Lipid metabolic changes in an early divergent fungus govern the establishment of a mutualistic symbiosis with endobacteria

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The recent accumulation of newly discovered fungal–bacterial mutualisms challenges the paradigm that fungi and bacteria are natural antagonists. To understand the mechanisms that govern the establishment and maintenance over evolutionary time of mutualisms between fungi and bacteria, we studied a symbiosis of the fungus *Rhizopus microsporus* (Mucoromycotina) and its *Burkholderia* endobacteria. We found that nonhost *R. microsporus*, as well as other mucoralean fungi, interact antagonistically with endobacteria derived from the host and are not invaded by them. Comparison of gene expression profiles of host and nonhost fungi during interaction with endobacteria revealed dramatic changes in expression of lipid metabolic genes in the host. Analysis of the host lipidome confirmed that symbiosis establishment was accompanied by specific changes in the fungal lipid profile. Diacylglycerol kinase (DGK) activity was important for these lipid metabolic changes, as its inhibition altered the fungal lipid profile and caused a shift in the host–bacterial interaction into an antagonism. We conclude that adjustments in host lipid metabolism during symbiosis establishment, mediated by DGKs, are required for the mutualistic outcome of the *Rhizopus–Burkholderia* symbiosis. In addition, the neutral and phospholipid profiles of *R. microsporus* provide important insights into lipid metabolism in an understudied group of oleaginous Mucoromycotina. Lastly, our study revealed that the DGKs involved in the symbiosis form a previously uncharacterized clade of DGK domain proteins.

mutualism evolution | antagonism | Mucoromycotina | oleaginous fungi | *Rhizopus–Burkholderia* symbiosis

The ubiquity of antagonistic interactions between fungi and bacteria in nature is widely appreciated due to medically important antimicrobials that mediate these relationships. In contrast, mutually beneficial associations of fungi with bacteria are only beginning to gain recognition for their abundance and role in human health, agriculture, food production, and general ecosystem functioning (1). Among these alliances, the mutualisms formed with various endobacteria by early divergent Mucoromycotina (2) and Glomeromycota (3), stand out as examples of a high level of coadaptation between the partners. However, little is known about the molecular mechanisms underlying establishment and maintenance of fungal–bacterial associations over evolutionary time, as the difficulties in cultivation and experimental manipulation of the symbiotic partners present a big impediment for elucidation of their biology. In this respect, the symbiosis of an early divergent fungus *Rhizopus microsporus* (Mucoromycotina) and its bacterial endosymbiont *Burkholderia* (β-proteobacteria) is emerging as a model for the study of fungal–bacterial mutualisms because both partners are easy to manipulate under laboratory conditions (4). *R. microsporus* is a soil saprotroph responsible for food spoilage and pathogenesis of plants and immune-compromised humans (2). Plant pathogenesis is facilitated by *Burkholderia*-mediated synthesis of a potent toxin, rhizoxin (2). The endobacteria, in turn, benefit from energy provision by the host (5). Several factors are known to contribute to the ability of *Burkholderia* endobacteria to form a stable association with the *R. microsporus* host (6–8). It remains an enigma, however, as to what fungal factors contribute to symbiosis formation.

In this study, we used the existence of closely related isolates of *R. microsporus* that differ in their interaction with *Burkholderia* endobacteria for the identification of the molecular underpinnings underlying fungal–bacterial symbiosis establishment. We discovered that unlike the symbiont-containing host isolates of *R. microsporus*, closely related but naturally symbiont-free nonhost isolates are antagonized by *Burkholderia* endobacteria and do not form symbioses with them. We compared fungal transcriptional responses during initial physical contact with *Burkholderia* endobacteria and found that host and nonhost fungi engaged different sets of genes in the reactions to endobacteria. In the host, the most striking response involved changes in the expression of lipid metabolic genes, whereas such changes were absent in the nonhost. Analysis of the host fungal lipidome revealed that symbiosis establishment was accompanied by an increase in the pools of triacylglycerol (TAG) and phosphatidylethanolamine (PE). When we altered the TAG:PE ratio with pharmacological inhibitors, the host–*Burkholderia* interaction

Significance

Mutually beneficial interactions of fungi with bacteria are increasingly recognized as ubiquitous and economically important. However, little is known about their establishment and maintenance. Utilizing the association between the fungus *Rhizopus microsporus* and its endosymbiont *Burkholderia* as a model, we provide first insights into fungal molecular mechanisms governing symbiosis establishment with bacteria. We show that specific changes in fungal lipid metabolism, mediated by diacylgycerol kinase enzymes, are required to maintain a mutualistic outcome of interaction with bacteria, a pattern consistent with the addiction model of mutualism evolution. We also offer insights into genetics and biochemistry of lipid metabolism in an understudied group of oleaginous fungi, which are a promising source of oils for biodiesel production.

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Data deposition: The Whole Genome Shotgun projects are deposited at GenBank under accession MCGZ00000000 (*R. microsporus* ATCC 52814) and MCOJ00000000 (*R. microsporus* ATCC 11559). The transcriptome data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE89305).

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shifted toward an antagonism, indicating that specific adjustments in lipid metabolism are required for the mutualistic outcome of this symbiosis. Collectively, we identified fungal genes important for symbiosis establishment with bacteria and a metabolic landscape favoring a mutualism. In addition, we provide genetic and biochemical insights into lipid metabolism in an understudied group of fungi, the Mucoromycotina.

Results

Host and Nonhost Fungi Differ in How They Interact with *Burkholderia* Endobacteria. To explore the relationship between host and nonhost isolates of *R. microsporus*, we examined whether they meet the phylogenetic species recognition criterion (9). Using a partition homogeneity test (10), we compared genealogies of three genes (11) sampled across host and nonhost isolates, designated in culture collections as representatives of the *R. microsporus* species. We found evidence of a history of gene flow across these isolates ($P = 0.002$; *SI Appendix*, Fig. S1), supporting their placement within a single interbreeding species. Moreover, comparison of genomes of host [*R. microsporus* American Type Culture Collection (ATCC) 52813] and nonhost (*R. microsporus* ATCC 11559), sequenced for this study and summarized in *SI Appendix*, Table S1, revealed that they share a 92% whole-genome average nucleotide identity, ANI (*SI Appendix*, Fig. S2), which offers an important insight into the intraspecific levels of ANI in fungi.

Nonhost isolates differ from host isolates in being naturally endosymbiont-free and in control of their asexual reproduction, which in the host isolates is dependent on endobacteria. As host isolates of *R. microsporus* are capable of establishing symbioses with nonnative *Burkholderia* isolated from other host *R. microsporus* (12), we tested whether nonhosts had the ability to form associations with *Burkholderia* endobacteria. Cocultivation of cured host fungi with endobacteria reestablishes a functional symbiosis whereby bacteria populate fungal hyphae and spores (*SI Appendix*, Fig. S3*A*). When we cocultivated nonhost *R. microsporus* ATCC 11559 and ATCC 52807 with endobacteria isolated from host *R. microsporus* ATCC 52813 and ATCC 52814, we found that nonhost isolates did not take up the bacteria into their hyphae (*SI Appendix*, Fig. S3*C*). Moreover, we noticed that nonhost fungi were antagonized by bacteria and changed their growth pattern by reducing hyphal extension around the site of bacterial inoculation, creating a "zone of inhibition" (*SI Appendix*, Fig. S3*D*). Similar growth changes were observed in other nonhost Mucoromycotina [*Rhizopus oryzae* ATCC 11423 and ATCC 13440 as well as *Mucor circinelloides* Centraalbureau voor Schimmelcultures (CBS) 277.49] during cocultivation with endosymbionts of host *R. microsporus* (*SI Appendix*, Fig. S4). Such phenotypic alterations were never observed in the host during interaction with endobacteria (*SI Appendix*, Fig. S3 *B*).

We conclude that two types of interactions are possible between mucoralean fungi and *Burkholderia* endobacteria: a mutualistic interaction arising as a consequence of symbiosis establishment in which bacteria populate fungal hyphae, and an antagonistic interaction in which bacteria do not populate fungal hyphae and cause phenotypic alterations in fungal growth. This finding prompted us to analyze the genetic underpinnings governing the interactions of the closely related host and nonhost isolates of *R. microsporus* with endobacteria with the aim of identifying molecular determinants of symbiosis establishment.

Different Sets of Genes Are Engaged by Host and Nonhost Fungi in the Interaction with Endobacteria. Using RNA sequencing (RNA-seq), we analyzed global gene expression changes in host and nonhost *R. microsporus* during cocultivation with *Burkholderia* endobacteria at a time point when the fungal colony has just come into physical contact with bacteria. This time point was expected to represent symbiosis establishment. Differentially expressed (DE) genes were identified by comparing: (*i*) cured host *R. microsporus* ATCC 52813 growing with its native *Burkholderia* endobacteria to (*ii*) cured host ATCC 52813 growing alone, as well as (*iii*) nonhost *R. microsporus* ATCC 11559 growing with endobacteria isolated from the host to (*iv*) nonhost ATCC 11559 growing alone. We reasoned that a portion of DE genes would exhibit similar expression patterns in host and nonhost, representing a nonspecific response to bacteria. Genes involved in this nonspecific response could then be subtracted from the pool of DE genes in the host, and the remainder would represent genes specifically involved in symbiosis establishment.

Using a threshold false discovery rate of 0.01, we found 508 DE genes in the host in response to interaction with its native endobacteria (representing fungal response during symbiosis establishment), and 183 DE genes in the nonhost in response to interaction with the same endobacteria. To directly compare host and nonhost gene expression changes, we identified orthologs by clustering all protein-coding genes from the host and nonhost. To discover genes involved in the nonspecific response to bacteria, we focused on genes that were commonly DE in both fungi. Contrary to our expectations, only 10 common DE genes exhibited the same expression patters (either up- or down-regulated), indicating a very limited nonspecific response (*SI Appendix*, Table S2). Clearly, establishment of symbiosis was governed by a very specific set of genes. The minimal overlap in host and nonhost transcriptomic responses was not due to expression of genes exclusively present in either of the two fungi. In fact, 97% of the DE host genes were also present in the nonhost genome, and vice versa, 95% of the DE genes in the nonhost were present in the host genome. The remaining 3% of the genes without homologs in the nonhost were also absent from another host *R. microsporus* ATCC 52814, and therefore could not represent symbiosis-specific genes. As the majority of DE host genes were unique to symbiosis establishment, we focused on these genes in our analysis of the genetic underpinnings of symbiosis.

Up-Regulation of Genes Responsible for the HOG Pathway and Lipid Metabolism During Symbiosis Establishment. Of the 508 host genes DE during symbiosis establishment, 298 were up- and 210 were down-regulated. To gain insight into the function of these genes, we conducted orthologous clustering across seven fungal genomes, including *Saccharomyces cerevisiae* (yeast), *Aspergillus nidulans*, and members of Mucoromycotina, followed by gene ontology (GO) enrichment analyses. Consistent with enrichment of the signal transduction category (*SI Appendix*, Fig. S5), we observed overexpression of many protein kinase genes (19 up, 8 down). Two of these protein kinase genes, encoding proteins with identification nos. 300418 and 286014, are homologous to the yeast gene encoding Hog1 MAPK (Fig. 1). The yeast Hog1 controls responses to a wide range of environmental stimuli, and signaling through Hog1 MAPK activates accumulation of glycerol, which counterbalances water molecule outflow from the cell under hypertonic conditions (13). Known upstream regulators of the Hog1 MAPK include the two-component histidine kinase phosphorelay system, involving Ssk1 (13). In *R. microsporus*, we identified an up-regulated two-component response regulator (201417) homologous to the *A. nidulans* AN3101, closest match to *SSK1* in yeast, that could have acted upstream of this MAPK. The yeast Hog1 activates glycerol synthesis by controlling the expression of a NAD-dependent glycerol-3-phosphate dehydrogenase, Gpd1 (13). In our RNA-seq experiment, a homolog of *GPD1* and another gene with the same function (222441) were up-regulated, together with homologs of genes *STL1*, encoding a glycerol symporter, *SKO1*, encoding a transcription factor, and *GUT1*, encoding a glycerol kinase. In yeast, *GDP1*, *STL1*, and *SKO1* are known to be regulated by Hog1 (13). The glycerol symporter functions to transport glycerol into the cell, whereas glycerol kinase and NAD-dependent glycerol-3-phosphate dehydrogenases convert different precursors into glycerol-3-phosphate (Gro-3-P). Gro-3-P can be further converted to glycerol via a glycerol-3-phosphatase. However, we did not detect differential expression of genes with this function. Therefore, based on

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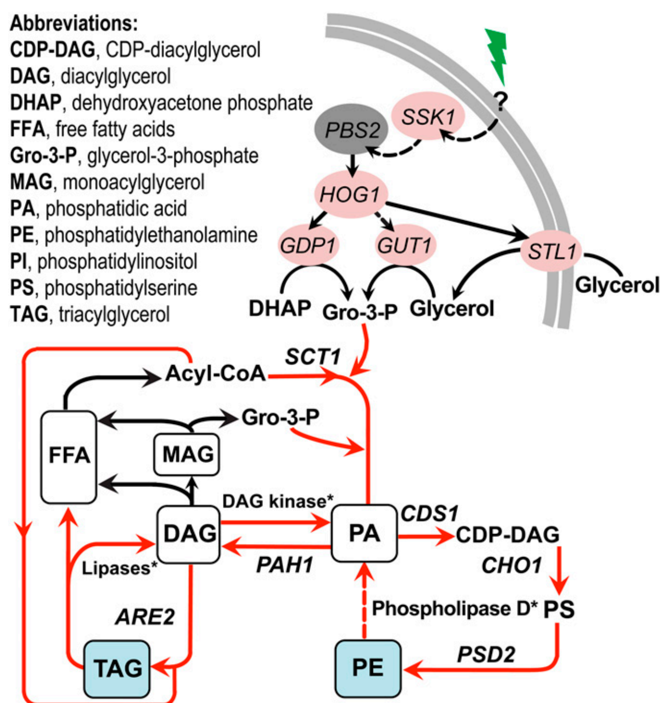

Fig. 1. Hypothetical representation of the link between the Hog1 MAPK and the lipid metabolic pathways activated in *R. microsporus* during symbiosis establishment. Genes are named according to *S. cerevisiae* nomenclature. Red circles and arrows represent genes up-regulated during symbiosis establishment; gray circles and black arrows depict genes not DE; dashed arrows represent unknown links, or uncertainty of precursor. In the metabolic pathway, names of the up-regulated genes are written adjacent to the metabolic conversions in which they are involved. Asterisks denote *R. microsporus* ATCC 52813 predicted protein annotations: DAG kinases (protein ID 315343 and 249146), phospholipases D (protein ID 277488 and 209457), and lipases (protein ID 249672, 281243, and 250139). The well-characterized yeast lipid metabolic pathway was used in the construction of this figure (14, 16, 18).

gene expression patterns in *R. microsporus*, which we mapped to known signaling networks in yeast, we hypothesize that the HOG pathway was activated in the host during symbiosis establishment, and in turn, likely activated synthesis and accumulation of Gro-3-P, but not that of glycerol.

In yeast, Gro-3-P serves as a precursor for the synthesis of glycerolipids (14) by becoming converted into phosphatidic acid (PA) via a Gro-3-P acyltransferase, Sct1 (15), whose homolog was also up-regulated in our RNA-seq experiment (Fig. 1). Therefore, based on these gene expression patterns, we hypothesized that establishment of symbiosis was mediated by signaling through the *R. microsporus* homologs of yeast Hog1 MAPK, which ultimately activated Gro-3-P synthesis. Gro-3-P was then likely channeled into glycerolipid synthesis by becoming acylated via a homolog of the yeast Sct1. Consistent with this hypothesis, the lipid metabolism GO category was enriched among the DE host genes during symbiosis establishment (*SI Appendix*, Fig. S5). To elucidate what pathways in lipid metabolism were important, we annotated the lipid-related genes in the *R. microsporus* genomes based on homology to functionally characterized lipid metabolic genes in *S. cerevisiae* (16). Of the 92 yeast genes, 77 had homologs in *R. microsporus*, of which 14 were DE. Additionally, we identified another 26 DE genes that had no homologs in yeast lipid metabolism but were annotated as being part of the "lipid metabolism" GO category (*SI Appendix*, Table S3). By mapping all of these DE lipid-related genes onto known pathways of lipid metabolism (16), we inferred that de novo biosynthesis of PE and turnover of TAG were likely initiated during symbiosis establishment (Fig. 1). Central intermediates of this pathway are diacylglycerol (DAG) and PA, which, besides being precursors for the biosynthesis of lipids, have important signaling function in eukaryotes (17, 18). They are interconvertible, i.e., PA can be dephosphorylated by a PA phosphatase to produce DAG (19), and DAG can be phosphorylated by DAG kinase (DGK) to yield PA (20). Interestingly, two DGKs were among the top most up-regulated genes due to bacteria (*SI Appendix*, Table S3), indicating their importance in symbiosis establishment. Additionally, two genes encoding phospholipase D (PLD), which are not homologs of the yeast *PLD1*, were upregulated; PLD makes PA through the breakdown of phospholipids (21, 22). Finally, a homolog of yeast *SCT1*, a gene encoding an acyltransferase that synthesizes PA de novo was also up-regulated. Based on these patterns, we hypothesized that the activities of PA-producing enzymes, DGK, PLD, and Gro-3-P acyltransferase, govern the lipid metabolic changes during symbiosis establishment.

Pharmacological Inhibition of DGK Activity, but Not PLD Activity, Shifts the Host–Endosymbiont Interaction into Antagonism. To determine whether the activities of fungal PA-producing enzymes were important during symbiosis establishment, we set up interactions of host and nonhost fungi with *Burkholderia* endobacteria in the presence of chemical inhibitors of PLD and DGK; there are no commercially available Gro-3-P acyltransferase inhibitors. We chose to work with chemical inhibitors because, at present, no genetic transformation technology is available for *R. microsporus*. Fungi were inoculated into liquid media and allowed to grow for 24 h before bacteria were added. Following a 24-h incubation with bacteria, fungal colony diameter was measured. DGK and PLD inhibitors were not expected to affect endobacteria, as their genome does not encode either a DGK or a PLD gene (5), although it is possible that inhibitors had undetected effects on bacterial physiology.

In the absence of bacteria, the inhibitors did not affect the growth of either cured host or nonhost fungi (*SI Appendix*, Fig. S6). In contrast, in the presence of bacteria, the growth of nonhost fungi was significantly reduced relative to growth without bacteria ($P < 0.001$), and this growth reduction occurred regardless of the presence or absence of chemical inhibitors (Fig. 2*A*). This was expected, considering that we observed a reduction in nonhost growth during interaction with the same bacteria on solid media (*SI Appendix*, Fig. S1*D*). Growth of cured host fungi was unaffected by bacteria in the absence of inhibitors and in the presence of the PLD inhibitor (Fig. 2*B*). However, when cured host fungi were grown with bacteria in the presence of DGK inhibitors, their growth was significantly reduced relative to growth without bacteria in the presence of DGK inhibitors ($P < 0.001$) in a way that resembled the reduction in nonhost growth during interaction with bacteria (Fig. 2*B*). Lastly, growth of host fungi that were not cured of endobacteria was also significantly reduced in the presence of DGK inhibitors ($P < 0.0001$), but was unaffected by inhibition of PLD (*SI Appendix*, Fig. S7).

Together, these results indicate that the activity of DGKs is important for the outcome of the interaction between host *R. microsporus* and *Burkholderia* symbionts, whereas PLD activity is dispensable. Inhibition of DGK appeared to shift the interaction toward an antagonism with a reduced fitness outcome in the fungus. Consistent with our transcriptomic observations that the expression of DGK and PLD remained unchanged in the nonhost during interaction with endobacteria, DGK and PLD inhibitors had no affect on the interaction between nonhost and endobacteria, which remained antagonistic. Lastly, the negative effect of DGK inhibition on the host fungus in an already fully established symbiosis indicates that DGK activity may be important not just for symbiosis establishment but also for the continued maintenance of the mutualism between the host and endobacteria (*SI Appendix*, Fig. S7).

Changes in the Host Lipid Profile Accompany the Shift to an Antagonistic Interaction with Endobacteria During Symbiosis Establishment. Pharmacological inhibition of DGK activity was expected to affect fungal lipid metabolism. In particular, we hypothesized that changes in

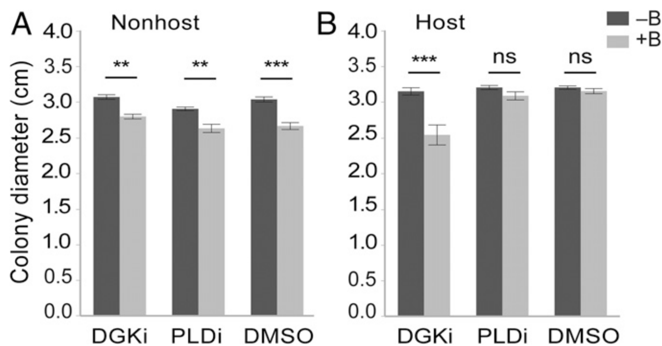

Fig. 2. Pharmacological inhibition of DGK and PLD activity. Effect of bacteria on growth of (*A*) nonhost and (*B*) host in the presence and absence of inhibitors. +B, with bacteria; –B, without bacteria; DGKi, diacylglycerol kinase inhibitors; PLDi, phospholipase D inhibitor; and DMSO indicates no inhibitor control. $^{***}P < 0.0001$, $^{**}P < 0.001$, n.s., not significant, from post hoc Student's *t* test, corrected for multiple comparisons. Error bars represent 1 SEM.

lipid metabolism might account for the shift in the host–symbiont interaction into antagonism. Furthermore, gene expression analysis of host fungi during symbiosis establishment pointed to specific changes in lipid metabolism, namely activation of de novo synthesis of PE and turnover of TAG (Fig. 1). Therefore, we analyzed the lipid profiles of cured host *R. microsporus* during symbiosis establishment in the absence and in the presence of DGK inhibitors. Growth conditions and timing of bacterial addition were the same as during the pharmacological inhibition experiment described in the previous section. Total lipids were extracted and separated by one-dimensional TLC, followed by quantification of individual phospholipids and neutral lipids.

In the absence of DGK inhibitors, we found a significant increase in the pools of TAG and PE in the host fungi during symbiosis establishment (Fig. 3 *C* and *D*). This increase was consistent with our interpretation of the RNA-seq data whereby genes involved in de novo synthesis of PE and turnover of TAG were overexpressed (Fig. 1). The DGK inhibitors alone also caused alterations to the fungal lipid profile. With inhibitors, we observed a significant increase in TAG and a significant decrease in monoacylglycerol (MAG) and free fatty acid (FFA) (*SI Appendix*, Fig. S8). Such changes are consistent with inhibition of DGK activity, which would cause an accumulation of DAG. In yeast, DAG is normally present in small amounts (23). Consequently, we hypothesize that in *R. microsporus*, to maintain physiological levels of DAG, it was channeled into biosynthesis of TAG, a storage molecule. A similar pattern of TAG accumulation was observed in the yeast DGK deletion mutant *Δdgk1* (24). Accumulation of TAG would explain the observed decrease in levels of FFA, as they become used up in the biosynthesis of TAG from DAG. Finally, inhibition of DGK activity was expected to decrease the pool of PA; however, we detected no such decrease (*SI Appendix*, Fig. S8 *A* and *B*). This could be explained by the existence of alternative pathways of PA synthesis, such as de novo biosynthesis of PA from the breakdown products of MAG, i.e., FFA and Gro-3-P, consistent with the observed decrease in the MAG pool.

Addition of bacteria to cured host fungi grown in the presence of inhibitors, which had a significant effect on fungal growth, did not induce any further alterations to the fungal lipid profile relative to fungi grown with inhibitors but without bacteria (Fig. 3 *A* and *B*). In other words, an increase in TAG and a decrease in MAG and FFA during interaction with endobacteria in the presence of DGK inhibitors (*SI Appendix*, Fig. S8) was the effect of the inhibitors alone. Finally, comparison of lipid profiles of host fungi grown with bacteria in the presence and absence of inhibitors revealed a significantly larger pool of TAG in the presence of inhibitors (*SI Appendix*, Fig. S8 *C* and *D*).

Together, these results indicate that during symbiosis establishment, fungi accumulated TAG and PE. During the interaction with an antagonistic outcome, suggested by fungal growth decline brought about by inhibition of DGK activity, fungi accumulated additional TAG, but maintained the same levels of PE. These patterns suggest that a specific TAG:PE ratio may be important for the interaction. We calculated these ratios for all conditions tested in our experiment and found that, in the absence of DGK inhibitors, TAG:PE ratio was 1.1, whereas in the presence of DGK inhibitors, this ratio increased significantly to 1.7 ($P < 0.0001$; *SI Appendix*, Fig. S9 and Table S4). Based on these results, we propose that a TAG:PE ratio close to 1 in *R. microsporus* during symbiosis establishment with *Burkholderia* endobacteria is important for the mutualistic outcome of the interaction, and a higher TAG:PE ratio shifts the interaction toward an antagonism, marked by a fitness cost to the fungus.

Phylogenetic Analysis of DGKs Implicated in the Interaction with Endosymbionts. The two DGK genes overexpressed in host *R. microsporus* during symbiosis establishment were not homologs of the yeast gene encoding diacylglycerol kinase, *DGK1*. A homolog of *DGK1* is present in *R. microsporus* but it was not DE during symbiosis establishment. The two genes overexpressed due to bacteria were, instead, homologs of each other. Annotation of the protein domains of these genes revealed the presence of a DGK catalytic domain (PF00781, IPR001206) and a DGK accessory domain (PF00609, IPR000756), both absent from the yeast *DGK1* gene. Phylogenetic analyses of representative DGK domain proteins indicated that the two DGKs encoded by genes DE during symbiosis establishment belong to a cluster that is unique to some members of early divergent fungi (Fig. 4 and *SI Appendix*, Fig. S10). Overall, fungal DGKs separate into two main clusters: All Fungi and Early Divergent Fungi, with a single chytrid sequence grouping with the animal DGKs as part of the Animal/Chytrid cluster. The All Fungi cluster contains DGKs from a diverse group of fungi, including Ascomycota, Basidiomycota,

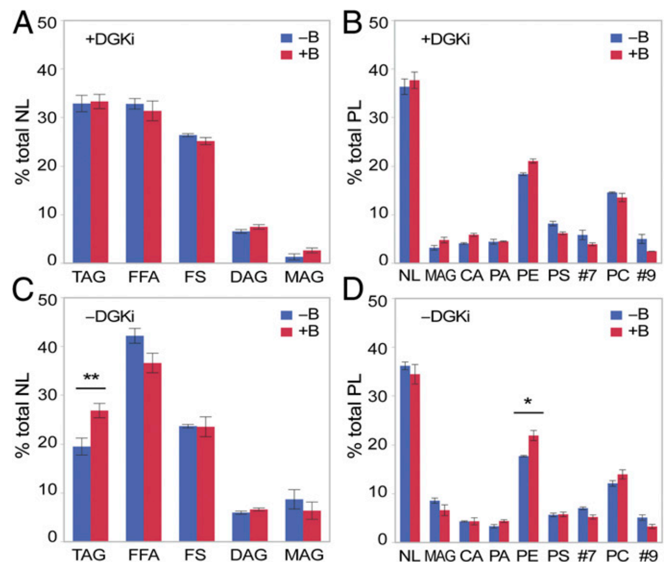

Fig. 3. Effects of DGKi on relative abundance of phospholipid (PL) and neutral lipid (NL) species in cured host *R. microsporus* ATCC 52813 grown with (+B) and without (–B) endobacteria. (*A*) Percent of total NLs in the presence of DGKi. (*B*) Percent of total PLs in the presence of DGKi. (*C*) Percent of total NLs in the absence of DGKi. (*D*) Percent of total PLs in the absence of DGKi. $^{**}P < 0.001$, $^{*}P < 0.01$, from post hoc Student's *t* test, corrected for multiple comparisons. FFA, free fatty acids; MAG, monoacylglycerol; DAG, 1,2- diacylglycerol; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; TAG, triacylglycerol; CA, cardiolipin; #7 and #9, unknown. Error bars represent 1 SEM.

Glomeromycota, and Mucoromycotina. The Early Divergent Fungi cluster, which harbors the symbiosis-activated DGKs, forms a sister group to the All Fungi cluster and harbors only representatives of the early divergent Mucoromycotina, Kickxellomycotina, and Chytridiomycota. Genomes of Mucoromycotina, including *R. microsporus*, encode DGKs that belong to both fungal DGK clusters, whereas genomes of Dikarya and Glomeromycota encode only representatives of the All Fungi cluster. Remarkably, with the exception of one DGK from chytrids, fungal DGKs are more closely related to DGKs in alveolates than to those in animals, even though, based on the organismal phylogeny (25), fungi and animals are part of the Opistokont supergroup. Collectively, we conclude that the two DGK genes overexpressed during symbiosis establishment represent a previously uncharacterized group of DGK genes.

Discussion

Antagonism–Mutualism Shift in the Course of Evolution of the *Rhizopus–Burkholderia* Symbiosis. The existence of *R. microsporus* host isolates that harbor *Burkholderia* and nonhost isolates that are endobacteria-free presents a unique opportunity to understand evolution of mutualisms in fungi. In particular, the *Rhizopus–Burkholderia* symbiosis was proposed to exemplify the addiction model of mutualism evolution (26), based on the obligate dependence of host fungi on endobacteria for asexual reproduction (4). However, the specific mechanism that keeps fungi cured of endobacteria from sporulation is unknown. The addiction model postulates that, after developing mechanisms to compensate for negative effects of an antagonistic symbiont, a host population may become dependent on, or addicted to the symbiont's continued presence (27). In the present study, we demonstrate that the nonhosts, including mucoralean fungi other than *R. microsporus*, exhibit growth inhibition when confronted by endobacteria derived from host fungi. These antagonistic outcomes suggest that endosymbiont-free nonhosts represent a preaddiction stage of the fungus. Whereas we did not identify specific compensatory mechanisms responsible for *Burkholderia* control of *Rhizopus* sporulation, we show that symbiosis establishment is governed by a set of fungal genes expressed uniquely by the host, with lipid metabolic genes playing a central role in this process (*SI Appendix*, Fig. S11). Specifically, analysis of fungal lipid profiles revealed that symbiosis establishment was accompanied by the accumulation of two lipid molecules, TAG and PE at a ratio of ∼1. Perturbation of this ratio caused a shift in the fungal–bacterial interaction during symbiosis establishment from mutualism to antagonism. From this pattern, we propose that accumulation of TAG and PE at a specific ratio is part of the fungal addiction syndrome to endobacteria.

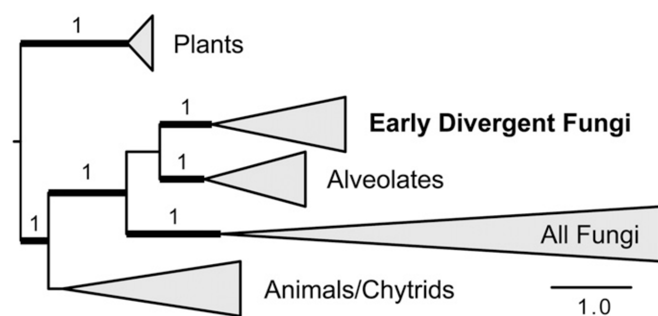

Fig. 4. Cartoon representation of the DGK phylogeny. DGKs encoded by genes DE during symbiosis are in the Early Divergent Fungi cluster. Bayesian posterior probability values >0.8 are displayed above branches; branches with maximum likelihood bootstrap support over 70% are thickened. The complete phylogeny is displayed in *SI Appendix*, Fig. S10, with strain and sequence IDs listed in *SI Appendix*, Table S5, and sequence alignment in *SI Appendix*, Dataset S1.

HOG-Mediated Activation of Lipid Metabolism During Symbiosis Establishment Leads to Accumulation of TAG and PE. The HOG signaling pathway has not been studied in early divergent fungi such as the Mucoromycotina. Based on gene expression analysis, we showed that in *R. microsporus* the HOG pathway was involved in mediating symbiosis establishment with bacteria and likely activated Gro-3-P accumulation. We further linked Gro-3-P accumulation to the observed activation of lipid metabolism. Analysis of lipid metabolic genes that were DE during symbiosis establishment revealed the likely activation of de novo biosynthesis of PE and the turnover of TAG. Examination of the fungal lipidome confirmed that symbiosis establishment was accompanied by a significant accumulation of PE and TAG.

PE is a ubiquitous component of eukaryotic membranes and is essential for growth of *S. cerevisiae* (28). However, accumulation of PE during interaction with bacteria cannot be explained by the need to synthesize new fungal membranes, because *Burkholderia* endosymbionts of *R. microsporus* are not housed within fungus-derived vesicles nor does host *R. microsporus* undergo increased rates of hyphal growth that would require biogenesis of new membranes. PE is also directly involved in the autophagic process (29) and has been implicated in affecting membrane curvature, fluidity, and polarized organization of actin (30). It is, therefore, more conceivable that increased PE levels might play a role in altering the biophysical properties of the membrane (31), although how that might be important for symbiosis establishment remains to be elucidated.

TAG is an important storage molecule that is typically localized to lipid droplets. It is likely that TAG serves as an energy reservoir for endobacteria, which, once inside the hyphae, must rely on fungus-derived carbon. Indeed, the genome of endobacteria revealed their ability to use glycerol, a breakdown product of TAG, as a sole carbon source (5). It is unclear, however, why fungi would accumulate TAG during symbiosis establishment, when a more plausible progression would be to break down TAG to release the energy source for bacteria. An alternative explanation is that the accumulation of TAG and PE during symbiosis establishment could be a result of transient accumulation of their precursor PA. PA is known to have signaling roles in eukaryotes (18) and it is possible that an accumulation of PA, undetected in this study, serves a signaling function in the coordination of symbiosis establishment. Accumulation of TAG and PE could thus be the end result of transient accumulation of PA, which, upon serving its signaling function, could become converted into PE through the cytidine diphosphate (CDP)–DAG pathway and channeled into biosynthesis of TAG by first becoming converted into DAG via a PA phosphatase. This hypothesis is supported by the up-regulation of PA-producing genes during symbiosis establishment.

Lipid Metabolism in Early Divergent Fungi. In addition to unraveling the mechanisms of mutualism establishment, we provide insights into the lipid metabolism in an understudied group of oleaginous fungi, the Mucoromycotina, which are a promising source of microbial oils for biodiesel production. We found that the genomes of *R. microsporus* encode homologs of well-characterized lipid metabolic genes from *S. cerevisiae*, which is the best-studied fungus in terms of lipid metabolism (16). Moreover, we analyzed the neutral lipid and phospholipid composition of *R. microsporus* and found that pharmacological DGK inhibitors affect its lipid profile in a way that is consistent with inhibition of DGK activity. This serves as evidence for the conservation of DGK enzyme activity between fungi and mammals, for which the inhibitors were developed (32).

We showed that fungal DGKs separate into two major groups, with one cluster unique to some members of early divergent fungi and a sister cluster containing representatives of all fungi. Such expansion of the DGK gene family in the Mucoromycotina genomes appears to mirror the enlargement of gene families involved in signal transduction in *M. circinelloides* and *Phycomyces blakesleeanus*, where it was attributed to a whole genome duplication

event predating the diversification of the Mucoromycotina (33). Moreover, with the exception of a DGK from chytrids, fungal and alveolate DGKs cluster together to the exclusion of animal DGKs, which is inconsistent with the organismal phylogeny uniting fungi and animals in the Opistokont supergroup (25). This pattern could be interpreted as a result of an ancient horizontal gene transfer event, although it is hard to speculate about the directionality and conditions that would favor such transfer, given how little is known about the function of DGKs in alveolates and early divergent fungi.

Conclusion

Overall, we identified a mechanism governing the transition from an antagonistic to mutualistic interaction between fungi and bacteria. We showed that symbiosis establishment is accompanied by specific changes in the lipid metabolic landscape of the fungus and perturbations of this landscape shift the interaction toward an antagonism during symbiosis establishment. Additionally, we provide first insights into the pathways and genetic basis of lipid metabolism in an understudied group of early divergent fungi, the Mucoromycotina.

Materials and Methods

Detailed descriptions of materials and methods are provided in *SI Appendix*. In brief, fungi were cultivated, cured, and endobacteria were isolated and visualized as previously described (2) with slight modifications. Infection success was confirmed by reestablishment of asexual sporulation in the host (4), PCR with *Burkholderia*-specific primers (3), and microscopy. A partition homogeneity test (10) with markers described in ref. 11 was performed to resolve the *R. microsporus* host and nonhost isolate relationship. Sequencing, assembly, and annotation of host and nonhost genomes was conducted at the Joint Genome Institute. In the RNA-seq experiment, mycelia were harvested from the interaction zone during initial physical contact with bacteria. RNA was extracted and rRNA was removed using commercial kits, followed by sequencing on the Illumina platform. Reads were mapped to the draft assemblies of host (JOSV00000000) and nonhost genomes. DE analysis was performed using standard tools. DGK inhibitor I (8 μM) and II (1 μM) (EnzoLife), and PLD inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (1 mM) (Sigma) were used. Total lipids were extracted with chloroform:methanol (2:1 vol/vol). Phospholipids were visualized as in ref. 34. Neutral lipids were separated in hexane:diethyl ether: formic acid (80:20:2, vol/vol) solvent system. Phylogenetic analyses of amino acid sequences at DGK loci were conducted using Bayesian and maximum likelihood methods.

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