Mitochondrial Carnitine-Dependent Acetyl Coenzyme A Transport Is Required for Normal Sexual and Asexual Development of the Ascomycete Gibberella zeae

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Fungi have evolved efficient metabolic mechanisms for the exact temporal (developmental stages) and spatial (organelles) production of acetyl coenzyme A (acetyl-CoA). We previously demonstrated mechanistic roles of several acetyl-CoA synthetic enzymes, namely, ATP citrate lyase and acetyl-CoA synthetases (ACSs), in the plant-pathogenic fungus Gibberella zeae. In this study, we characterized two carnitine acetyltransferases (CATs; CAT1 and CAT2) to obtain a better understanding of the metabolic processes occurring in G. zeae. We found that CAT1 functioned as an alternative source of acetyl-CoA required for lipid accumulation in an ACS1 deletion mutant. Moreover, deletion of CAT1 and/or CAT2 resulted in various defects, including changes to vegetative growth, asexual/sexual development, trichothecene production, and virulence. Although CAT1 is associated primarily with peroxisomal CAT function, mislocalization experiments showed that the role of CAT1 in acetyl-CoA transport between the mitochondria and cytosol is important for sexual and asexual development in G. zeae. Taking these data together, we concluded that G. zeae CATs are responsible for facilitating the exchange of acetyl-CoA across intracellular membranes, particularly between the mitochondria and the cytosol, during various developmental stages.

The homothallic ascomycete fungus Gibberella zeae (anamorph: Fusarium graminearum) is a major causative agent of Fusarium head blight (FHB) on small grains such as wheat, barley, and rice, in addition to causing ear and stalk rot on maize worldwide (35). Plant diseases caused by this fungus lead to severe yield losses and to accumulation of harmful mycotoxins in infected cereals (10). As a hemibiotrophic pathogen, G. zeae exhibits biotrophic growth during the initial stages of floral infection before finally causing plant cell death and necrosis (6, 26). Asexual spores (conidia) present on infected crops cause secondary infections of neighboring heads following periods of rainfall. Sexual spores (ascospores) produced in the perithecia at milder temperatures and with moderate moisture are forcibly discharged into the air (45, 59) and function as the primary inocula for FHB epidemics in cereal crops (59, 63). In addition, perithecia (or associated hyphae) provide the means for fungi to survive between seasons (11, 17).

Previous genome-wide transcriptional analyses of G. zeae demonstrated that dynamic transcriptional changes occurred during various developmental stages, including sexual and asexual development, mycotoxin production, and plant infection (15, 17, 18, 19, 36, 49). In addition, a recent genome-wide functional analysis revealed transcription factors that were closely related to these processes (55). These transcriptional changes were caused mainly by altered metabolic processes that were precisely regulated depending on the available carbon source and the developmental stage of the pathogen.

Acetyl coenzyme A (acetyl-CoA) is the key metabolite connecting catabolism and anabolism. Acetyl-CoA is generated through several metabolic processes and is used for energy production, synthesis of various cellular components and metabolites, and acetylation of proteins (13, 43). Since fungi encounter diverse environmental conditions during their unique differentiation processes, they have evolved highly efficient metabolic processes that allow for the exact temporal (developmental stages) and spatial (organelles) production of acetyl-CoA. Previous studies of acetyl-CoA synthetic enzymes in G. zeae have demonstrated that the roles and relationships of these enzymes are quite different from those in Aspergillus nidulans, an organism commonly used in fungal metabolism research (32, 52, 53). Therefore, functional characterization of the key acetyl-CoA synthetic enzymes is necessary for basic and applied studies of G. zeae.

Previously, we studied the G. zeae ATP citrate lyase (ACL) (52). ACL is known to mediate translocation of acetyl groups from the mitochondrial matrix to the cytosol by catalyzing the degradation of citrate into oxaloacetate and cytosolic acetyl-CoA in eukaryotes (33). Unexpectedly, ACL was not involved in de novo lipid synthesis but played a role in histone acetylation by supplying nucleocytoplasmic acetyl-CoA, similar to the case observed in mammalian cells (52, 68). These results led us to hypothesize that acetyl-CoA synthetases (ACSs) are required for supplementary acetyl-CoA production that is absent in ACL deletion mutants. ACS2 has functions similar to those of ACL, in addition to facilitating ACS1 function. Furthermore, ACS-dependent acetyl-CoA production is important for sexual development and lipid production in G. zeae (32). Additional studies of pyruvate decarboxylase 1 (PDC1), an upstream enzyme of ACS in the pyruvate-acetaldehyde-acetate

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(PAA) pathway, revealed that the ACS1 deletion mutant phenotype was caused mainly by endogenously generated toxic metabolites, such as acetate, acetaldehyde, and ethanol, in G. zeae (53).

In fungi, acetyl-CoA is produced in peroxisomes via peroxisomal β-oxidation (22, 28), in the mitochondria via the mitochondrial-associated pyruvate dehydrogenase complex or mitochondrial β-oxidation (37), and in the nuclei and cytosol by ACL and ACSs (32, 33, 52, 57, 60). Moreover, eukaryotic cells have an efficient shuttle system that allows acetyl-CoA to be transported between the cytosol and organelles via a process mediated by carnitine acetyltransferases (CATs) (27). Reversible CAT enzyme actions between acetyl-CoA and acetyl carnitine enable efficient exchange of acetyl-CoA across the intracellular membranes in eukaryotes (12, 66).

CATs have been shown to be important metabolic enzymes in A. nidulans. Neither AcuJ, a mitochondrial and peroxisomal CAT, nor FacC, a cytosolic CAT, is crucial for fungal development, although both are involved in the utilization of various carbon sources (25, 39, 56). In Magnaporthe oryzae, peroxisomal CAT was shown to be required for host invasion (3, 47). However, the biological roles of cytosolic CAT and its relationship with other acetyl-CoA synthetic enzymes have not been examined in detail.

In this study, we identified two CATs (CAT1 and CAT2) encoded by the G. zeae genome and characterized their cellular functions. Based on previous studies, we hypothesized that CAT-dependent acetyl-CoA transport between organelles has important physiological roles and that these CATs are closely related to other acetyl-CoA synthetic enzymes in G. zeae.

MATERIALS AND METHODS

Fungal strains and media. G. zeae wild-type strain Z-3639 (4) and transgenic strains derived from this strain are listed in Table 1, with the exception of the Δgzeicl1 mutant, which was generated previously from G. zeae wild-type strain Z-3643 (4, 31). Conidial suspensions of the respective strains were stored in 20% glycerol at −70°C. Carboxymethyl cellulose (CMC) medium (7) and yeast extract-malt extract agar (YMA) (21) were used for production of conidia as previously described (52). Minimal medium (MM) (35) was supplemented with 2% glucose, 50 mM sodium acetate, 50 mM ethanol, 10 mM sodium butyrate, 0.5% oleate, or 0.5% Tween 80 as the sole carbon source. MM containing 5 mM aminoguanine (MA) was used for trichothecene production (15). Other media used in this study were prepared and used according to the protocols in The Fusarium Laboratory Manual (35).

Nucleic acid manipulation and PCR. Standard procedures for fungal genomic DNA extraction and Southern and Northern hybridizations using 32P-labeled probes were performed as previously described (35, 48). Total RNA was extracted using an Easy-Spin Total RNA extraction kit (Intron Biotech, Seongnam, Republic of Korea) following the manufacturer’s instructions. PCR primers used in this study were synthesized by Bionics (Seoul, Republic of Korea) (see Table S1 in the supplemental material).

Fungal transformation. Constructs used for targeted gene deletion and complementation were generated by the double-joint (DJ) PCR method (70). To generate deletion constructs for the CAT1 and CAT2 genes, a Geneticin resistance cassette (gen), amplified from the plP99 vector by use of primers Gen-For and Gen-Rev (42), was fused to the 5′- and 3′-flanking sequences of each target open reading frame (ORF), amplified using the corresponding primer pairs (see Table S1 in the supplemental material).

For cellular localization analysis and complementation, the DNA fragment containing the native promoter and ORF of each gene was fused to the green fluorescent protein (GFP) gene, and the hygromycin resistance cassette (hyg), amplified from the pIGPAPA vector by use of primers pIGPAPA-sGFP F and HYG-F1 (24), was fused to the 3′-flanking region of each gene as previously described (52). All fungal transformations for gene deletion and complementation procedures were carried out as previously described (20).

Lipid staining. Mycelial lipids were stained with Nile red solution (0.01 mg/ml in acetone) (49), and fluorescence microscopy with a DE/Axio Imager A1 microscope with filter set 15 (excitation wavelength, 546/12 nm; emission wavelength, 590 nm) (Carl Zeiss, Oberkochen, Germany) was used to examine samples.

Sexual crosses and genotyping. Mycelia grown on carrot agar for 5 days were removed with the back of a surgical blade (surgical blade 11; Feather Safety Razor, Osaka, Japan) in the presence of 2.5% sterilized Tween 60 solution for self-fertilization (35). For outcrosses, heterothallic strains carrying the MAT1-1 deletion were used as female strains (Table 1) and spermatized with 1 ml of conidial suspension from each corresponding male strain. After sexual induction, all cultures were incubated under near-UV light (wavelength of 365 nm; HKIV Import & Export Co., Ltd., Xiamen, China) at 25°C. Dozens to hundreds of ascospores were randomly isolated from each cross, and the genotype of the progeny was determined via antibiotic resistance testing and PCR using appropriate primers (see Table S1 in the supplemental material). Forcible ascospore discharge was observed as previously described (64).

Conidium induction, germination, trichothecene production, and virulence testing. Conidium production was measured by counting the number of conidia produced after culturing 10 μl of conidium suspension (1 × 107 conidia/ml) in 5 ml of CMC medium for 72 h at 25°C on a rotary shaker (150 rpm). Germination of conidia was rated as previously de-

| TABLE 1 G. zeae strains used in this study | G. zeae strain | Genotype | Source or reference |
|--------------------------------------------|---------------|----------|---------------------|
| Z-3639 Wild type                           | 4             |          |
| Z-3643 Wild type                           | 4             |          |
| HK22 Δacs1::gen                            | 32            |          |
| HK36 Δpdc1::gen                            | 53            |          |
| HK34 gen-RFP-SKL                           | 32            |          |
| HK61 Δpdc1::gen Δacs1::gen                 | 53            |          |
| HK29 Δmat1-2::GFP-hyg Δacs1::gen           | 32            |          |
| HK53 Δcat1::gen                            | This study    |
| HK54 Δcat1::CAT1-GFP-hyg                   | This study    |
| HK91 Δcat1::CAT1-GFP-AKL-hyg               | This study    |
| HK55 Δcat2::gen                            | This study    |
| HK56 Δcat2::CAT2-GFP-hyg                   | This study    |
| Δmat2 mutant                               | 30            |          |
| HK57 Δmat1-2::GFP-hyg Δcat1::gen           | This study    |
| HK58 Δmat1-2::GFP-hyg Δcat2::gen           | This study    |
| HK59 Δcat1::gen; Δcat2::gen                | This study    |
| Δmat1 mutant                               | 30            |          |
| HK69 Δmat1-1::gen; Δcat1::CAT1-GFP-hyg     | This study    |
| HK92 Δmat1-1::gen; Δcat1::CAT1-GFP-AKL-hyg | This study    |
| HK70 Δmat1-1::gen; Δcat2::CAT2-GFP-hyg     | This study    |
| HK71 Δacs1::gen; Δcat1::CAT1-GFP-hyg       | This study    |
| HK72 Δacs1::gen; Δcat2::CAT2-GFP-hyg       | This study    |
| Δcat1::gen; Δcat2::CAT2-GFP-hyg            | This study    |
| HK74 Δcat2::gen; Δcat1::CAT1-GFP-hyg       | This study    |
| HK75 gen-RFP-SKL; Δcat1::CAT1-GFP-hyg      | This study    |
| HK76 gen-RFP-SKL; Δcat2::CAT2-GFP-hyg      | This study    |
| HK77 Δpec1::gen; gen-RFP-SKL; CAT2-GFP-hyg | This study    |
| HK78 Δacs1::gen; Δcat1::gen                 | This study    |
| HK79 Δacs1::gen; Δcat2::gen                 | This study    |
| HK80 Δacs1::gen; Δcat1::gen; Δcat2::gen     | This study    |
| Δgzeicl1 mutant                            | 31            |          |
deleted (52). Briefly, 3-day-old mycelia were harvested from the complete medium (CM) and washed twice with distilled water. The harvested mycelia were spread on YMA medium and incubated for 48 h under near-UV light (HKiv Import & Export Co., Ltd.). Conidia were then collected with distilled water, filtered through cheesecloth, washed, and resuspended in distilled water to a concentration of 1 × 10⁸ conidia/ml. Discharged ascospores were collected from petri dish covers, washed, and resuspended in distilled water to a concentration of 1 × 10⁸ conidia/ml. After inoculating 1 ml of a spore suspension in 10 ml of CM and MM, the number of conidia germinated was counted.

Total trichothecene (deoxynivalenol and 15-acetyldeoxynivalenol) production in MMA was measured with a Shimadzu QP-5050 gas chromatograph-mass spectrometer (GC-MS; Shimadzu, Kyoto, Japan) with selected ion monitoring and quantified on the basis of the biomass produced by each strain (52).

The wheat cultivar Eunamipil was used for virulence testing as previously described (52). Briefly, 10 μl of a conidial suspension (1 × 10⁶ conidia/ml) was injected into a center spikelet of wheat head at mid-anthesis, and the plants were placed in a humidity chamber for 3 days and then moved to a greenhouse for further incubation. Spikelets presenting with FHB symptoms were counted at 14 and 21 days postinoculation.

**Microscopy.** A Stereo Lumar V12 (Carl Zeiss) dissecting microscope was used for observations of perithecia. Mitochondria were stained with MitoTracker (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Fluorescence microscopic observation was performed using a DE/Axio Imager A1 microscope (Carl Zeiss) with filter set 38HE (excitation wavelength, 470/40 nm; emission wavelength, 525/50 nm) for GFP. Red fluorescent protein (RFP) and MitoTracker were observed at a filter setting of 15 (excitation wavelength, 546/12 nm; emission wavelength, 590 nm). Cytological procedures were adapted for observation of rosettes of asci (54).

**RESULTS**

**Identification of CAT genes and phylogenetic analysis.** We identified two CAT genes, CAT1 (FGSG_01924.3) and CAT2 (FGSG_00840.3), in the G. zeae genome from the Fusarium graminearum database (69) following a BLASTp search using A. nidulans AcuJ and FacC sequences (39, 56). The CAT1 and CAT2 protein sequences shared 63% and 67% identity with the protein sequences of AcuJ and FacC of A. nidulans, respectively.

Phylogenetic relationships among CAT homologs were estimated using the MEGA program, version 4.0 (61). A phylogenetic tree was constructed using a neighbor-joining algorithm, with bootstrap values calculated from 1,000 iterations. The G. zeae CAT1 protein was grouped with PTH2, AcuJ, and Cat2p of M. oryzae, A. nidulans, and Saccharomyces cerevisiae, respectively. CAT2 was grouped with FacC of A. nidulans (see Fig. S1 in the supplemental material).

**Gene deletion and genetic complementation.** We individually deleted CAT1 and CAT2 by homologous recombination. Each CAT gene ORF was successfully replaced with the gen gene. For genetic complementation, each ORF fused with GFP was introduced into a corresponding deletion mutant. All deletion and complementation mutants were confirmed by Southern blot hybridization (see Fig. S2 in the supplemental material).

For generation of the HK59 double deletion mutant (Δcat1 Δcat2), a Δmat2 strain was outcrossed with a CAT2 deletion mutant to select for the HK58 (Δmat2 Δcat2) strain, which was then outcrossed with HK53 (Δcat1). Numerous ascospores were isolated from the HK58 and HK53 outcross, and antibiotic resistance tests and PCR screenings were performed to obtain the double deletion mutant HK59.

**Developmental defects of CAT deletion mutants.** The CAT1 deletion mutants produced fewer conidia than the wild-type and complemented strains in CMC medium. Double CAT1 and CAT2 deletion mutants presented a more severe conidium production defect than that observed for the CAT1 single deletion mutant (Table 2). Germination of conidia and ascospores occurred earlier in the CAT1 and/or CAT2 deletion mutant than in the wild-type and complemented strains (Table 2).

All deletion mutants showed different pigmentation patterns on carrot agar (Fig. 1A). CAT1 deletion mutants presented with markedly reduced levels of red and yellow pigments, while the CAT2 deletion mutants were impaired only with respect to yellow pigments at mid-anthesis. For those mutants, carrot agar cultures were mainly covered with aerial mycelia, and perithecia were produced sporadically (Fig. 1A). Normal perithecia forcibly discharged ascospores, and some ascospore cirri were formed at the ostioles of the perithecia (64). Cirri were more prominent in Δcat1 and Δcat1 Δcat2 mutants than in the wild-type and complemented strains (Fig. 1B). Asci and ascospores matured normally (Fig. 1C), but forcible ascospore discharge was markedly reduced in CAT1 deletion mutants (Fig. 1D). Double deletion mutants discharged fewer ascospores than the CAT1 single deletion mutant.

**Trichothecene production and virulence test.** The mutants carrying a CAT1 deletion produced significantly fewer trichothecenes than the wild-type and other strains (Table 2). Virulence tests showed that the wild-type, CAT2 deletion, and complemented strains caused severe blight symptoms 14 days after inoculation, while the mutants with the CAT1 deletion caused significantly reduced blight symptoms in wheat heads (Fig. 2A). After 21 days, however, CAT1 single and double deletion mutants also produced severe blight symptoms, similar to those caused by the wild-type strain (Fig. 2B).

**Colocalization of CATs within peroxisomes and mitochondria.** To determine if CAT1-GFP and CAT2-GFP localized to the peroxisomes, HK54 (CAT1-GFP) and HK56 (CAT2-GFP) were outcrossed with the Δmat1 strain to generate HK69 (Δmat1; CAT1-GFP) and HK70 (Δmat1; CAT2-GFP). The resulting isolates were outcrossed again with strain HK34 (RFP-SKL) (32),

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**TABLE 2** Germination, conidium formation, and trichothecene production in G. zeae strains

| Strain        | Germination (%) | Conidium formation of conidia (no. of conidia/ml) | Total trichothecene concn (mg/g) |
|--------------|----------------|-----------------------------------------------|-------------------------------|
|              | Conidia        | Ascospores                                    |                               |
|              | CM | MM | CM | MM |                               |                               |
| Z-3639 (WT)  | 53.2A | 10.0A | 39.2A | 4.0A | 1.1 × 10⁶A | 37.7A                         |
| HK53 (Δcat1) | 74.7B | 76.0C | 82.2B | 75.6C | 7.3 × 10⁶B | 19.0B                         |
| HK59 (Δcat1 Δcat2) | 77.3B | 79.0C | 85.0B | 77.8C | 2.9 × 10⁶C | 14.6B                         |
| HK55 (Δcat2) | 80.8B | 63.8B | 77.0B | 13.2B | 1.1 × 10⁶A | 37.6A                         |
| HK54 (CAT1c) | 46.0A | 7.7A | 44.2A | 4.5A | 1.1 × 10⁶A | 35.4A                         |
| HK53 (CAT2c) | 51.8A | 8.5A | 38.0A | 4.2A | 1.2 × 10⁶A | 31.3A                         |

- a Percentage of germination measured after 4 h (conidia) or 5 h (ascospores) of incubation, CM, complete medium; MM, minimal medium.
- b Number of conidia counted after a 3-day incubation in CMC medium.
- c Total trichothecene production was measured after a 7-day incubation in minimal medium complemented with agmatine.
- d All experiments were repeated 3 times in triplicate. Values within a column that do not share a letter are significantly different from each other according to Tukey’s test (P < 0.05).
- CAT1c and CAT2c represent Δcat1 and Δcat2 mutant-derived strains complemented with corresponding CAT1-GFP and CAT2-GFP cassettes, respectively.
carrying the RFP gene fused with the peroxisomal targeting signal SKL at the C terminus (Table 1). The SKL tripeptide resulted in peroxisome localization of the RFP-SKL fusion protein (5). After isolating numerous ascospores from the outcrosses, HK75 (RFP-SKL; CAT1-GFP) and HK76 (RFP-SKL; CAT2-GFP) were selected based on their antibiotic resistance profile and on results of PCR screening, as well as their fluorescence microscopic characteristics.

CAT1 contains an N-terminal mitochondrial targeting sequence (MTS; MLASARSPRQFTKIRAAQTLSMAPKRA) (8) similar to those of other fungal CAT1 orthologs (25). CAT1-GFP was highly fluorescent in the mitochondria when acetate, butyrate, or oleate (but not glucose) was used as the sole carbon source (Fig. 3A). RFP-SKL signals did not match CAT1-GFP signals because the CAT1 C-terminal type 1 peroxisomal targeting signal (PTS1; also known as AKL) was masked by GFP at the C terminus in the HK54 strain (CAT1-GFP) (Fig. 3B; see Fig. S2B in the supplemental material). Overall, this observation suggested that an N-terminal MTS still worked to target CAT1-GFP to the mitochondria, whereas a C-terminal PTS1 did not function as a GFP fusion protein.

To confirm the peroxisomal localization of CAT1, a GFP-tagged CAT1-complemented strain (HK91; CAT1-GFP-AKL) carrying both the N-terminal MTS and C-terminal PTS1 was generated as described previously (see Fig. S3 in the supplemental material) (3, 25, 47). Strain HK92 (RFP-SKL; CAT1-GFP-AKL) was obtained by subsequent outcrosses. Unexpectedly, CAT1-GFP-AKL fluorescence was similar to that of RFP-SKL, and neither mitochondrial nor cytosolic localization was observed (Fig. 3C).

Although CAT2 does not contain any predictable subcellular localization signals (including PTS1) (23), CAT2-GFP colocalized within peroxisomes and/or cytosol when glucose, butyrate, or oleate was used as the sole carbon source. In acetate medium, however, CAT2-GFP was detected only in the cytosol (Fig. 4).

**PTS2-dependent peroxisomal localization of CAT2-GFP.** Since CAT2 does not contain PTS1, we hypothesized that CAT2 contains a type 2 peroxisomal targeting signal (PTS2) (46). To confirm this hypothesis, HK70 (Δmat1; CAT2-GFP) was outcrossed with strain Pex7SKL (Δpex7; RFP-SKL) (unpublished data) to generate strain HK77 (Δpex7; CAT2-GFP, RFP-SKL). PEX7 mediates intake of peroxisomal proteins that have PTS2 but not PTS1 (SKL). In strain HK77, therefore, RFP-SKL should localize within peroxisomes, and proteins containing PTS2 should not. While CAT2-GFP localized to peroxisomes in the wild-type background strain, CAT2-GFP in the PEX7 deletion mutant localized within peroxisomes but not to the mitochondria.

**FIG 1** Sexual development of *G. zeae* strains. (A) Vegetative growth (top) and perithecium production (bottom) on carrot agar. Arrowheads indicate unevenly formed perithecia. (B) Each strain was inoculated on carrot agar and self-fertilized. Cirri at the ostioles (arrows) were more prominent in Δcat1 and Δcat1 Δcat2 mutants than in the wild-type and complemented strains. Photographs were taken 10 days after sexual induction. Bar = 20 μm. (C) Asc and ascospores were produced normally in CAT mutants. Bar = 20 μm. (D) Forcible ascospore discharge. Photographs were taken 48 h after the assay was initiated. White cloudy material (arrows) is indicative of ascospore discharge. WT, *G. zeae* wild-type strain Z-3639; Δcat1, CAT1 deletion mutant; Δcat1 Δcat2, CAT1 and CAT2 double deletion mutant; Δcat2, CAT2 deletion mutant; CAT1c, Δcat1-derived strain complemented with CAT1-GFP; CAT2c, Δcat2-derived strain complemented with CAT2-GFP.
both single and double lipid bodies were easily detectable in the wild-type strain and in resistance tests and PCR screening. Nile red staining revealed that HK80 (notypes of the HK78 (32) was outcrossed with strain HK59 (ACS1 deletion mutant, we generated HK72 (CAT2-GFP (Fig. 7B) were highly expressed in the aerial mycelia of conidia of HK71 (ACS1 and ACS1 deletion mutant than in strains carrying the wild-type mutant, expressing both CATs were expressed more highly in the aerial mycelia of strains HK74 (CAT1-GFP), respectively. Strains HK72 (Δacs1; CAT2-GFP) and HK73 (Δcat1; CAT2-GFP), expressing CAT2-GFP but missing ACS1 or CAT1, were generated similarly (Table 1). Both CAT1-GFP (Fig. 7A) and CAT2-GFP (Fig. 7B) were highly expressed in the aerial mycelia of ACS1 and CAT2 deletion mutants compared to those of strains carrying wild-type ACS1 and CAT2 alleles.

Role of CATs in lipid production in an ACS1 deletion mutant. Both CATs were expressed more highly in the ACS1 deletion mutant than in strains carrying the wild-type ACS1 allele (Fig. 6 and 7). To confirm the supplementary role of CATs in lipid production in the ACS1 deletion mutant, we generated CAT deletion mutants carrying the ACS1 deletion. Strain HK29 (Δmat2 Δacs1) (32) was outcrossed with strain HK59 (Δcat1 Δcat2), and the genotypes of the HK78 (Δacs1 Δcat1), HK79 (Δacs1 Δcat2), and HK80 (Δacs1 Δcat1 Δcat2) strains were confirmed by antibiotic resistance tests and PCR screening. Nile red staining revealed that lipid bodies were easily detectable in the wild-type strain and in both single and double CAT deletion mutants, but mutants carrying both ACS1 and CAT1 deletions did not contain visible lipid bodies in aerial mycelia (Fig. 8).

Carbon source utilization. Single CAT1 deletion mutants presented with severe defects in carbon utilization, even on minimal medium supplemented with glucose as the sole carbon source. CAT2 deletion mutants rarely used acetate and ethanol but grew normally using butyrate, similar to the wild-type strain. Long-chain fatty acid utilization of the CAT2 deletion mutant was affected slightly (Fig. 9). In contrast, the Δcat2 mutant-derived, CAT2-GFP-complemented strain regained the wild-type phenotype. However, the ability to utilize butyrate and oleate was severely impaired in the CAT1-GFP-complemented mutant (HK54). Strain HK91 (CAT1-GFP-AKL) presented with a minimally reduced growth profile on glucose medium and grew slowly in the presence of other carbon sources. Strain HK74 (Δcat2; CAT1-GFP), carrying both the CAT2 deletion and CAT1-GFP, showed a similar growth pattern to that of the Δcat1 mutant-derived, CAT1-GFP-complemented HK54 strain (Fig. 9).

ACS1 single mutants and double deletion mutants missing both ACS1 and PDC1 utilized carbon sources similarly to the CAT2 deletion mutant. The PDC1 deletion mutant, however, was not deficient in carbon utilization. The Δgicl1 strain, carrying a deletion of the isocitrate lyase gene (GzICL1; encodes a key enzyme involved in the glyoxylate cycle) (31), grew poorly on carbon sources other than glucose compared to the wild-type Z-3643 strain (Fig. 9).

DISCUSSION

Gibberella zeae utilizes various carbon sources depending on its developmental stage and environmental conditions. During biotrophic or saprophytic growth, this fungus uses carbon sources from plant tissues or debris and accumulates large amounts of triacylglycerides (16, 32). During sexual development, accumu-
lated triacylglycerides composed mainly of 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol (32) serve as primary carbon sources, providing building blocks and serving as an energy source for peritheciium and ascospore development (52). In this regard, G. zeae has evolved unique and efficient carbon utilization systems that allow it to adapt to its environmental conditions (38). The present study provides data that expand our understanding of carbon utilization of this fungus via CATs.

Sufficient production of acetyl-CoA in G. zeae is required for the development of this fungus. Acetyl-CoA is produced in the

FIG 3 Cellular localization of CAT1-GFP (A and B) and CAT1-GFP-AKL (C) following growth on different carbon sources. Mycelia were harvested for microscopic observation 12 h after inoculation of conidia into liquid MM supplemented with glucose, sodium acetate, sodium butyrate, or Tween 80 (oleate source). DIC, differential interference contrast microscopy images. The middle two rows of panels show MitoTracker and RFP-SKL staining, representing the mitochondria and peroxisomes, respectively. The yellow areas in the merged images indicate colocalization in the bottom panels. Bars = 10 μm.

FIG 4 Cellular localization of CAT2-GFP in the presence of different carbon sources. Mycelia were harvested for microscopic observation 12 h after inoculation of conidia into liquid minimal medium supplemented with glucose, sodium acetate, sodium butyrate, or Tween 80 (oleate source). The yellow areas in the merged images indicate colocalization in the bottom panels. Bar = 10 μm.
nuclei by ACL, and ACS2 is responsible for histone acetylation to regulate perithecium development and ascospore delimitation (32, 52). Further phenotypic analyses of ACS1 and PDC1 deletion mutants demonstrated that acetate is readily generated in the mycelia for cytosolic acetyl-CoA production via the PAA pathway and that PAA pathway intermediates are responsible for the phenotypic changes observed in the ACS1 deletion mutant (32, 53).

Both CATs were highly expressed in an ACS1 deletion mutant (Fig. 6 and 7), but only CAT1 was responsible for lipid accumulation in aerial mycelia in the ACS1 deletion mutant (Fig. 8). Because ACS1 deletion triggers acetate accumulation (32) and CATs have been shown to be required for acetyl-CoA transport between the cytosol and peroxisomes and/or mitochondria, accumulation of high levels of acetate must be avoided by converting acetate to acetyl-CoA via an alternative enzyme reaction, such as that of acetyl-CoA hydrolase, which seems to be the alternate acetate user for acetyl-CoA production. In fact, carnitine-independent acetyl-CoA hydrolase (and its role in acetate detoxification) has been proposed to occur in various organisms, including fungi (9, 14, 29, 34).

Besides the supplementary role of CAT1 in lipid production in the ACS1 deletion mutant, CAT1-dependent acetyl-CoA transport is particularly important to the G. zeae life cycle. CAT1 deletion resulted in severe defects in vegetative growth, asexual/sexual reproduction, virulence, and toxin production in G. zeae. However, the CAT1 homolog Cat2p/Ctn2p of the hemiascomycete Candida albicans contributes to biofilm formation only, not to glucose-dependent growth or pathogenesis (58, 71). Moreover,

**FIG 6** Transcript analysis of CAT and ACS1 genes. Total RNAs were extracted from 5-day-old carrot agar cultures before sexual induction. Gene names are denoted on the left. An ethidium bromide-stained gel is shown as a loading control. WT, G. zeae wild-type strain Z-3639; Δacs1, ACS1 deletion mutant; Δcat1, CAT1 deletion mutant; Δcat2, CAT2 deletion mutant; Δcat1 Δcat2, double deletion mutant lacking CAT1 and CAT2.

**FIG 7** Expression of CAT1-GFP (A) and CAT2-GFP (B). Differential interference contrast (DIC) and fluorescence microscopic observations were conducted 3 days after inoculation of conidia onto carrot agar. CAT1-GFP, Δcat1 mutant-derived strain complemented with CAT1-GFP; Δacs1 CAT1-GFP, strain HK33 (Δacs1) carrying CAT1-GFP; Δcat2 CAT1-GFP, strain HK35 (Δcat1) carrying CAT1-GFP; CAT2-GFP, Δcat2 mutant-derived strain complemented with CAT2-GFP; Δacs1 CAT2-GFP, strain HK33 (Δacs1) carrying CAT2-GFP; Δcat1 CAT2-GFP, strain HK53 (Δcat1) carrying CAT2-GFP. Bars = 20 μm.

**FIG 8** Lipid accumulation analysis of G. zeae strains. Lipid accumulation in 3-day-old hyphae from carrot agar cultures was visualized with Nile red staining. WT, G. zeae wild-type strain Z-3639; Δacs1, ACS1 deletion mutant; Δcat1, CAT1 deletion mutant; Δcat2, CAT2 deletion mutant; Δcat1 Δcat2, double deletion mutant lacking CAT1 and CAT2; Δacs1 Δcat1, double deletion mutant lacking ACS1 and CAT1; Δacs1 Δcat2, double deletion mutant lacking ACS1 and CAT2; Δacs1 Δcat1 Δcat2, triple deletion mutant lacking ACS1, CAT1, and CAT2. Bar = 20 μm.
Traditionally, \( \beta \)-oxidation in fungi was thought to occur exclusively in the peroxisomes (22, 28). However, a previous study revealed that mitochondrial \( \beta \)-oxidation also exists in filamentous fungi (37). Fungi exhibit diverse \( \beta \)-oxidation pathways, and the combined presence of mitochondrial and peroxisomal pathways is the most common situation (50). As mentioned above, we suspect that mitochondrial \( \beta \)-oxidation of \( G. \) zeae is responsible for oleate utilization in strain HK74. Alternatively, CAT-independent pathways might be involved in acetyl-CoA export, which is general for peroxisomal CAT function (Fig. 9 and 10D and F).

Strain HK74 (\( \Delta \)cat2; \( \Delta \)cat1-GFP), which had no peroxisomal CAT activity, still utilized oleate to some degree, suggesting that peroxisomal CAT is not essential to oleate utilization by \( G. \) zeae (Fig. 9 and 10D). Butyrate utilization was observed only in the wild-type and CAT2 deletion (HK55) strains, in which both peroxisomal and mitochondrial CATs were functional (Fig. 9 and 10A and C). Overall, short-chain fatty acids such as butyrate seem to be metabolized exclusively via peroxisomal \( \beta \)-oxidation, whereas both peroxisomal and mitochondrial \( \beta \)-oxidation metabolizes long-chain fatty acids (Fig. 10A). Peroxisomal localization of CAT is necessary for fatty acid growth in \( A. \) nidulans (25). Strain HK74 utilized acetate and ethanol, whereas the CAT2 deletion mutant could not (Fig. 9), suggesting that CAT1-GFP additionally colocalizes to both the cytosol and mitochondria (Fig. 10D and F).

Traditionally, \( \beta \)-oxidation in fungi was thought to occur exclusively in the peroxisomes (22, 28). However, a previous study revealed that mitochondrial \( \beta \)-oxidation also exists in filamentous fungi (37). Fungi exhibit diverse \( \beta \)-oxidation pathways, and the combined presence of mitochondrial and peroxisomal pathways is the most common situation (50). As mentioned above, we suspect that mitochondrial \( \beta \)-oxidation of \( G. \) zeae is responsible for oleate utilization in strain HK74. Alternatively, CAT-independent pathways might be involved in acetyl-CoA export, which is general.
erated from peroxisomal β-oxidation. For example, the intermediates of the glyoxylate cycle are possible candidates for acetyl group translocation between peroxisomes, cytosol, and/or mitochondria, because some components (such as citrate and succinate) are key metabolites of both the glyoxylate and tricarboxylic acid cycles and are able to permeate subcellular membranes (1, 2, 33). Our results show that one of the key enzymes involved in the glyoxylate cycle is crucial for acetate and fatty acid utilization (Fig. 9).

It was supposed that CAT mutants presented with abnormalities associated with perithecium formation because lipid utilization is closely linked to sexual development in *G. zeae* (16). However, CAT double deletion mutants rarely used fatty acids as a sole carbon source (Fig. 9) yet produced almost normal perithecia. This result suggests the presence of complex metabolic processes for fatty acid degradation during perithecium formation in this fungus.

Through sexual development, ascospores produced in the perithecia are forcibly discharged into the air. The main force responsible for the observed discharge is turgor pressure within the extended asci (62). In *G. zeae*, buildup of K⁺, Cl⁻, and polyols (including glycerol) drives the influx of water that causes the turgor pressure to stretch the asci (40, 64). In this study, deletion of the *CAT1* gene greatly reduced the number of discharged ascospores (Fig. 1D). This result suggested that CAT1 is involved in turgor pressure formation, because the fungi produced more cirri when active discharge was not optimal (65). Influx of ions is coupled with the efflux of protons, which demands larger amounts of ATP (62) to be generated via the mitochondrial respiratory chain, which is closely linked to mitochondrial acetyl-CoA production (33). In addition, many transport ATPases are highly expressed during sexual development in *G. zeae* (19). Therefore, the defect in ascospore discharge observed in CAT1 deletion mutants might be due to deficient ATP generation.

Peroxisomal β-oxidation and carnitine-dependent transport of peroxisomal acetyl-CoA are important to *M. oryzae* virulence (3, 47, 67). To examine these properties, mutants carrying a mislocalized CAT1 chimeric protein were generated. Strain HK54, carrying only mitochondrial CAT1, not peroxisomal CAT1 (Fig. 1D), had a wild-type phenotype. However, strain HK91, with peroxisomal CAT1 (Fig. 1E), was severely defective in the ability to carry out vegetative growth on glucose (Fig. 9).
GFP-AKL cassette of HK91 did not localize to mitochondria even though the chimeric protein had an N-terminal MTS. Therefore, further studies will be needed to determine whether transcriptional or translational initiation sites were shifted as a result of GFP tagging, resulting in changes to protein structure that in turn affected protein localization. Taken together, our results demonstrate that CAT1-mediated transport of acetyl-CoA between the mitochondria and the cytosol plays important roles during various developmental stages, as well as in conferring virulence. Furthermore, peroxisomal acetyl-CoA transport seems to be achieved by alternative pathways. During mycelial growth, the FAA pathway of G. zeae functions to translocate carbon from embedded mycelia to aerial mycelia via subplasmalemmal mycelia to aerial mycelia for lipid body formation and energy generation by embedded mycelia (53). Cytosolic acetyl-CoA generated via these processes likely enters the mitochondria via subsequent reversible reactions mediated by cytosolic CAT2 and mitochondrial CAT1 and used in the production of energy sources (Fig. 10A). Reduced mycelial growth and the degree of virulence observed in the CAT1 deletion mutants might be affected by deficient energy generation.

In this study, we found that CAT1 functions as an alternative acetyl-CoA source that is needed for lipid accumulation in an ACS1 deletion mutant. Moreover, both CAT deletions resulted in various defects in development and virulence. CAT1 mislocalization experiments demonstrated that the role of CAT in acetyl-CoA transport between the mitochondria and the cytosol/peroxisomes is particularly important for G. zeae development. Based on the current results, we conclude that G. zeae CATs facilitate acetyl-CoA transport across the intracellular membranes of peroxisomes, mitochondria, and cytosol at various developmental stages.

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