

The molecular biology of appressorium turgor generation by the rice blast fungus *Magnaporthe grisea*

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Abstract

The rice blast fungus *Magnaporthe grisea* develops specialized infection structures known as appressoria, which develop enormous turgor pressure to bring about plant infection. Turgor is generated by accumulation of compatible solutes, including glycerol, which is synthesized in large quantities in the appressorium. Glycogen, trehalose and lipids represent the most abundant storage products in *M. grisea* conidia. Trehalose and glycogen are rapidly degraded during conidial germination and it is known that trehalose synthesis is required for virulence of the fungus. Lipid bodies are transported to the developing appressoria and degraded at the onset of turgor generation, in a process that is cAMP-dependent. A combined biochemical and genetic approach is being used to dissect the process of turgor generation in the rice blast fungus.

Introduction

Rice blast disease is the most serious disease of cultivated rice, and causes serious, recurrent epidemics throughout the rice growing regions of the world [1]. The disease is particularly prevalent in temperate-flooded and tropical-upland (rain fed) rice cropping systems, where serious crop losses of approx. 10–30% of the rice harvest can occur. The fungus that causes blast disease is an ascomycete called *Magnaporthe grisea* (Hebert) Barr. Individual host-limited forms of *M. grisea* are also responsible for causing blast disease on a large number of grass hosts, including important cereals, such as barley and wheat [2]. Wheat blast, in particular, has been a particularly serious problem in Brazil in the recent years [2,3].

To bring about rice blast disease, *M. grisea* undergoes a series of defined morphogenetic developmental steps, leading to the production of a specialized infection structure called the appressorium [1]. These cells are produced on the surface of rice leaves, and bring about plant infection primarily by physical breakage of the leaf cuticle. Experiments performed in the early 1990s demonstrated that appressoria of the rice blast fungus generate substantial turgor [4]. Incipient cytorrhysis experiments were performed by applying increasing concentrations of polyethylene glycol to appressoria of *M. grisea*, and then determining the rate of cell collapse [4]. In this way, the equivalent turgor within appressoria was estimated. These experiments provided evi-

dence that appressoria of *M. grisea* generate up to 8 MPa of pressure during plant infection. As a result of this enormous turgor, the appressorium produces a narrow penetration hypha at the base of the cell, which is forced through the underlying cuticle and later develops into invasive hyphae that fill the epidermal cells of the leaf [1,2]. Rice blast symptoms become apparent 4–5 days after initial infection [1,2]. In this review, we focus on the process of turgor generation by appressoria of the rice blast fungus, and describe the probable biochemical pathways that lead to generation of appressorium turgor and the genetic determinants responsible for pressure generation by infection structures of this important plant pathogen.

Appressorium development by *M. grisea*

Rice infection by *M. grisea* is initiated when three-celled, teardrop-shaped conidia land on the surface of a rice leaf. These spores germinate immediately on contact with the rice leaf, and adhere tightly to the hydrophobic surface by means of a spore tip mucilage that is released from the apex of the spore [5]. Germination proceeds by extension of a narrow germ tube that emerges from the conidium within an hour of its landing on the leaf surface [1,5]. Within 4 h, the germ tube starts to swell at its apex, and flattens against the surface of the rice leaf. The germ tube apex then develops into a swollen dome-shaped cell, called the appressorium [1,2,6]. The process of appressorium morphogenesis is tightly coupled to cell division, as mitosis is always observed within germ tubes of the fungus before the appressorium development [6]. *M. grisea* appressoria form in response to hard, hydrophobic surfaces, the absence of external nutrients, and the presence of constituents of cuticular wax [6]. The signalling pathways responsible for regulating appressorium formation have been

Q1 Key words: appressorium, fungal pathogenicity, *Magnaporthe grisea*, morphogenesis, turgor generation, trehalose.

Abbreviations used: MAP kinase, mitogen-activated protein kinase; PKA, cAMP-dependent protein kinase A; T6P, trehalose-6-phosphate.

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investigated in *M. grisea* and it is known that a cAMP response pathway is triggered soon after the attachment of the fungus on the leaf surface. Mutants lacking adenylate cyclase, which are unable to accumulate cAMP, do not form appressoria [7]. This mutation is subject to frequent reversion by a second-site mutation in the regulatory subunit of PKA (cAMP-dependent protein kinase A), which leads to cAMP-independent triggering of PKA and restoration of appressorium development [8]. Consistent with this, mutants lacking the catalytic subunit of PKA form small, mis-shaped and non-functional appressoria [9,10]. A MAP kinase (mitogen-activated protein kinase) cascade involving the Pmk1 MAP kinase is also required for the production of appressoria, and mutants lacking the *PMK1* gene arrest the growth before development of infectious structures [11].

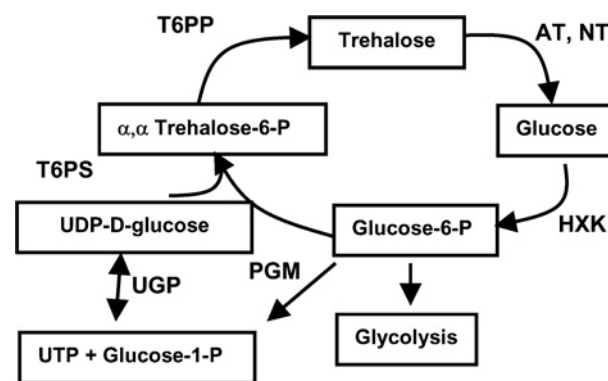
Based on these studies (reviewed in detail in [1,6]), appressorium development can be divided, broadly, into two different stages: the initiation of appressorium development, which requires cAMP signalling, and appressorium morphogenesis, which requires the presence of the Pmk1 MAP kinase pathway. Once formed, appressoria become separated from the germ tube and conidium by a thick septum, and become melanin-pigmented. Melanin is deposited in a layer within the cell wall of the appressorium. Mutants lacking melanin, due to mutations in gene-encoding enzymes in the dihydroxynaphthalene melanin biosynthetic pathway, are readily identified in *M. grisea* because of alteration in the colour of the fungus, which is normally grey. The *buf1* mutant, for example, is due to mutation of a gene encoding dihydroxynaphthalene reductase, and it was so called because of the buff colour of mutants lacking this enzymatic activity. Similarly, the *M. grisea* *RSY1* gene encodes scytalone dehydratase, and mutants are identified because of their pink colour. Finally, mutations in a polyketide synthase encoded by the *ALB1* gene, lead to an albino mutant phenotype. All three of these mutants are non-pathogenic because the appressoria that they produce are unable to accumulate turgor [6].

The process of appressorium turgor generation

Appressorium turgor generation in *M. grisea* occurs due to the rapid influx of water into the infected cell. Appressorium formation occurs, normally, in dewdrops on the surface of a rice leaf, and free water is an essential pre-requisite for the generation of these cells. Water flows into the appressorium against a concentration gradient that is generated by the accumulation of a compatible solute within the appressorium. Biochemical analysis of *M. grisea* appressoria demonstrated that a number of solutes accumulate within these cells, including, most notably, glycerol that accumulates to concentrations in excess of 3 M [12]. How the fungus is able to accumulate such high concentrations of the solute remains, as yet, unknown. Accumulation of high concentrations of glycerol was shown by vapour-pressure psychrometry, to be required for generation of the substantial turgor pressure that had previously been observed in *M. grisea* appressoria [12]. Because

Figure 1 | Trehalose metabolism in *M. grisea*. Synthesis of trehalose occurs from uridine-5-diphosphoglucose and glucose-6-phosphate

UDP-glucose is synthesized by uridine phosphoglucose pyrophosphorylase EC 2.7.7.9 (UGP) and glucose-1-phosphate by phosphoglucomutase EC 5.4.2.2 (PGM). Trehalose-6-phosphate synthase EC 2.4.1.15 (T6PS) is used to synthesize T6P that is dephosphorylated by T6P phosphatase EC 3.1.3.12 (T6PP). Trehalose hydrolysis occurs through acidic or neutral trehalases (AT, NT). Glucose is phosphorylated for entry to glycolysis by hexokinase (HXK).



appressoria form in free water on the leaf surface in the absence of external nutrients, glycerol and other solutes within the appressorium must be accumulated *de novo* from storage products present in conidia of the fungus. Analysis of conidia has revealed that they accumulate lipids, glycogen and the disaccharide trehalose as the predominant storage products [13,14]. Additionally, mannitol has previously been reported to accumulate within the spores [15]. At the onset of conidial germination, trehalose, glycogen and lipid have all been found either to be degraded rapidly or transported to the germ tube apex [13,14]. Recent investigations have focused on trehalose, lipid and glycogen as potential sources of glycerol and other compatible solutes during appressorium turgor generation in *M. grisea*.

Trehalose metabolism in *M. grisea*

The non-reducing disaccharide trehalose (α -D-glucopyranosyl- α -D-glucopyranoside) is a common storage product within microbial cells. Its major roles are to act as a stress metabolite and cellular protectant from desiccation [16]. However, it has become increasingly apparent that accumulation of trehalose in many eukaryotes is a means by which sugar metabolism can be regulated [17,18]. Analysis of the *M. grisea* genome sequence (<http://www.broad.mit.edu/annotation/fungi/magnaporthe/>) and recent biochemical analysis [14] indicate that trehalose synthesis occurs as illustrated in Figure 1. T6P (Trehalose-6-phosphate) is synthesized in *M. grisea* using UDP-glucose and glucose-6-phosphate as substrates and is then directly converted into trehalose. In *Saccharomyces cerevisiae*, it has been established that a

multienzyme complex is responsible for the synthesis of T6P [19], the catalytic subunit of which is encoded by a gene called *TPS1*. The homologous gene in *M. grisea* has been characterized and shown by targeted gene replacement to be required for rice blast disease. *M. grisea* $\Delta tps1$ mutants were found to be non-pathogenic and, on closer examination, it was found that appressorium turgor generation was severely affected in these strains [14]. However, $\Delta tps1$ mutants also displayed a number of pleiotropic effects. For example, $\Delta tps1$ mutants were unable to grow on glucose or a variety of other rapidly fermentable carbon sources, and were also unable to utilize acetate or lipids as sole carbon sources. In *S. cerevisiae*, it has been demonstrated that T6P synthase activity is a means by which the influx of glucose into glycolysis is regulated [17]. Yeast *tps1* Δ mutants are unable to grow on glucose due to the absence of T6P synthase activity leading to an uncontrolled influx of glucose into glycolysis. This leads to rapid utilization of ATP in the first two steps of glycolysis and a subsequent drop in ATP and phosphate levels within the cell [17,19]. There are, however, a number of key differences between yeast *tps1* Δ mutants and *M. grisea* $\Delta tps1$ mutants [14]. Whereas *S. cerevisiae* *tps1* Δ mutants are unable to grow in the presence of glucose under any conditions – glucose is effectively toxic to these cells – in *M. grisea*, glucose utilization can be restored by the presence of alternate carbon sources, and in particular, the presence of complex nitrogen sources such as yeast extract or peptone [14]. Moreover, *M. grisea* $\Delta tps1$ mutants are unable to grow on acetate or lipids, indicating that gluconeogenesis may also be affected by loss of T6P synthase activity [14].

To determine why *M. grisea* $\Delta tps1$ mutants are non-pathogenic, the potential for trehalose metabolism to lead to glycerol synthesis in the appressorium was explored. The *M. grisea* genome sequence revealed the presence of two trehalase-encoding genes, *TRE1* and *NTH1*. *NTH1* is predicted to encode a neutral trehalase, and has been shown to be highly expressed during conidiogenesis and spore germination [14]. Mutants lacking *NTH1* are reduced in virulence, although this appears to be due to a decreased ability to perform invasive growth within rice tissues. In contrast, *TRE1*, which is responsible for encoding the main trehalase activity during *M. grisea* spore germination, appears to be completely dispensable for pathogenesis. Mutants lacking *TRE1* produce rice blast symptoms that are identical to those of an isogenic wild-type strain of the fungus [14]. *TRE1* encodes an unusual trehalase that does not show significant similarity to other fungal neutral or acidic trehalases. *TRE1* is similar, however, to a novel trehalase gene found in the *Neurospora crassa* genome (NCU00943).

When considered together, the phenotypes of the *M. grisea* $\Delta tps1$ mutant and those of *Anth1* and *Atre1* trehalase mutants strongly suggest that trehalose synthesis is required for appressorium function but that trehalose metabolism is not. It is possible, therefore that either trehalose accumulates as an accessory-compatible solute in appressoria, or that the sugar signalling role of T6P synthase is essential for the regulation of appressorium turgor generation in *M. grisea*.

Lipid metabolism during appressorium turgor generation

During *M. grisea* spore germination, lipid bodies are mobilized quickly from the conidium, and can be observed accumulating in the germ tube apex [13]. Lipid bodies are surrounded by a single unit membrane in *M. grisea*, and appear highly refractile by phase contrast microscopy, but can be readily visualized by staining with Nile Red [13,20]. During appressorium development, lipid bodies accumulate at the germ tube apex and in the incipient appressorium. Subsequently, during appressorium maturation, lipid bodies coalesce and, eventually, are taken up by vacuoles, by a process that resembles autophagocytosis [20]. Lipolysis appears to occur in vacuoles that also coalesce to form a large central vacuole within the appressorium during turgor generation [6,20]. Biochemical analysis has shown the presence of a triacylglycerol lipase activity, which is induced during appressorium maturation [13]. Triacylglycerol lipase activity in *M. grisea* appressoria is cAMP-regulated, because in a $\Delta cpkA$ mutant, lacking the catalytic subunit of PKA, lipase activity is substantially decreased and, additionally, lipid bodies fail to coalesce or undergo degradation during appressorium morphogenesis [13]. In contrast, a regulatory subunit PKA mutant [8] carries out rapid lipid degradation in the appressorium, which is completed before melanization of the infected cell [13]. The initial trafficking of lipid bodies to the appressorium appears to be under the control of the Pmk1 MAP kinase, because in mutants lacking *PMK1*, lipid bodies fail to move to the germ tube apex during the initiation of appressorium development [13].

The genome of *M. grisea* encodes at least four genes that are predicted to encode intracellular triacylglycerol lipases. These enzymes may provide a means of rapidly producing glycerol from lipid droplets, which are transported to the developing appressorium. In *S. cerevisiae*, a recent report has provided evidence for a novel triacylglycerol lipase that is directly associated with lipid bodies [21]. This would provide the most probable candidate for the intracellular lipase responsible for the rapid lipolysis observed during appressorium maturation in *M. grisea*. Interestingly, there are two homologues of this gene (YMR313c) in the *M. grisea* genome, which are currently being investigated (Z.-Y. Wang and N.J. Talbot, unpublished work).

A consequence of appressorium lipolysis and glycerol generation [13,20] is likely to be the generation of fatty acids. Therefore a requirement for fatty acid β -oxidation and subsequent activation of the glyoxylate shunt and gluconeogenesis has been proposed [13]. The glyoxylate cycle has been shown to be required for pathogenicity in *M. grisea* [22]. Mutants lacking the *JCL1* gene encoding isocitrate lyase were recently shown to be reduced in virulence. This was due to a temporal regulation effect on the development of appressorium; spore germination was retarded in $\Delta icl1$ mutants, as was the subsequent germ tube extension and appressorium morphogenesis. This was taken as evidence for the fact that cell-wall biosynthesis and compatible solute generation required the glyoxylate cycle to be active in the

fungus during appressorium development. Consistent with this idea, *ICL1* gene expression was shown to be very high during appressorium morphogenesis, penetration peg formation and invasive growth of *M. grisea* [22]. The fact that *Δicl1* mutants were capable of causing rice blast symptoms, albeit in a delayed manner when compared with a wild-type strain of *M. grisea*, showed that alternative pathways for appressorium maturation and penetration hyphae development must be present in the fungus. Interestingly, the glyoxylate shunt has been shown to be required for pathogenicity of several other phytopathogenic and human pathogenic fungi [23–25], perhaps reflecting their shared need to develop initially within a glucose-deficient environment. Peroxisomal function has been shown to be required for appressorium-mediated plant infection by the antracnose fungus, *Colletotrichum lagenarium* which also produced melanin-pigmented appressoria [26], suggesting that fatty acid β -oxidation may be generally important for the physiology of appressoria.

Glycogen metabolism in *M. grisea*

Glycogen is abundant within the spores of *M. grisea*, and is mobilized very quickly on germination [13]. Glycogen rosettes are then found to accumulate within appressoria during their development [6], but at the onset of turgor generation, glycogen quickly disappears from the appressoria during melanization and turgor generation [6,13]. Glycogen mobilization also appears to be regulated by the cAMP response pathway because *ΔcpkA* mutants show retarded degradation of glycogen during conidial germination and during initiation of appressorium development [13]. Conversely, the regulatory subunit PKA mutant *Δmac1 sum1-99* showed rapid degradation of glycogen before the onset of melanin production within appressoria [8,13]. Glycogen is degraded by two major enzyme activities: glycogen phosphorylase and amyloglucosidase. Mutants lacking the genes encoding these enzymes have recently been generated in *M. grisea* and are currently being characterized. Preliminary evidence suggests that both of these enzyme activities are required for full virulence of the fungus (L.J. Holcombe and N.J. Talbot, unpublished work). Significantly, the *pls1* mutant, which is non-pathogenic and produces completely non-functional appressoria, accumulates glycogen deposits within infected cells [27]. *PLS1* encodes a tetraspanin, a novel membrane protein that may be required for controlling the translation of turgor into physical force for penetration hypha production. It is clear, however, that penetration hyphae emergence, which requires the *MST12*-encoded transcription factor, occurs independent of turgor generation [28]. How glycogen, trehalose and lipid metabolism each contribute to glycerol formation in the appressorium is, as yet, unresolved. Genetic analysis has now demonstrated that a number of genes encoding enzymes of the biosynthetic and metabolic pathways of the conidial storage products are required for virulence of the fungus. However, the relative contribution of glycogen and lipid, in particular, to glycerol synthesis

will require further biochemical analysis. Interestingly, the enzymes by which glycerol could be synthesized from storage carbohydrates – NADH-dependent glycerol-3-phosphate dehydrogenase and NADPH-dependent glycerol dehydrogenase – are present in appressoria but are not induced during turgor generation. In contrast, triacylglycerol lipase activity is rapidly activated during the onset of turgor generation [13]. Radioisotope labelling experiments, utilizing each of the currently generated *M. grisea* mutants, are currently in progress attempting to resolve this issue.

Conclusions

To bring about rice blast disease, *M. grisea* has evolved a remarkable mechanism involving production of a cell that is required for attachment to the rice leaf surface and for generation of mechanical force to penetrate the rice leaf cuticle. In addition, based on analysis of the genome sequence of *M. grisea* and cytological studies of plant infection, it seems highly probable that extra-cellular depolymerizing enzymes act to accelerate the process on living plant tissue [6]. However, the fact that *M. grisea* is capable of penetrating into inert plastic membranes highlights the importance of turgor generation to appressorium function. *M. grisea* appressoria accumulate high concentrations of compatible solutes such as glycerol, and current evidence indicates that this is responsible for bringing about the rapid generation of turgor. However, there remain several unresolved issues: for example, how does the fungus secure attachment of the appressorium to the leaf surface during the generation of such massive mechanical forces, which would otherwise lift the cell away from the underlying substrate? What is the nature of the adhesives that attach appressoria so tenaciously to the hydrophobic substrate? How is glycerol generated, and which of the storage products of the spore are most important for the generation of turgor? How is the process of turgor generation spatially and temporally regulated, and what are the precise downstream transcription factors that act to bring about expression of the genes involved in turgor generation? The availability of the full *M. grisea* genome sequence, coupled with the ability to perform gene functional analysis at a relatively high throughput, means that there is a clear opportunity to perform a systems biology approach to the dissection of fungal pathogenesis in *M. grisea* and, in particular, to resolve the mechanism by which appressorium turgor is generated.

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