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Growth in rice cells requires *de novo* purine biosynthesis by the blast fungus *Magnaporthe oryzae*

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Increasing incidences of human disease, crop destruction and ecosystem perturbations are attributable to fungi and threaten socioeconomic progress and food security on a global scale. The blast fungus *Magnaporthe oryzae* is the most devastating pathogen of cultivated rice, but its metabolic requirements in the host are unclear. Here we report that a purine-requiring mutant of *M. oryzae* could develop functional appressoria, penetrate host cells and undergo the morphogenetic transition to elaborate bulbous invasive hyphae from primary hyphae, but further *in planta* growth was aborted. Invasive hyphal growth following rice cell ingress is thus dependent on *de novo* purine biosynthesis by the pathogen and, moreover, plant sources of purines are neither available to the mutant nor required by the wild type during the early biotrophic phase of infection. This work provides new knowledge about the metabolic interface between fungus and host that might be applicable to other important intracellular fungal pathogens.

Rice (*Oryza sativa*) provides 23% of the calories consumed by humankind, but harvests are frequently lost to the blast fungus *Magnaporthe oryzae*¹. Rice blast disease is a severe threat to food global security^{2–4}, prompting the development of *M. oryzae* as a model phytopathogen⁵. Plant infection starts when a three-celled asexual spore of *M. oryzae* lands on the surface of a rice leaf and germinates. After a period of polarized growth, the tip of the germ tube begins to swell and form a specialized dome-shaped structure called the appressorium^{1,6,7}. Maturation of the appressorium results in the generation of enormous hydrostatic turgor pressure¹ that acts on a thin penetration hypha, forcing it through the leaf cuticle. In the plant, *M. oryzae* displays an infection strategy known as a hemibiotrophy such that it colonizes living host cells as a symptomless biotroph for the first 4–6 days of infection before becoming necrotrophic⁸. The biotrophic phase begins when the penetration peg expands to become a thin filamentous primary hypha in the cell lumen before elaborating into bulbous invasive hyphae (IH)^{3,8}. The IH pushes into host cells surrounded by the invaginated plant-derived plasma membrane, termed an extra-invasive hyphal membrane (EIHM). After 8 to 12 hours of growth – and following constriction of the IH – the fungus moves from the first infected cell into neighbouring cells surrounded by the EIHM at points thought to be plasmodesmata⁸. The IH grows as thin primary hyphae in the second set of invaded cells before enlarging again into bulbous IH. Movement to subsequent cells is more rapid, requiring only 2–3 hours of IH growth. A novel structure known as the biotrophic interfacial complex (BIC) is formed in primary hyphae as it enters each cell, and remains behind the IH as it elaborates and grows^{9–11}. Secreted effector proteins, some with roles in suppressing plant defenses, accumulate in the BIC. These include the biotrophy-associated secreted (BAS) proteins⁹, the known effectors Avr-Pita, PWL1, PWL2¹⁰ and AvrPiz-t¹², and the secreted protein MC69¹³.

Biotrophic growth requires nutrient acquisition. Recently, rice infection has been shown to be dependent on the *M. oryzae* glucose 6-phosphate (G6P) sensor Tps1, a signaling protein that couples carbon and nitrogen metabolism in response to available glucose^{3,14–17}. Loss of Tps1 function abolishes pathogenicity, in part due to the reduced expression of known Tps1-dependent virulence genes^{15,17} and the early expression of genes normally repressed by Tps1 in response to G6P sensing, such as those encoding some cell wall-degrading enzymes¹⁷. Tps1 thus presents as an important link between primary fungal metabolism – i.e. the exploitation of available carbon and nitrogen sources – and the pathogenic process.

Despite elucidating G6P sensing by Tps1 as a central regulator of infection, a significant gap remains in our understanding of the metabolic requirements of *M. oryzae* during biotrophic growth. In particular, how hyphal morphogenesis is influenced and/or controlled by available nutrients in host cells is little understood. The identity and origins (pathogen or host) of important metabolites during infection also remains largely unknown^{3,18}. Such



scarcity of knowledge impedes the development of a robust understanding of the physiology of rice blast disease that would link the cellular descriptions⁸ and genetic dissections¹⁷ of *M. oryzae* biology and point the way to new, sustainable strategies for combating this problem.

Here, we sought to shed more light on *M. oryzae* metabolic requirements during the biotrophic phase of rice infection by targeting purine metabolism in the fungus. We deleted the SAICAR synthetase-encoding gene *MoADE1* to show that *de novo* purine biosynthesis is not required for appressorium formation or rice cuticle penetration but is essential for establishing biotrophic growth in rice cells. We thus reveal a new metabolic constraint for rice blast disease: the absolute dependency of rice blast disease on fungal purine metabolism. The implications for understanding pathogen nutrient utilization *in planta* are discussed.

Results

The *M. oryzae* SAICAR synthetase orthologue *MoADE1* is required for *de novo* adenine biosynthesis. Guy11, the wild type isolate of the rice blast fungus *M. oryzae* used in this study, synthesizes purines and pyrimidines *de novo*. Following completion of the *M. oryzae* genome⁵, putative purine biosynthetic pathways in *M. oryzae* have been delineated in the Kyoto Encyclopedia of Genes and Genomes (KEGG) by homology to purine metabolic genes characterized in other fungi. We sought to disrupt purine biosynthesis in *M. oryzae* by targeting the SAICAR synthetase-encoding gene, named *ADE1* in *Saccharomyces cerevisiae*¹⁹. Interrogating the *M. oryzae* genome, we found the locus MGG_12537 encoded a SAICAR synthetase orthologue, and we subsequently deleted the *MoADE1* coding region from the *M. oryzae* genome using our high-throughput split marker method¹⁵. Like *ADE1* mutants in yeast,

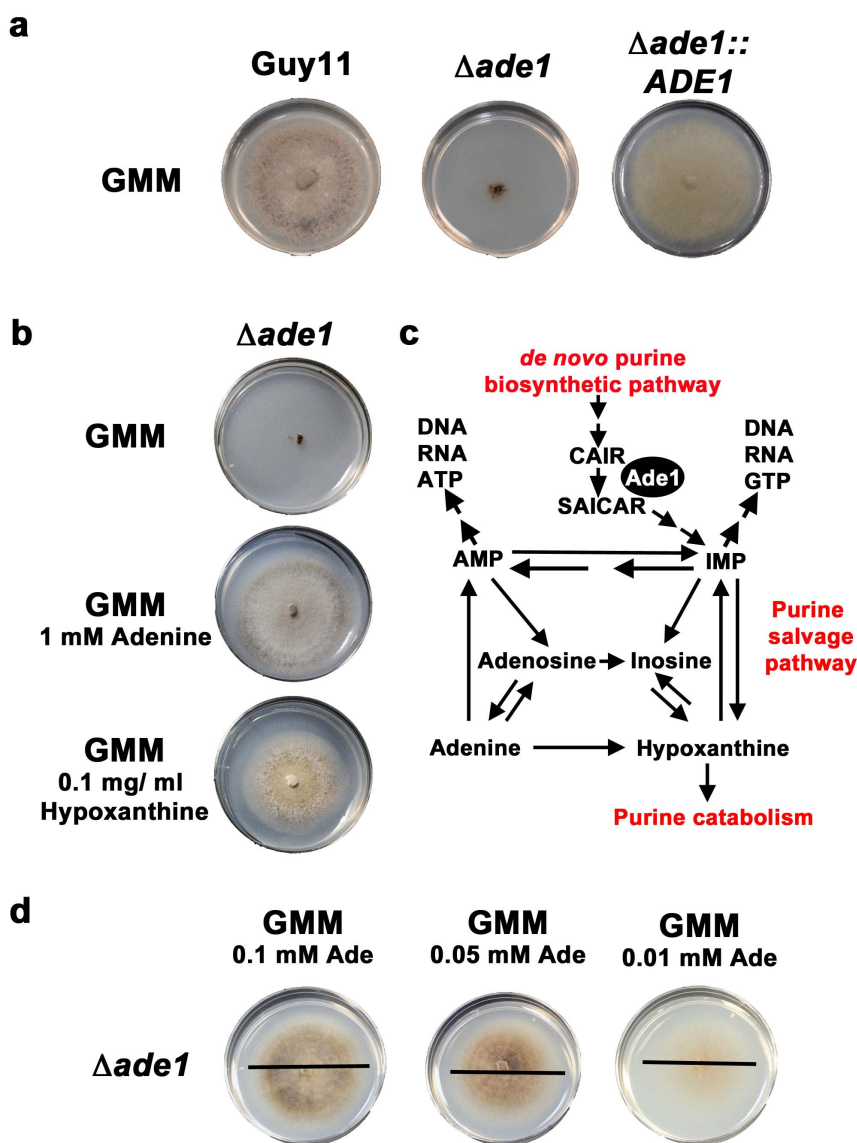


Figure 1 | Deleting *MoADE1*, encoding SAICAR synthetase, from the *M. oryzae* genome generates a conditionally lethal, purine-requiring mutant. (a) $\Delta ade1$ strains, unlike wild type Guy11 and $\Delta ade1$ complementation strains carrying a full-length copy of *MoADE1*, were unable to grow on glucose minimal media (GMM) lacking an exogenous source of purines such as adenine. (b) Growth of $\Delta ade1$ strains was restored on GMM supplemented with 1 mM adenine or 0.1 mg/ml hypoxanthine. (c) Model of purine metabolism in *M. oryzae*, based on KEGG and adapted from³¹, with steps omitted for clarity. Ade1 functions to convert CAIR to SAICAR in the purine *de novo* biosynthetic pathway. $\Delta ade1$ growth remediation by adenine and hypoxanthine on GMM shows the purine salvage pathway is active in *M. oryzae*. Hypoxanthine can also be metabolized as a sole nitrogen source by *M. oryzae*¹⁴, confirming the purine catabolism pathway is also operational in *M. oryzae*. (d) Adenine added to GMM at the concentrations shown did not affect the radial growth of $\Delta ade1$ strains, suggesting adenine uptake from the media is not impaired in this strain.



the resulting $\Delta ade1$ strain could not grow on defined glucose minimal media (GMM) lacking adenine (Fig. 1a). This was solely due to loss of MoAde1 function because growth was restored on GMM by re-introducing the wild type copy of *MoADE1* into $\Delta ade1$ strains (Fig. 1a). Growth of $\Delta ade1$ strains on GMM was also restored if 1 mM adenine or, interestingly, 0.1 mg/ml (final concentration) hypoxanthine was added to GMM (Fig. 1b). Restored growth with both adenine and hypoxanthine supplementation suggests the purine salvage pathway is operational in *M. oryzae* (Fig. 1c). Next, we grew $\Delta ade1$ on diminishing amounts of adenine and found mycelial radial growth was not reduced at very low concentrations of adenine (ie. 0.01 mM adenine), suggesting adenine uptake from the media is not impaired in adenine-requiring $\Delta ade1$ strains (Fig. 1d).

MoADE1 is required for rice leaf pathogenicity but not appressorium formation. We applied spores of wild type Guy11 and $\Delta ade1$ strains - harvested from fungal mycelia grown on complete media

(CM) supplemented with 1 mM adenine - to the leaves of whole plants of the susceptible cultivar CO-39. Fig. 2a shows $\Delta ade1$ strains were greatly reduced for pathogenicity on rice leaves compared to Guy11, indicating *de novo* adenine biosynthesis by *M. oryzae* is essential for rice blast disease. Supplementary Figure S1a shows that complementing $\Delta ade1$ strains with a wild type copy of *ADE1* restored the ability of these strains to infect rice but adding 1 mM adenine to suspensions of $\Delta ade1$ spores and applying them to rice leaves did not remediate infection.

Despite being non-pathogenic, Fig. 2b shows that $\Delta ade1$ strains could form appressoria on the surface of detached rice leaf sheaths that were indistinguishable from Guy11. Fig. 2c shows that the rates of appressorium formation on detached rice leaf sheaths of $\Delta ade1$ and Guy11 strains were not significantly different.

$\Delta ade1$ strains can penetrate rice leaf cuticles at the same rate as Guy11 but *in planta* development is delayed. Next, we used live-cell imaging to determine if the appressoria formed by $\Delta ade1$ were

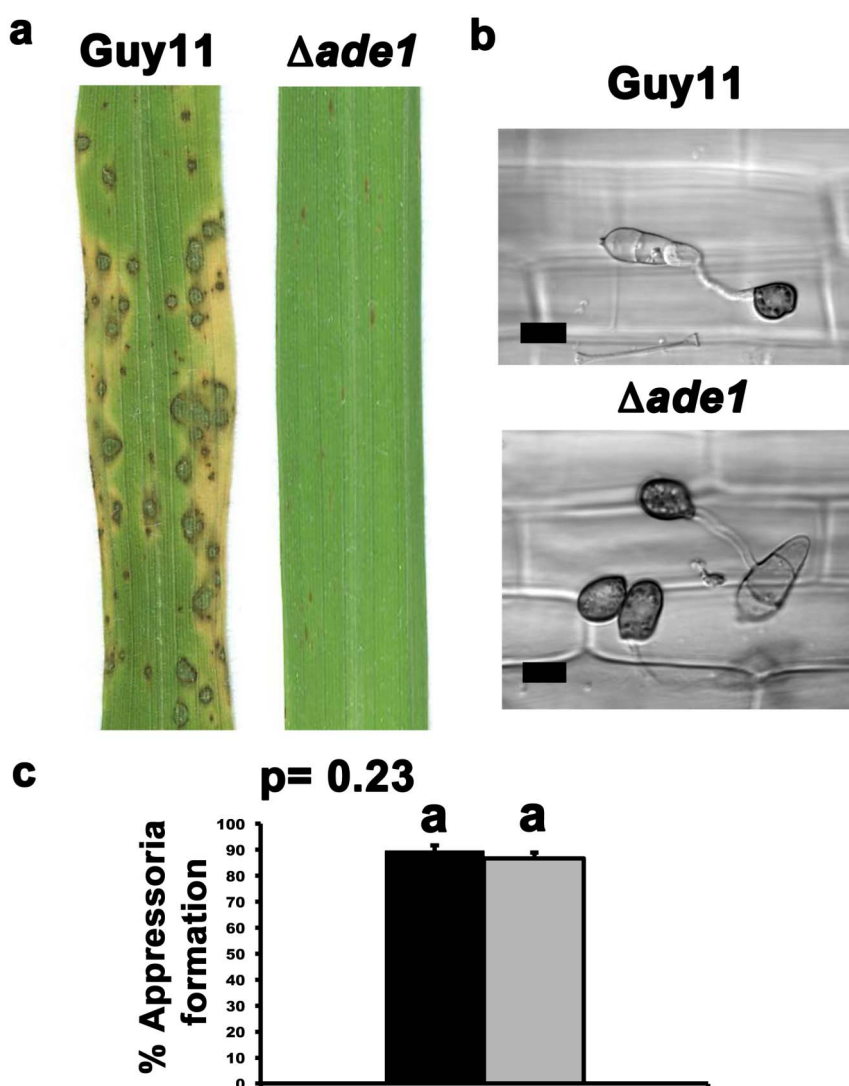


Figure 2 | Disrupting *MoAde1* abolishes infection but not appressorium formation. (a) Spores of $\Delta ade1$ strains were applied to three-week old plants of the susceptible cultivar CO-39 at a rate of 5×10^4 spores ml^{-1} . Compared to Guy11, $\Delta ade1$ strains were abolished for pathogenicity. Images were taken after 144 hpi. (b) Despite being non-pathogenic, $\Delta ade1$ strains were shown to form appressoria on detached leaf surfaces at 24 hpi. Scale bar is 10 μm . (c) The average rate of appressorium formation on the rice leaf surface was determined. 5×10^4 spores of each strain were inoculated onto rice cuticles. At 24 hpi, the number of appressoria that had developed from 50 conidia of each strain was analyzed. This was repeated three times for each strain to determine the mean value. Error bars are standard deviation. Closed bars are values for Guy11 and grey bars are values for $\Delta ade1$ strains. Bars with the same letter are not significantly different (*Student's t-test* $p \leq 0.05$).



functional. Spores of Guy11 and $\Delta ade1$ were harvested from fungal mycelia grown on CM plates supplemented with 1 mM adenine, applied to detached rice leaf sheaths, and analyzed for their ability to penetrate rice cuticles at 24 hr post inoculation (hpi). Fig. 3a shows there was no statistical difference (*Student's t-test* $p = 0.21$) between the rate of $\Delta ade1$ penetration into rice cells compared to Guy11, indicating loss of adenine biosynthesis does not impact the function of $\Delta ade1$ appressoria to breach rice cuticles. However, Fig. 3b shows that at 24 hpi, the subsequent *in planta* development of $\Delta ade1$ strains lagged that of Guy11. By 24 hpi, both Guy11 and $\Delta ade1$ strains had formed primary hyphae from the penetration peg, but only those from Guy11 had proceeded to elaborate bulbous IH which, although they had not yet begun to branch, contained a visible BIC structure (Fig. 3b).

IH growth is severely curtailed in $\Delta ade1$ strains. Fig. 4a shows that by 40 hpi, Guy11 strains - like $\Delta ade1 ADE1$ complementation strains (Supplementary Fig. S1b) - had filled the first infected cells and most had spread to adjacent cells. In contrast, $\Delta ade1$ strains had, by this time, undergone the morphogenetic transition to elaborate IH from the primary hyphae, including the formation of BICs, but further

growth was attenuated. Supplementary Figure S1b shows that adding 1 mM adenine to spores before applying to detached rice leaf sheaths does not improve the growth of $\Delta ade1$ strains in host cells.

By quantifying the differences in IH growth between Guy11 and $\Delta ade1$ strains at 40 hpi using the 4-point scale described in a previous study¹⁸, Fig. 4b shows that $\Delta ade1$ growth was significantly reduced (*Student's t-test* $p = 0.0002$) *in planta* compared to Guy11. Fig. 4c shows that by 40 hpi, and in contrast to Guy11, no $\Delta ade1$ strains had spread from the primary infected cell to adjacent cells. To determine if $\Delta ade1$ growth rates might change over longer time intervals, Fig. 5 shows that at 75 hpi, when Guy11 IH had spread to multiple cells, $\Delta ade1$ strains were still confined to the first infected cell. Taken together, these results suggest *de novo* purine biosynthesis is not required for elaborating appressoria from spores harvested from plates of CM supplemented with 1 mM adenine - nor required for appressorial penetration of the rice cuticle - but is essential for IH growth in and between host rice cells.

$\Delta ade1$ strains are alive in host cells at 48 hpi. Plants initiate defense programs in response to pathogen recognition, and these have to be avoided or overcome by the invading pathogen in order for a

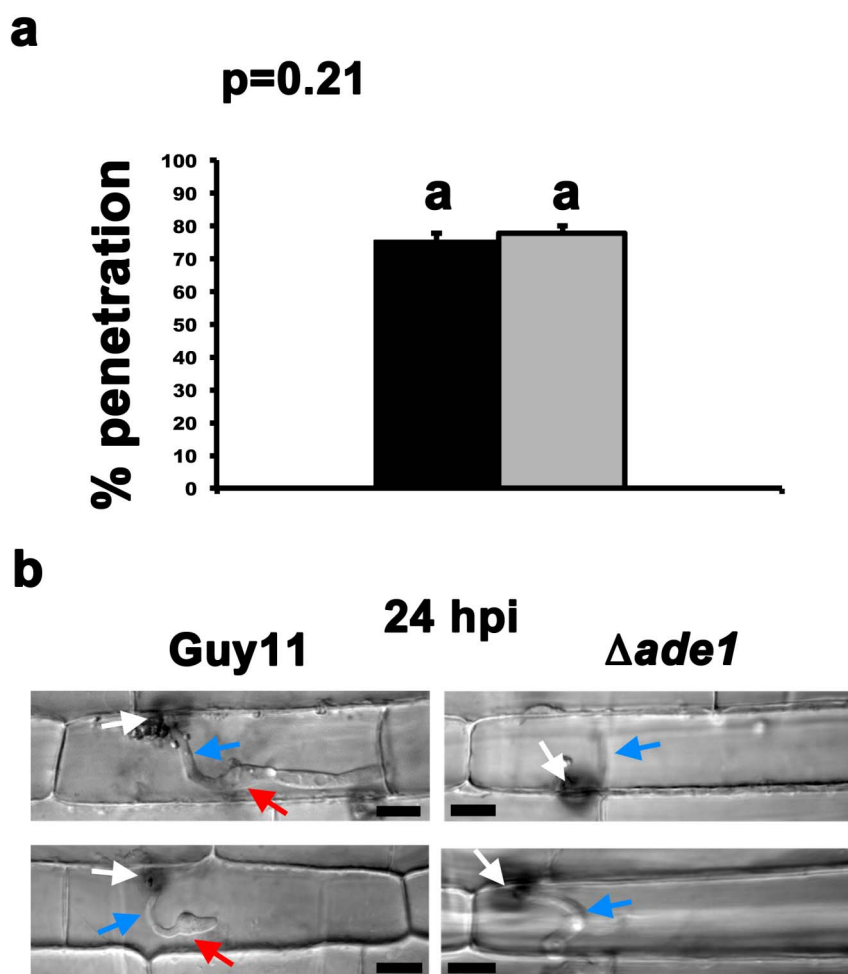


Figure 3 | Appressoria of $\Delta ade1$ strains are functional and penetrate leaf cuticles at the same rate as Guy11. (a) The number of appressoria that had penetrated the leaf cuticle, from a total of 50 appressoria observed for each strain, was determined at 24 hpi. The average is calculated from three independent replicates. Error bars are standard deviation. Closed bars are values for Guy11 and grey bars are values for $\Delta ade1$ strains. Bars with the same letter are not significantly different (*Student's t-test* $p \leq 0.05$). (b) Representative microscopic observations of fungal development in penetrated leaf epidermal cells at 24 hpi. In these examples, Guy11 appressoria have penetrated the cuticle and formed primary hyphae that have pushed into the epidermal cells and undergone the morphogenetic transition to bulbous IH. BICs have formed between the primary hyphae and IH. In contrast, although $\Delta ade1$ appressoria have penetrated rice cuticles at the same rate as Guy11 and have formed primary hyphae in host cells, elaboration of IH was not observed at 24 hpi in $\Delta ade1$ strains. White arrows are appressoria. Blue arrows are primary hyphae. Red arrows are BICs. Scale bar is 5 μm .

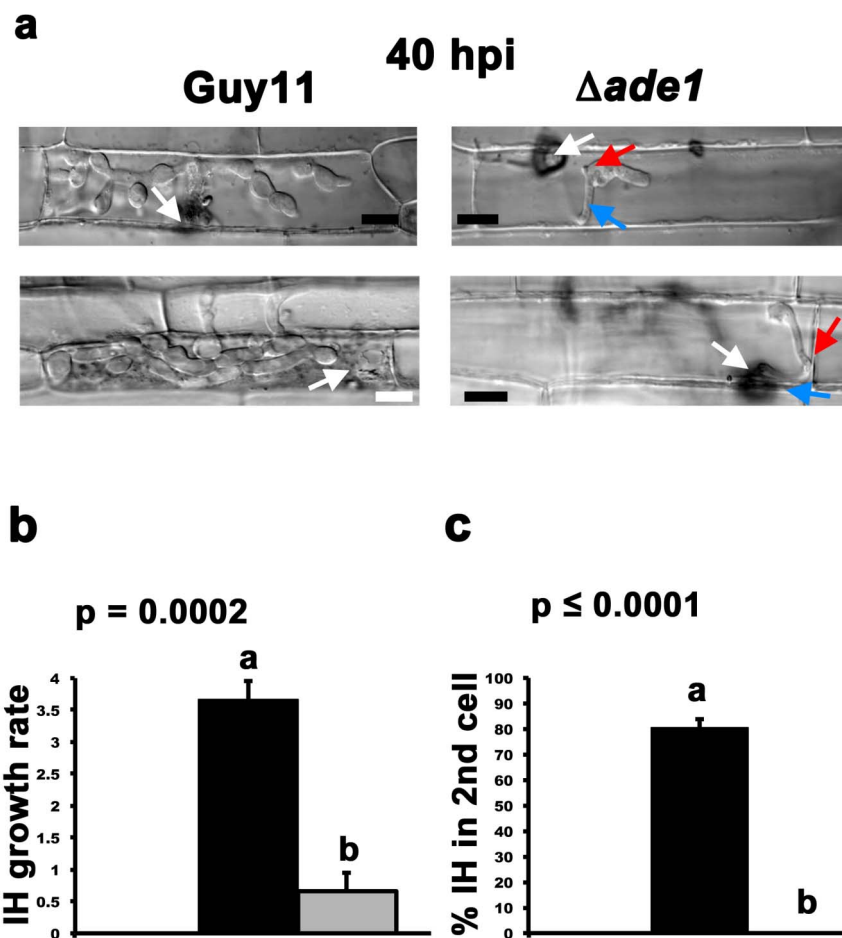


Figure 4 | Biotrophic growth in rice cells is attenuated in $\Delta ade1$ strains. (a) At 40 hpi, Guy11 strains had elaborated branching IH within the first infected cell and were moving to adjacent cells. In contrast, $\Delta ade1$ strains, although having undergone the morphogenetic transition to IH from primary hyphae including the formation of BICs, were greatly attenuated in growth. White arrows indicate appressoria and the point of penetration. Blue arrows indicate primary hyphae. Red arrows indicate BICs formed behind IH. Scale bar is 5 μm . (b) Quantification of the rate of IH growth in rice cells by Guy11 and $\Delta ade1$ strains. $\Delta ade1$ strains were significantly (*Student's t-test* $p \leq 0.05$) reduced in IH growth compared to Guy11. Growth scores were determined using a four-point scale¹⁸, where 1 = IH length shorter than 10 μm with no branching; 2 = IH length is 10–20 μm with 0–2 branches; 3 = IH length is longer than 20 μm and/or with more than 2 branches within one cell; 4 = IH has spread to adjacent cells. Three leaf sheaths were inoculated with each strain, and IH growth rates were determined for hyphae originating from 50 appressoria per leaf to generate a mean IH growth rate. Error bars are standard deviation. Closed bars are values for Guy11 and grey bars are values for $\Delta ade1$ strains. Bars with the same letter are not significantly different (*Student's t-test* $p \leq 0.05$). (c) At 40 hpi, no $\Delta ade1$ hyphae were observed spreading into adjacent secondary cells from primary infected cells. In contrast, approx. 75% of Guy11 hyphae had spread from the first infected cell into adjacent cells by 40 hpi. Three leaf sheaths were inoculated with each strain, and the average rate of spread of IH into adjacent secondary cells at 40 hpi was determined from a total of 50 primary infected cells per replicate. Error bars are standard deviation. Closed bars are values for Guy11 and grey bars are values for $\Delta ade1$ strains. Bars with the same letter are not significantly different (*Student's t-test* $p \leq 0.05$).

compatible interaction - such as the one that occurs between Guy11 and CO-39 - to occur^{3,20–22}. We considered that the attenuated growth observed for $\Delta ade1$ strains in rice cells (Fig. 4 and Fig. 5) could result from increased susceptibility to host defenses, relative to Guy11, due to the energetic costs of neutralizing plant defenses not being met in purine-requiring mutant strains. To address this, we sought to determine if $\Delta ade1$ strains elicited stronger rice defense responses than Guy11 during infection. Two rice pathogenesis-related (PR) genes, *PBZ1* and *PR1*, have been previously shown to be more highly induced during challenge by a *M. oryzae* mutant defective in neutralizing plant defenses, $\Delta des1$, than by wild type²⁰. In our study, Fig. 6a shows that rice *PBZ1* and *PR1* expression was not elevated during infection by $\Delta ade1$ strains compared to Guy11 at 48 hpi, as determined by quantitative real-time PCR (qPCR) analysis. 48 hpi was chosen because Guy11 spreads into neighboring cells at this time and $\Delta ade1$ strains have undergone the morphogenetic transition to IH formation. Moreover, Chi and associates²⁰ had

shown that $\Delta des1$ elicited an approximately 100-fold induction of *PBZ1* and *PR1* gene expression at 48 hpi compared to wild type. In contrast, Fig. 6a shows $\Delta ade1$ strains did not elicit more *PBZ1* or *PR1* induction compared to Guy11, and *PBZ1* expression was greatly reduced in rice cells challenged with $\Delta ade1$ compared to Guy11. This could indicate either that $\Delta ade1$ strains were not defective in neutralizing plant defense responses, or that the plant had successfully eliminated the threat from $\Delta ade1$ colonization by 48 hpi resulting in downregulated *PBZ1* expression. To distinguish between these two scenarios, we next sought to determine if $\Delta ade1$ strains were alive in rice cells at 48 hpi. Fig. 6b shows that expression of the *M. oryzae* actin gene *MoACT1* (Table 1) was detected by qPCR analysis of cDNAs resulting from rice leaves infected with either Guy11 or $\Delta ade1$ strains but not a mock-inoculated control at 48 hpi. The results were normalized against the expression of the rice actin gene *OsAct2* and hence, the reduced expression of *MoACT1* in $\Delta ade1$ strains relative to Guy11 reflects the reduced hyphal growth of

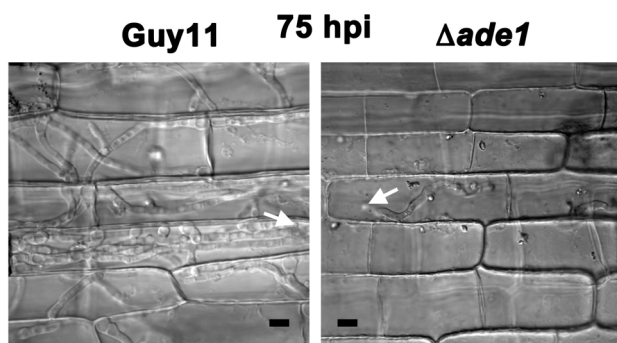


Figure 5 | No evidence for $\Delta ade1$ movement beyond the primary infected cell at 75 hpi. To determine if $\Delta ade1$ strains continue to grow, or if growth accelerates later in infection, we observed Guy11 and $\Delta ade1$ growth in leaf epidermal cells at 75 hpi. At this timepoint, Guy11 had spread to multiple adjacent cells, but $\Delta ade1$ remained confined to the primary infected cell. White arrows are appressoria and the point of infection. Scale bar is 5 μm .

this strain. Taken together, these results suggest that reduced growth of $\Delta ade1$ strains in rice cells does not occur due to increased susceptibility to rice defenses. Moreover, despite attenuated IH growth, $\Delta ade1$ strains were actively transcribing actin genes in host cells.

Putative nutrient transporters are upregulated in $\Delta ade1$ strains at 48 hpi compared to Guy11. On plates, $\Delta ade1$ strains can acquire adenine or hypoxanthine from the media (Fig. 1), but our results suggest that *in planta*, purines in amounts sufficient to remediate IH growth are not available, resulting in the poor growth of $\Delta ade1$ IH shown in Fig. 4. However, we do not consider this is because rice leaves lack sufficient sources or concentrations of purines, for two reasons. Firstly, we blended 55 g (wet weight) of 3-week old CO-39 leaves in 50 ml water, removed the plant debris and used the remaining liquid extract to make 100 ml of GMM. Fig. 7a shows that, compared to Fig. 1a, $\Delta ade1$ growth was remediated on plates of GMM containing this rice leaf extract. Secondly, Sato and associates²³ used capillary electrophoresis and mass spectrometry to show that, amongst other metabolites, the purines adenosine,

AMP, ADP, ATP, GMP, GDP and GTP were present in measurable quantities in leaf tissue. Concentrations of GMP, GDP and GTP in rice leaves were low (3.6, 4.7 and 4.3 nmol g^{-1} [fresh weight], respectively) when extracted in the daytime. Concentrations of AMP, ADP and ATP were higher at 89, 60 and 23 nmol g^{-1} (fresh weight), respectively, in daytime samples. Adenosine concentration was measured at 110 nmol g^{-1} (fresh weight), ie. 110 μM , in daytime samples²³. The concentration of adenine was not determined²³. Although this study did not specifically measure the concentrations of purines in rice leaf epidermal cells, Borisjuk and colleagues²⁴ showed that ATP levels in photosynthesizing cotyledons of faba bean embryos were highest immediately below the surface and were at comparable concentrations (100 μM – 400 μM) to those measured in rice leaf²³. It is therefore unlikely rice epidermal cells will be devoid of purines, and so we next sought to determine if the growth of $\Delta ade1$ strains could be remediated by utilizing the most abundant purine measured in rice leaf, adenosine, at the concentration determined by Sato and colleagues²³. Fig. 7b shows that $\Delta ade1$ strains could grow on GMM containing decreasing amounts of adenosine including, notably, growth on GMM supplemented with 0.01 mM adenosine, which is 10-fold less than the concentration of adenosine measured in the rice leaf²³. Therefore, rice leaf contains compounds that can suppress the purine requirement of $\Delta ade1$ on plates (Fig. 7a), and $\Delta ade1$ has the ability to acquire small amounts of adenosine from its environment in order to remediate growth (Fig. 7b), but growth does not occur during plant infection (Fig. 5).

To account for the observed deficiency in purine uptake from the plant by $\Delta ade1$ strains, we considered that purine transporter gene expression might be repressed in $\Delta ade1$ strains, relative to Guy11, during colonization of the host. *UAPI* and *FCY2* encode homologues of purine transporters from *Aspergillus nidulans*²⁵ and *Saccharomyces cerevisiae*²⁶, respectively (Table 1). Along with the expression of a putative sugar transporter gene unlikely to be involved in purine uptake, *RGT2* (Table 1), we determined the expression levels of *UAPI* and *FCY2* in $\Delta ade1$ and Guy11 strains *in planta* (Fig. 7c). At 48 hpi, *RGT2* expression was slightly (ie. less than two-fold) reduced in $\Delta ade1$ strains compared to Guy11. In contrast, the expression of *UAPI* and *FCY2* was upregulated approximately 4.5- and 3-fold, respectively, in $\Delta ade1$ strains compared to Guy11 at 48 hpi. No cross-hybridization was observed using the *M. oryzae* gene

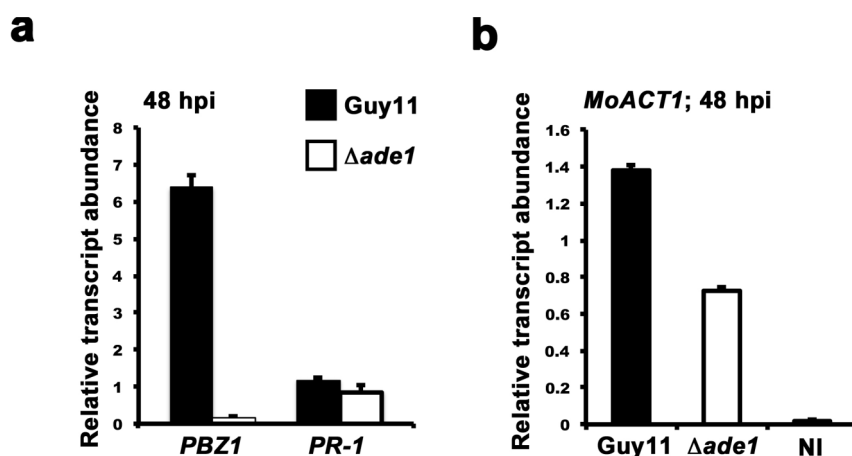


Figure 6 | $\Delta ade1$ strains do not elicit strong plant defense responses compared to Guy11. Detached rice leaf sheaths were inoculated with spores of Guy11 or $\Delta ade1$ and RNA was harvested at 48 hpi. (a) The expression of the rice pathogenesis-related genes *PBZ1* and *PR1* was analyzed by qPCR and normalized against the expression of the rice actin-encoding gene *OsACT2*. $\Delta ade1$ did not elicit responses greater than those observed for Guy11, and *PBZ1* expression was greatly reduced in rice leaves challenged with $\Delta ade1$ strains compared to Guy11. Results are the average of three independent replications. Error bars are standard deviation. (b) *M. oryzae* actin gene expression was detected in rice leaves challenged with $\Delta ade1$ and Guy11 strains but not an uninfected control (NI), suggesting these strains are actively transcribing in rice cells at 48 hpi. *MoAct1* expression was normalized against the expression of the rice actin gene *OsACT2*. Results are the average of three independent replications. Error bars are standard deviation.

Table 1 | Description of *M. oryzae* genes analysed by qPCR in this study

Locus ⁵	<i>M. oryzae</i> Gene Name	Putative molecular function	Function reference
MGG_03982	<i>MoACT1</i>	Actin	5
MGG_08056	<i>UAP1</i>	Uric acid-xanthine permease based on similarity to <i>UapA</i> and <i>UapC</i> of <i>Aspergillus nidulans</i> .	25
MGG_04868	<i>FCY2</i>	Purine-cytosine permease based on similarity to <i>Fcy2</i> and <i>Fcy22</i> in <i>Saccharomyces cerevisiae</i> .	26
MGG_01446	<i>RGT2</i>	High affinity glucose transporter	17, 30

specific qPCR primers in uninfected controls (not shown). Thus, although $\Delta ade1$ is unable to acquire remediate quantities of purines from the host, this is not likely to be due to the repression of purine transporter genes in this strain.

Taken together, the above results suggest that attenuated IH growth of $\Delta ade1$ strains in rice cells does not likely occur due to an inability to neutralize plant defenses or a reduced capacity to acquire purines compared to Guy11. Moreover, at least up to 48 hpi, $\Delta ade1$ strains are actively transcribing genes, including those that might be involved in purine uptake. Regardless, purine acquisition from the host is limiting.

Discussion

Plant-associated microbes can be beneficial to crops but many are devastating. Sustainable strategies against recalcitrant plant pathogens could come from identifying and exploiting metabolic pathways required for pathogenicity. Here, we characterized the function of *MoADE1*, encoding SAICAR synthetase in *M. oryzae*, in order to progress our understanding of what metabolic processes are intrinsic to the rice blast infection process, and whether the plant host satisfies those requirements. By disrupting *MoADE1* function, we have shown that *de novo* purine biosynthesis (Fig. 8a), but not purine scavenging or uptake from the rice cell (Fig. 8b), is essential for *M. oryzae* growth *in planta*. This is similar to what has been observed for the human pathogenic fungus *Candida albicans*, where adenine auxotrophy abolishes virulence in a murine model of systemic candidiasis²⁷, but differs from the obligate biotrophic plant pathogen *Blumeria graminis*, which diverts purines derived from the host to its own metabolome²⁸.

Interestingly, $\Delta ade1$ strains could form functional appressoria and elaborate IH from primary hyphae in the first infected rice cells, indicating these initial colonization processes do not require *de novo* purine biosynthesis (Fig. 8b). Moreover, following the morphogenetic transition to IH, $\Delta ade1$ strains were shown to be alive in host cells at 48 hpi and were actively transcribing actin and transporter genes, and neutralizing host defenses, despite being significantly reduced in growth compared to Guy11. Understanding how IH growth is curtailed, but transcription is permitted, following loss of *de novo* purine biosynthesis is a future fascinating challenge.

Our results suggest that early biotrophic growth is not dedicated to purine uptake from the host but is instead fuelled by *de novo* biosynthesis in *M. oryzae* (Fig. 8a). Additional transcriptional evidence for this strategy is shown in Supplementary Figure 2 where, relative to *RGT2*, the abundance of *UAP1* and *FCY2* transcripts were very low during Guy11 infection. This suggests the purine transporters *Uap1* and *Fcy2* (but not the sugar transporter *Rgt2*) might not play important roles under normal infection conditions. These observations are consistent with those of O'Connell and associates²⁹, who used transcriptional profiling of two hemibiotrophic plant pathogens, *Colletotricum graminicola* and *C. higginsianum*, to propose that biotrophic hyphae of *C. higginsianum* function in cell colonization rather than nutrient acquisition. Our results with *M. oryzae* provide the first experimental evidence to suggest that relying on the endogenous production of at least some central metabolites during

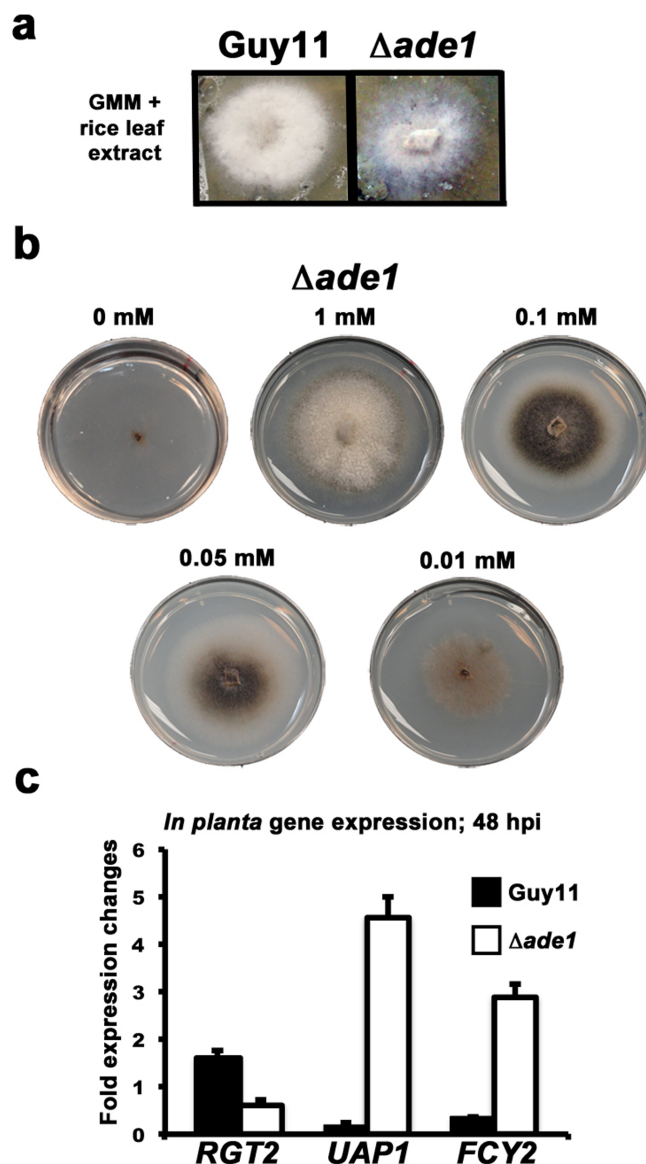


Figure 7 | Purine uptake from host cells is limiting. (a) $\Delta ade1$ strains were able to grow on GMM media containing rice leaf extracts. 55 g (wet weight) of 3-week old rice leaves were blended in 50 ml of water, pressed through cheesecloth, and the liquid extract used to make 100 ml GMM. (b) $\Delta ade1$ strains were grown on GMM media with adenosine added at the final concentrations shown. Growth was permissible on GMM supplemented with adenosine at concentrations down to ten-fold less (0.01 mM) than the 110 nmol g⁻¹ (0.11 mM) adenosine found in rice leaves²³. (c) The expression of two putative purine transporters (*UAP1* and *FCY2*), but not a putative sugar transporter (*RGT2*), were upregulated 4.5- and 3- fold in $\Delta ade1$ strains at 48 hpi compared to Guy11. Results were normalized against the expression of the *M. oryzae* actin-encoding gene *MoACT1* and are the average of three independent replications. Error bars are standard deviation.

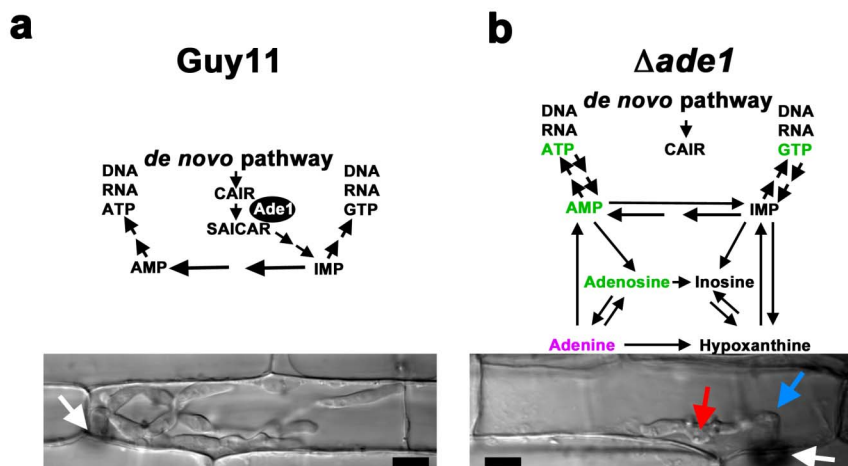


Figure 8 | *de novo* purine biosynthesis is essential for growth in rice cells. (a) Model of purine metabolism during biotrophic growth of Guy11 in rice cells (*Top*), resulting in successful host colonization by 40 hpi (*Bottom*). (b) Model of purine utilization during biotrophic growth of $\Delta ade1$ in rice cells (*Top*), resulting in attenuated growth at 40 hpi and loss of pathogenicity (*Bottom*). $\Delta ade1$ strains were still alive and transcribing genes at 48 hpi suggesting the recycling of adenine derived initially from the growth media (purple), and/or the limited uptake and salvage of purines known to be in the rice leaf (green), might contribute to survival in the host in the absence of the *de novo* purine biosynthetic pathway but is not sufficient to drive infection. White arrows indicate appressoria and the point of penetration. Blue arrow indicates primary hyphae. Red arrow indicates BICs formed behind IH. Scale bar is 5 μ m.

biotrophy, rather than acquiring them from the host, might be a common feature of hemibiotrophic pathogens.

What is the advantage to the fungus of having an infection strategy that relies on at least some metabolites being produced endogenously during biotrophy rather than acquired from the host? We do not consider this is because purines, for example, are not present in the plant host at the levels required for growth. Indeed, Sato et al.²³ have shown that adenosine was present in rice leaves in amounts that, when included in plate tests, were more than sufficient to satisfy the purine requirements for $\Delta ade1$ growth (Fig. 7b). Despite this, the failure of $\Delta ade1$ strains to grow *in planta* suggests only limiting amounts of purines, if any, are acquired from host cells (Fig. 8b). Thus, although early biotrophic infection by wild type *M. oryzae* does not require purine acquisition from the plant, $\Delta ade1$ strains that require purines are unable to obtain them despite the induction of purine transporter gene expression *in planta*.

How can we account for the inability of $\Delta ade1$ strains to acquire purines from the host, and how does this relate to the requirement for *de novo* purine biosynthesis during infection? During biotrophic growth, IH is surrounded by the plant-derived EIHM, a structure that the complex molecule FM4-64, a sterol dye, cannot cross⁸. Previously, we have noted that the nutrient utilizing capacity of *M. oryzae* is different in the host compared to axenic growth on media^{3,18}. We have suggested one reason for this could be due to the affect of the EIHM on the ability of *M. oryzae* to acquire nutrients from the host³. While glucose acquisition and utilization is a key driver of rice blast disease¹⁷, the purines discussed in this study, in addition to methionine and aspartate¹⁸, appear to be limited in availability from the perspective of the fungus during early biotrophic growth. Our data could fit a scenario whereby simple sugars might readily cross the EIHM to be acquired by the fungus from the apoplastic space using, for example, sugar transporters such as Rgt2 (Fig. 7c). On the other hand, more complex molecules such as purines, while abundant in rice leaves (Fig. 7a), might not readily cross the EIHM, resulting in the observed attenuated growth of $\Delta ade1$ strains. Although in this scenario, maintaining the EIHM inhibits nutrient acquisition and precludes that at least some metabolites must to be produced *de novo* from endogenous stores, the fungus might retain this strategy if the EIHM confers other growth advantages. Such advantages could include protection from plant defense

systems or, as in *Colletotricum* spp., a platform from which effectors can be delivered into the host cell during biotrophy²⁹. Indeed, the *M. oryzae* effectors Slp1²² and BAS4⁹ have been shown to accumulate in the space between the fungal cell wall and the EIHM during biotrophic growth. Thus, the EIHM and the requirement for *de novo* purine biosynthesis might represent necessary trade-offs in the *M. oryzae* lifestyle between the metabolic demands of the fungus on the one-hand, and effector manipulation of the host cell on the other.

In conclusion, our multi-level approach to understanding nutrient utilization by *M. oryzae* in rice cells – involving molecular genetics, transcriptional analysis and live-cell imaging of infected leaf epidermis – might have signposted the EIHM as a potential mediator of the metabolic interface between pathogen and host. Exploring this role will involve detailed characterizations of the molecular mechanisms by which *M. oryzae* acquires host nutrient across this barrier, characterizing the relationship between nutrient acquisition and the EIHM as biotrophy ends and the fungus becomes necrotrophic, and determining the consequences of perturbing the EIHM during early infection. Such analyses of this metabolic interface might reveal novel pathogenic processes that could be disrupted using chemical or biological means.

Methods

Fungal manipulations. $\Delta ade1$ was derived from Guy11¹⁵ and is stored as filter stocks at -20°C in the Wilson lab. Strains were maintained on complete medium (CM) containing 1% (W/V) glucose, 0.2% (W/V) peptone, 0.1% (W/V) yeast extract, 0.1% (W/V) casamino acids and supplemented with 1 mM adenine. Growth studies were performed using Cove's glucose minimal medium (GMM)¹⁴ with nitrate as sole nitrogen source. Adenine, adenosine or hypoxanthine was added to plates at the concentrations shown. For all plate growth tests, a 5 mm plug of each strain was transferred from CM to the appropriate media plates and allowed to grow at 26°C under 12 hr light/dark cycles for 10 days. 55 mm petri dishes were used throughout. Plate images were taken with a Sony Cyber-shot digital camera, 14.1 mega pixels.

Targeted gene replacement. Protoplasts were generated and transformed, as described previously¹⁵. To generate $\Delta ade1$ strains, the entire coding region of *MoADE1* (MGG_12537) was replaced with the *Bar* gene conferring bialaphos resistance using the PCR-based split marker method¹⁵ and the primers shown in Supplementary Table S1. Briefly, 1 Kb of the left flank (LF) and right flank (RF) of the *MoADE1* coding region were amplified using the primers LF5' & LF3' and RF5' & RF3', respectively. The 5' region of the *Bar* gene was amplified using the primer pair M13F:BA and BaSplit, and the 3' region of the *Bar* gene was amplified using the primer pair M13R:AR and ArSplit. The *MoADE1* left flank amplicon and the 5' region of the *Bar* gene were fused by amplifying with NesF and BaSplit. The *MoADE1*



right flank amplicon and the 3' region of the *Bar* gene were fused by amplifying with NesR and ArSplit. The two resulting fragments, which overlap in the *Bar* gene by approx. 300 bps, were transformed into protoplasts which were initially screened for bialaphos resistance. Targeted gene deletion was confirmed as described in¹⁵ using LF5' and RF3'. Complementation was achieved by amplifying the full length *MoADE1* gene, including 1 Kb of flanking sequences, using NesF & NesR primers, transforming the resulting PCR product into protoplasts of *Δade1* strains, and selecting for restoration of growth on GMM lacking adenine supplementation, as described previously¹⁸. Complementation strains retained bialaphos resistance.

Plant infection assays and live-cell imaging. Rice plant infections were made using a susceptible dwarf Indica rice (*Oryza sativa*) cultivar, CO-39, as described previously¹⁷. Fungal spores were isolated from 12–14 day-old plate cultures of CM supplemented with 1 mM adenine, diluted to a concentration of 1×10^5 spores ml⁻¹ in 0.2% gelatin and spray-inoculated onto 3-leaf stage rice plants using an artist's airbrush. Disease symptoms were allowed to develop under conditions of high relative humidity for 96–144 hrs.

Live-cell imaging was performed as described previously¹⁸ using 3 cm-long leaf sheath segments from 3–4 week-old rice plants and injecting one end of the sheath with a spore suspension of 5×10^4 spores ml⁻¹ in 0.2% gelatin. At the time points indicated, leaf sheaths were trimmed and observed using a Nikon Eclipse 50i microscope and a Nikon D100 digital net camera.

Physiological studies. The average rates of appressorium formation, penetration and IH growth were determined for each strain, in triplicate, by analyzing 50 spores or appressoria per rice cuticle. *In planta* biotrophic growth was determined using the 4-point scale described by Wilson and associates¹⁸, where 1 = IH length shorter than 10 μm with no branching; 2 = IH length is 10–20 μm with 0–2 branches; 3 = IH length is longer than 20 μm and/or with more than 2 branches within one cell; 4 = IH has spread to adjacent cells.

Gene transcript analysis. For *Δade1* and Guy11 *in planta* gene transcript analysis, rice leaf sheaths were used and RNA was extracted at 48 hpi. For transcript analysis of Guy11 during infection of whole plants, leaves were detached from the plant at the indicated time points, weighed, and approximately 100 mg of tissue was frozen in liquid nitrogen and ground in a mortar and pestle. RNA was extracted from each sample, and uninfected controls, using the RNeasy mini kit from Qiagen. Following treatment with DNase I (Invitrogen), RNA was converted to cDNA using qScript (Quanta). Quantitative real-time PCR (qPCR) was performed on an Eppendorf Mastercycler Realplex using the recommended reagents with primers designed using the netprimer software program (Supplementary Table S1). qPCR data was analyzed using the Realplex software package. Thermocycler conditions were: 10 min at 95°C, followed by 40 cycles of 95°C for 30 sec, 63°C for 30 sec and 72°C for 30 sec.

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Author contributions

R.A.W. designed the experiments. J.F., K.T.Y., K.M.C., J.D.W. and R.A.W. performed the experiments. R.A.W. interpreted the results. R.A.W. wrote the paper.

Additional information

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