Functional Analyses of Two Acetyl Coenzyme A Synthetases in the Ascomycete Gibberella zeae

Seunghoon Lee,1 Hokyoung Son,1 Jungkwan Lee,2 Kyunghun Min,1 Gyung Ja Choi,3 Jin-Cheol Kim,3 and Yin-Won Lee1*

Department of Agricultural Biotechnology and Center for Fungal Pathogenesis, Seoul National University, Seoul 151-921, Republic of Korea1; Department of Applied Biology, Dong-A University, Busan 604-714, Republic of Korea2; and Biological Function Research Team, Korea Research Institute of Chemical Technology, Daejeon 305-343, Republic of Korea3

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Acetyl coenzyme A (acetyl-CoA) is a crucial metabolite for energy metabolism and biosynthetic pathways and is produced in various cellular compartments with spatial and temporal precision. Our previous study on ATP citrate lyase (ACL) in Gibberella zeae revealed that ACL-dependent acetyl-CoA production is important for histone acetylation, especially in sexual development, but is not involved in lipid synthesis. In this study, we deleted additional acetyl-CoA-synthetic genes, the acetyl-CoA synthetases (ACS genes ACS1 and ACS2), to identify alternative acetyl-CoA production mechanisms for ACL. The ACS1 deletion resulted in a defect in sexual development that was mainly due to a reduction in 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol production, which is required for perithecium development and maturation. Another ACS coding gene, ACS2, has accessory functions for ACS1 and has compensatory functions for ACL as a nuclear acetyl-CoA producer. This study showed that acetate is readily generated during the entire life cycle of G. zeae and has a pivotal role in fungal metabolism. Because ACSs are components of the pyruvate-acetaldehyde-acetate pathway, this fermentation process might have crucial roles in various physiological processes for filamentous fungi.

The homothallic ascomycete fungus Gibberella zeae (anamorph Fusarium graminearum) is a prominent plant pathogen of major cereal crops, such as wheat, barley, maize, and rice. Fungal disease caused by this fungus leads to yield loss, and the harvested grains are frequently contaminated with mycotoxins that threaten human and animal health (15, 42). This fungus reproduces both sexually and asexually, and ascospores produced by sexual reproduction are believed to play a role in fungal survival and disease initiation. Many studies have been performed to characterize the mechanisms of sexual reproduction (4, 24, 28, 32, 35, 38, 39, 47, 55, 68, 72, 75). Recently, we showed that acetyl coenzyme A (acetyl-CoA) is an important metabolite for sexual development in this fungus (57).

Acetyl-CoA is a crucial metabolite in energy metabolism and the biosynthesis of many cellular components. First, mitochondrial acetyl-CoA supplied from the mitochondrion-associated pyruvate dehydrogenase complex or β-oxidation is used to replenish the mitochondrial tricarboxylic acid (TCA) cycle. Therefore, mitochondrial acetyl-CoA is essential in the production of energy sources for the survival and normal growth of eukaryotic cells (40). However, in some eukaryotes, including the protozoan parasite Trypanosoma brucei and some plants, mitochondrial acetyl-CoA is crucial for lipid biosynthesis through the conversion to acetate for either cytosolic acetyl-CoA generation or direct mitochondrial fatty acid synthesis (20, 50, 51).

Second, peroxisomal acetyl-CoA, which is mainly produced via β-oxidation, enters the glyoxylate cycle for acetyl unit utilization. Plants and several microorganisms have a glyoxylate cycle for the utilization of nonfermentable carbon sources, such as fatty acids, ethanol, and acetate. In consideration of the function of peroxisomes as places for the biosynthesis of lipids and amino acids, it is also possible to predict that peroxisomal acetyl-CoA takes part in lipid synthesis (14). Moreover, recent studies of fungi have revealed a connection between peroxisomal acetyl-CoA and the early steps of mycotoxin production (11, 31).

Lastly, cytosolic acetyl-CoA is an essential building block for the biosynthesis of fatty acids and numerous secondary metabolites. Acetyl-CoA carboxylase mainly controls fatty acid synthesis via malonyl-CoA production from cytosolic acetyl-CoA. The mevalonate pathway, which is required for the synthesis of various metabolites including sterol, is also created from cytosolic acetyl-CoA through the composition of 3-hydroxy-3-methylglutaryl-CoA by two enzymatic reactions (29, 40). Acetyl-CoA is also used for the acetylation of lysine residues in many proteins, and this posttranslational modification has a key role in protein stability and function (67, 74). Because the nuclear envelop is permeable to acetyl-CoA, citrate, and even cytosolic acetyl-CoA, histone acetylation is also strictly regulated by nucleocytosolic acetyl-CoA levels and related genes (48, 57, 63, 70).

The origins of cytosolic acetyl-CoA vary and depend on the living conditions and developmental stages of organisms. In most cases, cytosolic acetyl-CoA is translocated from peroxisomes and mitochondria via specified shuttle systems. Carni-
tine acetyl-transferases (CATs) are known to transport acetyl groups between peroxisomes or mitochondria and the cytosol and have important roles in several metabolic pathways (6, 59, 62). Carnitine-independent acetyl group movement by peroxisomal thioesterase was also proposed to occur in mammalian cells (41). ATP citrate lyase (ACL)-dependent cytosolic acetyl-CoA generation is the most common pathway in eukaryotes and some prokaryotes (18, 25). Citrate is that exported from the TCA cycle in mitochondria through tricarboxylate carriers is cleaved into oxaloacetate and cytosolic acetyl-CoA by ACL. In the case of Saccharomyces cerevisiae and Candida albicans, however, cytosolic acetyl-CoA is synthesized through the pyruvate-acetaldehyde-acetate pathway, which is mediated by acetyl-CoA synthetases (ACSs) (10, 17, 60, 61, 63, 66). ACSs are conserved in most eukaryotes and are thought to evolve from a mitochondrial origin (34).

Because of the significant roles of acetyl-CoA, most organisms have evolved their own appropriate metabolic processes for the precise temporal (developmental stages) and spatial (organelles) generation of acetyl-CoA. One in silico survey revealed an astounding diversity of metabolic pathways, even within the fungal kingdom, and showed variant metabolic processes across fungi (54). Thus, for basic or applied studies of single species, an in-depth understanding of acetyl-CoA metabolism is important.

A previous study on the functional characterization of ACL in G. zeae revealed that both ACL subunits are essential for ACL function in glucose utilization, development, trichothecene production, and virulence. The ACL genes were not required for de novo lipid synthesis but were required for histone acetylation during sexual development and possibly during asexual growth (57). In this previous work, we have three questions: (i) Which pathways in acetyl-CoA production are required for lipid synthesis? (ii) What enzymes enable ACL mutants to survive and grow to some degree? (iii) What are the functions and functional relationships among the cytosolic acetyl-CoA generating enzymes?

In this study, we studied the two ACS genes (ACS1 and ACS2) as target candidates for addressing the first two questions. Phylogenetic analysis revealed that some fungal species, including G. zeae, have two ACS genes; however, Aspergillus nidulans and Neurospora crassa have only one ACS coding gene each, facA and aceU-5, respectively (2, 13, 19, 53). We hypothesized that two ACSs are major acetyl-CoA producers for lipid synthesis. However, since acetate is not a physiological carbon source for G. zeae, we also hypothesized that acetate-producing mechanisms, such as aerobic fermentation, are important for acetyl-CoA production in this fungus.

### MATERIALS AND METHODS

**Fungal strains and media.** G. zeae wild-type strain GZ3639 (8) and transgenic G. zeae strains derived from this strain were used in this study. All mutant strains used in this study are listed in Table 1. Conidial suspensions of all of the derived or received strains were stored in 20% glycerol at −70°C. Minimal medium containing 5 mM agmatine (MMA) was used for trichothecene production (21).

**Nucleic acid manipulation and PCR primers.** Fungal genomic DNA was extracted as previously described (42), and other standard procedures for Southern and Northern hybridization with 32P-labeled probes were performed following standard protocols (52). Total RNA was extracted using an Easy-Spin Total RNA Extraction Kit (Intron Biotech, Seongnam, South Korea) following the manufacturer's instructions. PCR primers used in this study were synthesized by an oligonucleotide synthesis facility (Bionics, Seoul, South Korea) (see Table S1 in the supplemental material).

| Strain     | Genotype                     | Source or reference |
|------------|------------------------------|---------------------|
| GZ3639     | Wild type                    | This study          |
| HK22       | Δacs1::gen                   | This study          |
| HK24       | Δacs2::gen                   | This study          |
| HK23       | Δacs1::ACS1-GFP-hyg          | This study          |
| HK25       | Δacs2::ACS2-GFP-hyg          | This study          |
| HK31       | Δacs1::gen Δacs2::gen        | This study          |
| Δmat2 strain | Δmat1-2::GFP-hyg             | 36                  |
| HK29       | Δmat1-2::GFP-hyg Δacs1::gen  | This study          |
| HK30       | Δmat1-2::GFP-hyg Δacs2::gen  | This study          |
| ac12       | Δacs1::gen                   | 57                  |
| HK32       | Δacs2::gen Δacs1::gen        | This study          |
| HK33       | Δacs2::gen Δacs2::hyg-Pear-ACS2 | This study              |
| Δmat1 strain | Δmat1-1::gen                 | 36                  |
| mat1r      | Δmat1-1::gen HH1-RFP-gen     | 57                  |
| HK34       | gen-RFP-SKL                  | This study          |
| HK35       | Δmat1-2::GFP-hyg gen-RFP-SKL | This study          |
| HK36       | Δacs1::ACS1-GFP-hyg HH1-RFP-gen | This study              |
| HK37       | Δacs2::ACS1-GFP-hyg HH1-RFP-gen | This study              |
| HK38       | Δacs1::ACS1-GFP-hyg gen-RFP-SKL | This study              |
| HK39       | Δacs2::ACS2-GFP-hyg gen-RFP-SKL | This study              |
| Pezar-GzmetE | GzmetE::hyg-Pear-GzmetE      | 37                  |

**Lipid analyses.** Total lipid extraction and analyses were done according to a previous study (57). In brief, each strain was inoculated on plates containing 20 ml of yeast extract and harvested at 5 days after inoculation. Harvested samples were dried in a ventilated hood for 5 days. The dried cultures were ground in a blender, and 1 g of powder was extracted with 8 ml of n-hexane. The extract was evaporated to dryness in a Speed Vac concentrator (Savant Instrument, Inc., Farmingdale, NY), and the total amount of lipid was weighed.

**Lipid characterization of POL.** Dried cultures (300 g) were extracted with 1 liter of n-hexane followed by 1 liter of n-hexane three times and concentrated to dryness. The residual extract was dissolved in 30 ml of n-hexane and was applied at homogeneity by chromatography on a column (1 m b y 5 cm [inside diameter]) containing silica gel 60 (70 to 230 mesh; Merck, Germany). The column was then eluted with n-hexane followed by n-hexane-ethyl acetate (10:1, vol/vol). The fractions containing 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol (POL) were combined, and the purity was confirmed by thin-layer chromatography (57).

**Lipid characterization of POL.** The authentic POL was purchased from Sigma-Aldrich Co. (St. Louis, MO). The chemical identification of POL was determined by using spectroscopic analyses. Atmospheric pressure chemical ionization (APCI)-mass spectrum was recorded on a quadrupole mass spectrometer equipped with an APCI source (HP 1100 series LC/MSD; Hewlett-Packard Co., Palo Alto, CA). The vaporizer was operated at 540°C, and the inlet capillary was operated at 550°C. The corona discharge needle was set to 4.0 μA. High-purity nitrogen was used for the sheath and auxiliary gases. Nebulizer pressure and drying gas flow were set to 60 lb/in2 and 4 ml/min, respectively. 1H-NMR (where NMR is nuclear magnetic resonance) spectra were recorded on a Bruker AMX500 (500 MHz) instrument.
during the regeneration, overlay, and mutant selection processes (37). Pzear
the final construct was amplified with primers ACS2-5N/ACS2-3N pzear. For
tively. Three fragments were fused according to the DJ PCR method (73), and
Gze metE
is
hyg-pzear
promoter with the zearalenone-inducible promoter (ACS2
supplemental material).
For double deletion between the
new and EF Pro-Rev. The
GEN vector (see Fig. S1 in the supplemental material) with primers Neo-For
factor 1
HK23 (ACS1c) or HK25 (ACS2c). Ascospores carrying
localization of ACS1-GFP and ACS2-GFP with nuclei, the mat1r strain carrying
14 days. Inoculated plants were placed in a greenhouse after 3 days of incubation in a
valenol) from MMA were analyzed with a Shimadzu QP-5050 GC-MS with a
a rotary shaker (150 rpm). Trichothecenes (deoxynivalenol and 15-acetyldeoxyni-
conidia produced after incubating 10
(MMACD). Conidium production was measured by counting the number of
mented with 40 mM potassium acetate as the sole carbon source (MMAc), MM
Radial growth was measured from mycelia grown for 5 days on MM supple-
10 days. Mitochondria were stained with MitoTracker (Invitrogen, Carlsbad, CA)
according to the manufacturer’s instructions. Microscopic observation was per-
dependent mutants were confirmed by Southern hybridiza-
Identification of ACS genes and phylogenetic analysis. The
AcS2 genes are named differently in many organisms, including
fungus species. We identified two AcS2 genes in G. zeae from the Fusarium Comparative Database (http://www
Species, Magnaportha oryzae, and S. cerevisiae had two AcS2s, while A. nidulans and N. crassa had only one AcS2 each. One of the AcS2s in Fusar-
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neighbor-joining algorithm with bootstrap values calculated from 1,000 iterations. The Fusarium species, Magnaporthe oryzae, and S. cerevisiae had two AcS2s, while A. nidulans and N. crassa had only one AcS2 each. One of the AcS2s in Fusar-
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TABLE 2. Radial growth and conidium production in G. zeae strains

| Strain                  | MMS   | MMAc  | MMEtOH | MMACD |
|-------------------------|-------|-------|--------|--------|
| Wild type               | 84.0 A| 84.6 A| 69.0 A | 4.0 A  |
| Δacs1 strain            | 83.3 A| 40.0 B| 35.3 B | 2.4 B  |
| Δacs2 strain            | 84.0 A| 84.0 A| 65.0 A | 4.2 A  |
| Δacs1 Δacs2 strain      | 73.3 B| 18.3 C| 10.7 C | 1.0 C  |
| ACS1c                   | 84.0 A| 84.6 A| 67.0 A | 4.3 A  |
| ACS2c                   | 84.3 A| 85.3 A| 65.3 A | 4.2 A  |

Conidiation (no. of conidia/ml)

| Strain                  |       |       |       |       |
|-------------------------|-------|-------|-------|-------|
| Wild type               | 2.31 × 10^6 A |       |       |       |
| Δacs1 strain            | 2.34 × 10^6 A |       |       |       |
| Δacs2 strain            | 2.40 × 10^6 A |       |       |       |
| Δacs1 Δacs2 strain      | 1.16 × 10^6 B |       |       |       |
| ACS1c                   | 2.31 × 10^6 A |       |       |       |
| ACS2c                   | 2.47 × 10^6 A |       |       |       |

* Radial growth was measured after 5 days of incubation. All data were repeated three times with three replications. Values within a column with different letters are significantly different (P < 0.05) based on a Tukey test. MMS, minimal medium supplemented with 2% of sucrose; MMAc, minimal medium supplemented with 40 mM potassium acetate as the sole carbon source; MMEtOH, minimal medium supplemented with 40 mM ethanol as the sole carbon source; MMACD, minimal medium supplemented with 5 mM acetaldheyde as the sole carbon source.

**Conidiation was measured by counting the number of conidia after 3 days of incubation.**

HK30 (Δmat2 Δacs2) and HK22 (Δacs1), we obtained three strains carrying a double deletion of ACS1 and ACS2 (HK31).

The ACS1 deletion mutant showed normal conidium production but a defect in mycelial growth on MMAc, MMEtOH, and MMACD. Normal mycelial growth resumed when the colony was transferred to MM supplemented with sucrose (MMS). The ACS2 deletion mutant did not show any defect in growth or conidium production. The double deletion mutant (HK31) had a more severe defect in growth than the ACS1 mutant (HK22) and even showed impairment in growth on MMS and conidium production (Table 2). The virulence of all of the mutants used in this study was similar to that of the wild-type strain (see Fig. S3 in the supplemental material), and trichothecene production of all of the mutants was not significantly different from that of the wild-type strain (P = 0.67) (see Fig. S4).

**Lipid quantification and characterization of POL.** Total lipid production in the ACS1 mutant and double deletion mutant was lower than that of the wild-type strain, and the double deletion mutant produced approximately 50% of the amount of lipid as the ACS1 mutant (Fig. 2). We purified 4.4 g of the major compound in the lipid extract from 300 g of fungal cultures. The compound showed a [M+Na]^+ ion at m/z 880 and major fragmentation ions at m/z 854, 604, 578, and 265 by APCI mass spectrometry. The 1H-NMR spectrum was identical to that of authentic POL (see Fig. S5 in the supplemental material). In the GC-MS analysis of FAMEs, three peaks appeared in the total ion chromatogram at retention times of 20.1 min, 25.8 min, and 27.4 min, and their mass spectra were identical to those of the methyl esters of palmitic acid, oleic acid, and linoleic acid, respectively (Fig. 3).**

**Fertility test.** The HK22 (Δacs1) strain produced fewer mature perithecia than the wild-type strain 7 days after sexual induction. HK24 (Δacs2) and complemented strains produced normally melanized mature perithecia containing viable ascospores. The defect in perithecial development was more severe in the double deletion mutant (HK31) (Fig. 4). Perithecial maturation of HK31 (Δacs1 Δacs2) was variable. In five independent sexual induction tests, the HK31 strain did not produce any visible perithecia in two of the trials and produced a few mature perithecia in the remaining three trials. After 14 days, a few perithecia from both the ACS1 and double mutants were matured and normally discharged ascospores. When 0.5% POL was exogenously added to carrot agar, the perithecial maturation of HK22, but not the HK31 (Δacs1 Δacs2) double deletion mutant, was mostly restored. Treatment of the wild-type strain with POL enhanced melanin accumulation and perithecial production (Fig. 4).

**Transcriptional analyses of ACSs.** To determine whether deletion of ACS1 or ACS2 affects the expression of the other ACS gene, we performed Northern hybridization in the wild-type strain and each deletion mutant. Transcription profiles of ACS1 and ACS2 were not altered in the ACS2 and ACS1 deletion mutants, respectively, suggesting that transcriptional regulations of the genes are independent of each other (see Fig. S6 in the supplemental material).

**Generation of double deletion mutants carrying the ACL2 deletion.** Each HK29 (Δmat2 Δacs1) and HK30 (Δmat2 Δacs2) strain was fertilized with strain acl2 (Δacl2) to generate a double deletion mutant carrying both ACL2 and ACS1 or ACS2. We isolated 60 ascospores from the HK29 × acl2 out-cross and obtained six progenies carrying both ACL2 and ACS1 deletions. From the HK30 × acl2 out-cross, however, we did not obtain any progeny carrying both ACL2 and ACS2 deletions even though we isolated 194 ascospores from two independent out-crosses. In these out-crosses, 19 isolated ascospores (9%) did not germinate. Repeated attempts to generate double deletion mutants of ACS2 and ACL2 by transformation were also unsuccessful.

Since we hypothesized that the Δacl2 Δacs2 mutant was lethal, we replaced the promoter of ACS2 with Pzear in the ACL2 deletion mutant using the previously reported strategy...
The hpy-pzeart construct amplified from the Pzeart-GzmE strain was successfully introduced into the promoter of ACS2 in the acl2 strain (Fig. 5A). The promoter-replaced mutant, HK33 (Δacl2 ACS2p), exhibited a severe defect in growth on complete medium, but the defect was restored by exogenous treatment with 30 μM ZEA (Fig. 5B).

Similar to the acl2 (Δacl2) mutant, HK32 (Δacl2 Δacs1) did not produce any initial structures for perithecia even when potassium acetate was added in the medium but produced immature perithecia with ZEA treatment. Similar to the sexual development observations, mycelium growth was somewhat recovered in the acl2 and HK32 mutants when potassium acetate was added, but the HK33 mutant did not grow (Fig. 6B).

**Cellular localization of ACS1-GFP, ACS2-GFP, and ACL2-GFP.** We successfully introduced constructs containing wild-type genes fused with GFP into the corresponding deletion mutants and generated HK36 (ACS1-GFP hH1-RFP), HK37 (ACS2-GFP hH1-RFP), HK38 (ACS1-GFP RFP-SKL), and HK39 (ACS2-GFP RFP-SKL) strains using out-crossing strategies as previously described (see Fig. S2 in the supplemental material) (57).

Even though both ACS1 and ACS2 do not contain any subcellular localization signals, they showed different subcellular localization patterns. ACS1-GFP was highly fluorescent in peroxisomes when acetate was used as the sole carbon source in submerged culture but was not visible when MMG and MMGAc liquid cultures were used. In aerial mycelia, ACS1-GFP localized in peroxisomes when MMAc was used but mainly localized to the cytosol when MMG, MMGAc, and carrot agar were used (Fig. 7A). The colocalization study of ACS1-GFP suggested that it did not notably localize in nuclei and mitochondria (see Fig. S7 in the supplemental material).

ACS2-GFP was also highly expressed and localized in the cytosol and nuclei of submerged mycelia regardless of the presence of either glucose or acetate (Fig. 7B). In aerial mycelia, ACS2-GFP was not detected in the cytosol, and addition of acetate in solid medium caused ACS2-GFP to mainly localize in nuclei (Fig. 7B). As previously reported, ACL2-GFP localized in the cytosol in hyphae and was highly expressed in mycelia grown in all media except for MMAc (57) (Fig. 7C).

**DISCUSSION**

Our current study demonstrated that two ACSs have both overlapping and distinct functions in G. zeae. ACSs are not required for virulence and trichothecene production (see Fig. S3 and S4 in the supplemental material). However, ACS1 is required for the utilization of several nonfermentable carbon sources, suggesting that ACS1 mainly functions in the pyruvate-acetaldehyde-acetate pathway in this fungus (Fig. 8). In addition, ACS1 is required for perithecium maturation. Lipid quantification and complementation with POL suggested that the sexual defect of the deletion mutant is mainly caused by reduced lipid production. Although the ACS2 single mutant did not show any phenotype, ACS2 also had some overlapping roles with ACS1 since the double mutant (Δacs1 Δacs2) showed more severe defects than the ACS1 mutant. Moreover, ACS2 had accessorial functions for ACL that were required for nuclear acetyl-CoA production in G. zeae (Fig. 8).

ACS1 is required for cytosolic and peroxisomal acetyl-CoA production in G. zeae (Fig. 8). Our lipid analysis and POL complementation test suggested that cytosolic acetyl-CoA produced by ACS1 is crucial for perithecium development (Fig. 2 and Fig. 4). However, since perithecium development of the double deletion mutant was not restored even with POL treatment, ACS-dependent acetyl-CoA production might be required for variable physiological processes in addition to lipid production. Considering that peroxisomes also take part in lipid synthesis, ACS1-derived lipid synthesis might be related to its localization (Fig. 7A) (14). Furthermore, one of the functions of the peroxisome is to detoxify toxic metabolites

FIG. 3. Total ion chromatogram of the fatty acid methyl esters of POL isolated from G. zeae (A) and mass spectra (B) of methyl esters of palmitic acid (1), oleic acid (2), and linoleic acid (3) with retention times of 20.1 min, 25.8 min, and 27.4 min, respectively.
produced by oxidative reactions (40). Because acetaldehyde, ethanol, and acetate are toxic intermediates, they may need to be detoxified in peroxisomes by ACS1 and proceed to the glyoxylate cycle simultaneously.

POL is a stimulatory compound for perithecium development in *G. zeae*. Previous studies have reported that *G. zeae* accumulates an abundance of triacylglycerides for sexual development (23, 57). Because perithecium formation requires enormous energy sources and building blocks, previous researchers hypothesized that triacylglycerides accumulated for storage. We characterized a major compound of triacylglyceride, POL, which enhanced melanin production and peritheciem development (Fig. 4).

ACS2 localized to both the cytosol and nuclei and exhibited functions that compensated for ACL since the double deletion of *ACL2* and *ACS2* was lethal (Fig. 8). The double deletion of the acetyl-CoA synthetase gene *facA* and the *acl* gene was also lethal in *A. nidulans* (29). However, ACS1 is not a compensatory enzyme for the function of ACL even though the roles of ACS1 are similar to those of FacA in *A. nidulans*, which is also required for acetate utilization (13). The conditional gene expression of *ACS2* was created using a ZEA-inducible promoter (37) to generate HK33 (*H9004acs1 ACS2p*) in order to confirm the roles of ACS2 in the *ACL* mutant. Chemical complementation of the *ACL* mutant by exogenous treatment with acetate during both sexual and asexual development was repressed only in the HK33 mutant without ZEA treatment (Fig. 6). This result suggests that ACS2 is required for acetate-derived acetyl-CoA

**FIG. 4.** Self-fertility of *G. zeae* strains grown on carrot agar. Dissecting microscopic pictures were taken 10 days after sexual induction. Each strain was inoculated on carrot agar without (upper) and with (lower) 0.5% POL. WT, *G. zeae* wild-type strain GZ3639; Δacs1, ACS1 deletion mutant; Δacs2, ACS2 deletion mutant; Δacs1 Δacs2, double deletion mutant of ACS1 and ACS2; and ACS1c, ACS1-derived strain complemented with ACS1-GFP. Scale bar, 0.5 mm.

**FIG. 5.** Replacement of the *ACS2* promoter by *Pzear* in the *ACL2* deletion mutant. (A) Strategy for promoter replacement. The promoter of *ACS2* was successfully replaced with the zearalenone-inducible promoter (*Pzear*). H, HindIII; hyg, hygromycin B resistance gene cassette. Lane 1, wild-type strain GZ3639; lanes 2 and 3, promoter-replaced mutant. The sizes of the DNA standards (kb) are indicated on the left of each blot.

(B) Growth of *G. zeae* strains in minimal medium supplemented with 2% glucose (MMG) and MMG supplemented with 30 μM ZEA (MMG-ZEA). When the promoter was successfully replaced, the growth of the Δacl2 *ACS2p* mutant was severely retarded on minimal medium but was restored by exogenous treatment with ZEA. Δacl2, *ACL2* deletion mutant; Δacl2 *ACS2p*, ACS2 promoter-replaced mutant in the Δacl2 strain.
production, which is deficient in \(ACL\) mutants. Because nuclear acetyl-CoA deficiency was caused by \(ACL\) deletion during sexual development and possibly during asexual growth in \(G. zeae\) (57), ACS2 seems to specifically participate in nuclear acetyl-CoA production. Nuclear localization of ACS2 in mycelia from both submerged and solid cultures also supported the function of ACS2 as a nuclear acetyl-CoA producer.

Differences in mycelial growth of the \(ACL\) mutant in liquid and solid MMG correlated with features of ACSs in catabolic repression. After germination, hyphal elongation of \(ACL\) mutants stopped when they were grown in liquid MMG (57). In solid MMG, however, the \(ACL\) mutant grew normally, suggesting that some pathways for acetyl-CoA compensation are more functional in solid MMG than liquid medium. Both subunits of \(ACL\) were constitutively expressed and localized to the cytosol regardless of the medium, with the exception of growth in MMAc. ACS1 is glucose repressible in \(G. zeae\), similar to other fungal species (16, 53), and the expression level of ACS2 was also reduced in submerged MMG. On the other hand, ACS1 was highly expressed in the cytosol when the strain was grown on solid MMG, indicating that aerial mycelia were not influenced by glucose repression. Because of the decreased expression of both ACSs in liquid MMG, the \(ACL\) knockout mutant may fail to counterbalance acetyl-CoA production but not when the mutant is grown in solid MMG.

The functions of ACSs in \(G. zeae\) have some similarities and differences with \(S. cerevisiae\). Similar to ACS1 in \(G. zeae\), ACS1 in yeast is required for growth on several nonfermentable carbon sources, such as acetate and acetaldehyde. ACS1 in yeast, however, is an alternative nuclear acetyl-CoA producer in acetate-utilizing medium and is not required for ethanol utilization, indicating the diverse metabolic roles of ACSs among different organisms (16, 63). As an epigenetic regulator through histone acetylation, yeast ACS2 is required for growth
on glucose and was shown to localize primarily to the nuclei, with a minor amount also observed in the cytosol (63, 66). Moreover, a recent study revealed that ACS2 is involved in replicative longevity in yeast (17). Involvement in glucose utilization and histone acetylation, which are lethal features of the double deletion mutant of $ACL$ and $ACS2$, suggests that $ACS2$ in $G. zeae$ also has similar functions as $ACS2$ in yeast (57, 63). However, lipid biogenesis is a process that involves $ACS2$ in $S. cerevisiae$ but is a function of $ACS1$ in $G. zeae$.

One ACS in mammalian cells (AceCS1) also has functions that compensate for $ACL$ (70) but does not have any significant phenotype when silenced (25, 46). The other ACS (AceCS2), however, has been shown to be functional under hypoxic conditions (71). Mammalian $ACL$, therefore, seems to have more important roles in cell proliferation and lipid biosynthesis than the ACSs (5, 70).

Here, we demonstrated that acetate-derived acetyl-CoA generation by ACSs is important for lipid synthesis and the nuclear acetyl-CoA supply in $G. zeae$. Since acetate is not a major bioenergetic substrate for $G. zeae$, acetate should be produced internally by pyruvate decarboxylase (PDC), which is a component of the fermentation pathway. Under anaerobic conditions, fermentation is an alternative pathway for several eukaryotic cells because mitochondria do not have major role in energy metabolism under those conditions (3, 7, 22). Moreover, some obligate aerobic fungi are even known to use the fermentation pathway for survival under hypoxic conditions (43).

In several eukaryotes, the fermentation pathway is also useful for other nonanaerobic conditions. For example, in $N. crassa$, the transcription of the PDC coding gene was strongly induced by sucrose or glucose, and PDC was hypothesized to be a key postglycolytic enzyme (1). The $pdcA$ (one PDC of $A. nidulans$) transcript was highly expressed under aerobic conditions in $A. nidulans$ (43). In plants, pyruvate decarboxylase is also required not only for hypoxic growth, such as waterlogged root and seed, but also for survival in an aerobic environment (22, 49). This “aerobic fermentation” produces acetaldehyde from glycolysis-derived pyruvate by PDC and, subsequently, acetate by acetaldehyde dehydrogenase.

An active glyoxylate cycle under aerobic conditions in many fungi also underscores the importance of aerobic fermentation. In fungi, glyoxylate bypass, which comprises isocitrate lyase and malate synthase, was exclusively involved in acetate utilization, indicating its linkage with ACS activity. The glyoxylate cycle has been shown to be crucial for sexual development and

**FIG. 7.** Cellular localization of ACS1-GFP (A), ACS2-GFP (B), and ACL2-GFP (C) depending on the culture conditions. Submerged mycelia were harvested for microscopic observation 24 h after conidium inoculation in liquid minimal medium supplemented with potassium acetate (MMAc), glucose (MMG), and both potassium acetate and glucose (MMGAc). Aerial mycelia were collected 3 days after inoculation on solid MMAc, MMG, MMGAc, and carrot agar (Carrot). The middle panels show RFP-SKL and hH1-RFP localized in peroxisomes and nuclei, respectively. All of the white arrows indicate peroxisomal localization (A) and nuclear localization (B and C). DIC, differential interference contrast. Scale bar, 20 μm.
fungal virulence (30, 33, 38, 44, 45, 56, 69). A previous study of enzymes related to the glyoxylate cycle in G. zae revealed that they are highly expressed in aerial mycelia (38). In addition, peroxisomal localization of ACS1 suggests that it may function as an acetyl-CoA producer for the glyoxylate cycle (Fig. 7A).

In conclusion, we had previously characterized ACL subunits that are functional in nucleocytosolic acetyl-CoA generation. ACL-dependent cytosolic acetyl-CoA production is not essential for lipid synthesis but is required for histone acetylation (57). Taken together, these findings suggest that ACS1 and ACS2 are alternative cytosolic and nuclear acetyl-CoA producers, respectively. ACS1 is required for acetate utilization and lipid biosynthesis, and ACS2 has some overlapping functions with both ACS1 and ACL. Moreover, ACS-mediated fermentation seems to have important roles in lipid synthesis in G. zae. Further studies will focus on the functional characterization of PDC homologs and the functions of the pyruvate-acetaldehyde-acetate pathway in G. zae.

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