Targeted Gene Disruption of Glycerol-3-phosphate Dehydrogenase in Colletotrichum gloeosporioides Reveals Evidence That Glycerol Is a Significant Transferred Nutrient from Host Plant to Fungal Pathogen*

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Unidirectional transfer of nutrients from plant host to pathogen represents a most revealing aspect of the parasitic lifestyle of plant pathogens. Whereas much effort has been focused on sugars and amino acids, the identification of other significant metabolites is equally important for comprehensive characterization of metabolic interactions between plants and biotrophic fungal pathogens. Employing a strategy of targeted gene disruption, we generated a mutant strain (gpdhΔ) defective in glycerol-3-phosphate dehydrogenase in a hemibiotrophic plant pathogen, Colletotrichum gloeosporioides f.sp. maliæ. The gpdhΔ strain had severe defects in carbon utilization as it could use neither glucose nor amino acids for sustained growth. Although the mutant mycelia were able to grow on potato dextrose agar medium, they displayed arrhythmicity in growth and failure to conidiate. The metabolic defect of gpdhΔ could be entirely ameliorated by glycerol in chemically defined minimal medium. Furthermore, glycerol was the only metabolite that could restore rhythmic growth and conidiation of gpdhΔ. Despite the profound defects in carbon source utilization, in planta the gpdhΔ strain exhibited normal pathogenicity, proceeded normally in its life cycle, and produced abundant conidia. Analysis of plant tissues at the peripheral zone of fungal infection sites revealed a time-dependent reduction in glycerol content. This study provides strong evidence for a role of glycerol as a significant transferred metabolite from plant to fungal pathogen.

Considerable attention has been focused on nutrient uptake in a wide variety of biotrophic associations between plants and microbes (1, 2). To address this topic, several experimental approaches have been pursued, most involving either the axenic culturing of fungal pathogens to examine the requirements of particular nutrients, or the feeding of the plant host with radiolabeled metabolites followed by analysis of label distribution in the pathogen (3). Efforts to date have identified amino acids and sugars as basic carbon requirements for pathogen metabolism (4–9). Recent progress on the molecular cloning of hexose and amino acid transporters localized in the haustorial plasma membrane of rust have further advanced our understanding of the mechanistic details of fungal nutrient uptake (10–12). However, uncertainty still exists as to what other metabolites also serve as significant carbon and energy sources in sustaining biotrophic fungal growth.

The filamentous ascomycete Colletotrichum is a large group of fungi, which cause serious anthracnose disease in many plant species (13). Colletotrichum gloeosporioides f.sp. maliæ is a hemibiotrophic pathogen that is biotrophic for a period of time before causing necrosis to its host, the round-leaved mallow (Malva pusilla). During vegetative growth, conidiation is initiated, beginning with the production of aerial conidiophores that eventually bud to give rise to the conidia. During the infection process, C. gloeosporioides conidia germinate on the host surface and form an elaborate and highly specialized infection structure called the appressorium. The appressorium employs a turgor-based mechanical force that mediates the direct penetration of a narrow penetration peg through the host cuticle and epidermal layer (14). After hyphal penetration of an epidermal cell, the fungus develops intracellular infection vesicles that produce large primary hyphae (LPH).1 The LPH ramify throughout the apoplastic of the leaf tissue, giving rise to the thin secondary hyphae. This generation of thin secondary hyphae from LPH has been associated with the conversion from biotrophic growth to necrotrophic infection by C. gloeosporioides. A necrotic lesion develops, and under conditions of high humidity, C. gloeosporioides produces acervuli that erupt into the cuticle and release conidia to reinitiate the infection cycle (15). Unlike obligate biotrophic pathogens, a C. gloeosporioides culture can be readily established in media, displaying robust growth and a normal conidiation process. C. gloeosporioides is amendable to gene disruption and thus to studying fungal metabolism and pathogenicity.

In the present study, we set out to investigate the metabolic and developmental significance of C. gloeosporioides glycerol-3-phosphate dehydrogenase (GPDH) (L-glycerol-3-phosphate: NAD oxidoreductase, EC 1.1.1.8), which catalyzes the reduction of dihydroxyacetone phosphate using NADH as a reducing equivalent to form glycerol 3-phosphate (Gly-3-P). Disruption of the gpdh gene in C. gloeosporioides was achieved by replacement of the gpdh locus on chromosome 4 by a gpdhΔ integrating cassette. A heterologous selection marker (aphII) was used to screen transformants for the desired disruption of the gpdh locus. Two independently isolated mutants with disrupted gpdh loci were characterized in this study.

1 The abbreviations used are: LPH, large primary hyphae; GPDH, glycerol-3-phosphate dehydrogenase; Gly-3-P, glycerol 3-phosphate; PDA, potato dextrose agar; PC, phosphatidylcholine; PE, phosphatidylethanolamine; hpi, hours post-inoculation.
Carbon Source Uptake in Plant Fungal Pathogen

of the GPDH gene (Cg-GPDH) in C. gloeosporioides resulted in an altered morphology of fungal hyphae and a failure to initiate conidiation when cultured on medium plates. The gene disruption strain was unable to use either glucose or pyruvate as the sole carbon source, and utilized amino acids poorly in defined culture medium plates. However, glycerol as a carbon source efficiently sustained the growth of the mutant on minimal medium. Conidia derived from gpdA grown on glycerol-supplemented plates, once inoculated into plants, developed normal appressoria and displayed normal pathogenicity on plants. Most significantly, in planta, gpdA was fully capable of initiating conidiation. We provide several lines of evidence in this study for a hitherto unknown role of glycerol as a nutrient transferred from plant to pathogen.

EXPERIMENTAL PROCEDURES

Fungal Strains and Growth Conditions—C. gloeosporioides (Penz.) Penz. & Sacc. fsp. malvae, kindly provided by Dr. Karen Bailey, was maintained on potato dextrose agar (PDA) plates (Difco Laboratories, Detroit, MI). Fresh PDA plates were inoculated with mycelial plugs of 3-mm diameter and incubated for 8 days at 24 °C to promote conidiation. Growth tests on plates were carried out on fungal minimal media modified from Czapek's medium (0.2% NaNO₃, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, 0.001% FeSO₄, and 1.5% agar) and supplemented with 10 mM of each sugar unless indicated otherwise. In bioassays of rhizomorph growth, plugs of 2-mm diameter were cut from the margins of actively growing colonies and were then used to inoculate the center of PDA plates in 8-cm diameter Petri dishes. The cultures were entrained at a 12:12-h light/dark cycle. The rhizomorphic periods were determined by monitoring daily colony growth from positions marked on the reverse of the plate.

Bacterial Strains and Functional Complementation Study—Escherichia coli gpaA strain BB20-14 (gpaEK 20 gpdD pdaAsX) was kindly provided by Dr. John Cronan Jr., Department of Microbiology and Biochemistry, University of Illinois. Standard recombinant DNA experiments were carried out using E. coli strain DH5α. Growth of the BB20-14 strain is dependent on supplementation of Gly-3-P or glycerol. The minimal medium used for the E. coli BB20-14 strain was M9 (16). Plasmid preparation and mass conversion of the phage library to the BB20-14 strain is dependent on supplementation of Gly-3-P or glycerol. Plasmids generated from a cdNA library constructed using mRNA isolated from mallow leaves infected with C. gloeosporioides at 72 h post-inoculation (18) were introduced into E. coli BB20-14 cells, and transformed cells were grown on M9 basal medium plates in the presence of 50 μg isopropyl-1-thio-β-D-galactopyranoside. Colonies that gained the ability to grow on M9 medium were isolated, purified, and used for further analysis.

Nucleic Acid Isolation, Manipulation, and Hybridization—Fungal conidia and mycelia grown on PDA plates from 15-day-old cultures were scraped and stored at −80 °C. Mallow leaves infected with C. gloeosporioides were harvested at 3 days post-inoculation and used for isolation of DNA and RNA. Fungal and plant materials were ground under liquid nitrogen with a mortar and pestle. Genomic DNA and total RNA were extracted according to Ausbel et al. (19) and Wilkins and Smart (20), respectively. DNA hybridization probes were prepared using the Random Primer-It kit (Stratagene). Southern and Northern blotting and hybridizations were carried out according to the methods of Sambrook et al. (17). DNA sequencing was performed using an ABI377 automated sequencer (PerkinElmer Life Sciences). DNA/protein sequence data bases were searched using the BLAST algorithm via the World Wide Web.

Fungal Transformation and Gene Disruption—Conidia from 6-day-old cultures grown on PDA plates were transferred to distilled water with a bacterial loop, washed twice, and harvested by centrifugation. Conidia were transferred and resuspended in a 250-ml flask with 100 ml of liquid medium (0.25% MgSO₄·7H₂O, 0.27% KH₂PO₄, 0.1% peptone, 0.1% yeast extract, and 1.0% sucrose) (21), and grown for 48 h at 22 °C with constant agitation (120 rpm). Mycelia incubated in liquid medium for 48 h were harvested by filtration through two layers of cheesecloth, washed with 0.6M MgSO₄, and used for fungal homogenization. Competent protoplasts were prepared from mycelia of C. gloeosporioides by a modification of the method of Yelton et al. (22). Mycelia from liquid medium were resuspended in osmotic medium (5 ml/g of mycelium) (22), containing Novozyme 234 (20 mg/g of mycelium) and β-glucuronidase (0.2 ml/g of mycelium). The cells were digested on a rotary shaker at 80 rpm for 1.5–3 h at 30 °C. The suspension was transferred to a centrifuge tube, overlaid gently with 5 ml of 5T buffer, and centrifuged at 2000 × g for 10 min at 4 °C. Protoplasts were withdrawn with a widebore pipette, and subsequently washed and resuspended in STC buffer (22) at a concentration of 1 × 10⁶ ml⁻¹⁻¹. Plasmid pCg-GPDH, pBluescriptTM SK containing the cdNA insert of the C. gloeosporioides Cg-GPDH bearing an internal BglIII site, was linearized by digestion with BglIII, and Klenow enzyme was used to create blunt ends. Plasmid pAN7-2 (23), which contains a hygromycin-B resistance cassette composed of the coding sequence of hygromycin-B phosphotransferase (hph) from E. coli, the Aspergillus nidulans gpdA promoter sequence, and the A. nidulans trpC terminator sequence, was digested with BglII and HindIII. The excised fragment was blunt-ended and subsequently ligated into the linearized plasmid pCg-GPDH to create plasmid pGPDHΔ.

The vector DNA (10 µg in 20 µl of STC) linearized by digestion with XbaI was added to 200 µl of competent protoplasts, mixed, and incubated on ice for 20 min. The protoplast/DNA combination was mixed with 150 µl of PEG solution (20% PEG 6000, 1 mM sorbitol, 50 mM Tris-HCl, pH 7.5, 50 mM CaCl₂) and incubated on ice for 10 min. 1 ml of PEG solution was added and the mixture was incubated at room temperature for a further 5 min. 200-μl aliquots from each transformation experiment were mixed with 5 ml of 45 °C molten top complete medium containing 0.2% yeast extract, 0.2% Bacto-agar (B-D), water (45 ml) (21), and overlayed onto 20-ml complete medium plates. Plates were incubated at 25 °C overnight, then overlaid with 5 ml of complete medium containing hygromycin-B at 100 µg/ml and incubated at 25 °C for 5 to 10 days. Hygromycin-B-resistant colonies were monitored and subcultured onto PDA medium amended with 100 µg/ml hygromycin-B.

Lipid Analysis—Fungal mycelia developed on PDA plates with and without 10 mM glycerol were harvested for total lipid extraction (24). TLC separation of phospholipid species, and fatty acid composition analysis of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were performed as described (25).

Glycerol 3-Phosphate and Glycerol Content Analysis—Gly-3-P content in trichloroacetic acid extracts (26) was measured enzymatically using glycerophosphate dehydrogenase as described by Lang (27) in an enzyme reaction mixture containing 0.5 mM glycine, 0.4 mM hydrazine buffer (pH 9.5), 1 mM NAD⁺, and 1 unit of glycerol-3-phosphate dehydrogenase (Sigma). Glycerol content analysis was performed using a glycerol determination kit from Roche Diagnostics.

Assay for Appressorium Turgor—50-µl droplets of conidial suspensions, at concentrations of 10⁵ ml⁻¹⁻¹, of wild-type (wt) and the gpdA Δ mutant harvested from PDA plates supplemented with 250 mM glycerol, were placed on the surface of plastic coverslides (Fisher Scientific) on a 25 °C stage. One minute after the addition of the droplets, the coverslides were withdrawn with a wide-bore pipette, and subsequently washed and resuspended in STC buffer (22) at a concentration of 1 × 10⁶ ml⁻¹⁻¹. The fixed material was subjected to SEM preparation. Conidia and infection hyphae on/in the leaf surface was examined using a cytorrhysis assay as described by Howard et al. (28) and Dixon et al. (29).

Inoculation and Pathogenicity Test—Round-leaved mallow (M. pusilla), the host plant for C. gloeosporioides fsp. malvae, was grown in a growth chamber at 25 °C. The third leaves from the top of one-month-old plants were excised and used for the pathogenicity assay. The detached leaves were inoculated with 15 µl of the conidial suspension (5 × 10⁵ conidia ml⁻¹⁻¹) and incubated on moist filter paper in a plastic box at 25 °C in the dark. The inoculated leaves were monitored daily for 7 to 10 days.

Scanning Electron Microscopy—Droplets of PDA medium were placed on plastic coverslides (Fisher Scientific) and allowed to dry under a sterile flow of air, forming a thin film. 15 µl of the conidial suspensions on a wild-type strain and the gpdA Δ mutant harvested from PDA plates supplemented with 250 mM glycerol were placed on the centers of the PDA films. These slides bearing inoculated PDA films were incubated in Petri dishes at 25 °C for 3–7 days. The fungus grown on the film was fixed in an ethanol/acetic acid (1:1) solution at room temperature overnight. The fixed material was subjected to S.E. ± mean examination.

Confocal Microscopy—Leaf tissues were fixed and decolorized in an ethanol/acetic acid (1:1) solution at room temperature overnight. Visualization of germplams and infection hyphae on/in the leaf surface was facilitated by staining for 5 min with 0.1% aniline blue in lactoglycerol (lactic acid/glycerol/water (1:1:1)) followed by a brief rinse in lactoglycerol. The stained leaf sections were then mounted in lactoglycerol for microscopy.
RESULTS

Molecular Cloning of a GPDH cDNA from C. gloeosporioides—We adapted a complementation-based screening procedure to isolate the GPDH cDNA from C. gloeosporioides using an E. coli mutant strain, BB20-14, which is unable to synthesize Gly-3-P because of a loss-of-function mutation in the GPSA gene encoding an NAD(P)H-dependent GPDH, and cannot grow on basal medium without glycerol or Gly-3-P supplement (18). A cDNA library was constructed with mRNA prepared from infected mallow leaves at 72 h post-inoculation with C. gloeosporioides. The library thus contains cDNA populations derived from both the plant tissue and the pathogen (30). Plasmids excised in vivo from the phage library were used to transform the E. coli BB20-14 strain. Twenty-one colonies that gained the ability to grow on basal medium without glycerol supplementation were isolated. Southern blot analysis and PCR amplification using gene specific primers confirmed that a group of plasmids, designated as pCg-GPDH (deposited into EMBL under accession number Y331190) was derived from the fungus. The Cg-GPDH transcript encodes a protein of 420 amino acid residues, contains a predicted NAD binding domain, and shows a high degree of homology to a recently reported GPDH (GPD1) sequence from A. nidulans (31). The sequence information and the functional complementation result led us to conclude that Cg-GPDH corresponds to a GPDH gene from C. gloeosporioides.

Southern blot analysis of C. gloeosporioides with the full-length Cg-GPDH cDNA as a probe revealed only a single hybridization band in EcoRI, EcoRV, and HindIII-digested genomic DNA (Fig. 1A), suggesting Cg-GPDH is a single copy gene. Northern blot analysis identified a 1.7-kb transcript of approximately equal abundance in mycelia and spores harvested from PDA plates (Fig. 1B). A transcript of the same size was also detected from total RNA extracted from infected mallow leaves (data not shown). Therefore, Cg-GPDH is expressed at all stages of C. gloeosporioides development.

Targeted Gene Disruption in Cg-GPDH Affects Fungal Morphology and Disrupts Conidiation under Standard Culture Conditions—To determine the functional significance of Cg-GPDH in C. gloeosporioides, a gene disruption strategy was employed. An XbaI-linearized gene disruption vector, pGPDHΔ, containing a hygromycin-resistance gene cassette flanked by 5’ and 3’ ends of the Cg-GPDH cDNA (Fig. 2, A and B) was introduced into the wild-type strain and HygR transformants were selected. Among 180 HygR transformants, two (GDDH41Δ and GDDH63Δ) were confirmed by PCR analysis to have the endogenous Cg-GPDH gene disrupted. There was an increase in the amplicon size from 601 bp for the uninterrupted gene to 4.5 kb for the interrupted version (Fig. 2C). This was also confirmed by Southern blot analysis of the genomic DNA of the two mutants digested with several restriction enzymes (data not shown). The strain GDDH41Δ, hereafter referred to as gpdhΔ, was used for further study. The targeted gene disruption abolished Cg-GPDH expression as shown by the lack of Cg-GPDH transcript in mycelia (Fig. 2D).

The PDA plate provides a standard culture medium sufficient for all stages of C. gloeosporioides development (30). During early stages of growth, the gpdhΔ strain displayed a slightly slower mycelial growth rate and poorly developed aerial hyphae on PDA plates as compared with wild-type. Colonies of the wild-type strain and gpdhΔ could also be distinguished based on pigmentation. Heavy melanization caused increased pigmentation and hence a darker appearance in gpdhΔ. Micro-

Fig. 1. Molecular characterization of Cg-GPDH gene in C. gloeosporioides. A, Southern blot of digested DNA of C. gloeosporioides l.s. malvae hybridized with the cloned Cg-GPDH cDNA. The size in kb of the DNA molecular weight marker is shown at the left. B, Northern blot of total RNA extracted from conidia and mycelia of the wild-type strain. Fifteen μg of total RNA was separated in a denaturing formaldehyde gel, blotted onto a nylon membrane, and probed with the cloned Cg-GPDH cDNA. The fungus was grown on PDA plates for 7 days, and conidia and mycelia were harvested by rinsing and scratching the plates, respectively. Equal loading of RNA was confirmed by ethidium bromide staining.

Fig. 2. Disruption and analysis of the Cg-GPDH gene. A, the full-length cDNA of Cg-GPDH was cloned into the EcoRI/XhoI sites of the pBluescriptTM SK plasmid. A hygromycin-resistance gene (hgr) cassette was released from plasmid pAN7–2 by BglII and XbaI, blunt-ended, and inserted into the BglIII site of Cg-GPDH. An XbaI linearized fragment was used directly for transformation of the C. gloeosporioides wild-type strain. Arrows indicate the location of PCR primers used for the knockout mutant screening. B, BamHI; E, EcoRI; P, PvuII; Xo, XbaI; Xo, XhoI. C, gene disruption in the gpdh gene. D, PCR analysis of genomic DNA from wild-type (wt) and gpdhΔ mutant using primers specific for the Cg-GPDH shown in A was employed to verify the mutation. XXo, EcoRI, and XbaI primers were used to verify the mutation. D, RNA blot verification of the mutation of gpdhΔ. Twenty μg of total RNA from C. gloeosporioides wild-type and gpdhΔ strains blotted onto a nylon membrane were probed with a 341-bp 3' end fragment excised from the Cg-GPDH cDNA plasmid.
scopical examination revealed abnormal hyphae along the edge of the gpdhΔ colony, which displayed swelling in the branching regions similar to the phenotype described previously in the A. nidulans gfdA mutant (31). On PDA plates, the wild-type strain readily developed conidiophores and produced abundant conidia that appeared pink in color (Fig. 3A, a). The gpdhΔ strain, however, was unable to conidiate on the same rich medium even after prolonged culture (Fig. 3A, b). To further investigate this developmental defect, gpdhΔ and the wild-type strains were cultured on dried PDA droplets on plastic coverslides incubated in a Petri dish (for 3 days), and subjected to scanning electron microscopic examination. In contrast to the wild-type strain, which generated acervuli with abundant conidia, the gpdhΔ strain produced aborted acervuli as indicated by the presence of lone setae with no conidia (Fig. 3B).

The gpdhΔ Strain Has Reduced Gly-3-P and Glycerol Content and Altered Fatty Acid Composition of Glycerolipids—A lack of GPDH is expected to have a direct impact on Gly-3-P generation. Because GPDH is also coupled to glycerol metabolism, the mutation may also affect glycerol production. We thus analyzed glycerol and Gly-3-P content of gpdhΔ and wild-type mycelia grown on PDA medium. As shown in Fig. 4A, deletion of the Cg-GPDH gene resulted in a severe reduction of the cellular Gly-3-P content. Accordingly, the glycerol content of the gpdhΔ strain was also diminished to about one-eighth of that of the wild-type strain (Fig. 4B). The low levels of glycerol and Gly-3-P detected in the mutant strain are most likely attributable to the composition of the PDA culturing medium.

Gly-3-P is an obligatory substrate for the synthesis of glycerolipids from which phospholipids are formed by the addition of one of several polar head groups. The most abundant phospholipid species are PC and PE. In the A. nidulans mutant defective in GPDH activity, it was reported that there was no change in the cellular ratio of phospholipid species. However, a limited Gly-3-P provision would likely reduce the overall rate of glycerolipid assembly, and not affect the relative proportions of different phospholipid species. More likely, reduced glycerolipid biosynthesis, and hence a reduced incorporation of fatty acids into lipid species, would permit the fatty acids of the fatty acyl-CoA pool to be further modified, such as an increase in desaturation. Such a change could be detected through analysis of the fatty acid composition of the abundantly existing phospholipids. We thus purified PC and PE from the gpdhΔ strain on chemically defined minimal medium, because the GPDH activity of the strain was below detection. The molar 16:0 content in PC and PE of the gpdhΔ strain on minimal medium was almost negligible (Fig. 4C).

Mycelium Growth of the gpdhΔ Strain on Minimal Medium Requires a Glycerol/Gly-3-P Supplement—We investigated the growth of the gpdhΔ strain on chemically defined minimal media containing various carbon sources, because the GPDH reaction is only one step away from the glycolytic pathway and may therefore affect carbon source utilization. Glucose (12) and amino acids (2) are two confirmed carbon sources significant in biotrophic nutrition. When glucose, pyruvate, glycine, glutamate, and histidine were each provided at a concentration of 10 mM, as the sole carbon source in a minimal medium, the wild-type strain showed an optimum growth rate with glucose, and slightly reduced growth rates with all other carbon sources.
None of the aforementioned carbon sources could sustain colony growth of the gpdhΔ strain (Fig. 5). Dihydroxyacetone phosphate was also tested as a carbon source, but it could be utilized by neither the wild-type nor mutant strains. Contrary to what was reported regarding A. nidulans (31), the presence of an osmotic stabilizer (1 m NaCl) in the medium did not have any impact on carbon source utilization in the gpdhΔ strain. Strikingly, both wild-type and the gpdhΔ strain grew robustly on minimal media plates containing 10 mM glycerol. Gly-3-P can be utilized by the wild-type strain to support growth at an optimum concentration of 1 mM, beyond which a significant reduction in growth rate is observed. At the optimum concentration of 1 mM, Gly-3-P could also support gpdhΔ growth, but at a rate dramatically lower than that of the wild-type strain.

We further examined the concentration of glycerol required to support fungal growth by performing growth assays for both the wild-type and mutant strains on minimal medium supplied with various concentrations of glycerol. No significant difference in the growth rate (colony diameter/incubation period) was observed on plates containing between 10 and 1 mM glycerol for either of the strains. We also found that glycerol at as low as 100 μM in the medium supports a substantial growth of the fungus, but at a markedly reduced rate.

Glycerol Is a Metabolite That Restores Conidiation and Rhythmic Growth of the gpdhΔ Strain on PDA Plates—Although mycelia of the gpdhΔ strain were able to propagate on PDA plates, there were two noticeable growth and developmental defects: they failed to conidiate (Fig. 6A), and the growth was arrhythmic (Fig. 6B). We tested whether the provision of additional carbon sources in PDA could reverse these defects. It became clear that a robust growth rhythm was restored in gpdhΔ when glycerol was provided in the PDA medium (Fig. 6C). Furthermore, the gpdhΔ mutant developed conidial mass after 7 days of incubation (Fig. 6A). Microscopic examinations confirmed that massive conidiation in gpdhΔ took place. Addition of Gly-3-P, on the other hand, restored neither rhythmic growth nor conidiation in gpdhΔ. Additions of other sugars, amino acids, and polyols including trehalose, sorbitol, and mannitol, failed to have any effect on gpdhΔ (data not shown). Thus glycerol is the only metabolite that not only supported mycelia growth, but also restored the developmental defect.

Host Plant-derived Nutrients Capacitated gpdhΔ to Overcome Its Metabolic Defects and Complete Its Life Cycle—GPDH-mediated glycerol production might also be relevant to appressorium development (29, 32). Conidia of the wild-type strain and those of the gpdhΔ strain produced from glycerol-supplemented PDA plates were germinated for 24 h at 25 °C on plastic coverslides. A similar germination rate (45%) was observed in both strains. Like the wild-type strain, gpdhΔ produced melanized appressoria 2 days after incubation. We then assessed the exhaustion of appressorium turgor, using a cytorrhysis assay on plastic coverslides, against various concentrations of glycerol (28). A >3 mM glycerol was required to collapse 50% of the developed appressoria in both the wild-type and gpdhΔ (data not shown). Hence, generation of appressorium turgor pressure was not impaired in gpdhΔ, and the gpdhΔ strain retained the ability to complete the initial stage of plant invasion.

We inoculated conidia of the gpdhΔ strain on excised mallow leaves in parallel with that of the wild-type strain. The gpdhΔ mutant displayed normal pathogenicity, causing chlorotic lesions and host tissues were heavily macerated. Light microscopic examinations further confirmed that the infection structures and process of gpdhΔ had a pattern similar to the wild-type strain (15). The gpdhΔ conidia germinated in the first 3–4 h and produced appressoria in ~20 h on mallow leaves. An infection peg formed from the appressorium, and an infection vesicle appeared within an epidermal cell beneath the peg 2 days after inoculation. The LPH emerged from the infection vesicle and grew intercellularly through several adjacent epidermal cells (Fig. 7B). Thereafter, thin secondary hyphae developed from LPH in 4–5 days. A few hours after the appearance of thin secondary hyphae, necrotic water-soaked lesions were visible. Moreover, on the macerated lesion, acervuli with abundant conidia developed (Fig. 7C). The fact that the gpdhΔ mutant hyphae could grow, cause disease symptoms, and complete conidiation in the host tissues indicated that it was able to obtain a carbon source that enabled it to circumvent its inability to utilize glucose or amino acids.

Glycerol Content of Infected Plant Leaf Displayed a Time-dependent Decline after Fungal Infection—Free glycerol is a metabolite widely distributed in plant tissues (33). We inoculated detached mallow leaves with wild-type C. gloeosporioides conidia, and harvested leaf discs surrounding the infection sites at different time points for glycerol content measurement. Mock inoculations with water were used as controls. Our microscopic examinations showed that 48 h after inoculation the infection was still at a biotrophic stage where no necrosis of plant tissues was evident. However, glycerol content of plant
tissues at this time was reduced by about 40% (Fig. 8). After 72 h, a necrosis of the plant tissues began to appear, and the glycerol content was about one-third of the control.

**DISCUSSION**

The GPDH reaction represents a junction of several major biochemical pathways, and plays a fundamental role in energy metabolism and biomass synthesis. In addition to phospholipid and triacylglycerol biosynthesis, GPDH is involved in the synthesis of glycerol and, in concert with a mitochondrial Gly-3-P shuttle system, is also known to be crucial for coupling glycolysis with activation of mitochondrial oxidative metabolism and ATP production (34, 35). The aim of this research was to investigate the functional significance of this enzyme in the development and pathogenicity of *C. gloeosporioides*. This entailed isolating the gene encoding GPDH in *C. gloeosporioides* and generating a strain defective in GPDH activity. The GPDH-deficient *gpdhΔ* strain generated in this study not only allowed us to assess the impact of GPDH deficiency on fungal development, but also offered insight into nutrient uptake during the biotrophic growth phase.

A direct metabolic effect of GPDH deficiency is on the production of Gly-3-P. Indeed, the *gpdhΔ* strain had a dramatically reduced content of this metabolite when grown on PDA, from which a limited amount of glycerol and/or Gly-3-P may be obtained. Because Gly-3-P is an obligate precursor for glycerolipid synthesis, it stands to reason that *de novo* glycerolipid biosynthesis may be limited in the mutant strain. The mutant grown on a PDA plate exhibited an alteration of the fatty acid composition in PC and PE, in particular, an increased molar ratio of 18:1Δ11 as compared with wild-type. The fatty acid 18:1Δ11 is the elongation product of 16:1Δ9-CoA, which is generated from palmitoyl-CoA (16:0) through desaturation by the stearoyl-CoA Δ9-desaturase. Normally, palmitoyl-CoA is either directly elongated to stearoyl-CoA, or immediately assembled into glycerolipid species without any further modification. A decreased channeling of palmitoyl-CoA into glycerolipid may render this fatty acid an inadvertent substrate of the stearoyl-CoA Δ9-desaturase, and lead to an increase in 18:1Δ11 content. Hence, the fatty acid composition changes suggest that *gpdhΔ* had a compromised glycerolipid biosynthesis, which may be the direct cause of the growth and developmental defects.

In *A. nidulans* and *Magnaporthe grisea*, glycerol and Gly-3-P can also be produced from dihydroxyacetone and glyceraldehyde by an NADPH-dependent reductase (31, 36). Dihydroxyacetone and glyceraldehyde can be derived from intermediates of the glycolytic pathway beginning with glucose or gluconeogenesis from pyruvate and amino acids. However, our results show that glucose can be used as a sole carbon source by wild-type but not *gpdhΔ* on minimal medium. Both pyruvate and amino acids were also used as carbon sources to support wild-type growth, indicating that there exists the gluconeogenesis process for Gly-3-P production (37). However, neither pyruvate nor amino acids could sustain continued growth of *gpdhΔ*. The *gpdhΔ* strain displayed some growth on minimal media with amino acids as sole carbon sources, yet the growth rate was extremely slow. Moreover, the colonies ceased to grow after a few days, and never reached sizes beyond a few millimeters in diameter. These results indicate that a deficiency in GPDH also crippled gluconeogenesis in *gpdhΔ*.

The *gpdhΔ* mutant generated appressorium turgor pressure similar to that of the isogenic wild-type strain. Genomic Southern analysis indicated that *C. gloeosporioides* contains a single
copy of the Cg-GPDH gene. Our results are thus consistent with reports of Dixon et al. (29) and Thines et al. (36) and provide direct evidence that GPDH-mediated glycerol production is dispensable for appressorium turgor generation. Nonetheless, if the plant host is incapable of providing nutrients that enable gpdhΔ to overcome its metabolic defects, the infection steps to follow would be stalled (38). Having discovered that the gpdhΔ mutant cannot utilize glucose and amino acids as carbon sources, it was surprising that gpdhΔ could complete its life cycle in planta without any retardation. This is particularly intriguing in light of the fact that, even though the mutant mycelium grew on the rich PDA medium, they were never able to produce conidia. Thus, there must be a plant-derived nutrient transferred to the mutant thereby capacitating the mutant to overcome its metabolic defect. It emerges that glycerol is a bona fide significant transport nutrient based on our data and known observations of other workers summarized below. 1) Glycerol is a metabolite existing in plant cells at a concentration of 1–2 μmol/g fresh weight (33). Consistent with this, our results showed that glycerol content in the inoculated leaf was about 1.8 μmol/g fresh weight, which can be roughly translated as 1–2 mM, a concentration sufficient to support both the wild-type and mutant growth in a minimal medium. Aquaglyceroporins capable of facilitating the efflux of glycerol to the apoplast have been identified in several plant species (39). In this context, it is interesting to note that one of the most abundant proteins in the symbiosome membrane of N2-fixation nodules, nodulin 26, is a member of the aquaglyceroporin family, which shows high permeability to glycerol (40). 2) Glycerol in the apoplast can be readily taken up by the pathogen. An early study showed that the non-melanized fungal cell wall is permeable to glycerol diffusion (32). Unlike sugars, which require elaborate membrane transport systems, glycerol as an uncharged compound with only three carbon atoms can penetrate the fungal membrane by simple diffusion (41). 3) Glycerol was the singular metabolite that re-established a robust rhythm and excellent growth of gpdhΔ. 4) Glycerol, not Gly-3-P, was able to restore condensation of gpdhΔ in vitro, thereby establishing that the root cause of its metabolic defect can be critically relieved only by glycerol. 5) The gpdhΔ mutant not only maintained full pathogenicity, but also successfully completed its life cycle in planta. 6) Plant tissue at the peripheral zone of fungal infection sites had a reduced glycerol content.

Glycerol can be utilized as a sole carbon and energy source for both bacteria and fungi. Indeed, we found that glycerol is a very efficient carbon source for both the wild-type and gpdhΔ strain. There can be several alternative glycerol dissimilation pathways, including the NADP-linked glycerol dehydrogenase route and the FAD-linked Gly-3-P dehydrogenase route (42). It has been suggested according to its phylogenetic distribution that glycerol metabolism can be taken back as far as the prebiotic era (43). It was recently demonstrated that glycerol supplied exogenously to extracellular hyphae of the symbiotic mycorrhiza is incorporated into triacylglycerol in the intracellular hyphae, and subsequently transferred to the extracellular hyphae as an energy source (44). Direct studies of metabolism in plant-fungal interactions often encounter difficulties in interpreting the biochemical data, and none of the methodologies employed to date are without pitfalls (3). Our research demonstrates that the application of molecular genetics is a useful tool in the study of metabolism exchange in plant-microbial interactions. The results presented in this study strongly argue that glycerol is a metabolite of significance in plant pathogen metabolism, and future studies in this direction may hold much promise for developing new knowledge about plant-microbial interactions.

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