Review

The eukaryotic protein kinase superfamily of the necrotrophic fungal plant pathogen, *Sclerotinia sclerotiorum*

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SUMMARY

Protein kinases have been implicated in the regulation of many processes that guide pathogen development throughout the course of infection. A survey of the *Sclerotinia sclerotiorum* genome for genes encoding proteins containing the highly conserved eukaryotic protein kinase (ePK) domain, the largest protein kinase superfamily, revealed 92 *S. sclerotiorum* ePKs. This review examines the composition of the *S. sclerotiorum* ePKs based on conserved motifs within the ePK domain family, and relates this to orthologues found in other filamentous fungi and yeasts. The ePKs are also discussed in terms of their proposed role(s) in aspects of host pathogenesis, including the coordination of mycelial growth/development and deployment of pathogenicity determinants in response to environmental stimuli, nutrients and stress.

Keywords: pathogenicity, protein kinases, *Sclerotinia sclerotiorum*, stress response.

INTRODUCTION

Sclerotinia sclerotiorum is a highly polyphagous, necrotrophic fungal plant pathogen (Boland and Hall, 1994). Its infection process is unique among necrotrophs in that the pathogenic phase is bounded by saprophytic phases (Hegedus and Rimmer, 2005). More recently, Kabbage *et al.* (2015) suggested that *S. sclerotiorum* is more accurately classified as a hemibiotroph, as they found evidence for a biotrophic phase immediately after cuticle penetration. *Sclerotinia sclerotiorum* ascospores are unable to directly infect leaves and stems and, in the first saprophytic phase, nutrients for initial colonization are acquired from pre-existing dead host tissue, often senescent flower petals, providing resources for fungal biomass accumulation and infection of healthy host tissue. Upon cuticle penetration, a brief biotrophic phase ensues in which oxalic acid temporarily suppresses host defences and allows the pathogen to establish within the apoplastic space (Kabbage *et al.*, 2015). An aggressive pathogenic

phase follows in which mycelia ramify throughout the host and release a plethora of hydrolytic enzymes (Bashi *et al.*, 2012; Kasza *et al.*, 2004; Li *et al.*, 2004b; Martel *et al.*, 1996; Poussereau *et al.*, 2001a,b; Riou *et al.*, 1991; Zhang *et al.*, 2014), oxalic acid (Godoy *et al.*, 1990) and necrosis-inducing proteins (Bashi *et al.*, 2010a, 2013) to destroy and break down host tissue. The second saprophytic phase is characterized by a decline in hydrolytic enzyme synthesis and acidification of the environment by the secretion of oxalic acid, which promotes sclerotial development and completion of the life cycle. Transitions between these states are coordinated by physical and biochemical events, which include the detection of contact with hard surfaces (Bashi *et al.*, 2012), glucose levels (Vautard *et al.*, 1999; Vautard-Mey *et al.*, 1999), cyclic adenosine monophosphate (cAMP) levels (Girard *et al.*, 2004; Jurick and Rollins, 2007; Rollins and Dickman, 1998), ambient pH (Cotton *et al.*, 2003; Favaron *et al.*, 2004; Rollins, 2003; Rollins and Dickman, 2001), oxidative stress (Kim *et al.*, 2011; Papapostolou *et al.*, 2014) and probably other factors. Interplay between the associated signalling pathways is critical and, in some cases, has been shown to involve various protein kinases (Bashi *et al.*, 2010b; Chen and Dickman, 2005; Chen *et al.*, 2004; Duan *et al.*, 2013; Erental *et al.*, 2008; Harel *et al.*, 2005; Jurick *et al.*, 2004; Vacher *et al.*, 2003; Vautard-Mey and Fevre, 2000) and phosphatases (Erental *et al.*, 2007; Harel *et al.*, 2006).

Reversible phosphorylation is a major mechanism through which environmental and biochemical stimuli can affect protein function and gene expression. The majority of eukaryotic protein kinases (ePKs) phosphorylate serine, threonine or tyrosine, and possess a well-conserved catalytic domain comprising 12 subdomains that make up the ATP binding lobe (subdomains I–V) and the peptide binding and phosphotransfer lobe (subdomains VI–XI). Subdomains VIB and VIII are involved in peptide substrate recognition, and conserved amino acids within them are used to classify ePKs into functional groups (Hanks, 2003; Manning *et al.*, 2002a; Miranda-Saavedra and Barton, 2007), although atypical protein kinases occur in some species. The seven groups of typical ePKs found in yeasts and fungi are as follows: the AGC Group of cyclic nucleotide- and calcium/phospholipid-dependent kinases; the CMGC Group consisting of the cyclin-dependent and cyclin-dependent-like kinases, mitogen-activated kinases and glycogen

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synthase kinases; the tyrosine kinase (TK) Group; the TK-like (TKL) Group; the calmodulin-dependent kinase (CAMK) Group; the casein kinase (CK) Group; and the STE Group, originally identified from sterile yeast mutants and which includes kinases upstream of mitogen-activated protein kinases (MAPKs). The number of genes encoding ePKs occurring in the genomes of eukaryotes varies considerably, but is loosely correlated with developmental complexity. The *Arabidopsis thaliana* genome has 973 protein kinase genes (Champion *et al.*, 2004) and *Homo sapiens* 518 (Manning *et al.*, 2002b), whereas *Saccharomyces cerevisiae* has only 115 (Hunter and Plowman, 1997) and *Plasmodium falciparum* only 65 (Ward *et al.*, 2004). TKs and TLKs are not typically found in lower eukaryotes, such as yeasts and fungi. Histidine kinases represent a separate group of kinases and are most often associated with response-regulator signalling pathways in bacteria and some fungi (Santos and Shiozaki, 2001; Wolanin *et al.*, 2002). A two-component histidine kinase, Shk1, which feeds into the high-osmolarity glycerol mitogen-activated kinase pathway (see below), has been described in *S. sclerotiorum* (Duan *et al.*, 2013), but these kinases are not discussed in this review.

Composition of the *S. sclerotiorum* ePK family

An analysis of genes encoding signalling pathway components, provided in the report of the *S. sclerotiorum* genome sequence by Amselem *et al.* (2011), described nine MAPK components and a small number of other kinases. A search of the *S. sclerotiorum* genome sequence (available at http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiHome.html) using BLAST (Altschul *et al.*, 1990) revealed 92 genes with significant similarity ($\geq e^{-5}$) to one or more of the 115 *S. cerevisiae* ePK encoding genes (Hunter and Plowman, 1997). The *S. sclerotiorum* ePKs can be further classified into functional groups on the basis of conserved residues implicated in peptide substrate binding within ePK domains VIB and VIII, according to the scheme of Hanks (2003) (Tables 1 and S1, see Supporting Information). Parsimony analysis using full-length proteins is ineffective in clustering ePKs from the same functional groups as many are compound proteins and contain additional, non-ePK domains that complicate the analysis. However, parsimony analysis using only the conserved protein kinase catalytic domain, comprising 12 subdomains, agrees well with the functional classification (Fig. 1). Four proteins, encoded by SS1G_14026, SS1G_03622, SS1G_08574 and SS1G_03661, had partial ePK domains and are not included in the parsimony analysis, although future improvements to the genome sequence may reveal the missing attributes. ePKs were assigned to orthologous groups using OrthoMCL (Li *et al.*, 2003; <http://www.orthomcl.org/orthomcl>) by comparison with 150 species, including those classified as Alveolates (15), Amoebozoa (4), Archaea (16), Euglenozoa (9), Firmicutes (6), Fungi (24), Metazoa (29), Other Bacteria (11), Other Eukaryota (6), Proteobacteria (19) and Viridiplantae (11). This

revealed several ePKs that are unique to *S. sclerotiorum*, as well as expansion/contraction of some orthologous groups relative to other filamentous fungi and yeasts (Tables 1 and S2, see Supporting Information). These are discussed in more detail as they are described below. Unless already named, in describing the *S. sclerotiorum* ePKs, we have adopted and extended the unifying nomenclature scheme developed by Hamel *et al.* (2012) for proteins within the MAPK cascade, which is based on similarity to yeast orthologues.

The CMGC Group

The *S. sclerotiorum* ePK CMGC Group contains 18 members and is noted for MAPKs that are involved in the transmission of external stimuli to direct gene expression. *Saccharomyces cerevisiae* possesses five distinct MAPK pathways, each with its own MAPK component: the pheromone response (FUS3), filamentous growth (KSS1), high-osmolarity/glycerol (HOG1), cell wall integrity (SLT2) and spore wall assembly (SMK1) pathways (Chen and Thorner, 2007; Gustin *et al.*, 1998). *Sclerotinia sclerotiorum* possesses only three MAPKs encoded by SS1G_05445, SS1G_11866 and SS1G_07590. Filamentous fungi, in particular phytopathogens, have a reduced repertoire of MAPKs consisting of only FUS3/KSS1-like, HOG1-like and SLT2/SMK1-like kinases and associated signalling pathways (reviewed in Hamel *et al.*, 2012; Turra *et al.*, 2014; Xu, 2000; Zhao *et al.*, 2007).

The MAPK encoded by SS1G_05445 (SMK3) is orthologous to *S. cerevisiae* SLT2 and SMK1. In *S. sclerotiorum*, disruption of the *SMK3* gene led to severely reduced virulence on intact host plant leaves, but not on leaves stripped of cuticle wax. This was attributed to alterations in mycelial apical dominance, leading to the inability to aggregate and form infection cushions. The mutation also caused a loss of the ability to produce sclerotia, increased aerial mycelia and altered mycelial hydrophobicity (Bashi *et al.*, 2010b, unpublished results). The corresponding protein in *Candida albicans*, Mkc1p, is involved in the regulation of surface contact-dependent processes leading to filamentous invasive growth (Kumamoto, 2005). MAPKs encoded by *SLT2* orthologues in *Botrytis cinerea* (*BMP3*) (Rui and Hahn, 2007), *Colletotrichum lagenarium* (*MAF1*) (Kojima *et al.*, 2002) and *Magnaporthe grisea* (*MPS1*) (Xu *et al.*, 1998) are required for germ tube elongation, leading to appressoria formation on solid surfaces and on the host plant. In *S. sclerotiorum*, hydrolytic enzyme synthesis, in particular the cutinases involved in the degradation of cuticular wax, is also induced by contact with solid surfaces (Bashi *et al.*, 2012), and infection ability and trichothecene production have been linked to an SLT2-like MAPK in *Fusarium* species (Hou *et al.*, 2002; Zhang *et al.*, 2011). Taken together, this suggests that, in phytopathogenic fungi, a single MAPK fulfils the role of the SLT2 (cell wall integrity) and SMK1 (spore wall assembly) MAPKs found in yeast, and is involved in the detection of surface integrity, leading to infection

Table 1 *Sclerotinia sclerotiorum* eukaryotic protein kinases*.

Protein ID (SS1G#)	<i>Saccharomyces cerevisiae</i> orthologue	Functional group†	Functional family	Orthologous group‡	Comment
08653	YPL209C (IPL1)	AGC		OG5_127024	
02271	YNL161W (CBK1)	AGC	NDR	OG5_129797	
08085	YFL033C (RIM15)	AGC	NDR	OG5_135400	SsRIM15
13528	YOL100W (PKH2)	AGC		OG5_128785	SsPHK1
05114	YGR092W (DBF2)	AGC	NDR	OG5_134449	SsDBF2
01194	YNL161W (CBK1)	AGC	NDR	OG5_126822	SsCBK1
09988	YNL161W (CBK1)	AGC		OG5_159571	
03171	YKL166C (TPK3)	AGC	PKA	OG5_126748	SsPKA1
13577	YPL203W (TPK2)	AGC	PKA	OG5_126748	SsPKA2
09566	YNR047W (FPK1)	AGC		OG5_129514	SsFPK1
14455	YMR104C (YPK2)	AGC	AKT	OG5_126635	SsYPK1
01124	YHR205W (SCH9)	AGC	AKT	OG5_134169	SsSCH9
09704	YKL116C (PRR1)	CAMK		OG5_136536	
04288	YPL150W	CAMK	CAMKL	OG5_129098	
12333	YOR233W (KIN4)	CAMK	CAMKL	OG5_135759	SsKIN4
05253	YCL024W (KCC4)	CAMK	CAMKL	OG5_126655	SsHSL1
10426	YDR477W (SNF1)	CAMK	CAMKL	OG5_126655	SsSNF1
02943	YLR096W (KIN2)	CAMK	CAMKL	OG5_128024	SsKIN1
04687	YMR001C (CDC5)	CAMK		OG5_127396	SsCDC5
00255	YOR351C (MEK1)	CAMK	CAMK1	OG5_137004	
01850	YPL153C (RAD53)	CAMK		OG5_128695	SsRAD53
04069	YDL017W (DUN1)	CAMK	CAMK1	OG5_128695	SsDUN1
11871	YOL016C (CMK2)	CAMK	CAMK1	OG5_126600	SsCMK2
10021	YOL016C (CMK2)	CAMK	CAMK1	OG5_126600	SsCMK1
06203	YLR248W (RCK2)	CAMK	CAMK1	OG5_135068	SsRCK2
00018	YHR082C (KSP1)	CK1		OG5_137984	SsKSP1
09429	YPL204W (HRR25)	CK1		OG5_126780	SsHRR25
03818	YNL154C (YCK2)	CK1		OG5_128520	SsYCK1
03455	YDL214C (PRR2)		HAL	OG5_155883	
12179	YBR274W (CHK1)			OG5_130454	
00583	YCR008W (SAT4)		HAL	OG5_135414	SsSAT4
08494	YOR267C (HRK1)		HAL	OG5_133778	SsHRK1
11231	YGL180W (APG1)	STE		OG5_127101	
05159	YAR018C (KIN3)	STE		OG5_129446	Unique
06598	YNR031C (SSK2)	STE	STE11	OG5_130049	SsSSK2
03713	YAR019C (CDC15)	STE	STE11	OG5_131527	
10983	YJL095W (BCK1)	STE	STE11	OG5_136136	SsBCK1
00606	YLR362W (STE11)	STE	STE11	OG5_128416	SsSTE11
00059	YOR231W (MKK1)	STE	STE7	OG5_136512	SsMKK1
14143	YJL128C (PBS2)	STE	STE7	OG5_133213	SsPBS2
10792	YDL159W (STE7)	STE	STE7	OG5_127304	SsSTE7
00637	YNL298W (CLA4)	STE	STE20	OG5_127124	SsCLA4
02450	YHL007C (STE20)	STE	STE20	OG5_127124	SsSTE20
02954	YDR523C (SPS1)	STE	STE20	OG5_155727	
12848	YDR523C (SPS1)	STE	STE20	OG5_127277	SsSPS1
01266	YDR523C (SPS1)	STE	STE20	OG5_127277	SsSPS1
07569	YPL031C (PHO85)	CMGC1		OG5_152679	
08042	YLR113W (HOG1)	CMGC1		OG5_155659	
10377	YJL106W (IME2)	CMGC1		OG5_127559	SsIME2
12372	YMR216C (SKY1)	CMGC1		OG5_169303	
06118	YMR216C (SKY1)	CMGC1		OG5_149777	
11138	YDL017W (CDC7)	CMGC1		OG5_129189	
12241	YIL035C (CKA1)	CMGC1		OG5_126901	SsCKA1
09079	YMR139W (RIM11)	CMGC1		OG5_126888	
07590	YLR113W (HOG1)	CMGC1	MAPK	OG5_128610	SsHOG1
11866	YBL016W (FUS3)	CMGC1	MAPK	OG5_126781	SsSMK1
05445	YHR030C (SLT2)	CMGC1	MAPK	OG5_126781	SsSMK3
01068	YPL042C (SSN3)	CMGC1	CDK	OG5_129229	
06051	YDL108W (KIN28)	CMGC1	CDK	OG5_129224	
07226	YPL031C (PHO85)	CMGC1	CDK	OG5_126712	SsPHO85
02296	YBR160W (CDC28)	CMGC1	CDK	OG5_126712	SsCDC28
07672	YKL139W (CTK1)	CMGC1	CDK	OG5_128263	

Table 1 Continued.

Protein ID (SS1G#)	<i>Saccharomyces cerevisiae</i> orthologue	Functional group†	Functional family	Orthologous group‡	Comment
00560	YKL139W (CTK1)	CMGC1	CDK	OG5_128520	
00199	YPR161C (SGV1)	CMGC1	CDK	OG5_130531	
12527	YLL019C (KNS1)	CMGC2		OG5_126990	
08157	YMR216C (SKY1)	CMGC2		OG5_187668	Unique
03277	YMR216C (SKY1)	CMGC2		OG5_127107	SsSKY1
06528	YJL141C (YAK1)	CMGC2		OG5_128795	
00098	YJL141C (YAK1)	CMGC2		OG5_127901	SsYAK1
00874	YJL141C (YAK1)	CMGC2		OG5_127439	
13027	YMR216C (SKY1)	CMGC2		OG5_149930	Unique
13978	YMR216C (SKY1)	CMGC2		OG5_149930	Unique
01433	YDL028C (MPS1)	OTHER-01		OG5_129339	SsMPS1
04461	YPL236C	OTHER-01		OG5_129834	
04996	YJL187C (SWE1)	OTHER-01		OG5_131219	SsSWE1
06961	YAL017W (PSK1)	OTHER-02		OG5_131169	SsPSK1
13275	YGR188C (BUB1)	OTHER-02		OG5_130700	SsBUB1
05139	YAR018C (KIN3)	OTHER-02		No group	Unique
04823	YHR079C (IRE1)	OTHER-03		OG5_128843	SsIRE1
14489	YPL031C (PHO85)	OTHER-03		OG5_176856	Unique
09511	YPL031C (PHO85)	OTHER-03		OG5_176856	Unique
06542	YPL026C (SHA3)	OTHER-04		OG5_134005	SsSKS1
00591	YGL179C (TOS3)	OTHER-04		OG5_127880	SsTOS3
10932	YER129W (SAK1)	OTHER-04		OG5_152662	SsSAK1
07203	YNL020C (ARK1)	OTHER-05		OG5_127425	SsARK1
10463	YAR018C (KIN3)	OTHER-05		OG5_129446	Unique
02644	YJL057C (IKS2)	OTHER-05		OG5_131896	
00622	YDR283C (GCN2)	OTHER-05		OG5_129525	SsGCN5
14026§	YBL105C (PKC1)			OG5_129314	SsPKC1
03622§	YPL153C (RAD53)			OG5_126600	
08574§	YBR097W (VPS15)			OG5_129368	
03661§	YDR283C (GCN2)			OG5_130490	Unique

*Details and GenBank accession numbers provided in Tables S1 and S2 (see Supporting Information).

†Functional groups based on conserved residues implicated in peptide substrate binding within eukaryotic protein kinase (ePK) domains VIB and VIII according to the scheme of Hanks (2003).

‡OrthoMCL analysis.

§*Sclerotinia sclerotiorum* ePKs lacking complete ePK domain and not included in the phylogenetic analysis.

structure (appressoria or infection cushions) formation and the deployment of virulence determinants, such as hydrolytic enzymes and toxins, that are required for infection initiation and establishment.

The MAPK encoded by SS1G_07590 is orthologous to *S. cerevisiae* HOG1 (denoted here as SsHOG1), but has not been studied in *S. sclerotiorum*. In the closely related phytopathogen, *B. cinerea*, the HOG1 orthologue (BcSAK1) is involved not only in osmotic stress, but also oxidative stress, mycelial differentiation leading to conidia formation, sclerotial development and host penetration (Segmüller *et al.*, 2007), as well as secondary metabolite production (Heller *et al.*, 2012). BcSAK1 is phosphorylated under osmotic and oxidative stress and migrates to the nucleus under the former condition; however, under the latter, it appears to activate transcription factors whilst they are still within the cytosol (Heller *et al.*, 2012). *Botrytis cinerea* regulator-1 (BcReg1) is a downstream target of BcSAK1, as *bcreg1* mutants phenocopy *Bcsak1* mutants (Michielse *et al.*, 2011). The basic leucine zipper

transcription factor (BcAtf1) also appears to be a target for BcSAK1, although in a complementary manner to BcReg1, as *Bcatf1* mutants exhibit the opposite phenotype of either *Bcsak1* or *Bcreg1* mutants (Temme *et al.*, 2012). SS1G_12241 is also within this clade and is orthologous to CKA1, an *S. cerevisiae* protein kinase that is also involved in the response to osmotic stress. Under high salt conditions, CKA1 activity is attenuated, resulting in increased activation of the plasma membrane transporter, Ycf1p, that effluxes toxins and other metabolites from the cell (Pickin *et al.*, 2010).

The MAPK encoded by SS1G_11866 (SMK1) is orthologous to *S. cerevisiae* KSS1 and FUS3, suggesting that, as in other filamentous fungi (Hamel *et al.*, 2012; Turra *et al.*, 2014), *S. sclerotiorum* also lacks one of these MAPK pathways found in yeast. In *S. sclerotiorum*, mycelial growth and differentiation are important aspects related to sclerotial development, and *SMK1* transcription increases sharply at the onset of sclerotigenesis. Furthermore, an *smk1* RNA interference line exhibited impaired sclerotial formation

Fig. 1 Dendrogram showing the *Sclerotinia sclerotiorum* eukaryotic protein kinase (ePK) superfamily. ePKs were assigned according to Hanks (2003) with groups indicated by colour and families by asterisks. The nomenclature for the kinases is based on the most closely related *Saccharomyces cerevisiae* orthologue. Information pertaining to similarity to yeast kinases, function and groupings is available in Table S1 (see Supporting Information). Parsimony analysis was conducted using PHYLIP v3.696 in SeaView v4.5.3 (Gouy *et al.*, 2010; <http://pbil.univ-lyon1.fr/software/seaview.html>) and the dendrogram was assembled using FigTree v1.4 (<http://tree.bio.ed.ac.uk/software/figtree>).

(Chen *et al.*, 2004). Inactivation of KSS1/FUS3 orthologues in other phytopathogenic fungi led to a loss of pathogenicity because of the inability to form infection structures, such as in *B. cinerea* (BMP1) (Zheng *et al.*, 2000), *C. lagenarium* (CMK1) (Takano *et al.*, 2000), *Cochliobolus heterostrophus* (CHK1) (Lev *et al.*, 1999) and *M. grisea* (PMK1) (Xu and Hamer, 1996), or the inability to secrete hydrolytic enzymes, as in *Alternaria brassicicola* (AMK1) (Cho *et al.*, 2007), on contact with hard surfaces or the host plant. The *B. cinerea* mucin-like protein, Msb2, has been implicated as the

hard surface sensor that activates the BMP1 MAPK pathway, leading to infection structure formation (Leroch *et al.*, 2015). The STE12 transcription factor is a downstream target of BMP1, although the phenotype of *ste12* mutants is not entirely the same as that of *bmp1* mutants as the expression of other transcription factors is dependent on the BMP1 MAPK cascade (Schamber *et al.*, 2010).

Based on an extensive phylogenetic analysis of MAPKs in fungi, Hamel *et al.* (2012) proposed an additional clade consisting of

orthologues of the MAPK-related protein IME2. The *S. sclerotiorum* kinase encoded by SS1G_10377 is orthologous to the *S. cerevisiae* IME2, although two less similar kinases (SS1G_12372 and SS1G_06118) are also in this clade. In *S. cerevisiae*, IME2 is essential for the initiation of meiosis (Honigberg, 2004) and positively regulates spore formation by promoting the phosphorylation of the SMK1 MAPK in response to nutrient levels and cAMP (McDonald *et al.*, 2009). In fungal ascomycetes, IME2 orthologues control sexual development in response to nutrients and light (Irniger, 2011). What role(s) is played by SsIME2 in *S. sclerotiorum*, which possesses a sexual phase, but appears to outcross only infrequently on the basis of the genetic analysis of field isolates (Atallah *et al.*, 2004), remains to be investigated.

The *S. sclerotiorum* kinases encoded by SS1G_08042 and SS1G_07569 have weak similarity to *S. cerevisiae* HOG1 and cyclin-dependent kinases (CDKs), respectively. The larger *S. sclerotiorum* CDK clade contains seven kinases which may be classified as those that bind multiple cyclins and regulate the cell cycle (SS1G_02296 and SS1G_07226) and those that bind single cyclin molecules and regulate transcription through phosphorylation of the RNA polymerase large subunit (SS1G_00199, SS1G_00560, SS1G_07672, SS1G_06051 and SS1G_01068). SS1G_02296 is the orthologue of yeast CDC28 and mammalian CDK1, the main regulator of cell division in eukaryotes (Malumbres, 2014). SS1G_07226 is orthologous to *S. cerevisiae* PHO85, a regulator of cell division in response to environmental stresses (Nishizawa, 2015).

A second group of *S. sclerotiorum* ePKs, denoted CGMC-like (CGMC Group 2), contains eight members. SS1G_03277 is orthologous to *S. cerevisiae* SKY1 with three other proteins (SS1G_08157, SS1G_13027 and SS1G_13978) exhibiting less similarity. SKY1 is a member of a novel class of serine–arginine protein kinases (SRPK) that are involved in mRNA maturation and other cellular activities, including chromatin reorganization, cell cycle regulation and metabolic signalling (Giannakouros *et al.*, 2011). OrthoMCL analysis reveals that SS1G_08157 orthologues are found only in *S. sclerotiorum* and *Aspergillus* species, but not in any other taxa used in the analysis. Another CGMC-like clade contains the *S. cerevisiae* YAK1 orthologue, SS1G_00098, and two less similar ePKs (SS1G_06528 and SS1G_00874). YAK1 is a type of dual-specificity tyrosine phosphorylation-regulated kinase (DYRK), whose members control cell proliferation, differentiation and homeostasis (Aranda *et al.*, 2011). A separate CGMC-like clade contains the ePKs encoded by SS1G_13027 and SS1G_13978, which is worth noting as orthologues of these ePKs occur in only a few other filamentous fungi and not in yeasts.

The STE Group

In the classical MAPK cascade, MAPKs are activated by phosphorylation by MAPKKs, which are phosphorylated by

MAPKKKs, which, in turn, are phosphorylated by a more diverse group of upstream kinases. The yeast pheromone response and filamentous growth pathways use the same MAPKK (STE7), MAPKKK (STE11) and upstream kinase (STE20) (Chen and Thorner, 2007; Gustin *et al.*, 1998). The *S. sclerotiorum* STE7-like MAPKK family consists of three members (SS1G_10792, SS1G_14143 and SS1G_00059) with SS1G_10792 being orthologous to the *S. cerevisiae* STE7. SS1G_14143 is orthologous to *S. cerevisiae* PBS2, the MAPKK upstream of HOG1, whereas SS1G_00059 is orthologous to the *S. cerevisiae* MKK1 and MKK2 MAPKKs, which act redundantly to activate SLT2 in the cell wall integrity pathway. The *S. sclerotiorum* STE11-like MAPKKK family consists of four members. SS1G_00606 is orthologous to *S. cerevisiae* STE11, SS1G_06598 to SSK2 and SSK22 in the HOG1 pathway, and SS1G_10983 to BCK1 in the yeast cell wall integrity pathway. These genes have not been studied in *S. sclerotiorum*; however, mutation of the *STE7* and *STE11* orthologues in *B. cinerea* (*BcSTE7* and *BcSTE11*) (Schamber *et al.*, 2010) and *M. grisea* (*MST7* and *MST11*) (Zhao *et al.*, 2005) yielded the same phenotype as the corresponding *bmp1* and *pmk1* MAPK mutants noted above.

The STE20-like kinases include the p21-activated kinases (PAKs) and the germinal spore kinases (GCKs), the former having a role in pathogenesis in fungi (Boyce and Andrianopoulos, 2011). The *S. sclerotiorum* STE20-like kinase family has five members. SS1G_02450 is orthologous to the yeast STE20 PAK, which is the upstream activator of STE11. STE11 and STE20 may also participate in the activation of the HOG1 pathway (Chen and Thorner, 2007). SS1G_14026 encodes the orthologue of the yeast PKC1, which is the upstream activator of BCK1 in the cell wall integrity pathway. The MAPKK and MAPKKK directly upstream of the *S. cerevisiae* SMK1 in the spore wall assembly pathway are unknown; however, two *S. sclerotiorum* genes (SS1G_01266 and SS1G_12848) encode kinases with strong similarity to the *S. cerevisiae* SPS1 GCK, which has been proposed to function upstream of SMK1 (Gustin *et al.*, 1998). SS1G_00637 is orthologous to the *S. cerevisiae* CLA4 PAK which is involved in cytokinesis. Mutation of *CLA4* orthologues in *M. grisea* (*CHM1*) (Li *et al.*, 2004a) and *Claviceps purpurea* (*CLA4*) (Rolke and Tudzynski, 2008) has profound effects on conidial morphology, mycelial growth and pathogenicity. Figure 2 compares the components of the *S. sclerotiorum* MAPK pathways with corresponding *S. cerevisiae* orthologues.

The *S. sclerotiorum* genome encodes two additional STE ePKs (SS1G_05159 and SS1G_11231) which form a separate clade. OrthoMCL analysis reveals that SS1G_05159 is paralogous to SS1G_10463, which clusters into Other Group 05 on the basis of phylogenetic analysis with the ePK domain only. This is noteworthy, as only a single paralogue is found in other filamentous fungi and haploid yeasts.

Fig. 2 Proposed *Sclerotinia sclerotiorum* mitogen-activated protein kinase (MAPK) pathways based on corresponding *Saccharomyces cerevisiae* pathways. Kinases are shown in ovals and named according to the most closely related *S. cerevisiae* orthologue. Colours indicate eukaryotic protein kinase (ePK) groups as in Fig. 1. Full lines indicate established kinase reactions, whereas proposed reactions are indicated by '?'. Non-kinase pathway components are shown in boxes with interactions indicated by broken lines.

The CAMK Group

The *S. sclerotiorum* CAMK Group has 13 members, five of which (SS1G_10426, SS1G_02943, SS1G_05253, SS1G_12333 and SS1G_04288) are similar to the broad group of *S. cerevisiae* CAMK-like (CAMKL) kinases. Of note is the kinase encoded by SS1G_10426, which encodes the catalytic subunit of the sucrose non-fermenting (SNF1) kinase complex described in *S. sclerotiorum* as being involved in carbon catabolite repression (Vacher *et al.*, 2003). Under low-glucose conditions, SNF1 is activated through phosphorylation and localizes to the nucleus, where it, in turn, phosphorylates the transcriptional repressor Mig1 (Sanz, 2003) or

the *S. sclerotiorum* orthologue CRE1 (Vautard *et al.*, 1999; Vautard-Mey *et al.*, 1999). Phosphorylation of CRE1 leads to de-repression of genes subject to catabolite repression, such as *S. sclerotiorum* polygalacturonase 1 (Reymond-Cotton *et al.*, 1996). In *S. cerevisiae*, three kinases, SAK1, TOS3 and ELM1, phosphorylate SNF1 (Hedbacker *et al.*, 2004a). Two *S. sclerotiorum* ePKs clustering in Other Group 04 (SS1G_00591 and SS1G_10932) are orthologous to TOS3 and SAK1, respectively. SS1G_02943 is most similar to *S. cerevisiae* KIN1 and KIN2 which regulate exocytosis through phosphorylation of the t-SNARE Sec9 (Elbert *et al.*, 2005). Figure 3 compares the components of the *S. sclerotiorum* protein kinase pathways that may be involved in

Fig. 3 Proposed *Sclerotinia sclerotiorum* eukaryotic protein kinases (ePKs) involved in response to nutrients and environmental stresses. Kinases are shown in ovals and named according to the most closely related *Saccharomyces cerevisiae* orthologue. Colours indicate ePK groups as in Fig. 1. Full lines indicate established kinase reactions, whereas proposed reactions are indicated by '?'. Non-kinase pathway components are shown in boxes with interactions indicated by broken lines.

responses to nutrient deprivation and environmental stress with corresponding *S. cerevisiae* orthologues.

Several CAMKs are involved in aspects of cell division. SS1G_05253 is similar to *S. cerevisiae* KCC4 and HSL1, which regulate entry into mitosis through phosphorylation of the ePK SWE1 (SS1G_04996; Other Group 01) and, in turn, phosphorylate the CDK CDC28 (SS1G_02296; CMGC Group) (Barral *et al.*, 1999). SS1G_12333 is orthologous to *S. cerevisiae* KIN4, which is a key regulator of the spindle position checkpoint for cells exiting mitosis (Caydasi *et al.*, 2010), whereas SS1G_04687 is orthologous to *S. cerevisiae* CDC5, which regulates Bfa1, an inhibitor of mitotic exit (Botchkarev *et al.*, 2014). SS1G_01850 is orthologous to *S. cerevisiae* RAD53, which is involved in DNA damage checkpoint control (Usui and Petrini, 2014). The *S. cerevisiae* DUN1 protein kinase (SS1G_04069) also participates in this checkpoint control pathway (Gardner *et al.*, 1999).

Two *S. sclerotiorum* ePKs within the CAMK1 family (SS1G_11871 and SS1G_10021) are similar to *S. cerevisiae* CMK1/CMK2 kinases. These CAMKs are involved in the control of cell wall integrity and the response to oxidative stress, and may function upstream of STE11 to functionally link separate MAPK pathways (Ding *et al.*, 2014) (Fig. 1). Similarly, the CAMK1 protein kinase encoded by SS1G_06203 is orthologous to *S. cerevisiae* RCK2. RCK2 is a downstream target of HOG1 and links the pheromone response and hyperosmotic stress MAPK pathways by disrupting the FUS3–STE12 feedback loop (Nagiec and Dohlman, 2012) (Fig. 2).

The AGC Group

The *S. sclerotiorum* AGC group has 12 members. Many ePKs direct signals through specific linear pathways; however, a subset of ePKs, such as the CMK1/CMK2 and RCK2 kinases noted above, functionally couple individual pathways to coordinate gene expression. The AGC group ePKs are particularly important in linking nutrient status to biochemical and physiological responses. In *S. cerevisiae*, the main players in this cascade are the highly conserved, atypical protein kinases TOR1 and TOR2 (target of rapamycin), which regulate the activity of several AGC kinases. In response to nutrient limitation and other stresses, TOR1 directly or indirectly affects the phosphorylation status of cAMP-dependent protein kinase A (PKA), SCH9, RIM15 and DBF2/20, whereas TOR2 affects YPK1/2 and PKC1 (Jacinto and Lorberg, 2008) (Fig. 3).

PKA consists of a catalytic and a regulatory subunit with two *S. sclerotiorum* genes encoding the former; SS1G_03171 encodes a catalytic subunit similar to *S. cerevisiae* TPK1 and TPK2, whereas SS1G_13577 encodes a second catalytic subunit more similar to TPK2. This apparent redundancy is consistent with the observation that an *S. sclerotiorum* *pka1* mutant (SS1G_03171) did not exhibit an altered phenotype (Jurick *et al.*, 2004). The cAMP level is a key regulator of many fungal developmental and metabolic processes, and mutation of the *S. sclerotiorum* adenylate cyclase (*SAC1*) affects sclerotia and infection cushion formation, and reduces virulence (Jurick and Rollins, 2007). Elevated glucose levels lead to an increase in cAMP levels and PKA activation through dissociation

of the regulatory subunit. Under these circumstances, PKA affects SNF1 activity (SS1G_10426) through a complex mechanism which involves the phosphorylation of SAK1 (SS1G_10932), a SNF1 activating kinase (Barrett *et al.*, 2012), and alteration of the localization of members of the SNF1 complex (Hedbacker *et al.*, 2004b). cAMP may also act independently of PKA, as *S. sclerotiorum* sclerotia development is associated with increased PKA levels (Harel *et al.*, 2005), but increased cAMP levels, which should activate PKA, reduce sclerotial development (Rollins and Dickman, 1998).

The relationship between cAMP and MAPK pathways was first noted in *Ustilago maydis* adenylase cyclase suppressor mutants which no longer required cAMP for filamentous growth. This led to the identification of UBC3, a MAPK orthologous to SMK1 (SS1G_11866) in the FUS3/KSS1 pathway (Mayorga and Gold, 1999). In *S. sclerotiorum*, sclerotia formation is also associated with SMK1 and *SMK1* transcription induced by low ambient pH, which is known to promote sclerotigenesis, but repressed by cAMP (Chen *et al.*, 2004). Furthermore, cAMP prevents SMK1 activation in a PKA-independent manner mediated through the Ras small GTPase (Chen and Dickman, 2005). These pathways and other factors that affect sclerotial development have been reviewed (Erental *et al.*, 2008).

Sclerotinia sclerotiorum SS1G_01124 encodes an ePK orthologous to *S. cerevisiae* SCH9. SCH9 is structurally related to PKA and has been proposed previously to be the second *S. sclerotiorum* PKA (Erental *et al.*, 2008). SCH9 and PKA work in concert to regulate the response to nutritional stress through the RIM15 protein kinase (SS1G_08085). SCH9 phosphorylation of RIM15 prevents its entry into the nucleus, so that it can be phosphorylated by PKA in the cytoplasm. Under nutrient starvation or stress, PKA and SCH9 are inactive, leading to activation of RIM15 and induction of the quiescence programme via MSN2/4 and GIS1-directed gene expression (Roosen *et al.*, 2005). Like RIM15, DBF2/DBF20 (SS1G_05114) are members of the AGC nuclear DBF2-related (NDR) subfamily. DBF2/DBF20 are members of the mitotic exit network which regulates CDK inactivation and is essential for cytokinesis (Yoshida and Toh-e, 2001).

TOR2 regulates YPK1/YPK2 (SS1G_14455) and PKC1 (SS1G_14026) activity (Roosen *et al.*, 2005). PHK1/PHK2 (SS1G_13528) is a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates members of the AGC class of protein kinases, including SCH9, YPK1/YPK2 and PKC1 (Roelants *et al.*, 2004). In *S. cerevisiae*, YPK1/YPK2 are involved in various aspects of lipid metabolism (Roelants *et al.*, 2011), actin polarization (Niles and Powers, 2014), cell wall integrity (Roelants *et al.*, 2002), endocytosis (Jacquier and Schneiter, 2010) and flippase activity (Roelants *et al.*, 2010). An immediate downstream target YPK1 is the FPK1 protein kinase (SS1G_09566), a flippase activator (Roelants *et al.*, 2010). Flippases are important in maintaining

sphingolipid content and aminophospholipid asymmetry in the plasma membrane. *Saccharomyces cerevisiae* PKC1 (SS1G_14026) is an upstream kinase in the cell wall integrity SLT2 MAPK pathway and targets the BCK1 MAPKKK (SS1G_10983) (Reinoso-Martin *et al.*, 2003). *Saccharomyces cerevisiae* CBK1 is the terminal protein kinase in the morphogenesis-related (MOR) signalling network that controls polarized growth and the maintenance of cell wall integrity. CBK1 affects SLT2 MAPK activation in response to heat shock and cell wall stress by regulating the activity of the MAPK phosphatase SDP1 (Kuravi *et al.*, 2011) (Fig. 2). Two *S. sclerotiorum* genes (SS1G_01194 and SS1G_09988) encode ePKs with a high degree of similarity to CBK1 and one to a lesser degree (SS1G_02271).

The CK Group

The *S. sclerotiorum* CK group has three members (SS1G_00018, SS1G_09429 and SS1G_03818). *Saccharomyces cerevisiae* KSP1 (SS1G_00018) is a negative regulator of autophagy, a process that allows the remobilization of energy reserves in response to starvation. KSP1 activates TOR1 through phosphorylation which, in turn, phosphorylates ATG13 to down-regulate autophagy (Umekawa and Klionsky, 2012) (Fig. 3). KSP1 may be activated by PKA, and the depletion of both PKA and SCH9 activity is required for autophagy (Yorimitsu *et al.*, 2007). SS1G_03818 encodes an ePK similar to the *S. cerevisiae* membrane-localized protein kinases YCK1 and YCK2. The STE11 MAPKKK can transmit signals to both the filamentous growth and the high-osmolarity MAPK pathways mediated by interaction with the STE50 scaffold protein via the OPY2 membrane anchor protein (Fig. 2). Under high-glucose conditions, YCK1/YCK2 phosphorylates OPY2 leading to membrane recruitment of STE50/STE11 and specific activation of the high-osmolarity pathway. Under poor nutrient conditions, STE50/STE11 remains in the cytosol and only the filamentous growth pathway is activated (Yamamoto *et al.*, 2010). SS1G_09429 is orthologous to *S. cerevisiae* HRR25, a multi-functional kinase also involved in autophagy (Tanaka *et al.*, 2014), as well as endocytosis (Peng *et al.*, 2015), DNA repair (Hoekstra *et al.*, 1991) and several other cellular processes.

Other ePK groups

Many ePKs do not cluster into the major groups noted above, although distant relationships to major groups have been detected for some using a multi-tiered process employing Hidden Markov Modelling (Miranda-Saavedra and Barton, 2007). ePKs in the HAL family are involved in the stress response and the transport of smaller molecules across the plasma membrane. Two *S. sclerotiorum* ePKs (SS1G_00583 and SS1G_03455) are similar to *S. cerevisiae* SAT4/HAL4, which, together with the HAL5 kinase, are involved in the regulation of the activity of membrane

permeases responsible for amino acid and glucose transport (Pérez-Valle *et al.*, 2010). HRK1 (SS1G_08494) regulates the activity of a membrane ATPase linked to glucose uptake (Goossens *et al.*, 2000).

Other Group 01 contains the orthologue of *S. cerevisiae* SWE1 (SS1G_04996), which is targeted by CAMKs to regulate entry into mitosis as noted above (Barral *et al.*, 1999). This group also contains SS1G_01433, which encodes the orthologue of *S. cerevisiae* MPS1, which, in conjunction with BUB1 (SS1G_13275) (Other Group 02), regulates mitotic spindle pole formation and chromosome orientation (Storchová *et al.*, 2011). Other Group 02 also contains SS1G_5139, which is phylogenetically related to SsBUB1, but could not be assigned to an OrthoMCL group and may therefore be unique to *S. sclerotiorum*. SS1G_07203 (Other Group 05) is orthologous to *S. cerevisiae* ARK1, a highly conserved kinase which is also involved in chromosome separation and cytokinesis (Leversson *et al.*, 2002).

Other Group 03 contains SS1G_04823, which is orthologous to *S. cerevisiae* IRE1, an ePK that attenuates the unfolded protein response during endoplasmic reticulum stress (Chawla *et al.*, 2011). Other Group 03 also contains two ePKs (SS1G_14489 and SS1G_09511) which are weakly similar to PHO85 (SS1G_07226), a CDK and key regulator of cell division in response to environmental stresses (Nishizawa, 2015). OrthoMCL analysis reveals that orthologues of these genes are found only in *Aspergillus oryzae*, *Neurospora crassa* and *Gibberella zeae*, but not in any other taxa examined. SS1G_00622 (Other Group 05) is orthologous to *S. cerevisiae* GCN5, which inhibits translation in response to oxidative and heat stress through phosphorylation of elongation factor 2 (Morano *et al.*, 2012).

Two *S. sclerotiorum* ePKs in Other Group 04 (SS1G_00591 and SS1G_10932) are orthologous to TOS3 and SAK1, respectively, which activate SNF1 in a PKA-dependent manner in response to glucose (Hedbacker *et al.*, 2004a,b). Other Group 02 contains SS1G_06961, which is orthologous to *S. cerevisiae* PSK1/PSK2. PSK1 is a direct target of the SNF1 protein kinase and regulates glucose partitioning during nutrient stress through phosphorylation of the TOR1 binding protein BPP1 (DeMille *et al.*, 2015) (Fig. 3). Other Group 04 also contains SS1G_06542, which is orthologous to SHA3/SKS1, a protein kinase that integrates the response to glucose with hyphal development (Johnson *et al.*, 2014).

CONCLUSIONS AND AVENUES FOR FURTHER STUDY

Successful infection of a host plant and completion of a pathogen's life cycle are dictated by the ability to undergo morphological and biochemical changes in response to nutrient levels, stress and other environmental stimuli. Like other fungal phytopathogens (Hamel *et al.*, 2012; Turra *et al.*, 2014), *S. sclerotiorum* employs a rather

minimal set of kinases (when compared with yeast) and pathways to sense these stimuli and translate them into cellular and genetic responses. Figure 4 summarizes the *S. sclerotiorum* life cycle and attempts to place key protein kinases in the context of disease progression. After the initial saprophytic phase, mycelia undergo morphological changes to form compound infection cushions. The ability to sense surface hardness, topology and hydrophobicity is requisite for the formation of infection structures. In *B. cinerea*, the membrane protein MSB2 has been implicated in surface sensing (Leroch *et al.*, 2015) and expression of the *S. sclerotiorum* cutinase gene is dependent on interaction of mycelia with hard, hydrophobic surfaces (Bashi *et al.*, 2012). Studies with these and other fungal phytopathogens have implicated each of the three MAPKs (SsSMK3, SsHOG1 and SSSMK1) in aspects of mycelial differentiation and host penetration. It will be important to understand how surface sensors, such as MSB2 or its *S. sclerotiorum* ortholog(s), link to these MAPK pathways to control the very earliest events in the infection process, where intervention is most likely to lead to resistance. Once the host cuticle has been breached, a brief biotrophic phase occurs, followed by mycelial ramification through host tissues and transition to the necrotic phase (Kabbage *et al.*, 2015). In mapping the biological interactions between kinases and kinase pathways (Fig. 4), it becomes apparent that these phase transitions involve interplay between many signalling networks; however, a few key players emerge. The HOG1 MAPK controls the response to cellular stresses, such as redox and osmotic potential, and its activity is influenced by upstream kinases linked to membrane-associated sensors (Fig. 2), as well as by the response-regulator kinase Shk1 (Duan *et al.*, 2013). It would be interesting to explore whether SsHOG1 is responsible for the transition from the recently reported biotrophic stage (Kabbage *et al.*, 2015) to the mycelial ramification stage. SsHOG1 may also impact the activity of the SsSMK1 MAPK through SsRCK2, and therefore may also be involved in the transition from the pathogenic phase to the necrotic phase. The SsSMK3 MAPK is involved in the control of mycelial differentiation, leading to infection cushion formation, as well as sclerotogenesis (Bashi *et al.*, 2010b), whereas, in other systems, SsSMK3 orthologues are responsive to nutrient levels through protein kinase C (SsPKC1)-directed signalling. Yeast CMK1 and CMK2 regulate signalling through the high osmolarity (SsHOG1) and cell integrity (SsSMK3) MAPK pathways, and the role of SsCMK1/SsCMK2 in fine tuning the response to nutrient levels, osmotic and redox potential during the phase transitions should be explored. SsPKC1 is a member of the TOR2 signalling complex, which is functionally linked to the TOR1 complex through PHK1. Kinases within the TOR1 complex (Fig. 3) are critical for the response to nutrient levels and probably play a major role in the transition from the pathogenic to the necrotic phase. Central to this tenet is PKA (SsPKA1 and SsPKA2) which links the TOR1 complex to the SsSNF1–CRE1 complex, as well as to the SsSMK1 MAPK. Now that both SsPKA paralogues have been identified, it should be

Fig. 4 Diagram depicting a generalized *Sclerotinia sclerotiorum* life cycle with assignment of eukaryotic protein kinases (ePKs) to various stages of pathogenesis in host plant tissues. Colours indicate ePK groups as in Fig. 1. Shk1 is a histidine kinase. Full lines between kinases indicate direct or indirect interaction through signalling networks.

possible to properly investigate their role(s) in phase transition and sclerotogenesis. Each of the MAPKs (SsSMK1, SsSMK3 and orthologues of SsHOG1) has also been implicated in sclerotogenesis; however, the link(s) between SsSMK3 and SsHOG1 to kinases involved in nutrient, redox and osmotic potential signalling remains to be established. Although the formation of host necrotic tissue, as facilitated by the release of oxalic acid and

necrosis-inducing proteins, is thought to provide the nutrients necessary for fungal proliferation, the onset of sclerotogenesis is likely to be stimulated by nutrient depletion as well as low pH. Fungal autophagy may therefore be an important mechanism to allow the recovery of internal resources to be redirected to sclerotia formation, and this concept could be explored in the context of the aforementioned signalling pathways.

Although the major ePKs involved in some of these events/pathways can be delineated from comparisons with yeast and other pathogenic fungi, the life cycle of *S. sclerotiorum* involves transitions from saprophytic to biotrophic to pathogenic to necrotic phases, which presents unique and interesting challenges. This review has described several genes encoding additional protein kinases that can be studied to ascertain their role(s) in pathogenesis.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 *Sclerotinia sclerotiorum* eukaryotic protein kinases (ePKs).

Table S2 Assignment of *Sclerotinia sclerotiorum* eukaryotic protein kinases (ePKs) to orthologous groups using OrthoMCL.