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The pheromone response module, a mitogen-activated protein kinase pathway implicated in the regulation of fungal development, secondary metabolism and pathogenicity

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ABSTRACT

Mitogen-activated protein kinase (MAPK) pathways are highly conserved from yeast to human and are required for the regulation of a multitude of biological processes in eukaryotes. A pentameric MAPK pathway known as the Fus3 pheromone module was initially characterised in *Saccharomyces cerevisiae* and was shown to regulate cell fusion and sexual development. Individual orthologous pheromone module genes have since been found to be highly conserved in fungal genomes and have been shown to regulate a diverse array of cellular responses, such as cell growth, asexual and sexual development, secondary metabolite production and pathogenicity. However, information regarding the assembly and structure of orthologous pheromone modules, as well as the mechanisms of signalling and their biological significance is limited, specifically in filamentous fungal species. Recent studies have provided insight on the utilization of the pheromone module as a central signalling hub for the co-ordinated regulation of fungal development and secondary metabolite production. Various proteins of this pathway are also known to regulate reproduction and virulence in a range of plant pathogenic fungi. In this review, we discuss recent findings that help elucidate the structure of the pheromone module pathway in a myriad of fungal species and its implications in the control of fungal growth, development, secondary metabolism and pathogenicity.

1. Introduction

In order for eukaryotic organisms to detect and respond to external stimuli, an array of signalling transduction pathways are utilized (Dhanasekaran et al., 2007; Elion, 2000). An example of highly conserved signalling cascades in eukaryotes are mitogen-activated protein kinase (MAPK) pathways (Marshall, 1994; Schaeffer and Weber, 1999; Widmann et al., 1999). These pathways become active in response to a wide range of environmental stimuli, such as growth factors, cytokines, cellular stressors and pheromones (Davis, 2000; Widmann et al., 1999). A general feature of MAPK pathways is that they consist of three kinases, often termed MAP3K (MAPKKK), MAP2K (MAPKK) and MAPK, which become co-localized in response to stimulus detection, allowing for their sequential phosphorylation and activation. These pathways also often consist of various adaptor, docking and scaffold proteins which are implicated in the spatial and temporal regulation of MAP kinase signalling. Scaffolds are large, multi-domain proteins that act as a physical platform and are capable of binding multiple members of a MAPK pathway, allowing for the regulation of kinase localization, complex assembly and signal propagation to the nucleus. (Brown and Sacks, 2009; Buday and Tompa, 2010; Good et al., 2011; Pan et al., 2012). Upon receptor activation, kinases are often assembled on a scaffold protein and tethered to locations like the plasma membrane, allowing for the MAP3K to become phosphorylated *via* upstream regulators such as GTPases or kinases directly downstream of the receptor (Cuevas et al., 2007). The MAP3K then transfers a phosphate group to the MAP2K, which subsequently phosphorylates the MAPK. Each kinase becomes phosphorylated primarily at the hydroxyl groups of serine (Ser), threonine (Thr) and tyrosine (Tyr) residues (Brautigan, 2013; Jin and Pawson, 2012). The MAPK is phosphorylated at a conserved tripeptide Thr-X-Tyr motif, allowing for its activation. The MAPK then migrates to the nucleus where it interacts with transcription factors to influence gene expression, eliciting a characteristic response to the detected stimulus (Marshall, 1994; Saito, 2010; Widmann et al., 1999; Yoshioka, 2004).

The fungal kingdom is one of the largest eukaryotic kingdoms,

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consisting of an estimated 1.5–5 million species, most of which are currently uncharacterised (Choi and Kim, 2017; Dang et al., 2005). Species that belong to this kingdom include moulds, rusts, lichens, mushrooms, smuts and yeasts. Fungi are ubiquitous in the environment and are of critical importance to humankind as many species can be manipulated for use in agricultural, industrial and clinical settings and can be regarded as beneficial or detrimental with respect to human and plant health (Alazi and Ram, 2018). Approximately 10% of known fungal species have been classified as pathogens of animals, plants or humans (Ziaee et al., 2018). For the most part, the relevance of fungi is due to the ability of many species, predominately filamentous fungi to undergo secondary metabolism. This is a process of producing an array of small, bioactive compounds, known as secondary metabolites (SMs), that exert various properties, acting as cytotoxic agents, mutagens, immunosuppressants, antibiotics and carcinogens. Many SMs also contribute to fungal virulence in both human and plant hosts (Bok and Keller, 2004). A myriad of significant SMs, both beneficial and harmful, have been isolated from fungal species. For example, the antibiotic penicillin is produced by species of *Penicillium* (Bills and Gloer, 2016), while *Aspergillus* species such as *A. nidulans, A. flavus* and *A. fumigatus* produce toxic compounds like sterigmatocystin, aflatoxins and gliotoxin respectively (Amaike and Keller, 2011; Bok et al., 2006; Hedayati et al., 2007; Hof and Kupfahl, 2009). Although many compounds produced by filamentous fungal species have been characterised, genome sequencing suggests that fungi are capable of producing SMs well in excess of previously predicted numbers as they possess a myriad of dormant SM gene clusters that are not activated under standard laboratory conditions (Sanchez et al., 2012). Due to this, fungal secondary metabolism and the mechanisms governing regulation of SM production have become attractive fields of study.

In order for fungi to regulate their growth and development, as well as processes such as SM production and virulence, a wide range of signal transduction pathways are required (Bayram et al., 2012, 2008; Elramli et al., 2019; Frawley and Bayram, 2020). Multiple MAPK pathways have been implicated in the regulation of biological responses in fungal species. In *S. cerevisiae*, five MAPK pathways have been identified, each regulating separate cellular processes (Qi and Elion, 2005). One of these pathways is known as the Fus3 pheromone module which is the most extensively studied MAPK pathway in any eukaryotic organism and is responsible for the regulation of cell–cell fusion, otherwise known as sexual development, in response to pheromone signalling (Bardwell, 2005). This pathway is considered a mechanistic paradigm for MAP kinase module signalling and regulation and since it's discovery, orthologous pheromone module MAPK pathways have been found to be highly conserved in the fungal kingdom. However, information regarding their mechanisms of signalling and their biological consequences is sparse. In this review, we will highlight recent findings in order to elucidate the implications of the pheromone module in the regulation of fungal development, SM production and pathogenicity. This will provide a comprehensive overview of the utilization of the pheromone module as a central signalling hub in fungal species, specifically in filamentous fungi.

2. The Fus3 pheromone module in *S. cerevisiae* regulates cell fusion

The unicellular fungus *S. cerevisiae* (baker's yeast) has been adapted for use as a model eukaryotic organism in many areas of genetics, molecular and cell biology research. The utilization of yeast for studying protein interactions and signalling pathways has allowed for the extensive characterisation of many pathways that contribute to cell adaptation, survival, reproduction and apoptosis in eukaryotes, ranging from fungi to humans. This organism has been critical in the elucidation of MAPK signalling mechanisms across the entire eukaryotic kingdom (Chen and Thorner, 2007).

Yeast have two opposite mating types, known as 'a' and 'α' genotypes. Both of these cell types exist as haploid cells and the fusion of two opposite mating types results in the formation of diploid cells. In order for two neighbouring yeast cells to fuse, they release opposite pheromone mating signal peptides. 'a' cells respond to 'α' factor pheromone produced by 'α' cells and vice versa (Bardwell, 2005). To ensure yeast cells efficiently detect and respond to pheromone mating signals, the Fus3 MAPK pheromone module becomes activated (Fig. 1). Pheromone signals are detected by G-protein coupled receptors (GPCR), such as Ste2 and Ste3 at the plasma membrane. When activated, these receptors undergo a conformational change. The Gα subunit of the GPCR then exchanges guanosine diphosphate (GDP) for guanosine triphosphate (GTP), which triggers dissociation of the Gβγ heterodimer from the Gα subunit. The Gβγ heterodimer then recruits the large, multidomain Ste5 scaffold protein to the membrane, as well as the p21-activated protein kinase Ste20 (Bardwell, 2005; Leeuw et al., 1998; Pryciak and Huntress, 1998). Ste5 is essential for assembling a three-tiered kinase cascade as it possesses a binding site for the Gβγ heterodimer as well as sites for binding three separate kinases (Choi et al., 1994; Whiteway et al., 1995). The activation of the kinase cascade commences upon phosphorylation of the MAP3K Ste11. This is dependent on the cooperation of Ste5, Ste20 and the adaptor protein Ste50. Ste5 and Ste50 bind Ste11 *via* Sterile Alpha Motif (SAM) domain interactions, localising it to the membrane and Ste20 phosphorylates the N-terminal regulatory domain of Ste11, resulting in its activation (Pryciak and Huntress, 1998; Wu et al., 1999; Xu et al., 1996). Binding of each kinase to sites on the Ste5 scaffold results in Ste11-dependent phosphorylation of the MAP2K Ste7, which then phosphorylates the terminal MAPK Fus3 at the Thr-X-Tyr motif (Hao et al., 2008; Kranz et al., 1994; Saito, 2010). Once activated, Fus3 is then capable of migrating to the nuclear envelope where it translocates into the nucleus to interact with the Ste12 transcription factor (van Drogen et al., 2001). This results in cells undergoing various physiological changes such as

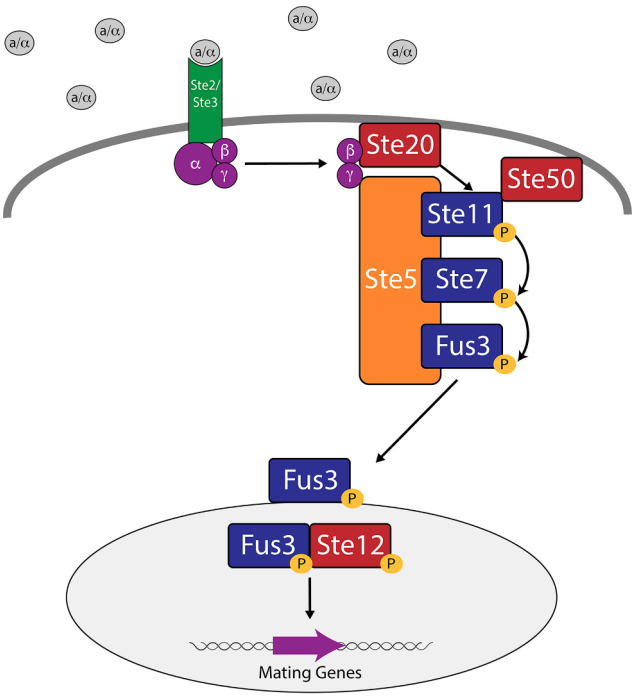

Fig. 1. The yeast Fus3 pheromone module. Detection of either 'a' or 'α' pheromones by GPCRs like Ste2 or Ste3 result in the assembly of a three-tiered kinase cascade consisting of the MAP3K Ste11, the MAP2K Ste7 and the MAPK Fus3. These kinases bind to the scaffold Ste5 and phosphorylate one another to enable Fus3 activation and translocation into the nucleus. This results in the activation of transcription factors like Ste12 which induces expression of mating genes and regulates cell fusion. 'P' represents phosphate groups.

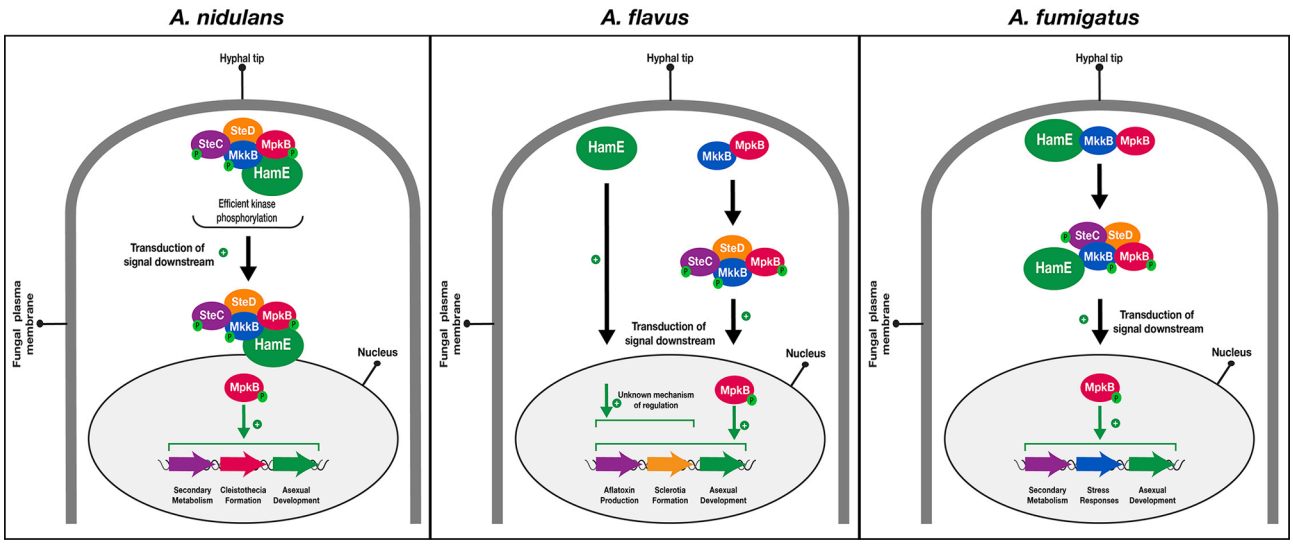

Fig. 2. The pheromone module pathway in *A. nidulans, A. flavus* and *A. fumigatus*. The *A. nidulans* pheromone module consists of the three kinases SteC, MkkB and MpkB, the adaptor protein SteD and the scaffold HamE. These 5 proteins co-localize to regions like the hyphal tip and plasma membrane. HamE mediates efficient kinase phosphorylation, resulting in MpkB activation and migration of the entire complex to the nuclear envelope. MpkB translocates into the nucleus and interacts with transcription factors like SteA and VeA to regulate various biological processes such as asexual sporulation, cleistothecia development, vegetative growth and the production of various SMs. 'P' represents phosphate groups. The *A. flavus* pheromone module consists of the three kinases and SteD. The MkkB-MpkB dimer localizes to the hyphal tip and interacts with the cytoplasmic SteC-SteD dimer to form a tetrameric complex. MpkB becomes phosphorylated and enters the nucleus where it positively regulates aflatoxin production, asexual sporulation and sclerotia development. HamE also localizes to the hyphal tip and regulates aflatoxin production and sclerotia development but the mechanism of regulation is unknown. The *A. fumigatus* pheromone module consists of the three kinases, SteD adaptor and the HamE scaffold. MkkB, MpkB and HamE localize at hyphal tips and interact with the cytoplasmic SteC-SteD dimer in the cytoplasm. MpkB translocates into the nucleus and positively regulates asexual sporulation, vegetative growth, stress responses and production of various SMs such as gliotoxin. Figure adapted from (Frawley, 2018; Frawley and Bayram, 2020; Frawley et al., 2020b).

cell cycle arrest in the G1 phase of growth, oriented growth towards the neighbouring cell and plasma membrane and nuclear fusion of the two mating partners (Bardwell, 2005; Wong Sak Hoi and Dumas, 2010).

3. The MAK-2 pheromone module in *Neurospora crassa* regulates germling and hyphal fusion, as well as sexual development

Since the incorporation of unicellular yeasts in fungal research, there has been an increasing focus on the use of filamentous fungi as these species are of high agricultural, medical and industrial importance. Discovery of the Fus3 pheromone module in yeast has led to the identification of many orthologous proteins and signalling pathways in eukaryotes, ranging from filamentous fungi to humans. In filamentous fungal species, pheromone module proteins have been shown to be implicated in the regulation of a wide variety of biological processes including vegetative growth, asexual conidiation, sexual development, pathogenesis and SM biosynthesis (Lev et al., 1999; Li et al., 2005; Zhao et al., 2005).

A MAP kinase pathway orthologous to the yeast Fus3 pheromone module has been identified and characterised in the filamentous ascomycete fungus *N. crassa*, which has been widely used as a model system for studying fungal genetics and development (Berlin and Yanofsky, 1985; Roche et al., 2014). This pathway is termed the MAK-2 module and has been shown to be crucial in the regulation of germling and hyphal fusion, as well as sexual development (Li et al., 2005; Pandey et al., 2004). The *N. crassa* MAK-2 pheromone module consists of three kinases, NRC-1 (MAP3K), MEK-2 (MAP2K) and MAK-2 (MAPK) and the STE-50 adaptor protein (Dettmann et al., 2014; Li et al., 2005; Pandey et al., 2004). Each of these proteins are orthologs of the respective proteins in the yeast Fus3 pheromone module. Interestingly, no Ste5 ortholog exists in *N. crassa* and it has been shown that orthologs of the *ste5* gene do not exist in the genomes of filamentous fungi (Rispail et al., 2009). Instead, the *N. crassa* pheromone module contains a scaffold protein known as HAM-5 (Dettmann et al., 2014; Jonkers et al., 2014). This protein does not exist in yeast and *ham5* orthologs are highly conserved in filamentous ascomycete fungi (Jamet-Vierny et al., 2007). During chemotropic interactions between either neighbouring germlings or hyphae, the HAM-5 scaffold physically associates with all three kinases and STE-50 to form a pentameric complex, which localizes to small puncta in opposing hyphal tips. During this polarised growth, the MAP kinase complex undergoes repeated cycles of assembly and disassembly every 4 min, during which the complex assembles in one hyphal tip, disassembles and then assembles in the opposite hyphal tip (Jonkers et al., 2014). Assembly of this complex and its interactions with STE-20 and the RAS-2 GTPase result in the sequential phosphorylation of each kinase, culminating in the activation of MAK-2. MAK-2 then translocates into the nucleus and activates the PP-1 transcription factor (Dettmann et al., 2014), which is an ortholog of yeast Ste12. This, in turn, leads to the regulation of hyphal extension, germling and hyphal fusion, ascospore germination and formation of sexual fruiting bodies known as protoperithecia (Leeder et al., 2013; Li et al., 2005).

To date, information is limited regarding the molecular roles of the *N. crassa* pheromone module in the regulation of secondary metabolism. However, it is postulated that at least 10 putative SM gene clusters exist in the *N. crassa* genome and several SMs isolated from this species have been characterised, such as coprogen (Toth et al., 2009), ergothioneine (Bello et al., 2012) and carotenoids (Bayram et al., 2019). It is known that the pheromone module is involved in the regulation of secondary metabolism in various fungal species, mostly due to cross-talk interactions with the velvet complex, a group of fungal specific proteins that are highly conserved among the ascomycetes (Bayram et al., 2012; Frawley et al., 2020a; Manfiolli et al., 2019; Ni and Yu, 2007). Interestingly, it has also recently been shown that the velvet complex is implicated in the regulation of SM production in *N. crassa* (Bayram et al., 2019). It can therefore be postulated that the pheromone module may also contribute to the regulation of SM production in *N. crassa* in a

similar manner. Thus, future work investigating the mechanisms of both fungal development and SM regulation in this species will provide insight on the biological consequences of pheromone module signalling in filamentous fungi.

4. The pheromone module in *Aspergillus* species regulates vegetative growth, asexual and sexual development and secondary metabolism

The filamentous ascomycete fungus *A. nidulans* has been used extensively in research as a model organism for eukaryotic biology. This species has been implicated in various fields such as cell biology, biochemistry, SM biosynthesis, fungal genetics and development, to name a few (Dasgupta et al., 2016; Harris, 1997; Morris and Enos, 1992). *A. flavus* and *A. fumigatus* are saprophytic fungi that are considered to be major threats with regards to human and animal health, as well as agriculture (Amaike and Keller, 2011; Latge, 1999). All three *Aspergillus* species are capable of producing a myriad of SMs such as the carcinogenic compounds sterigmatocystin and aflatoxin B1, produced by *A. nidulans* and *A. flavus* respectively (Amaike and Keller, 2011; Bok et al., 2006), as well as the immunosuppressant gliotoxin, produced by *A. fumigatus* (Hof and Kupfahl, 2009). Due to the production of aflatoxins, *A. flavus* is classified as a global threat as it can contaminate a range of crops, such as maize, peanuts, corns, cereals and cottonseed, to name a few (Lewis et al., 2005; Rushing and Selim, 2019; Yu et al., 2005). Ingestion of aflatoxin contaminated crops can pose significant threats to humans and animals and can result in the development of hepatocellular carcinomas or aspergillosis (Bhatnagar-Mathur et al., 2015; Hedayati et al., 2007; Kew, 2013). Gliotoxin production by *A. fumigatus* contributes to the development of invasive aspergillosis (IA) *via* the inhibition of NADPH oxidase and alcohol dehydrogenase activity, as well as macrophage and neutrophil functionality (Gardiner et al., 2005; Spikes et al., 2008). IA is a major risk to individuals who are immunodeficient, such as patients receiving chemotherapy or transplantations and the mortality rates in these patients ranges from 50 to 95% (Balloy and Chignard, 2009; Latge, 1999, 2001; Maschmeyer et al., 2007; McCormick et al., 2010).

In each of these three *Aspergillus* species, a pheromone module pathway, orthologous to those that exist in yeast and *N. crassa* has been identified and characterised (Fig. 2) and has been shown to be critical for the coordinated regulation of both development and secondary metabolism (Bayram, 2012; Frawley, 2018, 2020; Frawley et al., 2020b). This pathway consists of the MAP3K SteC, MAP2K MkkB, MAPK MpkB and the adaptor protein SteD which are orthologs of yeast Ste11, Ste7, Fus3 and Ste50, respectively (Bayram et al., 2012; Teague et al., 1986; Vallim et al., 2000; Wei et al., 2003). A unique protein of the pheromone module in *Aspergillus* species is the scaffold protein HamE (Frawley et al., 2018), which is an ortholog of the HAM-5 scaffold in *N. crassa* (Dettmann et al., 2014; Jonkers et al., 2014).

4.1. The A. nidulans pheromone module

In the *A. nidulans* pheromone module, SteC, MkkB and MpkB interact with SteD and HamE at sites such as the plasma membrane and the hyphal tips, forming a pentameric complex (Bayram et al., 2012; Frawley et al., 2018). It is postulated that this complex is assembled in response to pheromone signals released during chemotropic interactions between neighbouring hyphae. Following assembly of this complex, a phosphorylation cascade is triggered, which is dependent on HamE interactions with both MkkB and MpkB. Sequential phosphorylation and activation of each kinase is mediated and this results in the migration of the entire complex to the nuclear envelope (Bayram et al., 2012; Frawley et al., 2018). MpkB then translocates into the nucleus, where it interacts with various transcription factors such as the Ste12 ortholog SteA (Vallim et al., 2000) and the velvet protein VeA. SteA mediates regulation of developmental genes, specifically those that contribute to the regulation of sexual development, while VeA activation triggers assembly of the velvet complex to regulate secondary metabolism (Atoui et al., 2008; Bayram et al., 2012, 2008; Sarikaya Bayram et al., 2010). It has been shown that disruption of any of the pheromone module genes in *A. nidulans* results in developmental abnormalities, as well as defective secondary metabolism (Bayram et al., 2012; Frawley et al., 2018). Pheromone module mutants display reduced hyphal extension rates, reductions in asexual sporulation and a complete loss of cleistothecia formation, which are sexual fruiting bodies. These mutants also display significant reductions in expression of SM genes, such as those contributing to the biosynthesis of sterigmatocystin, penicillin and the antitumor compound terrequinone A (Bayram et al., 2012; Frawley et al., 2018). This is likely due to reduced activation of velvet complex components as it has been shown that the *veA, velB* and *laeA* genes are significantly downregulated in all pheromone module mutant strains (Frawley et al., 2018).

4.2. The A. flavus pheromone module

In the *A. flavus* pheromone module, it has been shown that a tetrameric complex consisting of the three kinases SteC, MkkB and MpkB and the adaptor protein SteD is assembled and that proteins of this complex physically interact at sites such as the hyphal tips, presumably in response to pheromone signalling between neighbouring hyphae (Fig. 2). This facilitates efficient MpkB phosphorylation and translocation into the nucleus where it interacts with transcription factors to regulate asexual and sexual development, as well as the production of various SMs (Frawley et al., 2020a). Deletion of either *steC, mkkB, mpkB* or *steD* results in significantly reduced levels of asexual sporulation and a complete inability to produce sclerotia. Each mutant was also incapable of producing aflatoxin B1. Interestingly, deletion of these genes results in the increased production of various SMs, such as leporin B, cyclopiazonic acid (CPA), aspergillicin A and aspergillicin F. This suggests that the pheromone module is implicated in both the positive and negative regulation of SM production, perhaps depending on the culture conditions or developmental state of the species (Frawley et al., 2020a). In *A. flavus,* contrary to what is observed in both *A. nidulans* (Frawley et al., 2018) and *N. crassa* (Dettmann et al., 2014; Jonkers et al., 2014), there is no experimental evidence to suggest that HamE acts as a scaffold in the pheromone module pathway as it was not found to interact with any of the pheromone module proteins. HamE was observed to localize to the hyphal tips and a *hamE* mutant is incapable of producing both sclerotia and aflatoxin B1. However, deletion of this gene does not impair asexual sporulation and the metabolic profile of this mutant resembles a wild type strain with regards to leporin B, CPA, aspergillicin A and aspergillicin F production (Frawley et al., 2020a). These data could suggest that HamE is required during cell–cell communication between hyphae but may exert it's regulatory roles independently of the pheromone module.

4.3. The A. fumigatus pheromone module

In the *A. fumigatus* pheromone module, it was found that the five pheromone module proteins physically interact to form a pentameric complex in *A. fumigatus* (Fig. 2), similar to what is observed in *A. nidulans* (Frawley et al., 2018) and *N. crassa* (Dettmann et al., 2014; Jonkers et al., 2014). Proteins of the *A. fumigatus* pheromone module localize to regions such as the hyphal apices, perhaps in order to respond to pheromone signals. Assembly of the complex enables MpkB phosphorylation and translocation into the nucleus, where it presumably interacts with transcription factors to regulate gene expression. The *A. fumigatus* pheromone module has been shown to be implicated in the regulation of various developmental programmes, such as vegetative growth, asexual sporulation and responses to both cell wall and oxidative stressors as mutants of this pathway exhibit increased sensitivity to both Congo Red and H_2O_2. This pathway was also found to regulate

secondary metabolism as deletion of any of the pheromone module genes results in dramatic reductions in gliotoxin production. Additionally, the deletion of *mkkB* resulted in reduced levels of pseurotin A, pseurotin D, fumagillin and pyripyropene A production (Frawley et al., 2020b).

Overall, it is evident that the pheromone module is essential for the regulation of development and secondary metabolism in *Aspergillus* species. However, the exact mechanisms of MpkB-mediated regulation of transcription factors in the nucleus are poorly understood. It is also not known which genes in particular are regulated *via* pheromone module signalling and whether this pathway contributes to the modulation of virulence. Future work involving characterisation of this pathway may elucidate the processes that govern complex activation and assembly, control of gene expression in response to environmental stimuli and regulation of fungal development, SM production and pathogenicity.

5. Proteins of the pheromone module pathway regulate development and virulence in plant pathogenic fungi

5.1. The UvPmk1 MAPK in Ustilaginoidea virens

The ascomycete fungal pathogen *Ustilaginoidea virens,* otherwise known as *Villosiclava virens* in it's teleomorphic state is a major cause of rice false smut, which is a critical disease worldwide in rice spikelets. During this disease, a white fungal mass protrudes from a rice spikelet, which then becomes a yellow smut ball before ultimately transitioning to a greenish-black phenotype (Bischoff et al., 2004; Fan et al., 2016; Zhang et al., 2014). Rice false smut disease results in the reduction of both rice yield and grain quality and *U. virens* produces various SMs that contaminate the grains and are toxic to humans and animals (Koiso et al., 1994; Nakamura et al., 1994). It has recently been discovered that *U. virens* contains an ortholog of the yeast MAPK Fus3 and this protein is known as UvPmk1 (Tang et al., 2020). In this study, it was found that UvPmk1 is required for the regulation of hyphal tip morphology and conidiation as a *UvPmk1* mutant produced sparse and wavy hyphae as opposed to the messy hyphae produced by the wild type and this mutant also exhibited significantly reduced levels of sporulation. It was also observed that the *UvPmk1* mutant displays increased resistance to both hyperosmotic and cell wall stress agents, whilst exhibiting increased sensitivity to oxidative stress. The expression levels of the *UvPmk1* gene were found to be significantly increased during infection, which implicates that this gene is required for virulence. This is further supported by the fact that a *UvPmk1* mutant was found to be completely unable to successfully colonize and infect susceptible rice spikelets and thus was unable to form false smut balls (Tang et al., 2020).

Overall, these data suggest that UvPmk1 is essential for the regulation of *U. virens* development, as well as host colonisation and the formation of infective hyphae. However, it is currently not known whether this MAPK functions as part of a conserved pheromone module in this species. Although it has been shown that *U. virens* contains an ortholog of yeast Ste12, known as Fst12 (Yu et al., 2016), it is unknown whether Fst12 acts as a downstream target for the pheromone module pathway in *U. virens*. However, it is possible that UvPmk1 may regulate various developmental programmes and virulence *via* phosphorylation and activation of Fst12, as is evident in other species.

5.2. The CfPMK1 MAPK in Colletotrichum fructicola

Colletotrichum fructicola is another ascomycete fungal pathogen that causes infections in more than 50 plant species such as strawberries, apples and pears (Liang et al., 2019; Weir et al., 2012). One notable disease caused by *C. fructicola* is *Glomerella* leaf spot disease which results in over 90% defoliation in apples (Wang et al., 2012). During the infection process, germination of *C. fructicola* leads to the production of germ tubes, which adhere to the surfaces of plants. A specialised infective structure known as an appressorium is then formed which penetrates the plant host (Liang et al., 2019). In the later stages of infection, this fungus produces necrotrophic hyphae which secrete a myriad of SMs that contribute to fungal virulence and necrotic symptom development (O'Connell et al., 2012). It has recently been found that *C. fructicola* possesses an ortholog of yeast Fus3 termed CfPMK1 and that this MAPK is essential for the regulation of development and virulence in this fungus (Liang et al., 2019). A *CfPMK1* mutant exhibited significantly reduced aerial hyphal differentiation and deformed perithecia, which are sexual reproductive structures. Perithecia produced by the wild type were globose and the outer layers were heavily melanised. However, the *CfPMK1* mutant exhibited compact, misshapen perithecia that had significantly reduced levels of melanisation. Deletion of *CfPMK1* results in increased sensitivity to osmotic stress agents but also increases resistance to both cell wall and plasma membrane stressors. This mutant was also incapable of forming appressoria during the infection process and failed to penetrate the cuticle of susceptible plant hosts, unlike the wild type which formed an abundance of necrotic lesions. Post-invasive colonization was also hindered in the *CfPMK1* mutant, as this strain is incapable of forming lesions in pre-wounded plant hosts (Liang et al., 2019).

Taken together, these data suggest that this MAPK is required for the regulation of *C. fructicola* development, stress responses and pathogenicity. However, information regarding signalling *via* a conserved pheromone module in *C. fructicola*, as well as the targets of regulation of CfPMK1 are poorly understood. Perhaps CfPMK1 interacts directly or indirectly with the *C. fructicola* Ste12 ortholog SteA, in a similar manner to what is observed in various related fungal species. To date, there have been no studies that have focused on the characterisation of *steA* mutant phenotypes in *C. fructicola* or the potential interactions between CfPMK1 and SteA. Thus, future work could help provide insight on the mechanisms of CfPMK1-mediated regulation of *C. fructicola* development and virulence and the transcription factors that are activated downstream of this MAPK.

5.3. The pheromone module proteins in Botrytis cinerea

Botrytis cinerea is an ascomycete grey mould fungus that causes diseases in over 200 crop hosts, leading to huge economic losses worldwide (Williamson et al., 2007). Germination of *B. cinerea* spores leads to the formation of appressoria-like hyphal swellings and penetration of host cells, resulting in the rotting of plant tissues (Schamber et al., 2010; Williamson et al., 2007). In this fungus, orthologs of yeast *ste11* (*Bc-ste11*), *ste7* (*Bc-ste7*), *fus3* (*bmp1*), *ste50* (*Bc-ste50*) and *ste12* (*Bc-ste12*) exist (Doehlemann et al., 2006; Schamber et al., 2010).

It has been shown that deletion of any of these kinases or the Bc-Ste50 adaptor results in various developmental defects. Each mutant displays significantly reduced rates of vegetative growth (30–70%) and more compact aerial mycelium that exhibit reduced proliferation (Schamber et al., 2010). With regards to asexual sporulation, each mutant produced conidia that are smaller, more spherical and contain reduced numbers of nuclei in comparison to the wild type conidia. Sexual development was also observed to be hindered as the ability to form sclerotia is completely abolished in each of these mutants (Doehlemann et al., 2006; Schamber et al., 2010). With regards to virulence, it was found that all mutants were incapable of forming lesions on tomato leaves, in comparison to the wild type which began to form lesions 1 day post-inoculation. These mutants were also unable to penetrate the epidermis layers of heat-killed onions and exhibited significantly slower lesion expansion in wounded apples, when compared to the wild type (Doehlemann et al., 2006; Schamber et al., 2010; Zheng et al., 2000). With regards to the *Bc-ste12* mutant, it was evident that this strain was incapable of producing sclerotia under standard culture conditions. This mutant also exhibited impaired invasion, lesion formation and expansion on tomato leaves and reduced penetration efficiency in heat-killed onions (Schamber et al., 2010).

Overall, these data suggest that the pheromone module proteins in *B. cinerea* are essential for the regulation of various developmental programmes and virulence in a range of plant hosts. Due to the phenotypical similarities observed in each of the mutants, it can be postulated that these proteins function in the same pathway or in a similar manner to coordinate the regulation of these biological processes, likely *via* phosphorylation and activation of the Bc-Ste12 transcription factor.

5.4. The PfFus3 MAPK in Pseudocercospora fijiensis

Pseudocercospora fijiensis is an ascomycete fungus that causes black leaf streak disease (BLSD) in the majority of bananas cultivated worldwide (Churchill, 2011). During the early stages of plant infection, *P. fijiensis* reproduces primarily *via* the production of conidia which germinate and penetrate the host plant. Hyphae are then formed which grow throughout the leaf, resulting in cell death. An early sign of infection is the formation of reddish-brown streaks along the length of the leaves, which eventually transition to large brown or black streaks. These streaks later form lesions and merge to induce severe leaf necrosis. This disease results in a significant reduction in both fruit quantity and quality (Churchill, 2011; Marín et al., 2003; Onyilo et al., 2018). It has recently been shown that *P. fijiensis* contains an ortholog of yeast Fus3, termed PfFus3 (Onyilo et al., 2018). It was found that deletion of the *PfFus3* gene results in reduced infection efficiency and delayed virulence. This was evident as the leaves of susceptible banana plants inoculated with mycelia from this mutant developed disease symptoms after 18–19 days, in comparison to 9–10 days for the wild type. Leaves inoculated with the *PfFus3* mutant also exhibited significantly reduced levels of necrosis, signifying that disease progression in this strain is hindered. Invasive growth in this mutant is also impaired as there is no hyphal growth observed in the intercellular spaces of inoculated leaf tissue and there is a significant reduction in fungal biomass observed in infected plant tissues, in comparison to the wild type (Onyilo et al., 2018).

Overall, this suggests that the MAPK PfFus3 is essential for the regulation of various developmental processes in *P. fijiensis* that contribute to pathogenicity in plants, such as invasive hyphal growth and host colonization. To date, there have been no studies that have focused on the characterisation of other pheromone module proteins in *P. fijiensis.* However, it is evident from BLAST searches that this species contains orthologs of yeast Ste11, Ste7, Ste50 and Ste12. Thus, it can be proposed that a conserved mechanism of signalling may be utilized by *P. fijiensis* to regulate development and virulence in response to environmental stimuli, such as pheromones. Future work may help elucidate the mechanisms of pheromone module assembly in this species and could identify downstream transcription factor targets for the PfFus3 MAPK.

5.5. The pheromone module proteins in Ustilago maydis

The basidiomycete pathogenic fungus *Ustilago maydis* is a major crop contaminant as it infects maize and leads to the formation of tumors, which is known as corn smut disease

(Matei and Doehlemann, 2016). Fusion of two compatible haploid cells in response to pheromone signalling results in dikaryon formation. This represents the pathogenic stage of growth for this species, which is strictly filamentous (Banuett and Herskowitz, 1994b). Hyphae and appressoria are capable of directly penetrating the surface of the plant and they produce various effector proteins to suppress the plant's immune system, allowing for the spread of hyphae from cell to cell (Doehlemann et al., 2008; Lo Presti et al., 2015). The fungus then induces tumor formation roughly four days post-infection. Inside these tumors, *U. maydis* undergoes hyphal branching and forms spore aggregates after 12 days, which are dispersed once the tumors break down, to enable spread of this species to other hosts (Skibbe et al., 2010). In *U. maydis,* an orthologous pheromone module pathway has been identified and it was shown to regulate cell fusion and pathogenicity in response to pheromone detection.

This pathway consists of the MAPKKK Kpp4/Ubc4, the MAPKK Fuz7/Ubc5, the MAPK Kpp2/Ubc3 and the Ubc2 adaptor, which has similarities to yeast Ste50 (Andrews et al., 2000; Banuett and Herskowitz, 1994a; Mayorga and Gold, 2001; Müller et al., 1999, 2003). Interestingly, according to BLAST searches, the genome of *U. maydis* does not contain an ortholog of yeast *ste12.* It is instead postulated that this MAPK pathway signals downstream to the transcription factor Prf1, which is a HMG box domain protein that is more closely related to the ROX1 transcription factor in yeast. Phosphorylation of Prf1 *via* the pheromone module and multiple other regulators is known to induce the expression of pheromone-responsive genes such as the *a* and *b* mating type genes (Chacko and Gold, 2012). It was found that deletion of any of the pheromone module genes results in the inability to form conjugation tubes. Appressoria production is also completely inhibited in these mutants and thus, these strains exhibit impaired mating and abolishment of pathogenicity, as is evident from the lack of tumors formed on host plants, in comparison to a wild type strain (Banuett and Herskowitz, 1994a; Mayorga and Gold, 1999; Müller et al., 1999, 2003). It has also been shown that a *prf1* mutant exhibits significantly reduced levels of expression from the *a* and *b* mating-type loci, even when exposed to pheromones. As a result, this mutant is sterile and is non-pathogenic (Hartmann et al., 1999), providing further evidence that Prf1 is a downstream target for the pheromone module pathway and that the response to pheromones is critical for the regulation of mating.

These data highlight that the pheromone module is highly conserved in *U. maydis* and that this pathway is utilized as a means of regulating various developmental programmes and virulence. However, it is not known why this pathway does not signal downstream to a Ste12 ortholog. It could be suggested that Prf1 orthologs may have evolved overlapping or redundant functions with Ste12 in certain species. Further work may provide useful information regarding the evolution of these transcription factors in fungi and the downstream targets of pheromone module signalling.

5.6. The pheromone module proteins in Magnaporthe oryzae

The filamentous ascomycete plant pathogen *Magnaporthe oryzae* is a major cause of blast diseases in cereals such as rice and wheat. Annually, this fungus contaminates enough rice to feed 60 million people and is a significant threat to wheat production in South America and Asia (Inoue et al., 2017; Wilson and Talbot, 2009). Infection is mediated initially *via* the production of an appressorium, which penetrates the outer cuticle of the host leaf (Dagdas et al., 2012). The fungus then produces invasive hyphae and a range of effector molecules to facilitate suppression of the host's immune system and colonization of host cells (Giraldo et al., 2013). Following initial infection, disease lesions are formed after about 4–5 days (Sakulkoo et al., 2018).

M. oryzae possesses pheromone module proteins orthologous to those that exist in yeast. These include the MAPKKK Mst11, the MAPKK Mst7, the MAPK Pmk1, the adaptor protein Mst50 and the transcription factor SteA (Xu and Hamer, 1996; Zhao et al., 2005). It has been shown that all three kinases are critical for the regulation of pathogenicity (Sakulkoo et al., 2018; Xu and Hamer, 1996; Zhao et al., 2005). This is due to the fact that each kinase mutant is incapable of producing necrotic lesions on susceptible rice seedlings and barley, suggesting that these strains cannot successfully penetrate the plant cuticle. Inoculation of each mutant directly into leaf sheaths of susceptible rice seedlings resulted in small areas of necrosis at sites of injection. However, unlike the wild type strain, lesions did not spread outside of these areas and it was found that mycelia and conidia of the *pmk1* mutant at injection sites were not viable. While each mutant undergoes successful germination, it is also evident that deletion of any of the kinase genes results in impaired germ tube formation and complete abolishment of appressoria production (Xu and Hamer, 1996; Zhao et al., 2005). Regarding the structure of

Table 1
Summary of the main functions of the pheromone module proteins in various fungi.

Organism	Cell/Germling fusion	Hyphal fusion	Vegetative Growth	Asexual Sporulation	Sexual Development	SM production	Virulence
Saccharomyces cerevisiae			*				
Neurospora crassa							
Aspergillus nidulans							
Aspergillus flavus						*	
Aspergillus fumigatus						*	
Ustilaginoidea virens							
Colletotrichum fructicola							
Botrytis cinerea							
Pseudocercospora fijiensis							
Ustilago maydis							
Magnaporthe oryzae							
Fusarium graminearum							

*= both positive and negative regulation observed

	Positive regulation
	Negative regulation
	No effect
	Not determined
	Not applicable

this pheromone module pathway, direct physical interactions between Mst11, Mst7 and Pmk1 are either only weakly detected or are not detected at all. However, it has been shown that both Mst11 and Mst7, but not Pmk1 interact with the Mst50 adaptor (Zhao et al., 2005), which may enable assembly of the complex, stabilisation of kinase interactions and mediation of kinase phosphorylation. While extensive phenotypical studies have not been performed using the *mst50* mutant, it has been shown that deletion of this gene results in phenotypes that resemble the kinase mutants, such as impaired appressorium development and decreased virulence (Zhao et al., 2005).

Overall, these data suggest that a conserved tetrameric pheromone module exists in *M. oryzae* and that this pathway is critical for the regulation of developmental processes that modulate pathogenicity, such as appressoria development and infective hyphal growth. However, information regarding the roles of the SteA transcription factor in *M. oryzae* is sparse and physical interactions between SteA and the pheromone module proteins have not been confirmed. However, it can be proposed that the pheromone module pathway signals downstream to facilitate the phosphorylation and activation of SteA to potentially mediate the regulation of fungal development and virulence, as is observed in other fungal species.

5.7. The pheromone module in Fusarium graminearum

The ascomycete plant pathogen *Fusarium graminearum* is a major crop contaminant that infects wheat, maize and barley and leads to destructive diseases such as Fusarium head blight (FHB), resulting in drastic reductions in crop yields (Khan et al., 2020; McMullen et al., 2012). Once fungal macroconidia or ascospores germinate on wheat spikelets, they are then capable of penetrating and disseminating throughout the host plant, which ultimately leads to cell necrosis and bleaching of plant tissues (Trail, 2009). A major concern regarding FHB is the production of various SMs by *F. graminearum* during the infection process. Consumption of crops contaminated with *F. graminearum* mycotoxins can cause a range of symptoms in both humans and livestock. In humans, SMs can lead to food poisoning, abdominal pain, diarrhea and headaches (McMullen et al., 1997; Ponts, 2015).

In *F. graminearum,* orthologs of the pheromone module exist. These include the MAPKKK FgSte11, the MAPKK FgSte7, the MAPK FgGpmk1, the adaptor protein FgSte50 and the transcription factor FgSte12 (Gu et al., 2015; Jenczmionka et al., 2003; Wang et al., 2011). It has been reported that FgSte50, FgSte11, FgSte7 and FgGpmk1 physically interact and that phosphorylation of FgGpmk1 is significantly reduced in *FgSTE11* and *FgSTE7* mutants (Ramamoorthy et al., 2007). This suggest that a tetrameric pheromone module could be assembled, enabling the phosphorylation and activation of FgGpmk1 (Gu et al., 2015; Wang et al., 2011). Each of these kinases have been shown to be critical for the regulation of specific developmental programmes and pathogenicity (Jenczmionka et al., 2003; Jenczmionka and Schäfer, 2005; Wang et al., 2011). On Czapek plates, a *gpmk1* mutant exhibits dramatically reduced levels of aerial hyphae formation, in comparison to the thick, white aerial hyphae produced by the wild type. It was also observed that this mutant displays significantly reduced levels of conidia and is incapable of producing perithecia, signifying that the deletion of *gpmk1* results in sexual sterility (Jenczmionka et al., 2003). With regards to virulence, it was observed that the *gpmk1* mutant showed initial growth when inoculated on wheat spikes. However, over a course of 3 weeks, no further signs of infection were evident, as opposed to the wild type, which causes bleaching of plant tissues 2–3 weeks post-inoculation (Jenczmionka et al., 2003).

Interestingly, the deletion of *FgSTE12* does not cause any defects in hyphal growth, germination or conidiation but this protein is critical for perithecia development, as the mutant strain is sexually sterile. The *FgSTE12* mutant is also significantly impaired with regards to virulence, as this strain is incapable of producing penetration structures. Consequently, the *FgSTE12* mutant was unable to infect flowering wheat heads even after 15 days and produced significantly smaller lesions in wounded tomatoes, in comparison to the wild type (Gu et al., 2015).

FgSte12 has been shown to physically interact with FgSte7, but not FgGpmk1 (Gu et al., 2015). Thus, it is possible that FgSte12 could be a downstream target of pheromone module signalling and this could contribute to the regulation of sexual development and pathogenicity. However, it is not currently understood how the pheromone module may regulate processes such as hyphal growth and conidiation, as defects in these developmental programmes were observed in the *gpmk1* mutant, but not in the *FgSTE12* mutant. Thus, future studies characterising the pheromone module may help identify direct targets of regulation, thus providing insight on how this pathway is implicated in the regulation of both fungal development and virulence.

6. Summary and outlook

In this review, we discussed the utilization of the pheromone module MAPK pathway as a central signalling hub in fungal species. This pathway is implicated in the regulation of various fungal developmental programmes, such as vegetative growth, asexual sporulation and sexual reproduction. Signalling *via* the pheromone module also modulates both secondary metabolism and pathogenicity in a wide range of fungal pathogens. A summary of the main functions of the pheromone module in the fungal species discussed in this review is provided in Table 1. It is evident that proteins of the pheromone module are highly conserved among the fungal kingdom. However, there is little known about how these proteins form signalling complexes in fungal species. Also, information regarding the stimuli required for activation of these pathways and the direct targets of regulation in the nucleus is sparse, specifically in filamentous fungi. Thus, future research may help provide insight on the chemical messengers required to induce complex activation, the mechanisms of signalling to the nucleus, the genes regulated *via* pheromone module signalling and the biological consequences of this pathway. By characterising this pathway as a critical regulator of fungal development and virulence, this could contribute to the identification of novel drug targets, allowing for the development of more selective and efficient anti-fungal therapies. This, in turn, could result in the prevention of crop spoilage and infections due to fungal species.

CRediT authorship contribution statement

Dean Frawley: Conceptualization, Visualization, Funding acquisition, Writing - original draft, Writing - review & editing. **Özgür Bayram:** Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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