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Recent advances in understanding the fungal G-protein-coupled receptors

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ABSTRACT

Transmission of extracellular signal across the plasma membrane into the cells of organisms is impossible without cell surface receptors. One of the most broadly studied receptor is the G-protein coupled receptor. This receptor is coupled with heterotrimeric G proteins with α, β and γ subunits that perceives external stimuli and transduces the signal into the cell for suitable physiological and biochemical responses. They have also been reported as potential receptors to sense light and fatty acids, but their exact mechanism remains unclear in fungi. Signalling and regulation via G proteins has been extensively studied in various models including pathogenic fungi. Fungal GPCRs are broadly required in fungal defence stimulation, vegetative growth, and pathogenicity mechanism. This review aims to highlight the research in fungal GPCRs including classification, physiological roles, mechanisms of action and signalling in GPCR function. Through fungal genome sequencing, mammalian GPCRs have been identified apart from fungal-specific GPCRs which adds another dimension to the classification. The deorphanisation of unclassified fungal GPCRs is necessary to further understand their role in fungi. While the mechanism of action has been well documented in mammals, the glucose and pheromone sensing are the only two well mapped systems in yeast. However, we are yet to ascertain if there are any additional mechanisms of signalling at work in fungi. Further we endeavour to compare and contrast between the eukaryotic GPCRs in various aspects of functionality. Through the information derived we hope to determine the gaps in knowledge and by so doing determine the future directions of GPCR research in fungi.

Keywords: G-Protein coupled receptors; eukaryotic; pathogenic fungi; plant- fungal interaction

INTRODUCTION

G protein and G-protein coupled receptors (GPCRs) are one of the largest protein families in eukaryotes and are widely discovered on the exterior layers of all cells of eukaryotic organisms (Premont and Gainetdinov, 2007). As the name implies, GPCRs are coupled with heterotrimeric G-protein with 3 different subunits namely α, β and γ, which can bind to GTP and GDP. The GPCRs play an integral role at the molecular level of biochemical reaction of almost all multicellular organisms (Drake *et al.*, 2006). The seven membrane-spanning domains are regarded as the unique structural architecture of GPCR which has a wide range of ligand binding specificity (Rosenbaum *et al.*, 2009; Tuteja, 2009). The range in ligand binding specificity is a consequence of the first cytoplasmic loop formation in GPCR which changes the conformation post ligand binding, bringing about the third loop which is responsible for G protein interactions and signal transmission (Stefan and Blumer, 1994).

Various ligands such as Ca^{2+}, fatty acids, odorants, amino acids, proteins, peptide pheromone, nucleotides and steroids (Maller, 2003) can activate GPCR for further intracellular signalling. The GPCRs signalling system is vital for most physiological processes in eukaryotes including vision, taste, smell, nervous system cardiovascular, endocrine, and reproductive functions. The involvement of GPCR in these important processes made them a well-studied major target for drugs development (Overington *et al.*, 2006).

Although GPCRs have been extensively studied in animal and plant systems (Urano and Jones, 2014), genome studies have identified many GPCRs in fungi where some have shown identity to mammalian like GPCRs. The fungal specific GPCRs include nutrient sensors (Gpr1), cAMP like receptors, pheromone receptors sensing peptide pheromones (Ste2), Stm1-like nitrogen sensors (Stm1), pheromone receptors sensing lipid modified peptide pheromones (Ste3), microbial opsins (Nop-1 and Orp-1), and glucose sensor (Gpr4) (Han *et al.*, 2004a; Han *et al.*, 2004b). The fungal specific GPCR such as pheromone sensing and glucose sensing receptors are well characterised in yeast. However, the fungal specific receptors do not follow exactly the standard GRAFS system and have been further classified into 6 classes, which will be discussed further.

Fungal GPCRs are required for various signalling and regulation of vegetative growth, mating, and development of the organism, pathogenicity and regulation of biosynthetic pathways. Through numerous sensing

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mechanisms such as pheromone, nutrient, nitrogen, carbon (glucose and amino acid sensing) and light sensing, GPCR initiates signalling cascades specific to the bound ligand. A GPCR known as GPCR3, found in the entomopathogenic fungi, *Beauveria bassiana*, was shown to be responsible for nutrient sensing, stress and developmental responses (Ying *et al.*, 2013). Besides, a seven-transmembrane protein known as Stm1, in *Schizosaccharomyces pombe* was required for nitrogen starvation signals under nitrogen-deficient condition (Chung *et al.*, 2001). Glucose sensing G protein-coupled receptors, Gpr1 in *Saccharomyces cerevisiae* is the first GPCR discovered that senses nutrient in fungi (Dijck, 2009). Two more receptors, Ste2 and Ste3 characterised in S. *cerevisiae* are important in pheromone signalling (Chen *et al.*, 1997). In *Neurospora crassa*, the GPR-4 GPCR (NcGPR-4) senses carbon sources by coupling to the Group I Gα GNA-1, which operates upstream of the cAMP signalling pathway to regulate growth and asexual development.

In addition, GPCRs are also involved in fungal-plant interactions. To infect the host plant, the fungal pathogen recognises various signal molecules or ligands from plant cells through the signalling from fungal GPCR. Homologs of the fungal specific receptor, cAMP receptors from *Dictyostelium*, and a steroid receptor mPR were found in the pathogenic fungi, *Magnaporthe grisea* (Kulkarni *et al.*, 2005). This is accompanied by the dicovery of PTH11, a novel class of fungal receptor that facilitates surface recognition during appressorium formation for pathogenesis in *M. grisea* (Kulkarni *et al.*, 2005).

Studies on *Aspergillus nidulans* revealed that GPCRs and regulators of G protein signalling (RGSs) functions in controlling development of appressorium and the attachment of spore on the host (Yu, 2006). In *N. crassa*, ten (10) GPCRs sequences were predicted and the newly identified sequences were responsible for extracellular cAMP signalling pathway during chemotaxis and multicellular development through the encoded proteins that control cAMP levels (Galagan *et al.*, 2005). *Aspergillus flavus* was identified to encode fifteen (15) putative GPCRs which controlled germination, biosynthesis of secondary metabolites, sporulation, and the production of spores at high and low cell densities (Affeldt *et al.*, 2014).

As mentioned above, the function of GPCR is largely dependent on binding and interaction between components of this protein with other proteins. Therefore, this protein-protein interaction is largely dependent on the protein structure.

Structure and classification of fungal GPCR

Over the years, many fungal specific GPCRs have been identified in various fungal species and have been classified into six classes based on the similarities in sequence and function (Xue *et al.*, 2008). The first class of fungal GPCR (Class I) consists of pheromone receptors for alpha-factor pheromone (Ste2-like). This class of GPCR senses alpha factor pheromones and interacts with G proteins to initiate the signalling cascades that leads to mating between haploid-a and alpha cells. The first ever discovery of the function of this receptor was shown in Ste2 receptor in *S. cerevisiae* (Burkholder *et al.*, 1985). Different regions within the fungal pheromone receptor Ste2 function in different ways to perform the role of Ste2. The N-terminal domain promotes the oligomerisation of Ste2 (Uddin *et al.*, 2012) whereas, the C-terminal domain is involved in the negative regulation of the receptor (Schandel *et al.*, 1994).

Class II of fungal specific GPCR includes pheromone receptors for a-factor pheromone (Ste3-like). GPCR in this class senses a-factor pheromone and transmits signalling cascades to conduct functions similar to Class I fungal GPCR. One of the earliest studies on this class of receptor revealed that the *STE3* gene in *S. cerevisiae* is likely to be a membrane protein and encodes a 7TM region like other GPCRs which concludes that it's a GPCR that is involved in pheromone signalling for a-factor pheromones (Hagen *et al.*, 1986). The fungal pheromone mating-factor receptors Ste 2 and Ste 3 (Class I and Class II) were originally classified into Class D of the general GPCR classification system. The other classes are discussed later.

The next class of fungal GPCRs (Class III) are carbon sensors or specifically known as homologs of glucose sensor Gpr1. The GPCR carbon receptor senses glucose, sucrose and methionine and activates cAMP signalling through α-subunit Gpa2 (Miwa *et al.*, 2004; Maidan *et al.*, 2005). Glucose sensing in *S. cerevisiae* is the most well represented mechanism in carbon sensing. Carbon sources are required for spore germination in *A. nidulans* and *Botrytis cinerea* and possibly many other fungi (d'Enfert, 1997; Doehlemann *et al.*, 2006).

Class IV of fungal GPCR consists of nutrient sensing GPCRs. Nutrient sensing GPCRs initiate signalling cascades for downstream signalling response to regulate virulence, metabolism and morphogenesis (Bölker, 1998; Lengeler *et al.*, 2000; Versele *et al.*, 2001). This class of receptor can be further subdivided into 3 classes. The first class contains GPCRs that resemble yeast Gpr1 receptor, the second class contains GPCRs closely related to the GprD receptor of *A. nidulans* and the third class exhibits putative nutrient sensing GPCRs that shows homology to *Sch. pombe* Stm1 receptor (Dijck, 2009) which is required for nitrogen starvation signals under nitrogen-deficient condition (Chung *et al*, 2001).

The cAMP receptor-like (CRL) constitutes class V of fungal GPCRs and was first characterised in ascomycete fungi, *N. crassa* which was known as Gpr1 (not to be confused with yeast Gpr1). It was thought to regulate the fruiting body formation and mating in fungi (Krystofova and Borkovich, 2006). The CRLs share similar homology to four cAMP receptors (cAR1-cAR4) and three cAMP receptor-like proteins (CrlA-CrlC) in *Dictyostelium discoideum*, and *Arabidopsis thaliana* GCR1 (Klein *et al.*, 1988; Plakidou-Dymock *et al.*, 1998; Raisley *et al.*, 2004). However, this class of receptor is absent in the yeasts *S. cerevisiae* and *Sch. pombe.*

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Class VI of GPCRs are microbial opsin which are represented by NOP-1 opsin from *N. crassa* (Bieszke *et al.*, 1999a) and ORP-1 opsin-related protein from *Leptosphaeria maculans* (Idnurm and Howlett, 2001). Opsin genes are divided into 2 different types, whereby type I are microbial opsins and type II are animal opsins (Spudich *et al.*, 2000). GPCRs from Class VI are not to be confused with animal opsins as these two groups of opsins do not share similar sequence homology. However, the members of this class share similarity to archaeal opsins. NOP-1 has been shown to bind all-trans retinal *in vitro* and may have signalling functions *in vivo* (Bieszke *et al.*, 1999b). Apart from these six classes, there are also a few putative GPCRs that remained unclassified (Li *et al.*, 2007). Table 1 shows the list of well characterised fungal specific GPCRs found in filamentous fungi and yeast according to their respective classes.

Apart from the fungal specific GPCRs, mammalian-like GPCRs were also found in fungi. The classification of metazoan GPCR is based on the ubiquitous GRAFS system (Fredriksson *et al.*, 2003; Fredriksson and Schioth, 2005; Lagerström *et al.*, 2008) which stands for Glutamate, Rhodopsin, Adhesion, Frizzled and Secretin (de Mendoza *et al.*, 2014). The mammalian GRAFS families are exceptionally characterised in Metazoa, but they were not represented in fungi until the most recent study on fungal genome which revealed that four mammalian families of GPCRs, except Secretin, were in fact present in fungi (Krishnan *et al.*, 2012).

Rhodopsin-like receptors are from Class A GPCRs and regarded as the largest and the most widespread group of GPCR. It has been sub-divided into 19 subfamilies (A1- A19) (Sgourakis *et al.*, 2005). These groups include hormones, neurotransmitters and light receptors which transduces external stimuli by interacting with G proteins. Rhodopsin-like receptors are characterised with short N-terminal domains and interact with a broad variety of ligands (Krishnan *et al.*, 2012). Three fungal species namely *Allomyces macrogynus* (Blastocladiomycota), *Batrachochytrium dendrobatidis* and *Spizellomyces punctatus* (Chytridiomycota) were found to contain 12 novel sequences of Rhodopsin family (Krishnan *et al.*, 2012).

The second largest GPCRs are from the *Adhesion* family (Class B) with 33 members in humans (Fredriksson and Schioth, 2005). This receptor has long N-terminal with different functional domains and is classified into eight main groups (I–VIII) (Lagerström *et al.*, 2008). In fungi, 30 novel Adhesion family members were found to be distributed among 22 species of the phylum Ascomycota and one in *A. macrogynus* from the phylum Blastocladiomycota (Krishnan *et al.*, 2012). The GPCRs from *Secretin* family also constitute Family B of GPCR and are found in human and animals but yet to be dicovered in fungi (Miller *et al.*, 2012)

The next class of GPCR is from *Glutamate* family (Class C) and is well-known as the metabotropic glutamate receptors (mGluRs) which includes GABA-B receptors, taste receptors, olfactory receptors and a few putative pheromone receptors coupled to the G-protein Go (VRs and Go-VN) (Chaudhari *et al.*, 2000; Heinbockel *et al.*, 2007; Tuteja, 2009; Padgett *et al.*, 2010). The N-terminal of this receptor is long and functions as endogenous ligand binding site (Krishnan *et al.*, 2012). Based on a study conducted by Krishnan *et al.* (2012), 96 putative Glutamate receptors were found in 4 fungal species from three different phyla. A total of 78 GPCRs were found in *A. macrogynus* which belongs to phylum Blastocladiomycota. Meanwhile 14 GPCRs were identified in *Sepsophis punctatus*, 3 GPCRs in *B. dendrobatidis* phylum Chytridiomycota and 1 GPCR was found in *Rhizopus oryzae* (Zygomycota). However, none were found in the phyla Ascomycota and Basidiomycota up till now.

Frizzled-receptors (Class F) are receptors for Wnt protein, an important secreted glycoprotein (Rao and Kühl, 2010) in tissue polarity and cell signalling (Lagerström *et al.*, 2008). This receptor has CRD_FZ domain with 10 conserved cysteine residues (Krishnan *et al.*, 2012). Frizzled proteins and the genes that encode them have been identified across diverse species of animals from sponges to humans (Huang *et al.*, 2004). Interestingly, two new Frizzled receptor sequences were found in *S. punctatus* (Krishnan *et al.*, 2012). One interesting thing to note here is that although none of the mammalian like GRAFS families were found in Ascomycota and Basidiomycota, they are the only fungal phylum to have fungal specific GPCRs. The identification of mammalian-like GPCRs in fungi is indeed a great breakthrough in fungal GPCR research; however, functional studies should be conducted to have a better understanding on these receptors in fungal biological functions.

Unclassified G-proteins are known as orphan GPCRs. The ligands of these orphan GPCRs are unidentified and their physiological role are yet to be determined (Tang *et al.*, 2012). A total of 16 GPCRs were identified in *A. nidulans*, but their ligands are unknown (Xue *et al.*, 2008). In *M. grisea*, 76 GPCR-like proteins have been identified based on the genome sequence analysis (Kulkarni *et al.*, 2005). More comprehensive studies on these orphan GPCRs in fungi may lead to the discovery of more signalling mechanisms that regulate different physiological responses. Besides, this existing classification system needs to be reconstructed to incorporate the fungal specific GPCR and mammalian like GPCR into a single integrated fungal GPCR classification system.

Physiological roles of GPCR in fungi

The interaction between cells with their environment is essential for the organism to conduct various functions for its survival. Cell surface receptors function as sensors to facilitate interaction between the cell and the environment. G protein-coupled receptors (GPCRs) are responsible in facilitating intracellular physiological processes by transducing extracellular signals into the cell. In fungi, G-proteins aid in regulating the cellular

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Table 1: List of well characterised fungal specific GPCRs in filamentous fungi and yeast according to respective classes.

Type of fungi	Species	Pheromone receptor		Carbon sensor (Class III)	Nutrient sensor (Class IV)	cAMP receptor-like (Class V)	Microbial opsin (Class VI)
		Ste2 like (Class I)	Ste3 like (Class II)				
Filamentous fungi	*N. crassa*	PRE-2	PRE-1	Gpr 4	Gpr5, Gpr6	GPR1, GPR2, GPR3	NOP-1, ORP-1
	A. nidulans	GprB	GprA	GprC, GprD GprE	GprF, GprG AN5720	GprH, GprI AN8262	AN3361
	C. neoformans	-	Ste3a/Ste3a, Cpr2		Gpr2, Gpr3	Gpr4, Gpr5	Ops1
	M. grisea	MGG_04711	MGG_06452	MGG_08803	MGG_04698MG G_02855	MGG_06738	MGG_09015
	U. maydis	-	Pra1, Pra2	-	UM06006 UM01546	UM03423	UM02629 UM04125
	C. cinerea	-	Rcb1, Rcb2 Rcb3	-	CC1G_07132 CC1G_04180	CC1G_02288 CC1G_02310	-
Yeast	*S. cerevisiae*	Ste2	Ste3	Gpr4	SCRG01312 SCRG02823 SCRG00179	-	-
	S. pombe	Mam2	Mam3	Git3	Stm1	-	-
	C. albican	Ste2	Ste3	Gpr1	CAWG02899 CAWG06059 CAWG02686	-	-

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Table 2: The differences of GPCR in fungi and other eukaryotes.

Kingdom / Component	Fungi	Animal	Plant
The requirement of GPCR in signalling	GPCR-dependent		GPCR-independent
G-protein subunits	Scarce 2Gα (yeast)/3Gα (filamentous fungi), 1 Gβ 1 Gγ (filamentous fungi)/ >1 Gγ (in some fungal species)	Abundant 23 Gα 5 Gβ 12 Gγ	Scarce Single Gα and Gβ subunits and 2 canonical Gγ subunits
Functions	Growth, asexual & sexual development, and virulence	Regulates diverse cellular responses to the majority of neurotransmitters and hormones	Stomatal aperture control, fungal defense, oxidative stress, development, sugar perception and phytochrome/cryptochrome-mediated responses
Effectors	cAMP-dependent and mitogen-activated protein kinase and adenylyl cyclases	Adenylyl cyclases and other well-known effectors	Protein interactors may function similar to effectors in animal and fungi.
Ligand	Different ligands stimulate different GPCR	Chosen ligand can stimulate GPCR for different intracellular signalling	Ligands inhibit the inhibitor

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functions of vegetative growth, conidiation, infection, structure differentiation and pathogenicity (Liu *et al.*, 2007) and to carry out these functions, specific sensing mechanisms are being carried out by fungal GPCRs.

Fungal GPCRs are especially important in pheromone sensing in fungi to attract their mates. In ascomycetes and basidiomycetes, the signalling mechanism for the pheromone sensing starts with the detection of pheromones from the opposite mate by the cell surface 7-TM GPCRs (pheromone receptors). Following pheromone binding, these receptors are activated to trigger the downstream-signalling pathways for the mating process (Xue *et al.*, 2008). In addition, GPCRs have been reported to sense nutrients in some fungal species. The Gpr1 G protein-coupled receptor in *S. cerevisiae* functions as an upstream component in the cAMP–PKA pathway (Yun *et al.*, 1997; Xue *et al.*, 1998). Lorenz *et al* (2000) revealed that Gpr1 G protein-coupled receptor regulates pseudohyphal differentiation in *S. cerevisiae*. Additionally, the activation of cAMP synthesis by glucose and sucrose was shown to be mediated by this receptor (Yun *et al.*, 1998). GPCRs also serve as a sensor for amino acid in fungi. For example, in *Candida albicans*, Gpr1 act as methionine sensor to control filamentation (Maidan *et al.*, 2005).

A nutrient sensing GPCR, Stm 1 protein in *Sch. pombe* is needed for a proper recognition of nitrogen starvation signals. Under nutritionally deficient conditions, the Stm 1 protein forces *Sch. pombe* cells to cease cell proliferation by meiosis (Chung *et al.*, 2007). Opsin, a member of the GPCR rhodopsin Family A, serves as a sensory receptor in light sensing which helps in the developmental stage specifically in mating and sporulation (Idnurm and Heitman, 2005). Based on a recent study on *L. maculans*, opsin protein can build an electrochemical transmembrane gradient of proton by forming of proton pump which suggests that opsins in fungi may conduct a similar mechanism as opsins in Archaea (Waschuk *et al.*, 2005).

GPCR might also be a potential receptor for free fatty acids (FFAs) in fungi although fatty acid sensors are not reported to date in fungi. FFAs have been demonstrated to be involved in the developmental process of fungus. For instance, oxylipins in *A. nidulans*, functions as a signalling molecule to promote the communication between fungus and host (Tsitsigiannis *et al.*, 2006). Novel GPCR candidates have been characterised in both *A. nidulans* and *Cryptococcus neoformans* and it is proposed that these novel GPCR candidates may sense fatty acids (Xue *et al.*, 2008). However, the mechanism by which these GPCRs can sense the FFAs remains unknown.

Gα subunits have conserved the signal transduction pathways governing mating, filamentous growth, and virulence. One Ga subunit, Gpa3 in the basidiomycete *Ustilago maydis* is important for both virulence and mating. In *Cryphonectria parasitica*, the causative agent of chestnut blight, virulence is regulated via a G-protein-linked signal transduction pathway. A hypovirus that infects this fungal pathogen has *CPG-1* gene that requires Ga subunits for mating, asexual sporulation and melanin production (Alspaugh *et al.*, 1997). The appressoria formation of *M. oryzae* is initiated by G protein α subunit MAGB which is triggered by MAP kinase following the activation of adenylate cyclase protein MAC 1 (Choi and Dean *et al.*, 1997).

Mechanism of action involving GPCR signalling

Glucose sensing mechanism

Glucose is a simple sugar that serves as important energy source in all living organisms. In fungi, glucose is utilised in various metabolic processes such as germination, (Beyer *et al.*, 2004), growth (Daynes *et al.*, 2008) and fermentation (Panagiotou *et al.*, 2008). To transport glucose inside the cell, fungi have developed various mechanisms to sense the glucose. Glucose sensing via GPCR signalling has been vastly studied in yeast (Bahn *et al.*, 2007). Generally, the GPCR signalling starts with binding of ligand which activates GPCR to undergo conformational change to activate the dissociation of G-protein for the transmission of the intracellular signal. In *S. cerevisiae*, the glucose molecules act as ligands that bind to glucose sensing GPCR receptor (Gpr1) which in turn activates it (Xue *et al.*, 1998) to undergo conformational change. This promotes the exchange of GDP to GTP at Gpa2 α subunit and its dissociation from the unknown βγ subunits. The Gpa2 is thought to stimulate the adenylyl cyclase (Cdc35/Cyr1) in *S. cerevisiae* which activates the cAMP-dependent protein kinase (PKA) pathway for various developmental and mating processes (Figure 1a) (Versele *et al.*, 2001). In other eukaryotes however, the common mechanism for glucose sensing through GPCR signalling is yet to be discovered. The above pathway involving GPCR Gpr1 in fungi sets an example for discovery of similar proteins and pathways in other eukaryotes.

Pheromone sensing mechanism

In living organisms, mating is an essential physiological process to ensure the survival of the species. Many organisms use substances known as pheromones to attract their mates (Gomez-Diaz and Benton, 2013). Fungi are not an exception in this matter. Again, the pheromone sensing mechanism in fungi is studied based on the model species *S. cerevisiae.* The yeast cells produce two types of mating factors known as a-factor and α-factor (peptide pheromone) which act as ligands that bind to pheromone receptor Ste2 and Ste3 (Jones and Bennett, 2011). Following the binding of pheromone, pheromone receptor changes their conformation from an inactive R state to an active R* state which stimulates the GDP-GTP exchange at Gpa1 α subunit. Even though the sequences of Ste2 and Ste3 are dissimilar, both activate the same Gpa1 for GDP-GTP exchange when they are in R* state (Xue *et al.*, 2008). The Gpa1 will then be dissociated from Ste4 and Ste18 dimer (βγ subunits). In

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most of the signalling pathways, both the dissociated Gα and Gβγ communicate with effectors for downstream signalling. However, in *S. cerevisiae* pheromone-signalling pathway, Gβγ complex plays a central role in inducing the downstream pheromone signalling cascades, where Ste4 and Ste18 aid in the transmission of the signal to Ste20 that activates MAP-kinase cascade consisting of Ste11, Ste7 and Fus3 (Versele *et al.*, 2001), leading to successful mating (Figure 1b). Hence, cells that do not have the Gβγ complex are unable to conduct mating responses (Whiteway *et al.*, 1989). In addition to this model, a study on yeast proposed that Gα dissociates from Gβγ dimer and moves into endosome to activate an endosome-localised phosphatidylinositol 3-kinase (PI3K) composed of four subunits including a catalytic subunit Vps34 and a regulatory subunit Vps15. Gα-GTP then binds to Vsp34 to activate PI3K activity producing phosphatidylinositol 3-phosphate, which serves as a binding site to recruit proteins to the endosome. Gα-GDP can also bind to Vps15, which resembles a Gβ subunit, without activating PI3K. It is proposed that Gα may undergo cycles of GTP binding and hydrolysis at the endosome, which causes Gα to alternate binding between the two subunits of PI3K (Koelle, 2006).

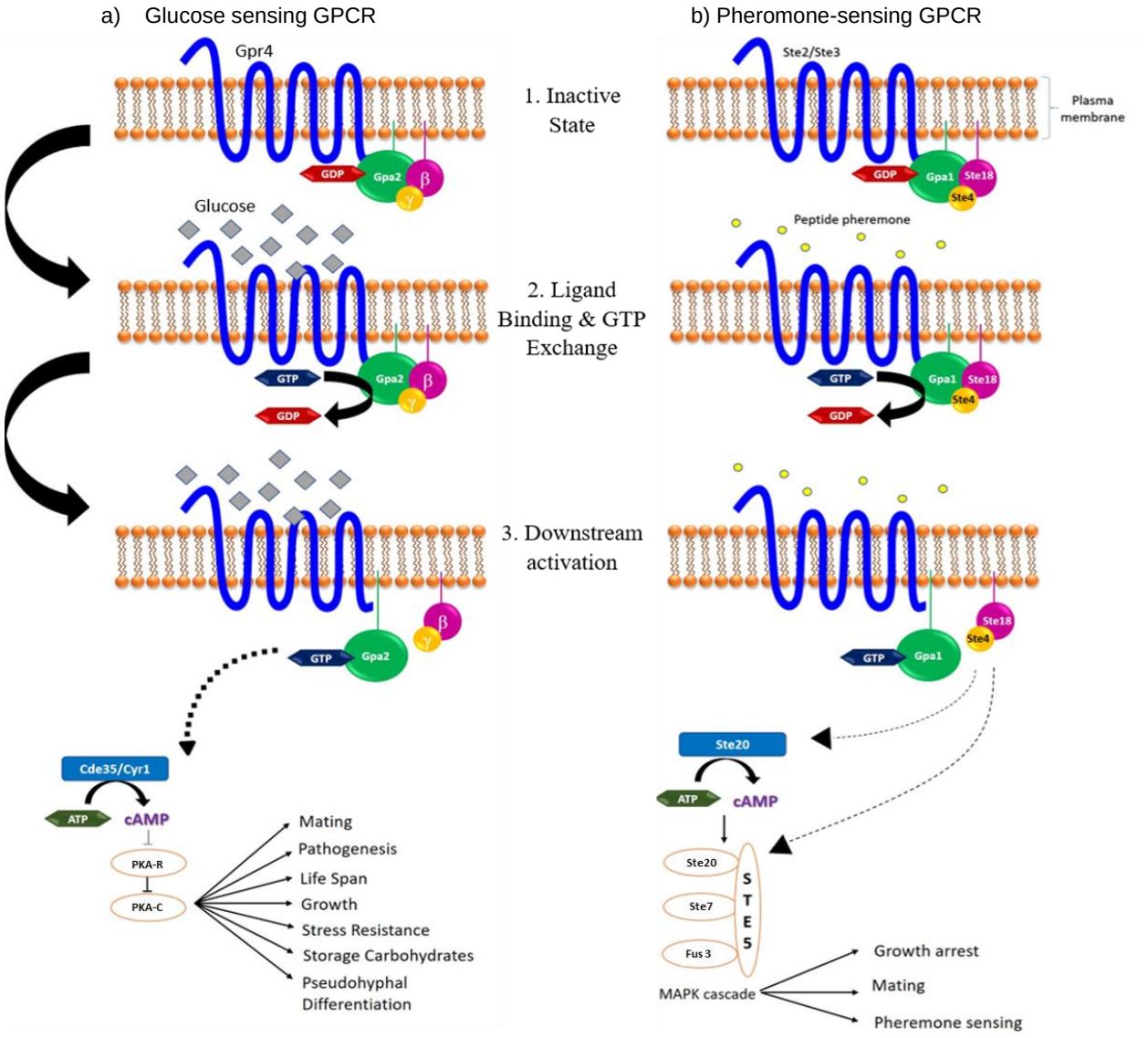

Figure 1: An overview of glucose and pheromone sensing GPCR signalling in yeast. a) Gpr1 activates Gpa2 upon ligand binding which subsequently activates Cde35/Cyr1. This leads to the downstream activation of cAMP-dependent protein kinase pathway which involves PKA-R and PKA-C that regulate responses for various developmental and mating processes. b) Ste2/Ste3 activates Gpa1 to dissociate from Ste18 and Ste4 dimer. Ste4 and Ste18 recruits Ste5 and Ste20 to the membrane and activate MAP kinase cascade (Ste11, Ste7 and Fus3) for mating process.

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Other mechanisms

Host sensing

Fungal pathogenesis is a biological process that involves the infection and development of disease in a host due to fungi. Diseases caused by fungal pathogens have resulted in devastating crop losses and has imposed alarming threat to human health. *M. oryzae*, a plant pathogenic fungus is believed to utilise GPCR mediated signalling to detect plant cell and start infection on them. The GPCR Pth11 in *M. oryzae* senses hydrophobic plant surfaces and promotes invasive growth. After the Pth11 receptor is activated through ligand binding, G-proteins will be dissociated and the βγ dimer activates adenylyl cyclase MAC I and MAPK pathway (Mst 11, Mst 7, Pmk 1) for appressorium formation and virulence (Kou *et al.*, 2017). Another pathogenic fungus known as *Fusarium oxysporum* can sense host factors such as haem-containing plant peroxidases in the roots before imposing attack on the plant host. The binding of the host factors to the sex pheromone receptor Ste2 activates the Mpk1 MAPK pathway for pathogenesis (Turrà *et al*., 2015). These well-characterised pathways may serve as a guide to study the host sensing pathways mediated by GPCR in other pathogenic fungi.

Amino acid sensing

Amino acids are building blocks of proteins which constitute most living tissue. It is also a source of nitrogen which is required for various developmental processes in fungi and to survive in nutrient-deficient condition. Gpr4 in *C. neoformans*, functions as amino acid sensor and senses methionine and stimulates capsule formation and mating filament through cAMP-PKA signalling. Gpr4 interacts with Gpa1 and the cyclase associated protein Aca1 to activate adenylyl cyclase (Cac1) to regulate downstream elements of the cAMP pathway which leads to the binding of cAMP to the regulatory subunit of PKA (Pkr1) and release of the PKA catalytic subunits (Pka1/2) (Xue *et al.*, 2006). In other eukaryotes like animals, the amino acids are sensed by class C GPCR, for example, ligand binding of amino acids to GPRC6A receptor activates phospholipase C (PLC) effector and promotes secretion of GLP-1 in enterocyte L-cells to regulate insulin secretion (Rolland *et al.*, 2001). The presence of pathways to sense amino acid via GPCR across the lower to higher eukaryotes shows that this mechanism is conserved to some level in all eukaryotes.

Nutrient sensing

Heterotrophic fungi depend on organic matter from other organisms for their nutrition, metabolism, and survival. To absorb these organic compounds from the environment, fungi must be able to sense them, and this is possible through GPCR signalling mechanism. In *C. albicans*, the Gpr1 receptor senses nutrients such as lactate, glucose and methionine and subsequently causes the dissociation of G protein. The dissociated Gpa2 activates the effector, Cyr1 which stimulates the cAMP-PKA pathway. The PKA activates Efg1 (transcription factor) to induce cell wall biosynthesis, filamentation, adhesion and biofilm formation (Dijck, 2009). Unlike this pathway, the Gpr1 involved in glucose sensing mechanism described earlier in *S. cerevisiae* is only activated by glucose and not amino acid or any other nutrient.

Comparison of fungal GPCR with other eukaryotes

In this portion we would like to elucidate if there are any differences in the role played or the mode of action amongst GPCRs in eukaryotic organisms. The following section will dissect the similarities and dissimilarities that have been observed for us to understand the diversity in the functionality of this group of proteins within the eukaryotes.

Fundamentally, all the GPCRs found in fungi and other eukaryotes such as plants and animals, share the same structure that is the portrayal of seven transmembrane (7TM) regions (Katritch *et al.*, 2013). G proteins have three subunits: Gα, Gβ, and Gγ. The GPCRs undergo conformational change due to the binding of ligand which triggers the G proteins to dissociate and in turn activates the effectors to initiate intracellular signalling responses. The wealth of evidence and knowledge accumulated in animal systems on GPCR has made them the ideal system to explain the GPCRs in other organisms. However, it's hard to ignore the fact that GPCRs in other systems are different in their own way and may conduct signalling in certain manner which can be slightly different in accordance to the physiological processes required for the survival of a particular group of organisms. The GPCRs in fungi are not well represented as compared to GPCRs from the other eukaryote system, but recent studies have provided the importance of GPCRs specifically to fungi. Through a series of inquiries below, we believe that the information derived will provide a better understanding of GPCRs and also serve to direct future research in this area.

How do the G-proteins differ in function between the eukaryotes?

In terms of the G-proteins, there are no reports hitherto stating that fungal GPCRs are structurally different from those in other eukaryotes. Nevertheless, the specific function of the G-protein differs across organisms. In fungi, G proteins are required for growth, asexual and sexual development, and virulence (Li *et al.*, 2007). To conduct these functions, the GPCRs are specifically required to sense light, nutrient, carbon, nitrogen and pheromones (Xue *et al.*, 2008). On the other hand, for other eukaryotes system, for example human and animals, the G-proteins are needed to regulate various cellular behaviours to the majority of neurotransmitters and hormones within the complex human and animal body (Heng *et al.*, 2013). As for plants, G proteins are important in some physiological roles such as control of

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stomatal aperture, defence against biotic stress, oxidative stress, developmental process, sugar perception, and some phytochrome/cryptochrome-mediated responses (Urano and Jones, 2014). While there are clear differences, there are also some underlying similarities where plant, fungi and animals all require hormone/pheromone sensing and nutrient sensing for survival. Therefore, from these already understood models, a leaf may be taken from them to hypothesise, test and develop similar or alternative sensing pathways that may be present in fungi.

Are all signalling mechanisms in eukaryotes GPCR dependent?

The one striking similarity between animal and fungal signalling is that both organisms are GPCR-dependent. However, that is not the case for plants as they are GPCR independent. Hydrolysis of GTP and G-protein dissociation is caused by GPCR in animal and fungi where as in plants the G-proteins are activated by the regulator of G-protein signalling protein (RGS) (Urano *et al*., 2012). Despite this, both GPCR dependent and GPCR independent signalling promotes the activation of effector systems for downstream signalling cascades for various physiological processes. Our immediate question would be if fungi are solely dependent on GPCR for G-protein signalling? Intriguingly, a study indicates that fungi may not fully depend on GPCR, as an intracellular guanine nucleotide exchange factors (GEF) found in *S. cerevisiae* is proposed to function in a same manner as the activator of G protein-signalling (AGS) which are thought to compete with GPCR to activate the G-protein signalling (Lee and Dohlman, 2008). Hence, more studies involving gene knockout or gene silencing is required to verify if signalling via G-protein can be mediated without GPCR across all the representative member of each phylum in fungi. Table 2 shows brief differences of GPCR in fungi and other eukaryotes.

How vast is the occurrence of the G-protein subunits in eukaryotes?

As compared to animals, G-protein subunits characterised in plants and fungi are scarce. In fungi, specifically in yeast, two α subunits were characterised whereas the most characterised filamentous fungi possess three α subunits that are constituted of different classes (Bölker, 1998; Kays and Borkovich, 2004; Li *et al.*, 2007). Nearly all the fungi possessed a single predicted β subunit, but recently in *R. oryzae*, it was predicted to have four β subunits (Li *et al.*, 2007). Filamentous fungi have a single γ subunit (Krystofova and Borkovich, 2005) but other fungal species like *C. neoformans*, *C. cinereus*, *Podospora anserina*, and *R. oryzae* possess more than one γ subunit (Palmer *et al.*, 2006). For each of the three subunits of the G-proteins, multiple family members existed (23 α, 5 β, 12 γ) in humans and animals and different combinations of these subunits allow specific G-protein based signalling pathways (Wettschureck and Offermanns, 2005; Trusov and Botella, 2016). Conversely, plants only have a single α (GPA1) and β (AGB1) subunit and two γ subunits (AGG1 and AGG2) in *Arabidopsis* (Ma *et al.*, 1990; Weiss *et al.*, 1994; Mason and Botella, 2000; 2001). Through further genome studies and mining of information on fungal G-proteins and their subunits, more information may be obtained to help understand and delineate the signalling mechanism for different pathways.

Do all the eukaryotes share the same effectors?

The activation of G-protein leads to the consequent activation of effectors to generate intracellular signalling (Lodish *et al.*, 2000). The main effectors of G proteins acting downstream in filamentous fungi are the cAMP-dependent and mitogen-activated protein kinase (MAPK) signalling cascades (Li *et al.*, 2007; Gupta *et al.*, 2014). Other specific effectors characterised in fungi includes adenylyl cyclases such as MAC 1 in *M. grisea*, Cac1 in *C. neoformans*, Cdc35 in *C. albicans* and Cyr1 in *S. cerevisiae*. As compared to fungi, the effector system in animal GPCR signalling are well defined and the signal is transmitted to various downstream effectors such as adenylyl cyclases, phospholipase Cβ, RGS-RhoGEFs (Wettschureck and Offermanns, 2005) and ion channels (Hille, 1992). The plant genome does not encode the effectors found in animal, however some protein interactors such as thylakoid formation 1 (THF1) and ACI-reductone dioxygenase 1 may function in a similar manner to the canonical G protein effectors (Jones and Assmann, 2004). Future research should emphasise on identifying other effectors besides adenylyl cyclase in fungi. Apart from that, analysis on sequence homology between the effectors found in fungi and effectors from other eukaryote system that are well characterised may provide a better understanding on how these effectors work similarly to conduct the downstream signalling.

Does ligand binding promote G-protein signalling differently across the eukaryotes?

Ligand binding induces the GPCR activation through the rearrangement of the transmembrane helices which triggers activation for downstream signalling with G proteins and modulation of cellular physiology (Rosenbaum *et al.*, 2009). In fungi, different ligands that bind to the GPCRs promote different intracellular signalling pathways. For example, mating-pheromone binds to Ste2/Ste3 for pheromone signalling whereas glucose binds to Gpr1 for glucose signalling. In animal systems, a chosen ligand can stimulate different intracellular signalling pathways independently through a single GPCR. In contrast, in plants, ligands appear to hamper the inhibitor in *Arabidopsis* whereby a ligand (D-glucose) impedes AtRGS1 GTPase-activating protein activity which allows AtGPA1 to be triggered without GPCR (Johnston *et al.*, 2007). It would be interesting to investigate whether a single ligand can activate different signalling pathways in fungi like in the animal system.

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CONCLUSION

GPCRs are multi-functional receptors which are crucial in mediating responses and in regulating the receptor expression. Our review is centred on the studies conducted so far in fungal GPCRs including the classification, physiological roles, mechanism of action in GPCR signalling as well as the differences of GPCRs between fungi and other eukaryotes. Based on this review, we have identified a few gaps in knowledge of fungal GPCR research. We have listed below some questions that may be addressed through future research to enhance our knowledge in fungal GPCR.

i) Are fungi solely dependent on GPCR for G-protein signalling?
ii) What are the specific functions of the mammalian like GPCRs in fungi?
iii) How are the fungal specific and mammalian-like GPCRs involved in mechanism of action?
iv) How do the receptors like Stm 1 sense nitrogen starvation and how are microbial opsins involved in sensory function?
v) If GPCR can sense fatty acids, how does this mechanism work?
vi) Can a single ligand activate different signalling pathways in fungi like the animal system?
vii) Can there be any other effectors besides adenylyl cyclase in fungi?
viii) Is the GPCR diversity conserved in fungi?
ix) Are the fungal specific GPCRs domains well characterised?
x) Is there any cross-talking in the GPCR signalling pathways of fungi?

Answering these questions may put together the missing pieces in the current knowledge about fungal GPCR. With further information and advancement in knowledge, we believe many key questions on how, where, and what of fungal GPCR may be determined to achieve the ultimate goal of having a well-defined and distinct characterisation of fungal GPCR system that stands out among other eukaryote systems. However, as we continue to answer these questions there will be others that crop up and thus ensue the continuous quest for more knowledge and information in this area.

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