EUKARYOTIC CELL, Aug. 2011, p. 1043–1052 Vol. 10, No. 8
1535-9778/11/$12.00 doi:10.1128/EC.05071-11

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

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Received 7 April 2011/Accepted 2 June 2011

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 accessorial 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, 65, 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 some 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 acetate, citrate, and even 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-

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† Supplemental material for this article may be found at http://ec.asm.org/.

▽ Published ahead of print on 10 June 2011.

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 that is 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 had 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 *acu-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). The other media used in this study were made and used according to the *Fusarium* laboratory manual (42).

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 ^{32}P-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).

TABLE 1. *G. zeae* strains used in this study

Strain	Genotype	Source or reference
GZ3639	Wild type	8
HK22	Δ*acs1*::*gen*	This study
HK24	Δ*acs2*::*gen*	This study
HK23	Δ*acs1*::*ACS1-GFP-hyg*	This study
HK25	Δ*acs2*::*ACS2-GFP-hyg*	This study
HK31	Δ*acs1*::*gen* Δ*acs2*::*gen*	This study
Δ*mat2* strain	Δ*mat1-2*::*GFP-hyg*	36
HK29	Δ*mat1-2*::*GFP-hyg* Δ*acs1*::*gen*	This study
HK30	Δ*mat1-2*::*GFP-hyg* Δ*acs2*::*gen*	This study
acl2	Δ*acl2*::*gen*	57
HK32	Δ*acl2*::*gen* Δ*acs1*::*gen*	This study
HK33	Δ*acl2*::*gen ACS2*::*hyg-Pzear-ACS2*	This study
Δ*mat1* strain	Δ*mat1-1*::*gen*	36
mat1r	Δ*mat1-1*::*gen hH1-RFP-gen*	57
HK34	*gen-RFP-SKL*	This study
HK35	Δ*mat1-2*::*GFP-hyg gen-RFP-SKL*	This study
HK36	Δ*acs1*::*ACS1-GFP-hyg hH1-RFP-gen*	This study
HK37	Δ*acs2*::*ACS2-GFP-hyg hH1-RFP-gen*	This study
HK38	Δ*acs1*::*ACS1-GFP-hyg gen-RFP-SKL*	This study
HK39	Δ*acs2*::*ACS2-GFP-hyg gen-RFP-SKL*	This study
Pzear-GzmetE strain	*GzmetE*::*hyg-pzear-GzmetE*	37

Fungal transformation for deletion and complementation. Constructs for targeted gene deletion and green fluorescent protein (GFP)-tagged complementation were generated according to the double-joint (DJ) PCR method (73). For deletion of either the *ACS1* or *ACS2* genes, a Geneticin resistance cassette (*gen*) was amplified with primers previously designed (26) and fused with 5′ and 3′ flanking sequences for each targeted region, which was amplified with appropriate primer pairs (see Table S1 in the supplemental material).

To complement each mutant, the DNA fragment carrying the promoter and the open reading frame (ORF) of each gene was fused with green fluorescent protein and the hygromycin resistance cassette (*hyg*) amplified with pIGPAPA-sGFP F/HYG-F1 primers from the pIGPAPA vector (27). This construct was fused with the 3′ flanking region of each gene as previously described (57). All of the fungal transformations for gene deletion and complementation procedures were followed as previously described (24).

Lipid analysis. 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 carrot agar and harvested 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.

Purification and characterization of POL. Dried cultures (300 g) were extracted with 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 by 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).

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 400°C, and the inlet capillary was operated at 350°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/in^2 and 4 ml/min, respectively. ^{1}H-NMR (where NMR is nuclear magnetic resonance) spectra were recorded on a Bruker AMX500 (500 MHz) instrument

(Bruker Analytische Messtechnik GmbH, Rheinstetten, Germany) in deuterochloroform with tetramethylsilane (TMS) used as an internal standard.

In order to determine the composition of fatty acids of POL, fatty acid methyl esters (FAMEs) were made by acid transmethylation according to a previously described method (12). FAMEs were analyzed by a capillary gas chromatograph-mass spectrophotometer (GC-MS) (Shimadzu GC-MS-QP5050; Shimadzu, Kyoto, Japan). The analytical conditions used were as follows: column, DB-5 fused-silica column (30 m by 0.32 mm [inside diameter], 0.25-μm film thickness (J & W Scientific, Folsom, CA); column temperature, 90°C for 5 min and then increased to 210°C at a rate of 1.5°C/min; injector temperature, 250°C (1 μl of a 10 mg/ml sample was injected).

Sexual crosses and genotyping. For self-fertilization, mycelia grown on carrot agar for 5 days were gently removed with a glass spreader in the presence of 2.5% of sterilized Tween 60 solution to induce sexual reproduction (42). For the chemical complementation with POL, carrot agar supplemented with 0.5% (vol/vol) purified POL was used. Female strains grown for 5 days on carrot agar were spermatized with 1 ml of conidial suspension of each male strain for out-crosses (36). After sexual induction, all of the cultures were incubated under near-UV light (wavelength, 365 nm; HKiv Import and Export Co., Ltd., Xiamen, China) at 25°C.

To generate the double deletion mutant HK31 (Δ*acs1* Δ*acs2*), the Δ*mat2* mutant was out-crossed with Δ*acs2* to obtain HK30 (Δ*mat2* Δ*acs2*). HK30 was then out-crossed with HK22 (Δ*acs1*) again to generate HK31 (Δ*acs1* Δ*acs2*). HK29 (Δ*mat2* Δ*acs1*) was selected from the Δ*mat2* × HK22 (Δ*acs1*) out-cross. For double deletion between the *ACS* genes and *ACL2*, both HK29 (Δ*mat2* Δ*acs1*) and HK30 (Δ*mat2* Δ*acs2*) were fertilized with strain acl2 (Δ*acl2*). Dozens of ascospores were randomly isolated from each out-cross, and the genotype of each progeny was determined by antibiotic resistance and PCR screening using the combination of primers ACS1-5F and Gen-with 5F (see Table S1 in the supplemental material).

Promoter replacement of *ACS2* with *Pzear* in the *ACL2* mutant. To replace the *ACS2* promoter with the zearalenone-inducible promoter (*Pzear*) in the *ACL2* deletion mutant, *hyg-pzear* was amplified from the *Pzear-GzmetE* strain (where *GzmetE* is *G. zeae metE* gene) (37) with HYG-F1 and zear-r2 primers, and the 5′ and 3′ flanking regions of the *ACS2* gene were amplified from GZ3639 with primers ACS2-3F/ACS2-5R pzear and ACS2-3F pzear/ACS2-3R pzear, respectively. Three fragments were fused according to the DJ PCR method (73), and the final construct was amplified with primers ACS2-5N/ACS2-3N pzear. For *Pzear* replacement in the *ACL2* mutant, 30 μM ZEA was added to the medium during the regeneration, overlay, and mutant selection processes (37).

Mycelial growth, conidium production, germination, trichothecene analysis, and virulence test. For mycelium growth, 1 ml of conidium suspension (1×10^5 conidia/ml) was inoculated in 50 ml of minimal medium (MM) supplemented with 2% glucose as the sole carbon source (MMG), MMG supplemented with 40 mM potassium acetate (MMGAc), or MMGAc supplemented with 30 μM ZEA (MMGAc-ZEA) and cultured for 72 h at 25°C on a rotary shaker (150 rpm). Radial growth was measured from mycelia grown for 5 days on MM supplemented with 40 mM potassium acetate as the sole carbon source (MMAc), MM supplemented with 40 mM ethanol as the sole carbon source (MMEtOH), or MM supplemented with 5 mM acetaldehyde as the sole carbon source (MMACD). Conidium production was measured by counting the number of conidia produced after incubating 10 μl of conidial suspension (1×10^5 conidia/ml) in 5 ml of carboxymethyl cellulose (CMC) medium (9) for 72 h at 25°C on a rotary shaker (150 rpm). Trichothecenes (deoxynivalenol and 15-acetyldeoxynivalenol) from MMA were analyzed with a Shimadzu QP-5050 GC-MS with a selected ion monitoring and quantified based on biomasses produced by each strain (57).

The virulence of fungal strains was determined on the wheat cultivar Eunpamil as previously described (57). In brief, 10 μl of conidial suspension (1×10^5 conidia/ml) was injected into a center spikelet of wheat head at midanthesis. Inoculated plants were placed in a greenhouse after 3 days of incubation in a humidity chamber, and spikelets with head blight symptoms were counted after 14 days.

Localization and expression of ACS1-GFP and ACS2-GFP. To observe the localization of ACS1-GFP and ACS2-GFP with nuclei, the mat1r strain carrying histone H1 fused with red fluorescent protein (RFP) was fertilized with either HK23 (ACS1c) or HK25 (ACS2c). Ascospores carrying *ACS1-GFP/hH1-RFP* (HK36) and *ACS2-GFP/hH1-RFP* (HK37) were selected by antibiotic resistance, genotyped by PCR, and observed under a fluorescent microscope. For generation of the HK35 (Δ*mat2 RFP-SKL*) mutant, the *gen-EF* (*gen* with elongation factor 1α promoter from *Fusarium verticillioides*) was amplified from the pSK-GEN vector (see Fig. S1 in the supplemental material) with primers Neo-For new and EF Pro-Rev. The *gen-EF* was then fused to *RFP-SKL* amplified from the pLC25FgH4Tomloxneolox vector provided by Michael Freitag (Oregon State University, Corvallis, OR) with RFP-For EF/RFP-Rev SKL tail primers. The final round of PCR was conducted with primers Neo-For 5N and RFP-Rev SKL 3N, and the construct was then randomly introduced into the wild-type strain. HK35 (Δ*mat2 RFP-SKL*) was then spermatized with HK23 (ACS1c) or HK25 (ACS2c) to generate HK38 (*ACS1-GFP RFP-SKL*) or HK39 (*ACS2-GFP RFP-SKL*). Mitochondria were stained with MitoTracker (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Microscopic observation was performed with a DE/Axio Imager A1 microscope (Carl Zeiss) using the filter set 38HE (excitation, 470/40 nm; emission, 525/50 nm) for GFP and the filter set 15 (excitation, 546/12 nm; emission, 590 nm) for RFP and MitoTracker.

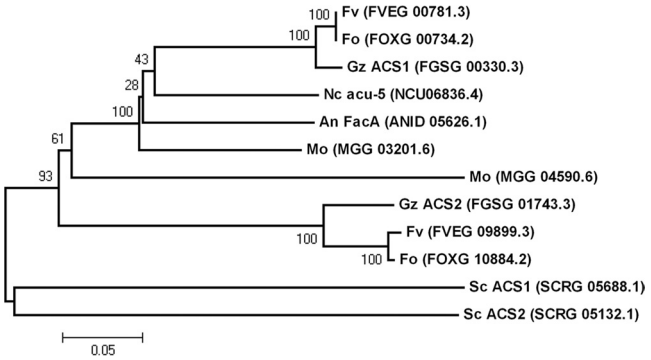

FIG. 1. Phylogenetic tree of acu-5/FacA homologs in several fungal species. The alignment was performed with ClustalW, and the MEGA program, version 4.0, was used to perform a 1,000-bootstrap phylogenetic analysis using the neighbor joining method. Fv, *F. verticillioides*; Fo, *F. oxysporum*; Gz, *G. zeae*; Nc, *N. crassa*; An, *A. nidulans*; Mo, *M. oryzae*; and Sc, *S. cerevisiae*.

RESULTS

Identification of *ACS* genes and phylogenetic analysis. The *ACS* genes are named differently in many organisms, including fungal species. We identified two *ACS* genes in *G. zeae* from the *Fusarium* Comparative Database (http://www.broadinstitute.org/annotation/genome/fusarium_group/) using both BLASTp and InterProScan (IPR011904 family) (58). The protein sequences of *ACS1* (FGSG_00330.3) and *ACS2* (FGSG_01743.3) had marked identity (80 and 71%) with the protein sequence of *acu-5* of *N. crassa*, respectively (19).

Phylogenetic relationships among the ACS homologs (IPR011904 family) were estimated using the MEGA program, version 4.0 (64). The phylogenetic tree was constructed using a neighbor-joining algorithm with bootstrap values calculated from 1,000 iterations. The *Fusarium* species, *Magnaporthe oryzae*, and *S. cerevisiae* had two ACSs, while *A. nidulans* and *N. crassa* had only one ACS each. One of the ACSs in *Fusarium* spp. and *M. oryzae* was grouped with FacA and acu-5, and the other was grouped outside the ACS1 group (Fig. 1).

Targeted gene deletion and genetic complementation. To elucidate the functions of *ACS*s in *G. zeae*, we deleted *ACS1* and *ACS2* individually through homologous recombination. Each gene was successfully replaced with the *gen*. For genetic complementation, each ORF fused with GFP was introduced into a corresponding deletion mutant. All deletion and complementation mutants were confirmed by Southern hybridization (see Fig. S2 in the supplemental material). After 30 discharged ascospores were isolated from an out-cross between

TABLE 2. Radial growth and conidium production in *G. zeae* strains

Strain	Radial growth by medium type (mm)[a]				Conidiation (no. of conidia/ml)[b]
	MMS	MMAc	MMEtOH	MMACD	
Wild type	84.0 A	84.6 A	69.0 A	4.0 A	2.31×10^6 A
Δ*acs1* strain	83.3 A	40.0 B	35.3 B	2.4 B	2.34×10^6 A
Δ*acs2* strain	84.0 A	84.0 A	65.0 A	4.2 A	2.40×10^6 A
Δ*acs1* Δ*acs2* strain	73.3 B	18.3 C	10.7 C	1.0 C	1.16×10^6 B
ACS1c	84.0 A	84.6 A	67.0 A	4.3 A	2.31×10^6 A
ACS2c	84.3 A	85.3 A	65.3 A	4.2 A	2.47×10^6 A

[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 acetaldehyde as the sole carbon source.

[b] Conidiation was measured by counting the number of conidia after 3 days of incubation.

HK30 (Δ*mat2* Δ*acs2*) and HK22 (Δ*acs1*), 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 spectrum. The ^{1}H-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).

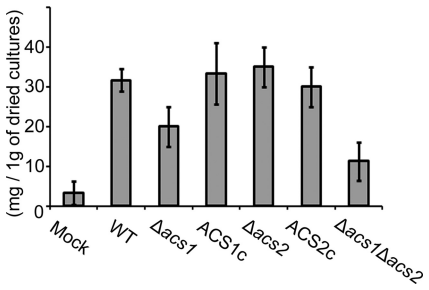

FIG. 2. Total lipid in 1 g of dried carrot agar cultures. WT, *G. zeae* wild-type strain GZ3639; Δ*acs1*, *ACS1* deletion mutant; ACS1c, *ACS1*-derived strain complemented with *ACS1-GFP*; Δ*acs2*, *ACS2* deletion mutant; ACS2c, *ACS2*-derived strain complemented with *ACS2-GFP*; and Δ*acs1* Δ*acs2*, double deletion mutant of *ACS1* and Δ*ACS2*.

Fertility test. The HK22 (Δ*acs1*) strain produced fewer mature perithecia than the wild-type strain 7 days after sexual induction. HK24 (Δ*acs2*) and complemented strains produced normally melanized mature perithecia containing viable ascospores. The defect in perithecium development was more severe in the double deletion mutant (HK31) (Fig. 4). Perithecium maturation of HK31 (Δ*acs1* Δ*acs2*) 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 perithecium maturation of HK22, but not the HK31 (Δ*acs1* Δ*acs2*) double deletion mutant, was mostly restored. Treatment of the wild-type strain with POL enhanced melanin accumulation and perithecium production (Fig. 4).

Transcriptional analyses of *ACS*s. 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 (Δ*mat2* Δ*acs1*) and HK30 (Δ*mat2* Δ*acs2*) strain was fertilized with strain acl2 (Δ*acl2*) 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 Δ*acl2* Δ*acs2* mutant was lethal, we replaced the promoter of *ACS2* with *Pzear* in the *ACL2* deletion mutant using the previously reported strategy

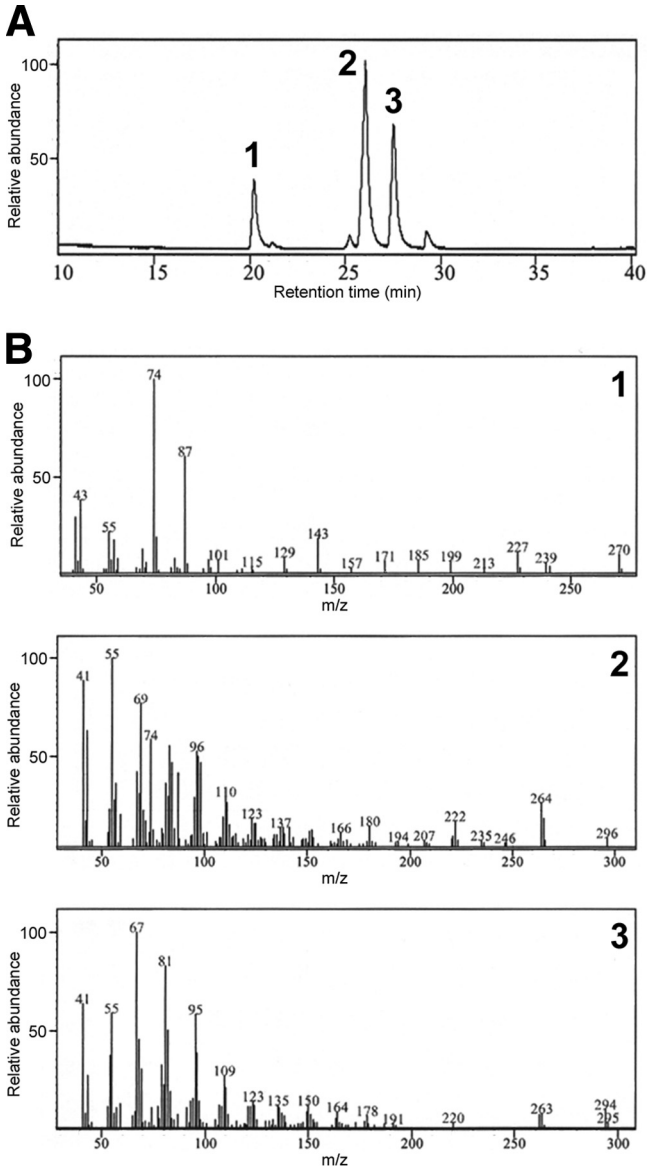

FIG. 3. Total ion chromatogram of the fatty acid methyl esters of POL isolated from *G. zeae* (A) and mass spectra (B) of methyl esters of palmitic acid (1), oleic acid (2), and linoleic acid (3) with retention times of 20.1 min, 25.8 min, and 27.4 min, respectively.

(37). The *hyg-pzear* construct amplified from the *Pzear-GzmetE* strain was successfully introduced into the promoter of *ACS2* in the acl2 strain (Fig. 5A). The promoter-replaced mutant, HK33 (Δ*acl2 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 (Δ*acl2*) mutant, HK32 (Δ*acl2* Δ*acs1*) did not produce any initial structures for perithecia but was somewhat restored by exogenous treatment with 40 mM potassium acetate in carrot agar (Fig. 6A). HK33 (Δ*acl2 ACS2p*) did not produce any initial perithecia even when potassium acetate was supplemented 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 (Δ*acs1* Δ*acs2*) 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

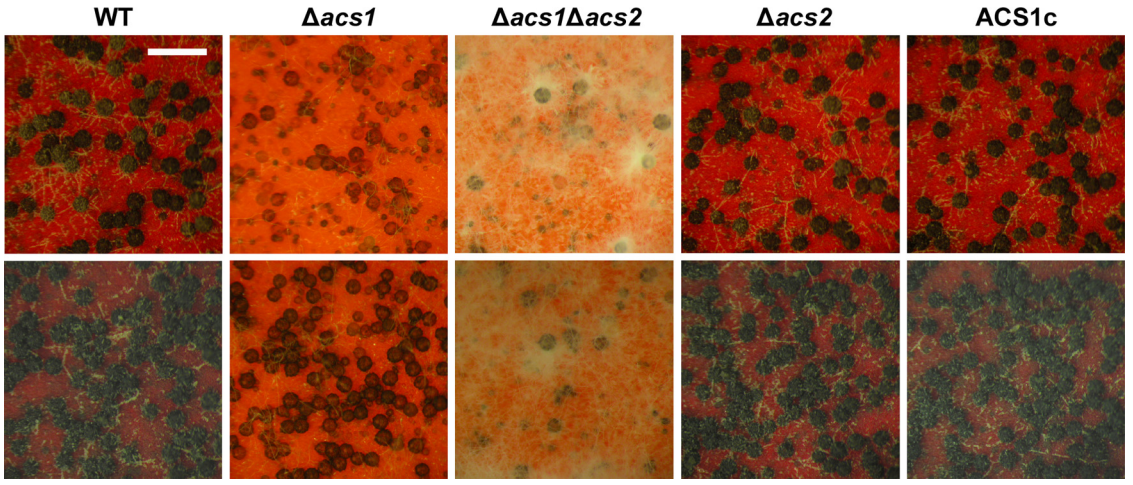

FIG. 4. Self-fertility of *G. zeae* strains grown on carrot agar. Dissecting microscopic pictures were taken 10 days after sexual induction. Each strain was inoculated on carrot agar without (upper) and with (lower) 0.5% POL. WT, *G. zeae* wild-type strain GZ3639; Δ*acs1*, *ACS1* deletion mutant; Δ*acs2*, *ACS2* deletion mutant; Δ*acs1* Δ*acs2*, double deletion mutant of *ACS1* and *ACS2*; and ACS1c, *ACS1*-derived strain complemented with *ACS1-GFP*. Scale bar, 0.5 mm.

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 perithecium 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 (Δ*acs1 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

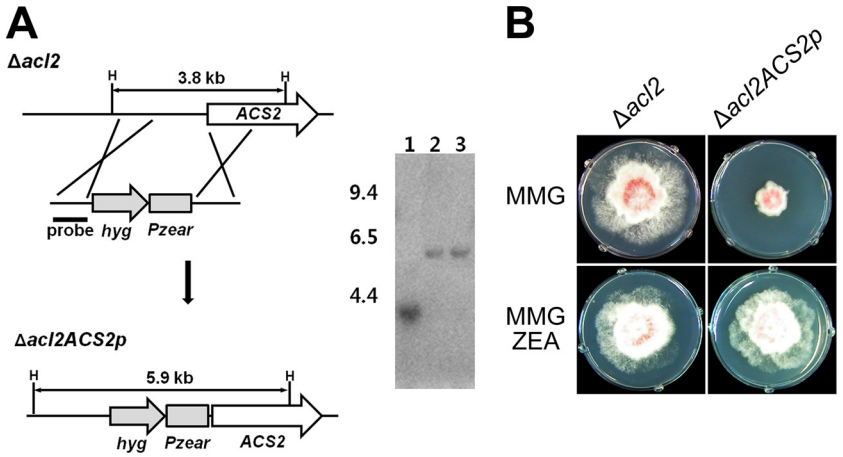

FIG. 5. Replacement of the *ACS2* promoter by *Pzear* in the *ACL2* deletion mutant. (A) Strategy for promoter replacement. The promoter of *ACS2* was successfully replaced with the zearalenone-inducible promoter (*Pzear*). H, HindIII; *hyg*, hygromycin B resistance gene cassette. Lane 1, wild-type strain 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 Δ*acl2 ACS2p* mutant was severely retarded on minimal medium but was restored by exogenous treatment with ZEA. Δ*acl2*, *ACL2* deletion mutant; Δ*acl2 ACS2p*, *ACS2* promoter-replaced mutant in the Δ*acl2* strain.

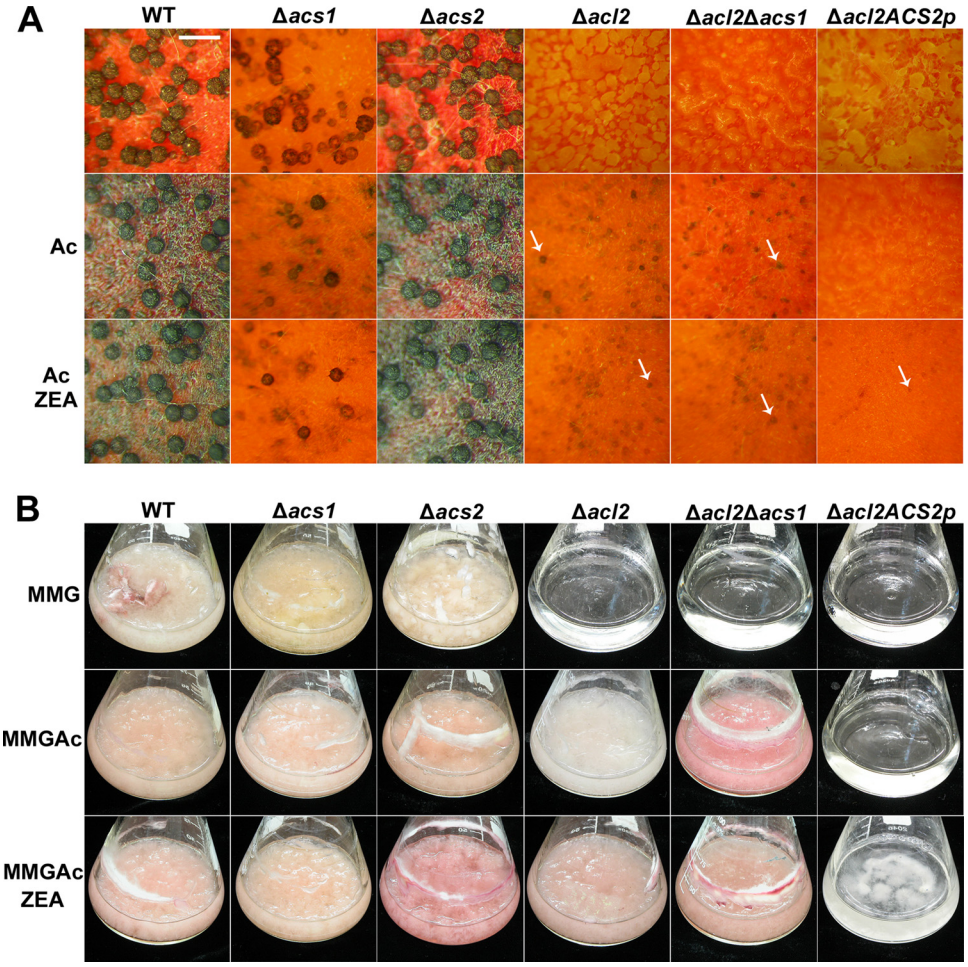

FIG. 6. Chemical complementation by potassium acetate in *G. zeae* strains. (A) Self-fertility of the *G. zeae* strains on carrot agar 10 days after sexual induction. Each strain was inoculated on carrot agar (upper), carrot agar supplemented with 40 mM potassium acetate (middle), and carrot agar supplemented with both 40 mM potassium acetate and 30 μM zearalenone (bottom). The white arrows indicate immature perithecia. Scale bar, 0.5 mm. (B) Mycelial growth of *G. zeae* strains in liquid minimal medium supplemented with 2% of glucose (MMG) 5 days after inoculation. The conidium suspension of each strain was inoculated in MMG (upper), MMG supplemented with 40 mM potassium acetate (middle), and MMG supplemented with both 40 mM potassium acetate and 30 μM ZEA (bottom). WT, *G. zeae* wild-type strain GZ3639; Δ*acs1*, *ACS1* deletion mutant; Δ*acs2*, *ACS2* deletion mutant; Δ*acl2*, *ACL2* deletion mutant; Δ*acl2* Δ*acs1*, double deletion mutant of *ACL2* and *ACS2*; Δ*acl2 ACS2p*, *ACS2* promoter-replaced mutant in the Δ*acl2* strain. Ac, exogenous treatment of 40 mM potassium acetate; MMGAc, MMG supplemented with 40 mM potassium acetate; ZEA, exogenous treatment of 30 μM ZEA.

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 (Fig. 7). 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

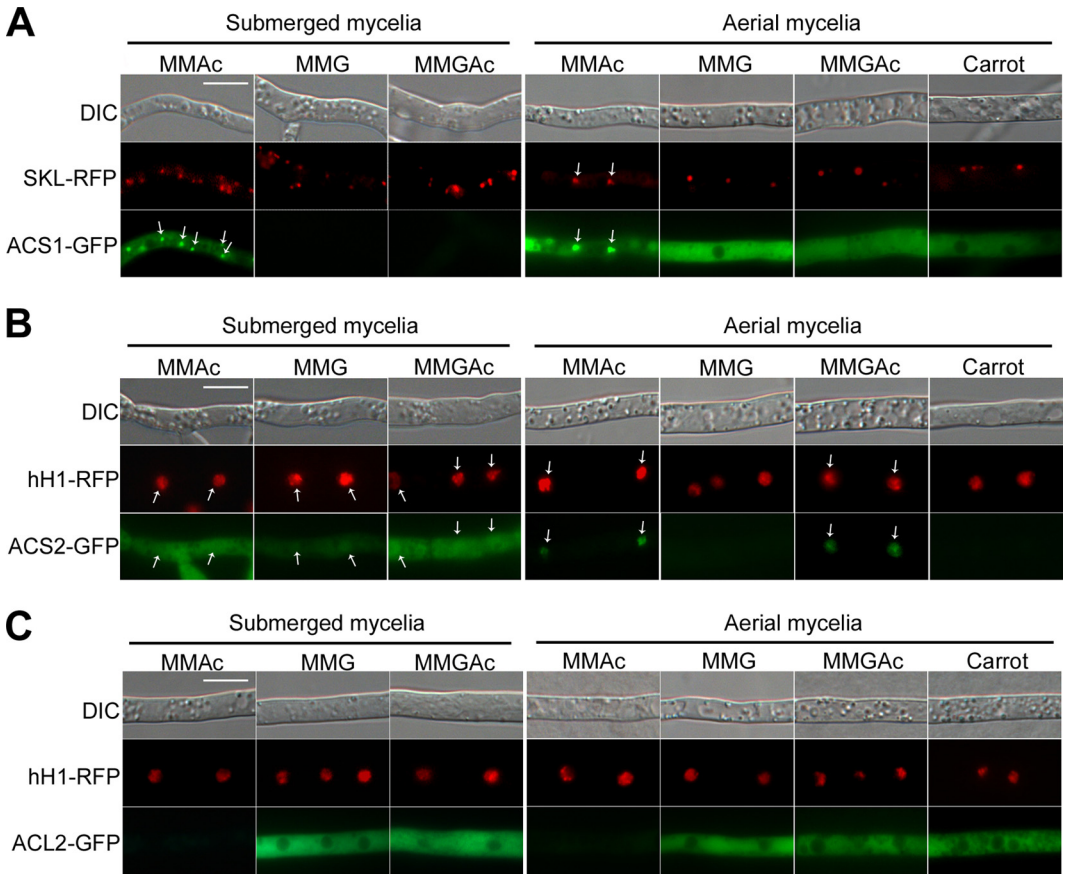

FIG. 7. Cellular localization of ACS1-GFP (A), ACS2-GFP (B), and ACL2-GFP (C) depending on the culture conditions. Submerged mycelia were harvested for microscopic observation 24 h after conidium inoculation in liquid minimal medium supplemented with potassium acetate (MMAc), glucose (MMG), and both 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.

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

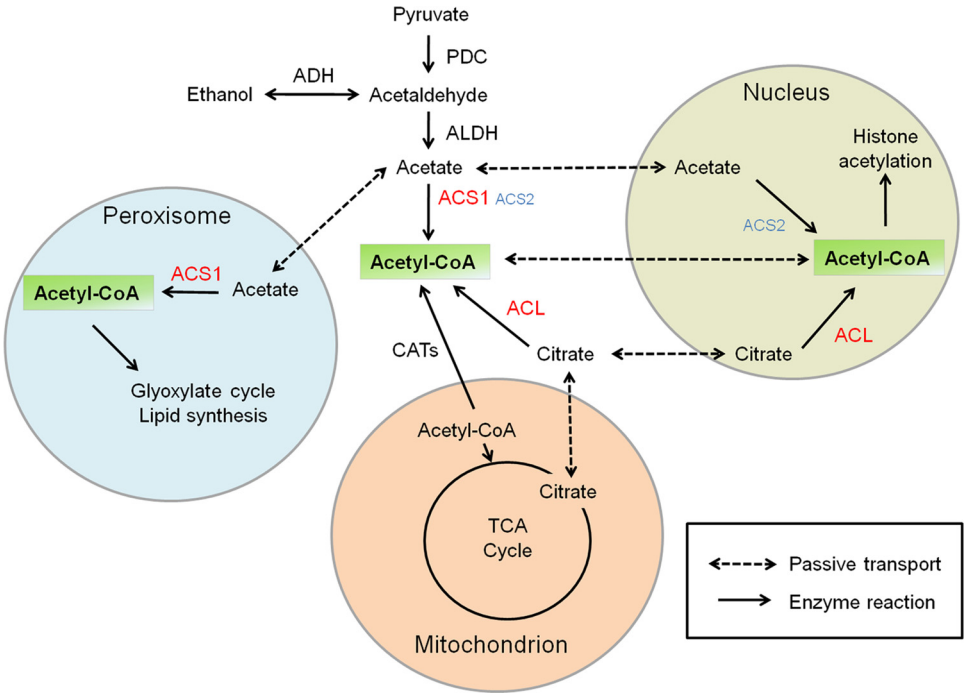

FIG. 8. Metabolic pathways for acetyl-CoA generation in *G. zeae*. The pyruvate-acetaldehyde-acetate pathway has vital roles in acetyl-CoA production. The full name and locus numbers of each enzyme are as follows: PDC, pyruvate decarboxylase (FGSG_09834.3); ACS1, acetyl-CoA synthetase (FGSG_00330.3); ACS2, acetyl-CoA synthetase (FGSG_01743.3); and ACL, ATP-citrate lyase (FGSG_06039.3 and FGSG_12857.3 for each subunit).

fungal virulence (30, 33, 38, 44, 45, 56, 69). A previous study of enzymes related to the glyoxylate cycle in *G. zeae* 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. zeae*. Further studies will focus on the functional characterization of PDC homologs and the functions of the pyruvate-acetaldehyde-acetate pathway in *G. zeae*.

ACKNOWLEDGMENTS

This work was supported by a National Research Foundation of Korea grant funded by the Korean government (MEST) (2010-0001826) and by a grant (CG 1141) from the Crop Functional Genomics Center of the 21st Century Frontier Research Program funded by the Korean Ministry of Education, Science, and Technology.

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