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WISH, a novel *CFEM GPCR* is indispensable for surface sensing, asexual and pathogenic differentiation in rice blast fungus

Nazmiara Sabnam, Subhankar Roy Barman*

Department of Biotechnology, National Institute of Technology, Durgapur, India

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ABSTRACT

We have selected and characterized a unique Conserved Fungal-specific Extra-cellular Membrane-spanning (CFEM) domain containing PTH11 like G-protein coupled receptor (GPCR), which is responsible for Water wettability, Infection, Surface sensing and Hyper-conidiation (WISH). The pathogenicity gene *WISH* is predicted to encode a novel seven transmembrane protein in the rice blast fungus, *Magnaporthe oryzae,* one of the deadliest pathogens of rice. We generated knockout mutants through a homologous recombination-based method to understand the function of the gene. These mutants are nonpathogenic due to a defect in sensing hydrophobic surface and appressorium differentiation. The mutant failed to undergo early events of pathogenesis, and appressorium development is diminished on inductive hydrophobic surface and was unable to penetrate susceptible rice leaves. The Δ*wish mutant* did not develop any appressorium, suggesting that WISH protein is required for appressorium morphogenesis and is also involved in host surface recognition. We examined various aspects of pathogenesis and the results indicated involvement of *WISH* in preventing autolysis of vegetative hyphae, determining surface hydrophobicity and maintenance of cell-wall integrity. *WISH* gene from *M. oryzae* strain B157 complemented the Δ*wish mutant*, indicating functional authenticity. Exogenous activation of cellular signaling failed to suppress the defects in Δ*wish mutants*. These findings suggest that WISH GPCR senses diverse extracellular signals to play multiple roles and might have effects on *PTH11* and *MPG1* genes especially as an upstream effector of appressorium differentiation. It is for the first time that a typical GPCR containing seven transmembrane helices involved in the early events of plant pathogenesis of *M. oryzae* has been functionally characterized.

1. Introduction

G-protein-coupled receptors (GPCRs) are presumed as a modern information technology system, which receives raw data in the form of diverse signals like photons, odorants, peptides, sugars, hormones etc. and processes these signals into useful information for the growth and development of the organism. These comprise the largest and most diverse group of membrane receptors in eukaryotes (Lagerström and Schiöth, 2008). Messages are conveyed to the cells about the presence or absence of life-sustaining nutrients or stress in their environment, or they serve as a conduit for other cells too. GPCRs represent a broad array of receptors and comprise one of the largest protein families found in nature exceeding 600 numbers in the genome of human (Lander et al., 2001; Venter et al., 2001). Discovery of new kind of GPCR is really challenging because of low similarity in the protein sequences; even within related classes, sequence conservation is limited only to the domain structure containing seven transmembrane helices that are tethered by alternating intracellular and extracellular loops (Schöneberg et al., 1999; Dohlman et al., 1991). The molecular series of heterotrimeric G-protein signaling is basically comprised of three components: a GPCR, a heterotrimeric G protein (α, β, γ subunits), and an effector (Neer, 1995). Binding of a ligand to the receptor brings about a conformational change, which leads to activation of the G protein with the subsequent exchange of GDP for GTP on the Gα subunit. The dissociation of GTP-bound α subunit from its $\beta\gamma$ companion allows regulation of downstream target genes (Kaziro et al., 1991; Birnbaumer, 1992; Neer, 1995; Gutkind, 1998).

A fungal pathogen must undergo a series of morphological and physiological programmes to cause a successful infection. During these developmental transitions, the pathogen also suppresses the plant's innate immune system and perturbs host metabolism and cell signaling to favorits own growth and development *in planta*. The fungus responds to a wide range of physical signals such as presence of hydrophobic surface, odorants, even photons and environmental stimuli as diverse as pheromones, protons, sugars, Ca^{2+}, nitrogen sources, amino acids, nucleotides, proteins, peptides, steroids, fatty acids etc. (Maller, 2003;

* Corresponding author.
E-mail address: subhankarroy.barman@bt.nitdgp.ac.in (S. Roy Barman).

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Xue et al., 2008). GPCRs with magnificent seven transmembrane helices are supposed to be engaged in the transduction of extracellular signals since heterotrimeric G proteins act as signal transducers that clamp cell surface receptors and cytoplasmic effectors together to activate secondary messengers. In fungi, signaling pathways such as cyclic AMP-dependent Protein Kinase (cAMP-PKA) or Mitogen-Activated Protein Kinase (MAPK) cascades generally transmit G protein signals to trigger a variety of cellular processes such as vegetative growth, mating, cell division, cell–cell fusion, morphogenesis, chemotaxis, and infection-related development (Bölker, 1998; Versele et al., 2001; Fredriksson et al., 2003).

M. oryzae is a superb model system to study the pathogen differentiation in response to plant cell surface cues and development of a specialized infection structure known as appressorium, which is required to penetrate the host cells (Dean, 1997; Tucker and Talbot, 2001). This amazing combination of rice and blast fungus has been transformed into a prototypical phytopathosystem by virtue of meticulous research and investigations that have been carried out in these two organisms over last decades. This filamentous, heterothallic, and haploid ascomycete fungus comprising of a reasonably small genome of ~40 Mb distributed over 7 chromosomes has been being treated as a model for investigations of molecular plant-fungal interactions (Talbot et al., 1993; Orbach et al., 1996). Infection begins, soon after attachment of a conidium to the plant surface, from which, a short germ tube emerges and differentiates into highly melanized appressorium. High turgor pressure is generated within the appressoria to breach the host cell wall and subsequently, invasive hyphae are produced from these infection structures.

The *M. oryzae* genome contains a large repertoire of *PTH11*-like GPCR genes (61 members) (Kulkarni et al., 2005). Twelve of these genes form a subfamily and contain a Conserved Fungal-specific Extracellular Membrane-spanning (CFEM) domain at the amino terminus that is unique to filamentous fungi but resembles the epidermal growth factor (EGF) module present in certain human GPCRs (Dean et al., 2005; Wilson and Talbot, 2009). The CFEM domain is composed of a conserved motif containing a consensus of eight cysteines. This domain is proposed to play an important role in fungal pathogenesis. The sequence of the domain is $PxC[A/G]x_2Cx_{8-12}Cx_{1-3}[x/T]Dx_{2-5}CxCx_{9-14}Cx_{3-4}Cx_{15-16}$, where x is any residue, and its range is indicated. The first identified CFEM protein in *M. oryzae* was ACI1, an adenylate cyclase (MAC1)-interacting protein (Kulkarni et al., 2003). *PTH11* gene is among one of the CFEM GPCRs, which is required for appressorium structure formation in response to inductive substrate cues and pathogenicity in *M. oryzae* (Talbot, 2003; DeZwaan et al., 1999). Similarly, CSA1 is such a CFEM domain containing protein found in *Candida albicans* (Lamarre et al., 2000). However, some CFEM proteins have also been found in non-pathogenic fungi. For example, CCW14 in *Saccharomyces cerevisiae* is involved is involved in cell-wall biogenesis and plays an important role in maintaining cell-wall integrity and stability (Moukadiri et al., 1997; Mrša and Tanner, 1999). Thus, the genes encoding CFEM-containing proteins may be involved in different developmental and infection processes. *M. oryzae* has the largest number of CFEM-GPCR proteins among sequenced fungi. Recently, a study on comparative genome analyses of three *Trichoderma* species reported distinguished variance of the genus- and species-specific differences in the putative GPCRs (Gruber et al., 2013). Only a few PTH11 GPCRs containing CFEM domains have been identified. For example, only 3 CFEM-PTH11 members each in *Trichoderma atroviride* and *T. reesei*, 4 in *T. virens* (Gruber et al., 2013) and only one in the saprophyte *Neurospora crassa* (Cabrera et al., 2015) have been reported. Limited amount of information is available on the characteristics and functions of fungal GPCRs as studied in *Saccharomyces cerevisiae* (Hagen et al., 1986; Blumer et al., 1988), *Schizosaccharomyces pombe* (Tanaka et al., 1993), *Aspergillus nidulans* (Lafon et al., 2006; Seo et al., 2004), *Cryptococcus neoformans* (Chang et al., 2003), and *Fusarium graminearum* (Li et al., 2007), *N. crassa* (Kim and Borkovich, 2004), or the basidiomycete fungi, *Ustilago maydis* (Bölker et al., 1992), and *Coprinus cinereus* (Olesnicky et al., 1999). Homologs of known fungal GPCRs other than CFEM-GPCRs were found in *M. oryzae* proteomes, such as the mating factor pheromone receptors STE2 and STE3 and GPR1 (glucose-sensing receptor). Recently, functional information of 15 GPCRs which belong to Classes I-IX in the filamentous fungus *Aspergillus flavus* was reported (Affeldt et al., 2014).

In addition, it became evident from the whole-genome microarray data that CFEM-GPCR-like proteins are actually expressed during infection-related development in *M. oryzae* (Dean et al., 2005). *M. oryzae* has maximum flexibility to react to the extracellular signals in contrast to saprobic fungi (Dean et al., 2005). At present, information on fungal genomes with respect to the number of identified GPCRs is also limited. However, hardly any experimental evidence is available for the genes with putative functions. GPCRs are likely involved in surface sensing and attachment of *M. oryzae* on the plant surface and they play an essential role during early events of pathogenesis. GPCRs from animals have immense importance as targets for drug discovery (Wise et al., 2002). Similarly, identification of fungal receptors would be equally important for understanding and control the disease caused by fungal pathogens like *M. oryzae* (Kulkarni et al., 2005). In addition to this, CFEM might be considered as a unique signature for those proteins functioning extracellularly (Kulkarni et al., 2003). Moreover, no authentic GPCRs have been functionally characterized, in detail, till now in the genome of *M. oryzae.* Thus, it is a hot area of research to uncover the involvement of selected putative GPCRs to understand the biology of *Magnaporthe,* especially in the process of pathogenesis using functional genomics tools.

Here, we report selection and characterization of a novel GPCR, from within the CFEM subfamily in the blast fungus *M. oryzae*, which has been designated as *WISH*. This gene is involved in surface sensing of the host and maintaining fungal surface hydrophobicity, in addition to its role in mycelia growth, cellular autolysis, maintenance of cell-wall integrity, conidiogenesis, appressoria development and virulence. “Hooking”, one of the most critical steps during early pathogenic differentiation, was not observed in the mutant even in presence of cAMP. Deletion of *WISH* led to the impairment of appressorium development both on artificial hydrophobic surfaces and rice plant surfaces and the mutant failed to respond to either cutin monomers or cAMP, with a significant defect in virulence. However, extracellular peroxidase and laccase activities were enhanced. Strikingly, cellular autolysis was induced from day-4 in the Δ*wish mutant*. Our data also indicate that *WISH* likely functions as an upstream sensor for the activation of the G-protein signaling pathway in a cAMP-independent manner. This work, thus, gives new insights into the physiological processes involved in the very early stages of rice infection. This is for the first time that a typical heptahelical serpentine GPCR, which is involved in the early events of plant pathogenesis, has been functionally characterized.

2. Materials and methods

2.1. Bioinformatics analysis

The FASTA file was retrieved from Broad Institute database of *M. oryzae* (version 6). TMHMM server version 2.0 was used for the hydropathy analysis and prediction of 7TM signature. GPCRdb (Isberg et al., 2016) BLAST was performed using default parameters. The presence of 7TM (transmembrane) helices was predicted and compared with different GPCR prediction web tools and servers such as DAS (Cserzö et al., 1997); TMHMM (Krogh et al., 2001); HMMTOP (Tusnady and Simon, 2001); Phobius (Käll et al., 2004) and TMpred (Hofmann and Stoffel, 1993). WISH protein was classified into GPCR family and subfamily by GPCRpred (Bhasin and Raghava, 2004) and GPCR-MPredictor (Naveed and Khan, 2012). *In silico* protein localization has been predicted by MultiLoc (Höglund et al., 2006) and Wolf Psort (Horton et al., 2007). For Multiple Sequence Alignment (MSA), at first, sequence