acid in an attempt
137 to create a constitutively active form of SMK3. The modified *Smk3* gene, denoted *Smk3u^{DED}* for
138 aspartic acid (D)-glutamic acid (E)-aspartic acid (D), was generated using SOEing PCR (Horton et al.
139 1990) as follows. The first SOEing PCR fragment was generated by amplifying genomic DNA with
140 MKCgenF (5'-GAAGGGTTCATTTTCGAGCCGAG-3') and MKCsoeingR (5'-
141 GCAACATCTTCATCCATGTATCCAGCATTCTCTTCAGGATC-3'), and the second fragment with
142 MKCsoeingF (5'- GCTGGATACATGGATGAAGATGTTGCTACAAGATGGTACCGTGCC-3') and MKCgenR
143 (5'- AAAATCCCAAAGCCTCTAAACACCG-3'). The mutated regions are located within the
144 overlapping SOEing primers and are underlined. The fragments were assembled in a second PCR
145 by first extending the annealed overlapping fragments for 5 PCR cycles followed by amplification
146 of the entire assembled region using the MKCgenF and MKCgenR primers. The ca. 5000 bp *Smk3*
147 region was cloned into pBARKS (Fungal Genetics Stock Center, www.fgsc.net) and sequenced. The
148 fungal transformation construct was prepared as follows. A 1549 bp fragment of the mutated
149 *Smk3* gene was amplified with primers MCK1 ca F 2565 Sal I (5'-
150 GTCGACGTAAGAAGATTTGGCAAAGC-3') located between exon 2 and exon 3 512 bp upstream of
151 mutation sit) and MKC1 ca R term 4085 (5'-TTCGCTGTTGCATTGCTATAGG-3') which overlaps the
152 stop codon. The hygromycin resistance cassette was amplified from pCB1004 (Fungal Genetics
153 Stock Center) using Hyg pCB1004 F (5'-AACTGGTCCCGGTCGGCATC-3') and Hyg pCB1004 R (5'-
154 AACTGATATTGAAGGAGC-3'). A 355 bp *Smk3* 3'untranslated region (UTR) was amplified using
155 MKC1 3' F amp PstI (5'-CTGCAGTTTGTGTATTGTCTTGTATCG-3') and MKC1 3' R amp 4509 NotI (5'-
156 GCGGCCGCGTGATTAAAATCCCAAAGCC-3'). All fragments were cloned into pGEM T-Easy
157 (Promega) and sequenced. The mutated *Smk3* fragment was excised by digestion with *SalI* and
158 *EcoRI* and cloned into pBluescriptKS (Promega). The hygromycin cassette was released by
159 digestion with *EcoRI* and *PstI* and inserted downstream of the mutated *Smk3* gene. The *Smk3*
160 3'UTR was released by digestion with *PstI* and *NotI* and inserted downstream of the hygromycin

161 cassette. This configuration allows replacement of the wild type *Smk3* gene with the modified
162 allele and selectable marker by homologous recombination. The construct was released by
163 digestion with *Sall* and *SstI* and cloned into pCB301 for *Agrobacterium*-mediated transformation
164 of *S. sclerotiorum* 1980 mycelia as described previously (Bashi et al. 2010b). Accurate
165 replacement of the endogenous *SMK3* gene with the *Smk3u^{DED}* allele was verified by amplification
166 of the locus followed by sequencing. Homokaryotic lines were selected as previously described
167 (Bashi et al. 2010b).

168

169 ***Effect of contact surface on MAPK phosphorylation and gene expression***

170 To examine MAPK phosphorylation in response to contact with various solid surfaces, *S.*
171 *sclerotiorum* strain 1980 was grown on potato dextrose agar (PDA) at 20°C. Thirty plugs, 3 mm in
172 diameter, were excised from the advancing margin of the colony and used to inoculate 200 mL of
173 minimal salts-glucose (MS-Glu) medium (10 g/L glucose, 2 g/L NH_4NO_3 , 1 g/L KH_2PO_4 , 0.1 g/L
174 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/L yeast extract, 3 g/L DL-malic acid, 1 g/L NaOH). The culture was incubated at
175 20°C in a 1 L flask with shaking at 80 rpm for 3 days. Mycelia were then harvested by
176 centrifugation at 10,000 rpm and placed on glass slides, glass slides coated with leaf cuticle wax,
177 Parafilm, or *B. napus* cv. Westar leaves. Leaf wax was isolated by dipping *B. napus* leaves in
178 chloroform for 30 sec and evaporating the chloroform under a stream of liquid nitrogen. Wax was
179 applied by dissolving the crystals in a small volume of chloroform, spreading it to form a thin film
180 on a glass Petri dish and allowing the chloroform to evaporate. Mycelia were incubated in contact
181 with the various surfaces for 10 min at 20°C and then immediately frozen in liquid nitrogen. Total
182 protein was extracted by grinding the mycelia in liquid nitrogen and incubating in extraction
183 buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 2% DTT) at a 1:5 w/v ratio for 2 min. Samples were
184 then centrifuged at 14,000 rpm for 5 min in a micro-centrifuge and the supernatant transferred to
185 a separate tube and stored at -20°C. Western blot analysis was conducted using a commercially
186 available antibody that can specifically detect phosphorylated ERK1/ERK2 type MAPKs (Cell
187 Signaling Technology). Western blot analysis was conducted according to the manufacturer's
188 protocol.

189

190 The effect of contact surface and carbon availability on *Smk1* and *Smk3* gene expression was
191 examined as follows. Mycelia grown as above were spread onto intact *B. napus* leaves or leaves
192 that had been stripped of wax by dipping in chloroform for 30 sec and then allowing the

193 chloroform to evaporate. The leaves were incubated for 24 h on wet filter paper in a sealed plastic
194 container with moistened Whatman No. 5 filter paper to provide adequate humidity for infection.
195 The mycelial mat was removed with a forceps and then immediately frozen in liquid nitrogen.
196 Northern blot analysis was conducted as described earlier (Bashi et al. 2010a). DNA fragments
197 corresponding to *Smk1* and *Smk3* were amplified from *S. sclerotiorum* genomic DNA and used as
198 probes. The following primers were used: *Smk1* forward (5'-CTTCTCGCATTTAATCCTG-3') and
199 *Smk1* reverse (5'-CCTTGGTGAGGTTGTCC-3') and *Smk3* forward (5'-CTTCTCGCATTTAATCCTG-3')
200 and *Smk3* reverse (5'-CCTTGGTGAGGTTGTCC-3').

201

202 **Microscopy**

203 A plug from the growing margin of a colony was used to inoculate a PDA plate which was then
204 incubated at 20°C for 3 days. To examine aerial hyphae formation, vertical sections were made
205 through the colony using a razor and a 1 mm thick slice laid on a glass slide. Samples were
206 examined at 20 X magnification using a dissecting microscope. To determine the branching
207 pattern, mycelia were cultivated in MS-Glu liquid medium and examined using a light microscope
208 at 200 X magnification.

209

210 **Incubation in cell wall degrading enzyme cocktail**

211 Mycelia were grown in 25 mL of potato dextrose broth for 3 days at 20°C and washed once with
212 sterile water and once with buffer (0.8 M MgSO₄·7H₂O, 0.2 M sodium citrate, pH 5.5). Mycelia
213 were then coarsely fragmented with a razor and incubated in 10 mg/mL *Trichoderma harzianum*
214 Lyase (Sigma-Aldrich) in buffer (1 M sorbitol, 50 mM sodium citrate, pH 5.8). The mixture was
215 incubated at 28°C with shaking at 100 rpm. Samples were collected every 30 min and observed
216 using a light microscope. Protoplasts were counted using a hemocytometer with 4 replicates at
217 each time point for each line. The data was normalized to 1 gram of tissue prior to analysis.
218 Average and standard deviations were calculated for each line at each time point. Differences
219 between the means at each of the four time points (30, 60, 90 and 120 minutes) were analyzed
220 using the General Liner Model (GLM) followed by Tukey's least significant test at a significance
221 level of 0.05%. Data analysis was done using SAS Enterprise version 5.1.

222

223 *Hydrophobicity measurement*

224 Mycelia hydrophobicity was determined as per Chau et al. (2009). Briefly, microscope slides were
225 sterilized using 70% ethanol, transferred to a sterile Petri dish and allowed to dry in a sterile flow
226 chamber. Liquid PDA at 37°C was poured onto the slide to form 1 mm thick layer and the media
227 allowed to solidify at room temperature. Using tweezers, a pin head size portion of mycelia was
228 transferred to the edge of the slide and incubated at 20°C for 2 days. Water droplets (10 µL) were
229 gently placed on the mycelia and analyzed immediately. Droplet images were analyzed using
230 ImageJ (<http://rsb.info.nih.gov/ij/>) and a plug-in for the analysis of drop shape used to measure
231 the contact angle (<http://bigwww.epfl.ch/demo/dropanalysis>). Measurements were taken from
232 two locations, the edge and the center of the colony, with three replications for each line.
233 Differences between means for the edge and center locations were determined for each line
234 using the Student T-test at a significance level of 0.05%. Analysis of the means at each of the two
235 locations over the three lines was analyzed using General Liner Model (GLM) followed by Tukey's
236 least significant test at a significance level of 0.05%. Data analysis was done using SAS Enterprise
237 version 5.1.

239 *Effect of temperature and compounds that affect cell wall integrity on growth*

240 To assess the effect of temperature on growth, PDA was inoculated using a 3 mm plug from the
241 edge of a growing colony. The plates were incubated at 20, 28, 37 and 52°C and colony diameters
242 measured daily. The ratio of growth at 28°C to that at 20°C was calculated from three
243 independent replicates. This calculation was used to account for the difference in colony
244 expansion rate between the wild type and the mutants and measures the change in expansion
245 ratio.

246
247 Radial expansion was also assessed on PDA supplemented with compounds that affect growth of
248 yeast and fungal mutants with altered cell wall integrity including 1.5 M glucose, 1.5 M sorbitol, 1
249 M KCl, 1M NaCl, 300 µM Congo Red, 1.5 mM Calcofluor White, 0.01% SDS and 10 mM caffeine.
250 Colony diameter was measured daily on each of four independent replicates.

252 *Pathogenicity assay and infection cushion examination*

253 Mycelia were grown in MS-Glu medium and placed on the adaxial surface of either intact *B. napus*
254 cv. Westar leaves or leaves stripped of wax. Leaf wax was removed by dipping leaves in

255 chloroform for 30 sec and then allowing the chloroform to evaporate. The leaves were incubated
256 on wet Whatman No. 5 paper for 24 h in a sealed plastic container. To observe infection cushion
257 development, 20 μ L MS-Glu media droplets containing a mycelial suspension were applied to the
258 leaf surface and allowed to incubate for 24 h under the same conditions as the pathogenicity
259 assay above.

260

Draft

261 Results

262 *Changes in MAPK phosphorylation in response to contact with different surfaces*

263 To better understand the role of MAPKs in sensing contact with a surface, *S. sclerotiorum* mycelia
264 were applied to different solid surfaces including leaf wax, glass, Parafilm, and leaves for 10 min.
265 Western blot analysis of total cell protein from these mycelia with an anti-phospho ERK1/ERK2
266 type MAPK antibody revealed that phosphorylation of ERK1/ERK2 type MAPKs increased upon
267 contact of mycelia with a solid surface. The patterns and levels of MAPK phosphorylation were
268 similar after contact with all the solid surfaces tested (Figure 1).

270 *Characterization of S. sclerotiorum pathogenicity and cell wall integrity MAPKs*

271 The western blot analysis above demonstrated that changes in MAPK phosphorylation accompany
272 contact with solid surfaces as would occur during the initial stages of infection. We focused our
273 efforts on the SLT-2 like MAPK involved in the cell wall integrity pathway in *S. cerevisiae*. A search
274 of the *S. sclerotiorum* genome using BLAST with the *B. cinerea* cell wall integrity MAPK *Bmp3*
275 (GenBank ABJ51957.1) identified a similar gene that was denoted *Sclerotinia MAPK3* (*Smk3*)
276 (SS1G_05445; GenBank XP_001594017.1). The *Smk3* gene was predicted to have 4 introns and 5
277 exons resulting in an open reading frame encoding a protein of 413 amino acids and a molecular
278 mass of 47 kDa as determined by the sum of the component amino acids. SMK3 was most similar
279 to *B. cinerea* BMP3 (99.8% identity) and possessed each of the 11 conserved subdomains in the
280 ATP binding and peptide binding/phosphotransfer lobes (Hanks 2003). The functionality of the
281 SMK3 MAPK was asserted by the presence of the conserved TEY motif upstream of subdomain VIII
282 (Figure 2) in which the threonine and tyrosine are phosphorylated leading to activation of the
283 kinase. The published assembly of the *S. sclerotiorum* genome (Amselem et al. 2011) is missing
284 information 5' and 3' to the *Smk3* region encoding the open reading frame. We obtained this
285 information during the construction of the transformation vectors and the complete *Smk3*
286 genomic region has been deposited in GenBank (Accession # 1891783). A phylogenetic analysis of
287 the *S. sclerotiorum* protein kinase superfamily, including the MAPK group, can be found in
288 Hegedus et al. (2015).

289
290 Northern blot analysis was used to examine changes in the expression of *Smk3* and the
291 pathogenicity MAPK *Smk1* in response to contact with different surfaces in the presence or
292 absence of glucose. Glucose was included to simulate the presence of organic carbon that would

293 be available from senescent plant materials. *Smk1* transcript levels did not change when mycelia
294 growing in liquid culture were transferred to intact leaves or leaves stripped of surface wax
295 (Figure 3). The presence of glucose reduced the expression of *Smk1* under these conditions,
296 indicating that it may be subject to catabolite repression. Conversely, *Smk3* expression was
297 induced on intact leaves in the presence of glucose, which simulates the conditions present during
298 infection. Finally, expression was unaffected on leaves stripped of wax indicating the importance
299 of natural conditions or context for regulation of *Smk3* expression.

300

301 ***Mycelial morphology and sclerotia formation***

302 To determine the roles of SMK3 in *S. sclerotiorum* development, *Smk3* disruption mutants (*Smk3* Δ
303 .3 and *Smk3* Δ .5) developed previously by Bashi et al. (2010b) were characterized. In this study,
304 an attempt was made to create a *S. sclerotiorum* line expressing a constitutively active form of
305 SMK3 by replacing the threonine and tyrosine residues in the TEY motif with aspartic acid, an acid
306 amino acid that has been shown to mimic phosphorylation and lead to constitutive activation of
307 some other kinases. This line was denoted *Smk3u^{DED}*. The phenotypes of the two independent
308 *Smk3* Δ disruption mutants and the *Smk3u^{DED}* mutant were found to be nearly identical (see
309 below) indicating the alterations to the TEY motif may have inactivated, rather than activated, the
310 SMK3 kinase.

311

312 Disruption of the *S. sclerotiorum Smk1* MAPK gene was found to impair the signaling pathway
313 involved in sclerotia formation (Chen et al. 2004). Similarly, while the wild type strain 1980
314 formed sclerotia within a week of inoculation on solid MS-Glu medium, the *Smk3* Δ mutants failed
315 to form sclerotia even after 3 weeks of incubation (Figure 4).

316

317 In liquid culture, both wild type and *Smk3* Δ mutant mycelia formed spherical aggregates. At the
318 edge of these aggregates, wild type hyphae radiated or projected outward, whereas *Smk3* Δ
319 mutant hyphae branched more frequently and closer to the growing tip which increased
320 aggregate density (Figure 4). Microscopic examination of a vertical section of a colony growing on
321 solid medium revealed that wild-type hyphae grew parallel to the surface, while the *Smk3* Δ
322 mutants produced a dense arrangement of highly branched, erect, aerial hyphae causing the
323 colony to rise from the surface of the medium (Figure 4). Aerial hyphae formation is promoted by
324 increased surface tension and hydrophobicity. Hydrophobicity of the central region and edge of

the colony was determined using a technique that measures water droplet contact angle on the mycelia surface. Wild type mycelia were more hydrophobic at the edge of the colony than in the centre with water droplet contact angles of 91° and 64°, respectively (Figure 4). The *Smk3 Δ* mutants had the opposite pattern as the center was more hydrophobic than the edge with water droplet contact angles of 33-71° and 95-96°, respectively. The increased hydrophobicity towards the center of the mutant colonies correlated with the higher density of aerial hyphae formation in this zone.

Cell wall integrity characteristics

Previous studies demonstrated that *Slr2* orthologs in other fungi played a role in cell wall biosynthesis and architecture as indicated by the decreased resistance to high temperatures and cell wall degrading enzymes in the corresponding mutants (Navarro-Garcia et al. 1995 Xu et al. 1998). To compare the cell wall integrity of wild type and *Smk3 Δ* , hyphae were fragmented and incubated in lyase, a solution of cell wall degrading enzymes from *T. harzianum*. Hyphal fragments from the *Smk3* mutants formed protoplasts at a much faster rate than fragments from wild type hyphae indicating that cell wall architecture was compromised and that lyase was able to more easily access or degrade cell wall components in the *Smk3 Δ* mutant hyphae compared to wild type (Figure 5).

The sensitivity of the *Smk3 Δ* mutants was also assessed in the presence of compounds that affect the growth of yeasts and fungi with altered cell wall integrity. The growth characteristics of the *Smk3 Δ* .3 mutant, a knockout generated by transposon mutagenesis, and the *Smk3 Δ ^{DED}* mutant, a point mutation that alters the ability of the MAPK to be phosphorylated, were very similar under all of the conditions tested. On solid PDA medium, the *Smk3 Δ* mutants grew slower and the rate of radial expansion was only 63-82 % that of the wild-type. Increasing the osmolarity of the medium with the addition of 1.5 M glucose or sorbitol did not affect the growth of the wild type or relative growth of the *Smk3 Δ* mutants appreciably (Figure 6). Conversely, the addition of 1 M KCl or NaCl impacted the growth of the *Smk3 Δ* mutants to a much greater extent than the wild type strain. In yeast and fungi, the chitin binding agents Calcofluor White and Congo Red inhibit cell division, but can also stimulate chitin synthesis (Roncero and Duran 1985). Addition of these compounds to the medium failed to inhibit or stimulate radial growth beyond that observed on PDA alone. Interestingly, the *Smk3 Δ* mutants were highly sensitive to SDS indicating that cell

357 membrane integrity may also be affected or that cell wall architecture is altered in such a manner
358 as to allow easier access of SDS to the plasma membrane. The *Smk3 Δ* mutants were also hyper-
359 sensitive to caffeine, a compound that induces the cell wall integrity pathway and involves the
360 SLT2 (SMK3) MAPK (Fuchs and Mylonakis 2009).

361

362 ***Temperature effects***

363 As noted above, earlier studies demonstrated that disruption of the cell wall integrity MAPK
364 pathway adversely affected the ability of mycelia to tolerate exposure to elevated temperatures,
365 but the effect on growth rate was not examined. To examine the latter, the radial expansion of
366 wild type and *Smk3 Δ* mutant colonies was compared at different temperatures (Figure 7). Neither
367 the *Smk3 Δ* mutants nor the wild type was able to grow at temperatures above 37°C. When the
368 ratio of linear colony expansion at 20°C and 28°C was compared, the mutant colonies were in fact
369 found to be more tolerant of the higher temperature. At 28°C, the wild type colony expanded at
370 22% of its rate at 20°C, whereas the *Smk3 Δ* mutant colonies grew at 47-51% of their rates at 20°C.
371 This indicated that SMK3 may be involved in regulating growth rate as well as cell wall formation
372 in response to temperature stress.

373

374 ***Host infection***

375 In other phytopathogenic fungi, loss of the cell wall integrity MAPK either rendered the mutant
376 non-pathogenic or reduced virulence. Similarly, the *Smk3 Δ* mutants exhibited reduced virulence
377 on intact *B. napus* leaves (Figure 8A). With the wild type strain, the entire area beneath the
378 mycelial inoculum became necrotic within 24 hours of inoculation, while the *Smk3 Δ* mutants only
379 formed infection initials, i.e. small necrotic spots around the leaf veins (Figure 8A). Interestingly,
380 when mycelia from the *Smk3 Δ* mutants were applied to leaves that had been stripped of cuticle
381 wax, necrosis development was equal to or even more so than of the wild type strain within 24
382 hours of inoculation (Figure 8A) indicating that SMK3 was important for infection initiation, but
383 not necessarily for lesion expansion. To further this observation, droplets of MS-Glu medium
384 containing mycelia were placed on an intact leaf surface and examined after 24 hours using a
385 dissecting microscope. Wild type hyphae radiated out of the droplet and formed infection
386 cushions that are responsible for cuticle penetration (Figure 8B) and a large necrotic area had
387 already begun to form around the infection cushions by this time. Conversely, *Smk3 Δ* mutant
388 hyphae radiated outward from the droplet and grew parallel to the leaf surface. The hyphae failed

389 to orient toward the leaf surface or aggregate to form infection cushions. No signs of necrosis
390 were observed.
391
392

Draft

393 Discussion

394 ***Identification of pathogenicity-related MAPKs in S. sclerotiorum***

395 MAPKs are highly conserved in fungi and yeasts and are functionally interchangeable between
396 different organisms, even though they may have evolved to regulate slightly different biological
397 processes (Fujioka et al. 2007; Mey et al. 2006). Two types of MAPKs, the pathogenicity and cell
398 wall integrity MAPKs, have been demonstrated to be important for pathogenesis by filamentous
399 phytopathogenic fungi. In *S. sclerotiorum*, the pathogenicity MAPK, *Smk1*, was found to be
400 involved in mediating cross-talk between cAMP and pH to coordinate sclerotia formation (Chen et
401 al. 2004). While the pathogenicity of the *Smk1* mutant was not examined, disruption of orthologs
402 in other phytopathogenic fungi severely reduced virulence and in some cases rendered them non-
403 pathogenic (Xu et al. 1998; Hou et al. 2002). In this study, the *S. sclerotiorum* *Slr2* ortholog, *Smk3*,
404 was characterized. SLT2-like MAPKs are involved in the signaling pathway that is activated in
405 response to extracellular factors or events that cause cell wall perturbation. *Smk3* was also up-
406 regulated at the onset of the infection which further supports the notion that SMK3 may play a
407 role in early *S. sclerotiorum*-host interactions. In another study, the expression of the gene
408 encoding a cutinase which is involved in cuticle penetration was dependent on contact with a
409 solid surface, while the expression of *SsPg1* was restricted to certain infection zones, such as the
410 expanding margin of the lesion (Bashi et al. 2012). Regulation of both genes requires that the
411 pathogen sense its environmental context and therefore the cell wall integrity MAPK pathway
412 may be involved.

413

414 ***Role of SMK3 in aerial hyphae formation and branching***

415 Disruption of *Smk3* caused visually detectable alterations in colony morphology. *Smk3*Δ mutant
416 colonies were less pigmented and more elevated than the wild type, which upon microscopic
417 examination was found to be due to a higher density of erect, aerial hyphae. Unlike *S.*
418 *sclerotiorum*, mutations in *Slr2* orthologues in *B. cinerea* (Rui and Hahn 2007), *F. graminearum*
419 (Hou et al. 2002), *M. grisea* (Xu et al. 1998) and *Aspergillus nidulans* (Bussink and Osmani 1999)
420 reduced aerial hyphae formation, while complete loss was observed in *Mycosphaerella*
421 *graminicola* (Mehrabi et al. 2006). Similar to the *Smk3*Δ mutant, a *S. sclerotiorum* adenylate
422 cyclase mutant formed dense aerial hyphae (Jurick and Rollins 2007).

423

The degree of aerial hyphae formation is a function of small (~ 100 amino acid), surface-active, secreted proteins with eight conserved cysteine residues known as hydrophobins (Wosten and Willey 2000; Wessels 1997). Once secreted, hydrophobins self assemble at the hydrophilic-hydrophobic interface into a 10 nm thick amphipathic protein film. This film increases water tension allowing formation of aerial hyphae. The increase in aerial hyphae in *Smk ν* mutants could be due to SMK3 interaction with transcription factors that regulate hydrophobin gene expression. *Smk ν* mutant colonies were also found to be more hydrophobic at the center of the colony than at the edge, while the wild type strain was more hydrophobic at the edge than the center. Fungal hyphae exhibit apical dominance which allows the colony to differentiate between the edge and the center of the colony. Lack of proper apical dominance may also be the reason for extensive branching in the *Smk ν* mutant hyphae. The shift in the hydrophobic zone towards the edge of the colony in the *Smk ν* mutants may be a secondary effect of the lack of apical dominance and extensive branching. Hyper-branching in the *Smk ν* mutants was consistent with an earlier finding in *C. purpurea* which exhibited a similar phenotype upon mutation of its *Slt2* ortholog.

Smk3 ν mutant colonies expanded on solid medium at a slower rate than wild type colonies. Disruption of *Slt2* orthologs in *F. graminearum* (Hou et al. 2002), *C. albicans* (Navarro-Garcia, 1995) and *B. cinerea* (Rui and Hahn 2007) caused reduced expansion on solid medium similar to the *S. sclerotiorum* mutant. In *Mycosphaerella graminicola*, no alterations in growth phenotype were observed until the fifth day on which cells showed polar growth with swollen cells forming by the eleventh day (Mehrabi et al. 2006). Unlike other fungi, the mutation had no effect on colony expansion rate in *M. grisea* (Xu et al. 1998) or *C. lagenarium* (Kojima et al. 2002). Reduced colony expansion rate has been suggested to be due to lack of osmotic tolerance. Addition of an osmotic stabilizer to cultures of a *B. cinerea* *Slt2* orthologue disruption mutant recovered 80% of the mycelial growth and 100% of the germling elongation rate (Rui and Hahn 2007). However, in this study the addition of glucose or sorbitol to the medium failed to accelerate radial expansion of *Smk3 ν* mutant colonies. It is also possible that the reduced colony expansion rate in the *Smk3 ν* mutants was due to hyper-branching which leads to dense rather than expansive growth.

Role of SMK3 in sclerotia formation

The *Smk3 ν* mutants were unable to form sclerotia or sclerotia initials. Proper branching of hyphae is known to be a prerequisite for sclerotia formation in the close relative *Sclerotinia rolfii*. Hyphae

of *S. rolfii* were classified as either leading hyphae with ca. 40 nuclei or lateral hyphae with ca. 10 nuclei. Lateral hyphae with shorter intrudes (240 μ m) were proposed to be responsible for sclerotia formation (Goujon et al. 1970). Apical dominance is regulated through a hormone-like substance and prevents lateral branching and therefore sclerotia formation (Burnett 1968). In *S. sclerotiorum*, apical dominance at the expanding colony margin may repress sclerotia formation in the center of the colony. Sclerotia form at the perimeter of the colony when mycelia reach the edge of the plate and only later in the interior of the colony. In all cases, sclerotia formation is strongly related to proper apical dominance and branching which is altered in the *Smk3 ν* mutant. Disruption of the *S. sclerotiorum Smk1* MAPK gene was also found to impair sclerotia formation (Chen et al. 2004) indicating that both MAPK pathways are required for normal sclerotia formation.

Role of *SMK3* in the response to elevated temperatures

Mutations in *Slr2* orthologues have been demonstrated to cause sensitivity to high temperatures. Cells of *F. graminearum* (Hou et al. 2002), *Schizosaccharomyces pombe* (Toda et al. 1996) and *C. albicans* (Navarro-Garcia et al. 1996) *Slr2* ortholog mutants burst upon exposure to high temperatures. Interestingly, the colony expansion rate was higher in the *Smk3 ν* mutants than the wild type strain when incubated at the more restrictive temperature of 28°C compared to 20°C. To our knowledge the role of MAPKs in the response to heat stress in filamentous fungi has not been studied. In tomato, MAPK phosphorylation led to activation of a heat stress transcription factor indicating that the MAPKs play a role in transducing the heat stress signal (Link et al. 2002). *SMK3* may be responsible for reducing growth rate at high temperatures to avoid lethal events, such as cell burst, which would explain why the wild type strain grew slower than the mutant. It is also possible that cell wall structure or components have been altered in an advantageous way that accommodates the growth of the *Smk3 ν* mutants at higher temperatures. It should be noted that the response to environmental stresses, including elevated temperatures, are coordinated through cross-talk between multiple signaling pathways including the TOR, calcineurin, MAPK and cAMP-protein kinase A pathways (Fuchs and Mylonakis 2009).

Role of *SMK3* in proper cell wall formation

The *Smk3 ν* mutant colonies were found to be more susceptible to cell wall degrading enzymes than the wild type. Similar results were found in *Slr2*-ortholog mutants in *M. grisea* (Navarro-

488 Garcia et al. 1998), *F. graminearum* (Hou et al. 2002) and *M. graminicola* (Mehrabi et al. 2006). An
489 *Slt2* ortholog and a second gene were found to play a redundant role in regulating cell wall
490 integrity in *C. lagenarium* as mutation of the *Slt2* ortholog alone did not increase the susceptibility
491 of the cell wall to these enzymes (Kojima et al. 2002). *Smk3 Δ* mutant cell walls may be less
492 compact or rigid due to the lack of certain components or to structural changes. This flexibility
493 could have allowed the *Smk3 Δ* mutant to grow better at high temperatures, while the associated
494 structural changes make it more susceptible to cell wall degrading enzymes than the wild type. In
495 this regard, the increased sensitivity of the *Smk3 Δ* mutants to SDS could be attributed to
496 alterations in cell wall architecture that permit access of the detergent to the plasma membrane.
497 *Smk3 Δ* mutants were also hyper-sensitive to caffeine, a compound that induces the cell wall
498 integrity pathway and involves the SLT2 (SMK3) MAPK (Fuchs and Mylonakis 2009).

499

500 ***Role of SMK3 in pathogenicity***

501 The *Smk3 Δ* mutants were found to be much less or even avirulent on intact leaves. Mutations in
502 the *Slt2* ortholog in *B. cinerea*, *Bmp3*, also resulted in reduced virulence on intact leaves and spore
503 germlings seemed unable to sense the leaf surface, elongated excessively and were unable to
504 form appressoria (Rui and Hahn 2007). In *C. lagenarium*, *Slt2* ortholog mutant spores also lost
505 their ability to form appressoria, but retained a low level of virulence due to the release of cell
506 wall degrading enzymes and mycotoxins (Kojima et al. 2002). *Magnaporthe grisea* MPS1 (Xu et al.
507 1998) is also required for germ tube elongation leading to appresoria formation on solid surfaces
508 and on the host plant. While a *M. graminicola* *Slt2* ortholog mutant was able to initiate infection
509 through the stomata, it was unable to expand in the plant tissue (Mehrabi et al. 2006). In our
510 study, *Smk3 Δ* mutants were at least as virulent as the wild type strain on leaves that had been
511 stripped of cuticle wax and lesion expansion occurred rapidly once this barrier was removed or
512 breached.

513

514 **Conclusion**

515 A wide range of pathogenicity factors may be affected by mutation of *Slt2* orthologs in
516 filamentous fungi. Only cuticle penetration appears to be impaired in *Smk3 Δ* mutants since
517 mycelia were able to expand in the leaf tissue. This could be due to lack of infection cushion
518 formation in the mutant which, interestingly, also occurred in the adenylate cyclase mutant
519 (Jurick and Rollins 2007). Failure to form infection cushions may be due to inability to sense the

leaf surface and respond accordingly or to loss of a physiological function that is necessary for infection cushion development, such as proper branching. The corresponding protein in *Candida albicans*, Mkc1p, is involved in the regulation of surface contact-dependent processes leading to filamentous invasive growth (Kumamoto 2005). Considering the overlap in functions between cAMP and SMK3 signaling, including aerial hyphae formation, branching and sclerotia formation, it is possible that both signaling mechanisms regulate infection cushion formation as well. In *S. sclerotiorum*, the production of hydrolytic enzymes (cutinases) required for cuticular wax degradation is induced by contact with solid surfaces (Bashi et al. 2012) and infectivity and trichothecene production are linked to an SLT2-like MAPK in *Fusarium* species (Hou et al. 2002; Zhang et al. 2011). This suggests that in *S. sclerotiorum* the detection of surface integrity leading to infection cushion formation and subsequent deployment of virulence determinants, such as hydrolytic enzymes and toxins, which are required for infection initiation, may be co-dependent on the SMK3 MAPK pathway. Interestingly, the FUS3-like MAPK is also associated with surface sensing and host penetration in fungal phytopathogens (Leroch et al. 2015; Perez-Nadales and Di Pietro 2015), suggesting that the two MAPK pathways may share common partners. In conclusion, the functions regulated by fungal MAPKs are diverse and vary even in closely related species; therefore, it is important to characterize the ortholog associated with each species of interest. The availability of a near complete *S. sclerotiorum* genome sequence (Amselem et al. 2011) and the ability to generate disruption mutations through homologous recombination or gene silencing should allow discovery and characterization of additional signaling components required for interaction with host plants.

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542

543 **Acknowledgements**

544 Funding for this work was provided by Sask Canola, the Province of Saskatchewan through the
545 Agriculture Development Fund and the Government of Canada through the Agriculture Innovation
546 Program.

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Draft

770 Figure Legends

771 **Figure 1. Alteration in the phosphorylation state of *S. sclerotiorum* ERK1/ERK2 type MAPKs in** 772 **response to contact with solid surfaces.**

773 Mycelia were grown in MS-Glu liquid medium and then placed in contact with various solid
 774 surfaces for 10 min. Western blot analysis was conducted using an antibody specific to
 775 phosphorylated forms of ERK1/ERK2 MAPKs. Asterisks (*) indicate the location of MAPKs
 776 exhibiting increased phosphorylation in mycelium placed in contact with solid surfaces (leaf,
 777 Parafilm, glass, or leaf wax) relative to mycelia grown in liquid culture. The bottom panel shows
 778 the same membrane treated with Ponceau stain to detect protein loaded in each lane.

779

780 **Figure 2. Alignment of SLT2-like MAPKs from filamentous fungi and yeasts.**

781 Alignment of *S. sclerotiorum* SMK3 (1G05445) with SLT2 orthologs including *Aspergillus nidulans*
 782 MPKA (AAD24428.1), *Botrytis cinerea* BMP3 (ABJ51957.1), *Blumeria graminis* MPK2
 783 (AAG53655.1), *Candida albicans* MKC1 (AAL50116.1), *Claviceps purpurea* CPMK2 (CAC87145.1),
 784 *Colletotrichum lagenarium* MAF1 (AAL50116.1), *Fusarium graminearum* MG1 (AAM13670.1),
 785 *Magnaporthe grisea* MPS1 (AAC63682.1) and *Saccharomyces cerevisiae* SLT2 (CAA41954.1).
 786 Identical amino acids (white on black background) and those with conserved properties (black on
 787 gray) are shown. The ERK1/ERK2 type phosphorylation site (TEY) is indicated by asterisks (*).

788

789 **Figure 3. Expression of *Smk1* and *Smk3* in response to contact with leaf surfaces.**

790 Northern blot analysis showing gene expression in liquid minimal salts medium and after contact
 791 with intact *B. napus* leaves (24 h) or leaves stripped of cuticular wax in the presence or absence of
 792 glucose. The lower panels show the rRNA loaded in each lane.

793

794 **Figure 4. The effect of *Smk3* mutation on *S. sclerotiorum* sclerotia formation, mycelial** 795 **development, and mycelial hydrophobicity.**

796 Panel A: Wild type (1980) and *Smk3^u* mutant colonies three weeks after inoculation of potato
 797 dextrose agar. Sclerotia were observed with the wild type, but not the mutant lines. Panel B: Light
 798 microscopic images of the wild type strain and *Smk3^u* mutants growing in minimal salt-glucose
 799 liquid medium. Mycelial branching is more frequent in the mutant mycelia than in the wild type.
 800 Bar = 50 μ m. Light microscopic images of the wild type strain and *Smk3^u* mutants growing on
 801 potato dextrose agar. Vertical sections from the colonies showed increased branching and aerial

802 mycelia formation in the mutants. Panel C: Water droplet contact angle on *S. sclerotiorum* wild
803 type and *Smk3* ν mutant mycelia surface in the center (black bars) and the edge (grey bars) of the
804 colony. Error bars indicate standard deviation of three replicates. Lower case letter indicate
805 significant differences in hydrophobicity between the edge and the center of the colony for each
806 line (Student t-test $p \leq 0.05$). Upper case letters indicate differences between lines at either the
807 edge or the center of the colony (Tukey's least significant test $p \leq 0.05$).

808

809 **Figure 5. The effect of *Smk3* mutation on *S. sclerotiorum* cell wall integrity as determined by**
810 **incubation with cell wall degrading enzymes.**

811 Time course study showing formation of protoplasts. Upper case letters indicate differences in
812 protoplast formation between lines at each time point (Tukey's least significant test $p \leq 0.05$).

813

814 **Figure 6. Radial expansion of *S. sclerotiorum* wild type (1980) and *Smk3* ν mutant colonies on**
815 **PDA supplemented with compounds that affect yeast and fungal mutants with altered cell wall**
816 **integrity.** PDA, PDA + 1.5 M glucose, PDA + 1.5 M sorbitol, PDA + 1 M KCl, PDA + 1M NaCl, PDA +
817 300 μ M Congo Red, PDA + 1.5 mM Calcofluor White, PDA + 0.01% SDS, PDA + 10 mM caffeine.
818 Error bars in graphs indicate standard deviation of four replicates.

819

820 **Figure 7. Effect of *Smk3* mutation on *S. sclerotiorum* growth at elevated temperature.**

821 Colony expansion of wild type (1980) and *Smk3* ν mutants on PDA at 20 or 28°C.

822

823 **Figure 8. Effect of *Smk3* mutation on *S. sclerotiorum* infection of *B. napus* leaves.**

824 Panel A: Intact *B. napus* leaves or leaves stripped of cuticle wax 24 hours after inoculation with
825 wild type *S. sclerotiorum* or *Smk3* ν mutants. The upper panels show leaves with the mycelial
826 inoculum, while in the lower panels mycelia has been removed to reveal the affected tissue
827 beneath. Panel B: Upper panels show *B. napus* leaves 24 hours after inoculation with droplets
828 containing wild type or *Smk3* ν mutant mycelia. Lower panels are magnified to better depict the
829 presence or absence of infection cushions (arrows).

Figure 1

157x223mm (300 x 300 DPI)

	1		100
<i>S. sclerotiorum</i> SMK3	MADLQG--RKVFQVFNQDFIVDERYTVTKELGGAYGIVCAATNSQTQEG--VAIKKVTNVFSKKILAKRALREIKLLQHFGRGHRNITCLYDMDIP--RP		
<i>A. nidulans</i> MPKA	MSDLQVQGRKVFQVFNQDFIVDDRYTVTKEIGGAYGIVCAATNVQTQEG--VAIKKVTNVFSKKILAKRALREIKLLQHFGRGHRNITCLYDMDIP--RP		
<i>B. cinerea</i> BMP3	MADLQG--RKVFQVFNQDFIVDERYTVTKELGGAYGIVCAATNSQTQEG--VAIKKVTNVFSKKILAKRALREIKLLQHFGRGHRNITCLYDMDIP--RP		
<i>B. graminis</i> MPK2	MSDMQG--RKVFQVFNQDFIVDERYTVTKELGGAYGIVCAATNISQTQEG--VAIKKVTNVFSKKILAKRALREIKLLQHFGRGHRNITCLYDMDIP--RP		
<i>C. lagenarium</i> MAF1	MCDLQG--RKVFQVFNQDFIVDERYTVTKELGGAYGIVCAAVNNQTQEG--VAIKKVTNVFSKKILAKRALREIKLLQHFGRGHRNITCLYDMDIP--RP		
<i>C. purpurea</i> CPMK2	MIDIPG--RRAFKCFNQDFVVDERYTVTKELGGAYGIVCAAVNNQTKES--VAIKKVTNVFSKKILAKRALREIKLLQHFGRGHRNITCLYDMDIP--RP		
<i>F. graminearum</i> MGV1	MCDLQG--RKVFQVFNQDFIVDERYTVTKELGGAYGIVCAAVNNQTNEG--VAIKKVTNVFSKKILAKRALREIKLLQHFGRGHRNITCLYDMDIP--RP		
<i>M. grisea</i> MPS1	MSDLQG--RKIFQVFNQDFIVDERYTVTKELGGAYGIVCAAVNNQTSEG--VAIKKVTNVFSKKILAKRALREIKLLQHFGRGHRNITCLYDMDIP--RP		
<i>S. cerevisiae</i> SLT2	MADKIE--RHTFKVFNQDFSVDKRFQLIKEIGHGAYGIVCSARFAEAAEDTTVAIKKVTNVFSKTLCKRSLRELKLLRHFRGHKNITCLYDMDIVFYPD		
	101	***	200
<i>S. sclerotiorum</i> SMK3	DNFNETYLYEELMECDLAAIIVRSQGPLTDAHQSFTIYQILGGLKYIHSANVLHRDLKPGNLLVNADCELKICDFGLARGFSVDPEENAGYMT EYVATRWY		
<i>A. nidulans</i> MPKA	DNFNETYLYEELMECDLAAIIRSGQPLTDAHQSFTIYQILGGLKYIHSANVLHRDLKPGNLLVNADCELKICDFGLARGFSIDPEENAGYMT EYVATRWY		
<i>B. cinerea</i> BMP3	DNFNETYLYEELMECDLAAIIVRSQGPLTDAHQSFTIYQILGGLKYIHSANVLHRDLKPGNLLVNADCELKICDFGLARGFSVDPEENAGYMT EYVATRWY		
<i>B. graminis</i> MPK2	ENFNETYLYEELMECDLAAIIRSGQPLTDAHQSFTIYQILGGLKYIHSANVLHRDLKPGNLLVNADCELKICDFGLARGFSVDPEENAGYMT EYVATRWY		
<i>C. lagenarium</i> MAF1	DNFNETYLYEELMECDLAAIIRSGQPLTDAHQSFTIYQILGGLKYIHSANVLHRDLKPGNLLVNADCELKICDFGLARGFSVDPEENAGYMT EYVATRWY		
<i>C. purpurea</i> CPMK2	DVFNETYLYEELMECDLAAIIRSGQPLTDAHQSFTIYQILGGLKYIHSANVLHRDLKPGNLLVNADCELKICDFGLARGFSADPEENAGYMT EYVATRWY		
<i>F. graminearum</i> MGV1	DNFNETYLYEELMECDLAAIIRSGQPLTDAHQSFTIYQILGGLKYIHSANVLHRDLKPGNLLVNADCELKICDFGLARGFSVDPEENAGYMT EYVATRWY		
<i>M. grisea</i> MPS1	DNFNETYLYEELMECDLAAIIRSGQPLTDAHQSFTIYQILGGLKYIHSANVLHRDLKPGNLLVNADCELKICDFGLARGFSVDPEENAGYMT EYVATRWY		
<i>S. cerevisiae</i> SLT2	GSINGLYEELMECDMHQIIRSGQPLTDAHQSFTIYQILGGLKYIHSADVLHRDLKPGNLLVNADCELKICDFGLARGYSENPEENAGYMT EYVATRWY		
	201		300
<i>S. sclerotiorum</i> SMK3	RAPEIMLSFQSYTKAIDVWSVGCILAEILGGRPFFFKGRDYVDQNLQILHILGTPNEETLSRIGSPRAQ EYVRNLPYMAKRPFETLFPNANPDALDLDLDM		
<i>A. nidulans</i> MPKA	RAPEIMLSFQSYTKAIDVWSVGCILAEILGGRPFFFKGRDYVDQNLQILHYLGTPNEETLSRIGSPRAQ EYVRNLPFMPKVPFQRLFPNANPDALDLDLDM		
<i>B. cinerea</i> BMP3	RAPEIMLSFQSYTKAIDVWSVGCILAEILGGRPFFFKGRDYVDQNLQILHILGTPNEETLSRIGSPRAQ EYVRNLPYMAKRPFETLFPNANPDALDLDLDM		
<i>B. graminis</i> MPK2	RAPEIMLSFQSYTKAIDVWSVGCILAEILGGRPFFFKGRDYVDQNLQILHVLGTPNEETLARIGSPRAQ EYVRNLPFMAKRPFQAIFPNANPDALDLDLDM		
<i>C. lagenarium</i> MAF1	RAPEIMLSFQSYTKAIDVWSVGCILAEILGGRPFFFKGRDYVDQNLQILHILGTPNEETLSRIGSPRAQ EYVRNLPFMPKKEPFQLFAQANPDALDLDLDM		
<i>C. purpurea</i> CPMK2	RAPEIMLSFQSYTKAIDVWSVGCILAEILGSRPFFFKGRDYVDQNLQILHVLGTPNEETLSRIGSPRAQ EYVRNLPVMPKKNFATLFPQANPHALDLDLDM		
<i>F. graminearum</i> MGV1	RAPEIMLSFQSYTKAIDVWSVGCILAEILGGRPFFFKGRDYVDQNLQILHILGTPNEETLSRIGSPRAQ EYVRNLPFMPKKEPFSLFPQANPDALDLDLDM		
<i>M. grisea</i> MPS1	RAPEIMLSFQSYTKAIDVWSVGCILAEILGGRPFFFKGRDYVDQNLQILHILGTPNEETLSRIGSPRAQ EYVRNLPFMAKKPFETLFPNANPDALDLDLDM		
<i>S. cerevisiae</i> SLT2	RAPEIMLSYQGYTKAIDVWSAGCILAELFLGGRPFIFKGRDYVDQNLQILQVLGTPPEETLRRIGSKNVCDYIHQLGFIKVFQVNLYPNANSQALDLDLEQM		
	301		400
<i>S. sclerotiorum</i> SMK3	LAFDPSSRIDVETALEHPYLIHWHDASDEPGCPTTFNEDFEVVEDVGEIRKILILEVHRFRQHVRVQPG-----QQGQSNGP--QVPIPDQA-----		
<i>A. nidulans</i> MPKA	LAFDPSSRISVEEALHHPYLAIWHDASDEPSCPTTFDEHFEVVDVQEMRRMIYEVEVFRFRQSVRMPPQ-----AQSLAQQQQIGNIPIEHQQG-----		
<i>B. cinerea</i> BMP3	LAFDPSSRIDVETALEHPYLIHWHDASDEPGCPTTFNEDFEVVEDVGEIRKILILEVHRFRQHVRVQPG-----QQGQSNGP--QVPIPDQA-----		
<i>B. graminis</i> MPK2	LAFDPASRISVEEALHHPYLQIHWHDVSDPCCPTTFNEDFEVTDVVGEMRMILDEVARFRHHVRVQPG-----MQSPSN-DPSVPTLQTSV-----		
<i>C. lagenarium</i> MAF1	LAFDPSSRISVEQALEHPYLIHWHDASDEPDCTPTTFNEDFEVVEDVGDMRKMILEVYFRFRQIVRTVPG-STQAGAAQAGQAGAGQVPIPSGNP-----		
<i>C. purpurea</i> CPMK2	LAFDPSSRISVEQALEHPYLQVWHDPADEPNCTPTTFNEDFEVLDDVGEMRMILDEVIRFRQMVRTASSAEPPTGQAQTAAG--QVEMPQAGG-----		
<i>F. graminearum</i> MGV1	LAFDPSSRISVEQALEHPYLQIHWHDASDEPDCTPTTFNEDFEVVEDVQMRGMILDEVQFRFRQIVRTVPGQ---SGGGLQGQG--VPVPLPQNG-----		
<i>M. grisea</i> MPS1	LAFDPSSRISVEQALEHPYLIHWHDASDEPDCTPTTFNEDFEVVEDVGEIRKILILEVYFRFRQIVRTAPG---AGGHGAPHAPQVPIBAGAGQG-----		
<i>S. cerevisiae</i> SLT2	LAFDPQKRITVDEALEHPYLIHWHDPADEPVCSEKFEBSFESVNDMEDLKQMVIOEQVDFRLFVRQPLLEEQRQLQLQQQQQQQQQQQQQQPSVDVNG		
	401		486
<i>S. sclerotiorum</i> SMK3	-----SWRAEDPRPQ-----EAYGQGPNDLEQDTLGGGLDAMRS		
<i>A. nidulans</i> MPKA	-----VWRQEEPRPQ-----AGAVGGNHNDDLESSLQRMGDA---		
<i>B. cinerea</i> BMP3	-----SWRAEDPRPQ-----EAYGQGPNDLEQDTLGGGLDAMRS		
<i>B. graminis</i> MPK2	-----AWGSEDPRPQ-----ENNDQEIITGLERDLTVGLDR---		
<i>C. lagenarium</i> MAF1	-----GWTSSDPRPQ-----EYVGQGG-GLEADTAGGLDHRR-		
<i>C. purpurea</i> CPMK2	-----QWKAEDPRPQ-----ENTPQGN-GLEQDLQAGLDAA--		
<i>F. graminearum</i> MGV1)	-----QWTAEDPRPQ-----EYAGHGNTGLEQDTLGGGLDASRR		
<i>M. grisea</i> MPS1	-----QWKAEDPRPQ-----EYVGQMN-DLEADTAGGLDQRR-		
<i>S. cerevisiae</i> SLT2	NAAASEENYPKQMATSNSVAPQQESFGIHSQNLPRHDADFPRPQESMMEMRPATGNTADIPPQNDNGTLLDLEKELEFGLDKRYF

Figure 3

96x123mm (300 x 300 DPI)

Figure 4.

170x267mm (300 x 300 DPI)

Figure 5.

147x141mm (300 x 300 DPI)

154x282mm (300 x 300 DPI)

Figure 7

150x131mm (300 x 300 DPI)

Figure 8

169x263mm (300 x 300 DPI)