*The 13th Symposium on Plant Biotechnology* **13:** 41-44, 1999.
*1999 Korea-Japan Join Symposium.*



# Signal transduction pathways for infection structure formation in the rice blast fungus, *Magnaporthe grisea*

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**Abstract**

*Magnaporthe grisea* (Hebert) Barr (anamorph: *Pyricularia grisea*) is a typical heterothallic Ascomycete and the causal agent of rice blast, one of the most destructive diseases on rice (*Oryza sativa* L.) worldwide. The interactions between cells of the pathogen and those of the host involve a complex of biological influences which can lead to blast disease. The early stages of infection process in particular may be viewed as a sequence of discrete and critical events. These include conidial attachment, germination, and the formation of an appressorium, a dome-shaped and melanized infection structure. Disruption of this process at any point will result in failure of the pathogen to colonize host tissues. This may offer a new avenue for developing innovative crop protection strategies. To recognize and capture such opportunities, understanding the very bases of the pathogenesis at the cellular and molecular level is prerequisite. Much has been learned about environmental cues and endogenous signaling systems for the early infection-related morphogenesis in *M. grisea* during last several years. The study of signal transduction system in phytopathogenic filamentous fungi offers distinct advantages over traditional mammalian systems. Mammalian systems often contain multiple copies of important genes active in the same tissue under the same physiological processes. Functional redundancy, alternate gene splicing, and specilized isoforms make defining the role of any single gene difficult. Fungi and animals are closely related kingdoms [3], so inferences between these organisms are often justified. For many genes, fungi frequently possess only a single copy, thus phenotype can be attributed directly to the mutation or deletion of any particular gene of interest.

## *Infection-related morphogenesis in M. grisea*

### *Environmental cues*

A number of environmental signals control both germination and the fate of the germ-tube tip in *M. grisea*. Conidia do not germinate when held in aqueous suspension unless nutrients are added or the conidia contact a solid surface [18]. Presumably, this prevents precocious germination. The destiny of the germ tube depends on additional signals - an infection signal, a vegetative growth signal, or both. Cellular response in *M. grisea* to the infection signal overrides signals for vegetative growth because appresssorium formation is not greatly influenced by the presence of nutrients such as sugars. Upon sensing the infection signal, such as the hydrophobic leaf surface, a fundamental change occurs: apical extension ceases and the region immediately behind the hyphal tip buckles up and away from the surface and the tip begins to swell into the appressorium. The surface on which conidia of *M. grisea* land has little effect on germination [18]. Suspensions of conidia germinate equally well and as rapidly on glass as they do on hydrophobic surfaces. On hydrophilic surfaces such as clean glass or the hydrophilic side of Gelbond (FMC BioProducts, Maine) the germ tube develops into typical vegetative mycelium.

Plant leaf surfaces release of variety of chemical signals such as sugars, phenolics, and various volatile metabolites to which plant pathogenic fungi are capable of responding, but their significance is uncertain, with a few exceptions. Components of leaf surface, particularly derivatives of the cuticle, have been shown to stimulate appressorium formation. The fatty alcohol fraction of plant wax components specifically induce appressorium formation in *M. grisea* [15, 33]. Addition of nanomolar concentration of cutin monomers to conidial suspension stimulates appressorium formation [12]. Structure function analyses of authentic compounds revealed that 1,16-hexadecanediol and 1,16-hexadecanedial to be the most active compounds in *M. grisea*.

### *Extracellular matrix proteins*

Proteins associated with the extracellular matrix (ECM) play a crucial role in cell-substratum adhesion,

differentiation and signal transduction in mammalian cells [11]. It is hypothesized that analogs of these proteins are present in *M. grisea* and are important for the triggering of conidial attachment and appressorium formation by hydrophobic surfaces. Western blotting using polyclonal antiserum raised to the human proteins reveals the presence of a vitronectin-like protein of approximately 95 kDa and a fibronectin-like protein of 60 kDa in protein extracts of *M. grisea* mycelium and spores. The addition of IgG purified from polyclonal antiserum raised to human vitronectin or fibronectin to conidial suspensions prevents both conidial attachment and appressorium formation, but does not affect germination.

*Intracellular signaling pathways for appressorium formation*

The mechanisms involved in recognition and adaptive response to stimuli are fundamentally similar in all eukaryotes invested [14, 25]. The transduction of environmental information within a cell is controlled by a number of small molecules, such as cyclic nucleotides, $Ca^{2+}$, and phosphoinositides. The transduced signal is relayed by a protein phosphorylation cascade which involves protein kinases and phosphatases.

*cAMP-dependent pathway*

cAMP / protein kinase A (PKA) pathway is used as a model system for understanding general kinase function [31, 32, 35]. Increases in intracellular cAMP levels occur in response to a variety of primary signals by activation of adenylate cyclase, usually through G-protein coupled receptor binding. The primary target of cAMP in eukaryotic cells is PKA [30, 32]. cAMP also plays a major role in morphogenesis in a diverse group of fungi. In *M. grisea*, the function of cAMP in growth and development has been well studied. *M. grisea* undergoes infection related morphogenesis in response to at least two environmental cues, hydrophobicity of the substratum [19] and chemical compounds (cutin monomers) derived from the plant host surface [12]. Both of these signals result in the production of appressoria. It was also found that exogenously added cAMP or the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), can trigger appressorium formation on hydrophilic surfaces at the high levels [17, 18]. Surface hydrophobicity is likely sensed by the hydrophobin protein MPG1 [4], leading to activation of adenylate cyclase (MAC1). MAC1 may perhaps be activated by means of the G-protein $\alpha$ subunit (magB), as the mutant of this gene could no longer form appressoria in response to surface hydrophobicity [21]. However, this mutant was rescued by application of endogenous cAMP and its analogs [1, 6], demonstrating the involvement of cAMP in both of these pathways. Gilbert *et al.* (1996) postulated the existence of a specific receptor for recognition of the cutin monomers responsible for the infection structure formation. Developmental mutants have shown that these two pathways are separate and distinct [6, 12, 38]. To further our understanding of cAMP mediated differentiation in *M. grisea*, the catalytic subunit gene (*cpkA*) of PKA was cloned and characterized [23]. Mutants of *cpkA* showed greatly delayed and reduced appressoria formation in response to surface hydrophobicity, cutin monomers, cAMP, or IBMX. The low numbers of appressoria that formed had altered characteristics and were unable to penetrate the host [23, 37]. The cloning of the *M. grisea* regulatory subunit of PKA has been accomplished and its functional role in appressorium formation is underway. More recently, much higher PKA activity was observed during appressorium formation than vegetative growth [13]. A role for cAMP in activation of a mitogen-activated protein (MAP) kinase pathway in *M. grisea* has also been demonstrated [36] and a cooperation of PKA and MAP kinase pathways have been suggested in appressorium formation [9, 36]. Thus, the cAMP signaling pathway, as defined in mammalian systems, is present and crucial in infection related morphogenesis in *M. grisea*.

*Calcium-dependent pathway*

Cytosolic $Ca^{2+}$ ion is regarded as a requisitive cation for cell growth and development in many organisms [5, 8]. Changes in cytosolic $Ca^{2+}$ gradients are implicated in cellular morphogenesis of fungi including hyphal extension and branching [26, 27]. Recently, extensive studies using a wide range of pharmacological agents which disrupt $Ca^{2+}$ fluxes or interfere with the calcium-binding protein have strongly implicated $Ca^{2+}$ signaling system in appressorium formation in *M. grisea* [16]. The addition of calcium-chelator (EGTA) and calcium ionophore (A23187) inhibited appressorium formation, but not conidial germination in *M. grisea*. EGTA inhibition of appressorium formation was completely recovered by exogenous addition of calcium. However, exogenous addition of $Ca^{2+}$ did not induce appressorium formation on a non-inductive surface. These strongly suggest that calcium homeostatis is more important than calcium influx for appressorium formation. In addition, these imply that appressorium differentiation in *M. grisea* requires sustained rather than transient increase of $Ca^{2+}$ concentration in the cytosol. The calmodulin antagonists also inhibited appressorium

formation, but at different dosages. This probably reflects the different affinities of antagonists to calmodulin in *M. grisea*. Although the precise mechanisms of calmodulin functions remain to be elucidated, it clearly demonstrates that calmodulin plays an important role in appressorium formation of *M. grisea*. Recently, calmodulin genes were cloned from *M. grisea* [22] and *Colletotrichum trifolii* [10]. Reducing the expression of this gene by expressing an antisense copy resulted in normal appressoria but at a reduced frequency in *C. trifolii* (Dickman, *et al.*, 1995). Neomycin, a phosphoinositide specific phospholipase C (PLC) inhibitor, inhibited appressorium formation over wide range of concentration, while conidial germination remained unaffected. This indicates that activation of PLC is required for appressorium formation in *M. grisea*. However, addition of $IP_3$ did not induce appressorium formation on a non-inductive surface. This suggests that diacylglycerol (DAG) generated by activated PLC may induce appressorium formation by activating protein kinase C (PKC). Genes encoding PLC and PKC of *M. grisea* were cloned and are being characterized. PLC gene resembles δ-type of mammalian PLC isoform. PKC gene was identified as new type of PKC. Both genes are present as a single copy in the haploid genome of *M. grisea* (unpublished dart). Targeted disruption of them will give more direct evidence of their function in appressorium formation of *M. grisea*. Although precise mechanisms of these signaling pathways remain to be elucidated, it is clear that calcium-dependent signaling system is involved in appressorium formation in *M. grisea*.

*Cross-talks*

Pathway cross talks in signal transduction are known to occur in a number of systems. For example, cAMP has been shown to activate the MAP kinase pathway in both mammalian and fungal systems [34, 36]. Calcium signaling in conidial germination and appressorium formation of *Metarhizium anisopliae* is believed to be influenced by cAMP levels [29], and activation of a PLC protein has been linked to PKA [20]. Many pathways share transcription factors [2] or activate antagonistic proteins [24]. Other forms of pathway cross talk include partial or complete redundancy. Evidence suggests that cross talk is occurring in the cAMP signaling pathway in *M. grisea*. Mutants of the catalytic subunit of protein kinase A (*cpkA*) showed greatly reduced and functionally abnormal appressoria [23]. These mutants were unaffected in colony growth, conidiation, germination, and sexual reproduction. However, mutants of both adenylate cyclase (*MAC1*) and the $G_\alpha$ subunit gene (*magB*) were virtually identical phenotypically (*magB* mutants germinated normally whereas *MAC1* mutants had reduced germination); demonstrating reduced growth, conidiation, and sterility [6, 21]. These data are indicative of a second pathway(s) for cAMP to control growth and development, perhaps through either the existence of other *cpkA* homolog(s) [1] or by the MAP kinase *pmk1* [36]. However, other possibilities exist. It has been shown in *Dictyostelium* that the regulatory subunit of PKA binds to, and stimulates, a cAMP phosphodiesterase (regA) independent of the catalytic subunit [28]. It is possible that the regulatory subunit could regulate other types of phosphodiesterases [7]. Cross talks between cAMP- and $Ca^{2+}$-dependent pathways in *M. grisea* are being elucidated. Exogenous addition of cAMP recovered the appressorium formation inhibited by EGTA, W-7, or neomycin. Furthermore, appressorium formation inhibited by calcium chelator or ionophores was also recovered by addition of a cutin monomer, 1,16-hexadecanediol.

*Future prospects*

It is becoming clear that the second messengers and kinase relay systems found in mammals exist and play important roles on infection-related morphogenesis in *M. grisea*, but what is still lacking in our knowledge is the beginning and endpoints of these systems - the identification of important receptors, the protein targets of kinases, and the identity of upregulated genes. Are the mammalian kinase substrates (e.g. CREB, CBP, ATF-1, AP-1, etc.) conserved in *M. grisea*? What genes are involved in appressorium initiation or infection and how are they controlled? Where do they come into play in the signaling system? Does the knowledge of specific gene function give us a means of blocking the infection process so as to increase crop production? And perhaps the most important, how can we use this knowledge to better understand the function of these genes in the more complex mammalian systems? The future of signal transduction research is ripe with opportunities and many possibilities. Not only to better understand fungal biology and pathogenicity, but in the broader aspect of eukaryotic cell function.

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