Roles of the Glyoxylate and Methylcitrate Cycles in Sexual Development and Virulence in the Cereal Pathogen Gibberella zeae

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The glyoxylate and methylcitrate cycles are involved in the metabolism of two- or three-carbon compounds in fungi. To elucidate the role(s) of these pathways in Gibberella zeae, which causes head blight in cereal crops, we focused on the functions of G. zeae orthologs (GzICL and GzMCL) of the genes that encode isocitrate lyase (ICL) and methylisocitrate lyase (MCL), respectively, key enzymes in each cycle. The deletion of GzICL (ΔGzICL) caused defects in growth on acetate and in perithecium (sexual fruiting body) formation but not in virulence on barley and wheat, indicating that GzICL acts as the ICL of the glyoxylate cycle and is essential for self-fertility in G. zeae. In contrast, the ΔGzMCL strains failed to grow on propionate but exhibited no major changes in other traits, suggesting that GzMCL is required for the methylcitrate cycle in G. zeae. Interestingly, double deletion of both GzICL and GzMCL caused significantly reduced virulence on host plants, indicating that both GzICL and GzMCL have redundant functions for plant infection in G. zeae. Thus, both GzICL and GzMCL may play important roles in determining major mycological and pathological traits of G. zeae by participating in different metabolic pathways for the use of fatty acids.

During the infection process, pathogenic fungi usually encounter nutrient deprivation in the host before gaining access to sufficient nutrients for successful colonization of the living tissue. To cope with a nutrient-limited environment, fungal pathogens seem to rely mostly on fatty acid metabolism for both energy supply and biosynthesis of essential molecules (29). The ability of fungi to use fatty acids as a carbon source for growth is based on the glyoxylate cycle. Fungal pathogens have been proposed to employ the glyoxylate bypass for the use of acetyl-coenzyme A (CoA) units produced by the enzymes of the glyoxylate pathway, such as isocitrate lyase (ICL) and methylisocitrate lyase (MCL), respectively, key enzymes in each cycle. The deletion of GzICL (ΔGzICL) caused defects in growth on acetate and in perithecium (sexual fruiting body) formation but not in virulence on barley and wheat, indicating that GzICL acts as the ICL of the glyoxylate cycle and is essential for self-fertility in G. zeae. In contrast, the ΔGzMCL strains failed to grow on propionate but exhibited no major changes in other traits, suggesting that GzMCL is required for the methylcitrate cycle in G. zeae. Interestingly, double deletion of both GzICL and GzMCL caused significantly reduced virulence on host plants, indicating that both GzICL and GzMCL have redundant functions for plant infection in G. zeae. Thus, both GzICL and GzMCL may play important roles in determining major mycological and pathological traits of G. zeae by participating in different metabolic pathways for the use of fatty acids.

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disease development caused by the devastating cereal pathogen Gibberella zeae (anamorph: Fusarium graminearum). G. zeae is a ubiquitously distributed ascomycete fungus that causes major disease in cereal crops such as corn, wheat, barley, and rice (33). Severe epidemics of these diseases result in serious economic consequences due to yield losses and contamination by fungal mycotoxins (32, 33). Wind-disseminated sexual spores (ascospores), which are produced in perithecia formed on plant debris, can infect plant spikes during anthesis (13, 39, 45). Detailed studies of the G. zeae infection process on wheat and barley heads have shown that fungal hyphae on the inner surfaces of the spike penetrate epidermal cells through pits or pores and grow into the carpoecysts through the pericarp (21). Thus, the glolyoxylate cycle, either alone or in conjunction with the methylcitrate cycle, is likely employed by G. zeae during the infection process, as in other fungus-plant interactions (20, 46). G. zeae genome searches have identified orthologs of fungal ICL and MCL genes, designated GzICL1 and GzMCL1, respectively. Here, we performed functional analyses of these genes to provide new insights into their importance in lipid metabolism during the G. zeae infection process in host plants.

MATERIALS AND METHODS

Strains, culture conditions, and fungal transformation. Gibberella zeae strain Z03643, a lineage 7 and self-fertile strain (37), was obtained from Robert L. Bowden, U.S. Department of Agriculture, Manhattan, KS. GzMAT1 is a self-sterile strain derived from a mut1-1-deleted G. zeae strain (T39ΔM1-1) (25), which is a mut1-1-deleted progeny carrying no germ from the outcross between T39ΔM1 and Z03643 (S.-H. Lee et al., unpublished data). Fungal strains from 25% glycerol stock cultures stored at −80°C were maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI). For both perithecial formation and total RNA extraction, the strains were grown on carrot agar plates (26). For vegetative growth and genomic DNA extraction, the strains were grown on either PDA or complete medium (26) or on basal medium (minimal medium without sucrose) (26). E. coli strains were grown on Luria-Bertani agar or in liquid medium supplemented with 75 μg/ml ampicillin. Fungal transformation was performed as previously described (25).

Vector construction. To construct the plasmid used for targeted deletion of GzICL1, we employed an inverse PCR strategy. A 4.0-kb DNA fragment carrying the inverse PCR entire gene GzICL1 open reading frame (ORF) (plus 1.016-bp 5′ and 801-bp 3′ flanks) was amplified from the genomic DNA of Z03643 using GzICL1-BglI-F and GzICL1-BglI-R primers (see Table S1 in the supplemental material) carrying the recognition sites for BglII at the 5′ ends. The PCR product was digested with BglII, self-ligated, and used as a template for inverse PCR with primers GzICL1-BglII-DelF and GzICL1-BglII-DelR (see Table S1 in the supplemental material). An 1.817-bp fragment carrying both the 5′ and 3′ flanks of GzICL1, but not the ORF, was inserted into the EcoRV site of the pCB1004 vector (kindly provided by Seogchean Kang, Penn State University, University Park, PA) carrying hygB as a selectable marker. For the deletion of the GzICL1 gene, the resulting 6.617-bp plasmid designated pCBGzICL1, was linearized with BglII and introduced into G. zeae protoplasts.

A transforming DNA fragment used for the deletion of GzMCL1 was constructed using the double joint PCR method (48). DNA fragments corresponding to 5′ (1,633 bp) and 3′ (1,672 bp) regions of the GzMCL1 ORF were amplified from the genomic DNA of Z03643 using the primer pairs GzMCL1-FG/MCL1-RGT and GzMCL1-R-GT/MCL1-G (see Table S1 in the supplemental material), respectively. A 1.8-kb fragment containing the gene fragment was amplified from the vector pBI99 (36) using primers Gen-F and Gen-R. Three amplicons (the 5′-flanking region of GzMCL1, the gen cassette, and the 3′-flanking region of GzMCL1) were mixed in a 1:2:1 molar ratio and used as a template for a second round of PCR with a new primer pair GzMCL1-NF and GzMCL1-NR (see Table S1 in the supplemental material), resulting in a 3,903-bp fragment of the fusion PCR product. Following purification, the final PCR products were mixed with fungal protoplasts for use in transformation for the deletion of GzMCL1.

For complementation analyses, intact copies of GzICL1 and GzMCL1 including the native promoter and terminator were amplified from genomic DNA of the Z03643 strain using primer pairs GzICL1-NF/GzICL1-NR and GzMCL1-NF/GzMCL1-NR, respectively, and that of A. nidulans acuD was amplified from A. nidulans FGSC A4 using the primer pair AnICL1-NF/AnICL1-NR (see Table S1 in the supplemental material). The amplified GzICL1, or acuD, product was directly added into the ΔGzICL1 protoplasts along with the pBIF9 vector carrying gen as a fungal selectable marker; the GzMCL1 PCR product was introduced into the ΔGzMCL1 protoplasts along with pUCH1 carrying hygB.

Phylogenetic analyses. Total RNA for RNA bands and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Fungal genomic DNA was extracted using the cetyltrimethylammonium bromide protocol, as described previously (26). Plasmid DNA was purified using a plasmid DNA purification kit (Intron Biotechnology, Seoul, Korea). Standard procedures were used for gel electrophoresis, restriction enzyme digestion, ligation, and blotting (42). RT-PCR was performed using the AccessoII-PCR kit (Intron, Daegu, Korea) according to the manufacturer’s protocol. Quantitative real-time PCR was performed with the SYBR green super mix (Bio-Rad, Hercules, CA) and a 7500 real-time PCR system (Applied Biosystems, Foster, CA). The PCRs were repeated three times with three replicates per run. The G. zeae gene encoding elongation factor 1-β (EF1B; FGSG_01088.3) was used as an endogenous control for normalization. The cycle threshold (CT) value of EF1B was subtracted from that of PKS12 (FGSG_02324.3) (23) encoding a polyketide synthase responsible for the production of the root-secreted pigment (aromatic) to obtain a ΔCt value. The ΔCt value of an arbitrary calibrator was subtracted from the ΔCt value, of each sample to obtain a ΔΔCt value. The PKS12 expression level relative to the calibrator was expressed as 2ΔΔCt.

Sexual crosses. Both a self-cross and an outcross of each G. zeae strain were performed in carrot medium, as described previously (25, 26). A self-cross was initiated by placing mycelial agar blocks of a fungal strain at the center of carrot agar plates and incubating at 25°C. After 10 days (during vegetative mycelial growth), the aerial mycelia that had grown on carrot agar were then removed by scraping the culture with a conidial suspension (10⁶ conidia/ml in 2.5% Tween 60) of the same strain, and the plates were incubated for an additional 10 to 14 days for perithecial induction at 25°C under a mixture of fluorescent cool white and black lights with a 12-h photoperiod. An outcross was initiated by placing mycelial agar blocks of the mut1-1-deleted strain (GzMΔMAT1) of G. zeae on carrot agar plates. After 10 days at 25°C, conidial suspensions (1 × 10⁶ conidia/ml) of the fungal strains were applied to the mycelia of GzMΔMAT1, and the plates were incubated as described above. Each outcross was set up in 10 carrot agar plates. More than 50 perithecia were randomly picked up on the mating plates of each cross, and two ascospores were isolated from each perithecium for genetic analysis.

Virulence tests. Virulence tests were performed using spikelets of the barley cultivar SangRok and the wheat cultivar Empamul, both of which are very susceptible to head blight. For fungal inoculation, two different methods were compared: G. zeae conidia solutions containing 1 × 10⁴ conidia/ml were prepared in CMC (carboxymethylcellulose) medium (8) and sprayed evenly onto barley and wheat heads, as described previously (18) (spray inoculation method), or approximately 10 μl of the G. zeae spore suspension (1 × 10⁶ spores/ml) was injected into a basal spikelet of the wheat head at midanthesis (point inoculation method). For each treatment, six barley or wheat heads were inoculated, and inoculated plants were placed in a humidity chamber for 3 days and then transferred to a greenhouse under normal conditions until disease symptoms appeared. At 14 days postinoculation, spikes inoculated by the spray method were visually rated and the average severity of head blight was calculated as follows. The sum of infected spikelets for each symptom severity on a 0 to 4 scale (0, no symptoms; 1, <25% necrotic regions on a spikelet; 2, <50%; 3, <75%; 4, 100% necrosis and/or bleaching) divided by the total number of spikelets inoculated. All data were obtained from two biological replicates, and the Tukey test using SPSS 12.0 software (SPSS Inc., Chicago, IL) was performed to examine the significant difference (P < 0.05) of disease severity among the mean values of each strain.

ICL and MCL assays. Isocitrate lyase activity was determined using cell extracts following the method of Dixon and Kornberg (10). The cell extracts were prepared as described by De Lucas et al. (9) and directly assayed for enzyme activity. The amount of protein in each mutant was quantified using Bradford’s method (3). The assay mixture consisted of 8 mM L-isocitrate, 6 mM MgCl₂, 4 mM of phenylhydrazine, 12 mM cysteine, and 62.5 mM potassium phosphate buffer (pH 7.0). The reaction was started by adding cell extract, followed by incubation for 5 min at 25°C. Formation of the enzymatic product was monitored at a wavelength of 324 nm using a spectrophotometer (Ultrospec 4000; Pharmacia Biotech, Tokyo, Japan). For the methylisocitrate lyase assay, we used 2-methylisocitrinate lactone, a kind gift from Matthias Brock, Leibniz Institute for Natural Product Research and Infection Biology, Hans Knöll Institute, Jena, Germany, as the enzyme substrate for measurement.
RESULTS

Sequence similarity. The GzICL1 and GzMCL1 genes were identified from the F. graminearum genome (http://www.broad.mit.edu/annotation/fungi/fusarium/index.html). The putative ORF of GzICL1 (FGSG_09896.3; annotated as an isocitrate lyase in the fungal genome database) is 1,893 bp long and contains four putative introns; the putative 1,800-bp ORF of GzMCL1 (FGSG_00176.3; annotated as another isocitrate lyase in the genome database) carries one putative intron. The deduced GzICL1 amino acid sequence exhibited significant similarity to other fungal ICLs, such as acu3 of Neurospora crassa (84% identity) (15), ICL1 of M. grisea (81% identity) (46), ICL of L. maculans (75% identity) (20), and acuD of A. nidulans (74% identity) (15). The putative polypeptide of GzMCL1 showed 69% identity to A. nidulans mclA (CAI65406) encoding MCL, as well as other hypothetical MCLs or ICLs (see Fig. S1 in the supplemental material). The putative GzMCL1 protein carried a predicted sequence motif (GFVLQLISLAGLH) specific for fungal MCLs (4). When we searched for a putative mitochondrial targeting sequence using the program MITOPROT (http://ihg.gsf.de/ihg/mitoprot .html), only the GzMCL1 protein was transported to mitochondria, with a probability of 0.9996. The two G. zeae genes, GzICL1 and GzMCL1, shared 43% identity with each other at the amino acid level.

Targeted gene deletions and complementation. To determine the role(s) of GzICL1 and GzMCL1 in G. zeae, we deleted one or both genes from the genome of strain Z03643 (Fig. 1). First, the putative GzICL1 ORF was replaced with the vector pCBGzICL1 carrying the fungal selectable maker hygB via double homologous recombination between both the 5′/H11032 and 3′/H11032 regions of the GzICL1 ORF on the vector and the corresponding genomic regions (Fig. 1A). Genomic DNA of strains with the deletion of GzICL1 (designated ΔGzICL1) carried a 9.4-kb hybridizing band when digested with BglII, instead of the 6.5-kb band found in the wild-type Z03643 strain, suggesting that the 1.9-kb GzICL1 ORF had been deleted and replaced with the hygB gene (Fig. 1A). For the deletion of GzMCL1 (ΔGzMCL1), the ORF of GzMCL1 was replaced with the gen gene by double homologous recombination between the double joint PCR product and the fungal genome (Fig. 1B). The ΔGzMCL1 strains derived from Z03643 showed a single 5.0-kb hybridizing band on a blot of HindIII-digested genomic DNA instead of the 2.7-kb band in the wild-type Z03643 strain (Fig. 1B). For the double deletion of GzICL1 and GzMCL1 (designated ΔGzICL1 ΔGzMCL1), we removed the GzMCL1 gene from the genome of a ΔGzICL1
strain (TdGI1-1) using the same method described above. Double gene deletion in the ΔGzICL1 ΔGzMCL1 strains was confirmed by DNA gel blot analysis (Fig. 1B). The insertion of the GzICL1, acuD, or GzMCL1 ORF at the genomes of the complemented transformants was also confirmed by DNA gel blot hybridization (Fig. 1C).

**ICL and MCL activities.** To determine the relative contribution of GzICL1 or GzMCL1 to the enzymatic activities of ICL and MCL, the fungal cell extracts obtained from the ΔGzICL1 and ΔGzMCL1 strains, as well as their wild-type progenitor, Z03643, were examined for ICL or MCL activity (Fig. 2). In both Z03643 and the ΔGzMCL1 strain grown for 24 h in liquid basal medium containing 5% sodium acetate as a sole carbon source, ICL specific activities increased approximately 18 to 20 times over those grown in 2% glucose. In contrast, the ICL activities in both the ΔGzICL1 and ΔGzICL1 ΔGzMCL1 strains grown in acetate were dramatically reduced to levels similar to those observed for growth in glucose (Fig. 2A). Under both growth conditions, the complemented ΔGzICL1::GzICL1 and ΔGzICL1::acuD strains showed ICL activities similar to those in the Z03643 and ΔGzMCL1 strains (Fig. 2A). Similarly, the MCL activities in the Z03643, ΔGzICL1, and ΔGzMCL1::GzMCL1 strains grown in 0.25% propionate were strongly induced (~10 times) compared with those grown in 2% glucose (Fig. 2B). However, the ΔGzICL1 and ΔGzICL1 ΔGzMCL1 strains showed approximately 20 times-reduced MCL activities in 0.25% propionate (Fig. 2B). In addition to the gene deletion strains mentioned above, sexual progeny for each gene deletion, which obtained from the sexual crosses described below, showed a similar pattern for the activities for the corresponding enzymes (see Fig. S2 in the supplemental material).

**Mycelial growth and other phenotypes of the deletion strains.** To assess the roles of GzICL1 and GzMCL1 in the metabolism of two- or three-carbon compounds or fatty acids, we tested the mycelial growth of the deletion strains on basal medium containing only nonfermentable carbon sources, such as sodium acetate, ethanol, C3 propionic acid, C12 fatty acid (monolaurate; Tween 20), and C18 fatty acids (Tween 60, linoleic acid, and oleic acid). The ΔGzICL1 strains exhibited about 60% of the wild-type growth on 2% acetate, but they were unable to grow on 5% acetate and other C2 compounds or fatty acids. However, they showed no significant difference from Z03643 on propionate (Table 1). When an intact copy of GzICL1 was reintroduced into the genome of the ΔGzICL1 strain, its growth defects on acetate and other fatty acids were fully restored; *A. nidulans* acuD also completely restored the hyphal growth of the ΔGzICL1 strain on the same carbon sources (Table 1). These data suggest that GzICL1 acts as a fungal ICL in the glyoxylate cycle in *G. zeae*. In contrast, the ΔGzMCL1 strains grew normally on acetate and other fatty acids but failed to grow on propionate, as did the *A. nidulans* mclA strain lacking MCL (4) (Table 1). However, the ΔGzMCL1 strains showed a different phenotype from the *A. nidulans* mclA strain when glucose was used as the sole carbon source or propionate was added into glucose medium. The *A. nidulans* mclA strain grew normally on glucose and was still able to grow on glucose plus propionate (up to 100 mM), although the degree of growth inhibition depended on the propionate concentrations (4). In contrast, the ΔGzMCL1 strains showed significantly reduced growth on glucose alone (Table 1), and their hyphal growth was completely inhibited when even a low amount of propionate (1 mM) was added into glucose. When we reintroduced an intact copy of the GzMCL1 gene back into a ΔGzMCL1 mutant, hyphal growth on propionate was completely restored (Table 1), suggesting that GzMCL1 encodes a putative MCL in the methylcitrate cycle for propionate catabolism. Mycelial growth of the double deletion ΔGzICL1 ΔGzMCL1 strains was abolished on both acetate and propionate (Table 1). All deletion strains showed...
pigmentation was observed on the agar surface of these deletion strains (Fig. 3A and B); mycelia of Z03643 usually began to produce red pigment 4 to 5 days postinoculation, eventually turning carmine red. The quantitative real-time PCR analysis revealed that the expression of PKS12, which is responsible for the production of the red pigment (aurofusarin) (23), decreased significantly in the ΔGzICL1 and ΔGzICL1 ΔGzMCL1 strains compared to Z03643 (Fig. 3C). The complemented ΔGzICL1 transformants (ΔGzICL1::GzICL1 and ΔGzICL1::acuD) produced red pigment normally, as did Z03643, confirming the effect of ΔGzICL1 on pigmentation of aerial mycelia in G. zeae.

Sexual reproduction of the deletion strains. To determine whether the gene deletions affected fungal self-fertility, both the ΔGzICL1 and ΔGzMCL1 strains were grown on mating plates (carrot agar). All of the ΔGzICL1 strains examined produced white aerial mycelia during the vegetative growth stage (up to 10 days postinoculation) (Fig. 3B). After the removal of aerial mycelia (i.e., perithecial induction stage), the ΔGzICL1 strains formed either none or few perithecia, indicating that GzICL1 is important for self-fertility in G. zeae. The defect in self-fertility of the ΔGzICL1 strains was completely restored in the ΔGzICL1::GzICL1 and ΔGzICL1::acuD strains. Unlike the ΔGzICL1 strains, the ΔGzMCL1 strains showed no difference from the wild-type strain in both vegetative growth and perithecial formation. The double deletion ΔGzICL1 ΔGzMCL1 strains were similar to the ΔGzICL1 strains in female fertility and produced few perithecia on carrot agar.

Outcroses. To determine the effect of ΔGzICL1 or ΔGzMCL1 on normal meiosis and male fertility in G. zeae, we forced either the ΔGzICL1 (hygromycin B-resistant [HygB⁺]) or ΔGzMCL1 (geneticin-resistant [Gen⁺]) strain to act as the male parent in outcroses to a mat1-1 self-sterile G. zeae strain, GzAMAT1 (HygB and Gen sensitive [HygB⁻ Gen⁺]) on the same mating plates. All of the outcroses produced similar numbers of mature fertile perithecia as did the outcross of the wild-type strain, indicating that ΔGzICL1 or ΔGzMCL1 had no effect on male fertility in G. zeae. Random ascospores obtained from the outcroses segregated into parental phenotypes in equal proportions (confirmed statistically by χ² test) for the corresponding drug resistance (HygB⁺:HygB⁻ = 38:38 in the outcross of the ΔGzICL1; Gen⁺:Gen⁻ = 55:45 in the outcross of the ΔGzMCL1). Among progeny carrying both intact MAT

### Table 1. Mycelial growth rate of the gene deletion strains grown on various carbon sources

| Carbon sourcea | WT | ΔGzICL1 | ΔGzMCL1 | ΔGzICL1 ΔGzMCL1 | ΔGzICL1::GzICL1 | ΔGzMCL1::GzMCL1 | ΔGzICL1::acuD |
|----------------|----|---------|---------|-----------------|-----------------|-----------------|-------------|
| Glucose (2)    | 100| 100     | 31 ± 2  | 29 ± 1          | 100             | 100             | 100         |
| Acetate (2)    | 100| 62 ± 3  | 96 ± 4  | 60 ± 2          | 98 ± 2          | 96 ± 3          | 96 ± 4      |
| Acetate (5)    | 100| 0       | 95 ± 1  | 0               | 95 ± 4          | 98 ± 2          | 96 ± 4      |
| Tween 60 (2)   | 100| 7 ± 1   | 99 ± 0  | 6 ± 2           | 97 ± 1          | 97 ± 3          | 99 ± 1      |
| Linoleic acid (0.25) | 100| 12 ± 2  | 96 ± 3  | 11 ± 2          | 98 ± 1          | 97 ± 3          | 99 ± 1      |
| Propionate (0.25) | 100| 98 ± 0  | 0       | 0               | 99 ± 1          | 96 ± 3          | 98 ± 2      |
| Propionate (0.01) | 100| 98 ± 2  | 0       | 0               | 98 ± 2          | 96 ± 2          | 96 ± 2      |

*a The growth rate was determined 7 days after inoculation on each agar plate. All data were obtained from three replicates.

*b The value obtained with the wild-type strain on each sole carbon source was set to 100%.

*c See also Fig. 2.

*d Numbers in parentheses indicate the percent concentration of each carbon source.
idiomorphs from the former outcross, all examined HygB’ progeny, when self-crossed, produced none or few perithecia (<1,000 on a 90- by 15-mm carrot agar plate), as did the \( \Delta GzICL1 \) parental strain TdGI1-1; all the HygB’ progeny formed >70,000 perithecia on the same plate, as did the wild-type Z03643 strain. This indicates that the significantly reduced female fertility of the progeny was caused by the targeted deletion of Gz\( \text{ICL1} \) from the \( G. \text{zeae} \) genome. In addition, the HygBr progeny showed the same phenotype as TdGI1-1 in other characters, such as mycelial growth on various carbon sources and ICL activity. Similarly, all examined Genr progeny from the latter outcross showed the same abolished and reduced growth pattern on propionate and glucose, respectively, and the reduced MCL activity as did the parental \( \Delta GzMCL1 \) strain, TdGI2-3, confirming that \( \Delta GzMCL1 \) is responsible for the altered phenotypes.

**Virulence test.** When single wheat spikelets were point inoculated with a macroconidial suspension of the wild-type Z03643 strain, the head blight symptoms hardly spread into the adjacent spikelets, which was an unexpected result. Instead, we infected both barley and wheat heads with the \( \Delta GzICL1 \), \( \Delta GzMCL1 \), or \( \Delta GzICL1 \ \Delta GzMCL1 \) strains by using the spray inoculation method. When inoculated with the \( \Delta GzICL1 \) strain TdGI1-1, the disease severity and progression on barley and wheat were similar to those when inoculated with the wild-type Z03643 strain (Fig. 4). Both strains produced typical head blight symptoms that began to appear as early as 3 to 5 days postinoculation and became obvious after 7 days. Similarly, the \( \Delta GzMCL1 \) strain, TdGI2-3, produced head blight symptoms on both host plants, but the disease severity on barley only seemed somewhat (~25%) reduced compared to that of Z03643 (Fig. 4). Unlike Z03643 and the single gene deletion \( \Delta GzICL1 \) or \( \Delta GzMCL1 \) strains, three independent double deletion \( \Delta GzICL1 \ \Delta GzMCL1 \) strains showed significantly reduced virulence on barley and wheat. The \( \Delta GzICL1 \ \Delta GzMCL1 \) strains caused only small necrotic spots on some spikelets of each barley or wheat head even after 10 days following fungal inoculation (Fig. 4). In addition to these transgenic strains, five independent double deletion progeny, which were obtained from an outcross of a \( \Delta GzICL1 \ \Delta GzMCL1 \) strain to Gz\( \text{MAT1} \), also showed a similar pattern of reduced virulence on both host plants (data not shown).

**Expression patterns of the Gz\( \text{ICL1} \) and Gz\( \text{MCL1} \) genes.** To determine the transcriptional regulation patterns of the

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**FIG. 4.** Virulence of the gene deletion stains of \( G. \text{zeae} \) on barley and wheat heads. (A) Head blight symptoms on barley and wheat heads inoculated with the \( G. \text{zeae} \) strain Z03643 (WT), a \( \Delta GzICL1 \) strain, a \( \Delta GzMCL1 \) strain, and a double deletion \( \Delta GzICL1 \ \Delta GzMCL1 \) strain. (B) Disease severities caused by the fungal strains, which were determined at 14 days after inoculation by the following formula: \( \frac{\text{[(number of infected spikelets) × (specific symptom scale)}]}{\text{(total number of spikelets inoculated)}} \). Different letters above the bars are significantly different according to the Tukey test (\( P < 0.05 \)).
GzICL1 and GzMCL1 genes, transcript accumulations were examined using Northern blot analysis after mycelial growth on various carbon sources (Fig. 5) during the developmental stages for sexual reproduction or pathogenesis (Fig. 6). Significant amounts of GzICL1 transcript were detected on the blot of total RNA extracted from mycelia grown on the two-carbon compounds and the fatty acids examined, but not on glucose. The highest levels of GzICL1 transcript accumulated in mycelia grown on basal medium containing either Tween 20 or 60 (Fig. 5A), whereas GzICL1 expression decreased when the fungal strain was shifted from these conditions to glucose as a sole carbon source (Fig. 5C). In contrast, the expression levels of GzMCL1 were much lower than those of GzICL1 under all of the conditions examined, and gene induction by the carbon sources was not so great (Fig. 5A). However, glucose-induced inactivation of GzMCL1 seemed to occur (Fig. 5C). Some effects of the deletion of one gene on the expression of the other gene were observed. The levels of GzICL1 transcripts decreased significantly when a ΔGzMCL1 strain was grown on the carbon sources, except for propionate, whereas those of the GzMCL1 transcripts were elevated in the ΔGzICL1 strain grown on most carbon sources, except Tween 60 (Fig. 5A). Note that GzICL1 was strongly induced until 4 days after the mycelial plugs of the ΔGzMCL1 strain were inoculated onto basal liquid medium containing propionate, after which time no further mycelial growth of the ΔGzMCL1 strain occurred (Fig. 5B).

In contrast to gene expression during vegetative growth, GzICL1 expression decreased significantly during the perithecial induction stage on carrot agar (Fig. 6A). Unlike GzICL1, a similar level of GzMCL1 transcripts accumulated during all incubation times, although the transcript level was much lower than that of GzICL1 (Fig. 6A). In barley plants infected with the G. zeae Z03643 strain, the transcription of GzICL1 was strongly induced 6 days after inoculation, but decreased dramatically after day 9 (Fig. 6B). This in planta expression pattern of GzICL1 was also confirmed using RT-PCR analysis. A putative RT-PCR product of GzICL1 was first detected on day 1, increased continuously, and reached the highest level on day 6 (Fig. 6C). The expression of GzICL1 by the ΔGzMCL1 strain in barley decreased similarly to that of vegetative growth (Fig. 6B). The second band of putative GzICL1 RT-PCR products, which was still smaller in size than the genomic DNA band, may indicate alternative splicing of the GzICL1 gene inside the plant, but it needs to be confirmed. Similarly, the in planta expression of GzMCL1 was much lower than that of GzICL1.
as in other cases; the expression level was too low to be detected using Northern blot hybridization, but a putative RT-PCR product of GzMCL1 was detected in barley 1 day after inoculation and the level increased continuously until day 9. GzMCL1 expression decreased when the ΔGzICL1 strain was inoculated into barley (Fig. 6C).

**DISCUSSION**

Several cases for the requirement of ICL genes in fungal virulence led us to investigate the roles of two different ICL orthologs, GzICL1 and GzMCL1, in *G. zeae*. Sequence similarity suggested that GzICL1 is a typical fungal ICL-encoding gene involved in the glyoxylate cycle for C2 metabolism, whereas GzMCL1 encodes a putative MCL, rather than an ICL, which is a key enzyme in the methylcitrate cycle for propionate (C3) metabolism (4, 30). These proposed roles of GzICL1 and GzMCL1 in the two metabolic pathways were genetically confirmed in this study based on the growth phenotypes of the ΔGzICL1 and ΔGzMCL1 strains on C2 or C3 compounds as sole carbon sources. The lack of hyphal growth and reduced ICL specific activity in the ΔGzICL1 strain on various C2 sources demonstrated the effect of ΔGzICL1 in disruption of the glyoxylate cycle, although its growth defect occurred at relatively high concentrations of acetate compared to the other ICL-deleted filamentous fungi (20, 46). The complete restoration of hyphal growth in the ΔGzICL1 strain carrying *A. nidulans* acuD as well as GzICL1 also support the role of GzICL as an ICL in the *G. zeae* glyoxylate cycle. The expression pattern of GzICL1 was consistent with those of typical fungal ICL genes. GzICL1 was strongly induced by most non-fermentable carbon sources examined but was repressed when glucose was the sole carbon source. In addition, GzICL1 showed glucose-induced repression, indicating the possibility of catabolite inactivation of the GzICL1 protein, as for other fungal ICLs (1). The absence of growth and reduced MCL activity in the ΔGzMCL1 strains on propionate as the sole carbon source clearly indicates that GzMCL1 encodes a MCL for the utilization of propionate in *G. zeae*, as for the *A. nidulans* mclA gene encoding MCL (4). However, the growth phenotypes of the ΔGzMCL1 strains on 2% (111.1 mM) glucose or acetate alone or 2% glucose containing propionate may suggest a different metabolic role for the GzMCL1 protein from *A. nidulans* mclA. In the *A. nidulans* ΔmclA strain, growth inhibition occurred only when propionate was added into the glucose or acetate medium, which was explained by the inhibitory effect of methylisocitrate accumulation on the enzymes involved in the tricarboxylic acid (TCA) cycle. This phenotype implies a specific role for MclA in the methylcitrate cycle in *A. nidulans* (4). In contrast, hyphal growth of the ΔGzMCL1 strain decreased significantly on 2% glucose alone, but not on acetate, indicating that the effect of ΔGzMCL1 on the TCA cycle in *G. zeae* may be different from that of ΔmclA in *A. nidulans*. The alterations in the expression pattern of one gene when the other gene is missing may suggest a compensatory network between the GzICL1 and GzMCL1 genes at the transcriptional level. In particular, strong induction of GzICL1 in the ΔGzMCL1 strain on propionate indicates that an inter-regulatory mechanism between the two genes has been evolutionarily conserved, even though the highly induced GzICL1 expression no longer compensates for the growth defect of the ΔGzMCL1 strain on propionate.

Unlike the effect on fatty acid metabolism, a pronounced
cumulative effect of the double deletion was observed for \textit{G. zeae} virulence on barley and wheat heads, which is clearly different from other fungal pathogens such as \textit{C. albicans}, \textit{L. maculans}, and \textit{M. grisea} (20, 27, 46). The wild-type level of disease development caused by \textit{ΔGzICL1} confirms that GzICL1 is not essential for fungal virulence, although its deduced protein sequence is highly similar to other fungal ICLs, which were solely responsible for virulence, and its gene expression is strongly upregulated in barley. The virulence of the \textit{ΔGzMCL1} strains on both host plants, similar to that of the wild-type strain, also indicates the functional dispensability of GzMCL1 in fungal virulence, although a slight reduction in disease severity was observed on barley heads (but not on wheat heads).

In contrast, the more dramatically reduced disease severity on two hosts inoculated with the double deletion strains clearly indicates that both GzICL1 and GzMCL1 genes (both the glyoxylate and methylcitrate cycles) likely have overlapping or reciprocally redundant functions for full \textit{G. zeae} virulence in host plants. Because of the difference of gene expression levels, however, it is likely that reciprocity between these two genes is somewhat unbalanced. In addition, the relatively independent contributions of each of these metabolic cycles to the fungal virulence may still exist. The induction of GzICL1 expression in barley 1 day after inoculation (based on RT-PCR) indicates that GzICL1 may be required for the use of C2 compounds available from plant cell membranes, intracellular stores of lipids, or stored fungal lipids before \textit{G. zeae} has gained access to nutrients from the host plant tissues (20, 46). The possible function of GzMCL1 in fungal virulence can be postulated based on the role(s) of GzMCL1 discussed above. Possible involvement of GzMCL1 in the TCA cycle may directly affect fungal glucose catabolism for energy acquisition, especially in nutrient-rich plant tissues. In addition, the impairment of propionyl-CoA assimilation throughout the methylcitrate cycle in the \textit{ΔGzMCL1} strain would disturb the fungal defense system against propionate or propionyl-CoA in host plants, which may be generated by β-oxidation of odd-chain-length fatty acids in plant lipids. However, it is yet unknown how these two genes (or two metabolic cycles) can redundantly function for only fungal virulence toward host plants. Because they cannot functionally compensate each other for the defects in other phenotypes (e.g., enzyme activity, carbon catabolism, and sexual development), it is unlikely that either of the gene products has a dual role, as ICL1 from \textit{Mycobacterium tuberculosis} does, as it can clearly function as an MCL as well as an ICL (17).

The functional compensation between GzICL1 and GzICL2 in \textit{G. zeae} pathogenesis raises a question regarding previous results demonstrating that a single ICL gene is required for fungal virulence. Would the deletion of the MCL or the second hypothetical ICL gene, if present, from the genomes of pathogens with the first ICL gene deleted result in a more severe reduction in or complete abolishment of fungal virulence? The Δicl1 mutants of \textit{M. grisea} obviously retain the capacity to cause appreciable typical lesions on rice leaves, although they were impaired in functions associated with the prepenetration stage of disease development (46). Therefore, the virulence of \textit{M. grisea} may be even more severely impaired if the MCL ortholog (MgMCL1; EAA47373) (see Fig. S1 in the supplemental material) is disrupted in the \textit{M. grisea} Δicl1 mutants. The same strategy could be applied to \textit{Cryptococcus neoformans}, in which a site-directed mutation in \textit{ICL} caused no apparent virulence defect in animal models (41), and even to \textit{A. fumigatus}, which is not dependent on the glyoxylate cycle for invasive aspergillosis (43).

Unlike fatty acid metabolism and fungal virulence, sexual development appears to be affected only by GzICL1 in \textit{G. zeae}. The formation of fewer perithecia in the \textit{ΔGzICL1} strains confirms that only GzICL1 is essential for female fertility in \textit{G. zeae}. The lack of GzICL1 expression during the later stages of sexual development in the wild-type strain indicates that inactivation of the glyoxylate cycle (or shutdown of an unknown fatty acid catabolic process mediated by GzICL1) may be a prerequisite for switching from the vegetative to the perithecial induction stage in \textit{G. zeae}. The greater importance of ICL in vegetative growth than in sexual development has been also reported for the brown rot basidiomycete \textit{Fomitopsis palustris}; ICL activity in mycelia increased markedly at an early stage of vegetative growth but thereafter dropped sharply during the fruiting stage (47). Therefore, the activation of the glyoxylate cycle during the vegetative stage may be necessary for the synthesis of essential cellular constituents that serve as biosynthetic precursors for the formation of perithecia and other sexual structures, as well as for energy supply during the sexual development stage in \textit{G. zeae}. However, further investigation should examine the importance of isocitrate carbon flow between the glyoxylate and TCA cycles for perithecial induction in \textit{G. zeae} because the ICL gene is upregulated during fruiting body formation in the ectomycorrhizal ascomycete \textit{Tuber borchii} (24). A second possibility involves a regulatory role of the metabolic pathway catalyzed by GzICL1 on the biosynthesis of polyketides that may be involved in sexual development. A reduced \textit{PKS12} gene transcript level, responsible for the production of the polyketide red pigment, aurofusarin (23), in the white aerial mycelia of the \textit{ΔGzICL1} strain, would support this speculation since aurofusarin deficiency leads to elevated production of zearealenone (22), another polyketide whose endogenous levels may affect perithecial induction in \textit{G. zeae} (Y. T. Kim et al., unpublished data). A third possibility for a role for GzICL1 in sexual development is its involvement in specific metabolism of some fatty acids essential for fungal sexual reproduction. Some specific endogenous fatty acids have long been speculated to be directly involved in fruiting body formation in ascomycetes. In \textit{Neotyra haematococcans}, the exogenous supply of linoleic acid enhanced perithecial development, and increased endogenous levels of linoleic acid were detected during perithecial formation (11).

In summary, the glyoxylate cycle mediated by GzICL1 is required for self-fertility, as well as fatty acid metabolism in \textit{G. zeae}, and plays an important role in fungal virulence toward host plants together with the methylcitrate cycle mediated by GzMCL1 in \textit{G. zeae}. Our results provide insights into the importance of these two cycles in determining major mycological and pathological traits of \textit{G. zeae} that were previously unanticipated or unappreciated.

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