The Methylcitrate Cycle is Required for Development and Virulence in the Rice Blast Fungus *Pyricularia oryzae*

Yuxin Yan,¹ Huan Wang,¹ Siyi Zhu,¹ Jing Wang,¹ Xiaohong Liu,² Fucheng Lin,² and Jianping Lu^{1,†}

¹ State Key Laboratory for Rice Biology, College of Life Sciences, Zhejiang University, Hangzhou 310058, Zhejiang Province, China

² State Key Laboratory for Rice Biology, Biotechnology Institute, Zhejiang University

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The methylcitrate cycle metabolizes propionyl-CoA, a toxic metabolite, into pyruvate. *Pyricularia oryzae* (syn. *Magnaporthe oryzae*) is a phytopathogenic fungus that causes a destructive blast disease in rice and wheat. We characterized the essential roles of the methylcitrate cycle in the development and virulence of *P. oryzae* using functional genomics. In *P. oryzae*, the transcript levels of *MCS1* and *MCL1*, which encode a 2-methylcitrate synthase and a 2-methylisocitrate lyase, respectively, were upregulated during appressorium formation and when grown on propionyl-CoA-producing carbon sources. We found that deletion of *MCS1* and *MCL1* inhibited fungal growth on media containing both glucose and propionate, and media using propionate or propionyl-CoA-producing amino acids (valine, isoleucine, methionine, and threonine) as the sole carbon or nitrogen sources. The $\Delta mcs1$ mutant formed sparse aerial hyphae and did not produce conidia on complete medium (CM), while the $\Delta mcl1$ mutant showed decreased conidiation. The aerial mycelium of $\Delta mcs1$ displayed a lowered $NAD^+/NADH$ ratio, reduced nitric oxide content, and down-regulated transcription of hydrophobin genes. $\Delta mcl1$ showed reduced appressorium turgor, severely delayed plant penetration, and weakened virulence. Addition of acetate recovered the growth of the wild type and $\Delta mcs1$ on medium containing both glucose and propionate and recovered the conidiation of both $\Delta mcs1$ and $\Delta mcl1$ on CM by reducing propionyl-CoA formation. Deletion of *MCL1* together with *ICL1*, an isocitrate lyase gene in the glyoxylate cycle, greatly reduced the mutant's virulence as compared with the single-gene deletion mutants ($\Delta icl1$ and $\Delta mcl1$). This experimental evidence provides important information about the role of the methylcitrate cycle in development and virulence of *P. oryzae* by detoxification of propionyl-CoA and 2-methylisocitrate.

Keywords: fungal development, fungus–plant interactions, mechanisms of pathogenicity

Propionyl-CoA is a metabolite produced by the degradation of propionate, amino acids (valine, isoleucine, methionine, and threonine) thymine, cholesterol, and odd-chain fatty acids (Graham and Eastmond 2002; Halarnkar and Blomquist 1989). In animals and some bacteria, propionyl-CoA is metabolized into succinyl-CoA via the methylmalonyl-CoA pathway and then enters the tricarboxylic acid (TCA) cycle (Halarnkar and Blomquist 1989; Peyraud et al. 2009). In fungi and some bacteria, propionyl-CoA is detoxified via the methylcitrate cycle (Pronk et al. 1994; Uchiyama et al. 1982). In the methylcitrate cycle (Fig. 1A), propionyl-CoA and oxaloacetate are condensed into methylcitrate by 2-methylcitrate synthase (Mcs), converted into 2-methyl-cis-aconitate by 2-methylcitrate dehydratase (Mcd), subsequently converted into 2-methylisocitrate by aconitase (Acn), and then cleaved into succinate and pyruvate by 2-methylisocitrate lyase (Mcl) (Uchiyama et al. 1982). Finally, succinate and pyruvate are transformed into oxaloacetate or other metabolites, respectively, via the TCA cycle or other metabolic pathways (Brock et al. 2000, 2002).

Propionyl-CoA, 2-methylcitrate, and methylisocitrate inhibit cell growth and are toxic to organisms. 2-Methylcitrate inhibits the cell growth of *Salmonella enterica* (Horswill et al. 2001). In *Aspergillus nidulans*, propionyl-CoA inhibits pyruvate dehydrogenase, ATP citrate lyase, and succinyl-CoA synthetase (Brock and Buckel 2004), and 2-methylisocitrate inhibits NADP-dependent isocitrate (Brock 2005). Therefore, sodium propionate is widely used to inhibit fungal growth (Brock 2005; Brock and Buckel 2004). In humans, propionyl-CoA noncompetitively inhibits pyruvate dehydrogenase complex activity (Schwab et al. 2006), and 2-methylcitrate competitively inhibits citrate synthase, aconitase, nicotinamide adenine dinucleotide⁺ (NAD^+)- and $NADP^+$ -linked isocitrate dehydrogenase, and phosphofructokinase (Cheema-Dhadli et al. 1975). Accumulation of propionyl-CoA, 2-methylcitrate, and metabolites of propionate oxidation led to mitochondrial dysfunction in propionic aciduria patients (Cheema-Dhadli et al. 1975; Schwab et al. 2006).

In *A. nidulans*, $\Delta mcsA$ and $\Delta mclA$, mutants in which the 2-methylcitrate synthase gene or 2-methylisocitrate lyase gene, respectively, was deleted could not utilize propionate as the sole carbon source, and propionate inhibited the growth of $\Delta mclA$ in media containing glucose, glycerol, or acetate (Brock 2005; Brock et al. 2000). Accumulation of propionyl-CoA inhibited the synthesis of polyketides such as carcinogens, mycotoxins, and sterigmatocystin in *A. nidulans* and *A. fumigatus* (Brock and Buckel 2004; Maerker et al. 2005; Zhang et al. 2004). In *A. fumigatus*, $\Delta mcsA$ could not grow on medium using propionate as the sole carbon source, propionate inhibited the growth of $\Delta mcsA$ in media containing glucose, and the virulence

[†]Corresponding author: J. Lu; jplu@zju.edu.cn

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Fig. 1. Methylcitrate cycle in *Pyricularia oryzae*. **A**, Homologous proteins of the methylcitrate cycle in *P. oryzae*. AcnA and AcnB = aconitases, Mcd1 = 2-methylcitrate dehydratase, Mcl1 = 2-methylisocitrate lyase, Mcs1 = 2-methylcitrate synthase, and Pcl1 = propionate-CoA ligase. **B**, Propionate inhibits the growth of *P. oryzae*. The wild type was cultured on complete medium (CM) and minimal medium (MM) (1% glucose) containing different concentrations of sodium propionate at 25°C for 9 days. Bar = 10 mm. **C**, Relative expression levels of *MCS1* and *MCL1* in response to propionate. The wild type was cultured in MM containing different concentrations of sodium propionate at 25°C for 6 h. Asterisks indicate significant differences between two columns as estimated by Tukey's honestly significant difference test, $P < 0.05$. **D**, Relative expression levels of *MCS1* and *MCL1* in hyphae cultured in CM, conidia (as 1), and appressoria at 4, 6, 8, and 18 h postinoculation. In C and D, β -TUBULIN and α -ACTIN were used as reference genes.

of $\Delta mcsA$ in animals was reduced (Ibrahim-Granet et al. 2008; Maerker et al. 2005). In *Gibberella zeae*, the virulence of $\Delta mcl1$ on barley and wheat was also reduced (Lee et al. 2009). In *Trichoderma atroviride*, $\Delta icl2$, in which the 2-methylisocitrate lyase gene (*ICL2*) was deleted, could not utilize propionate as the sole carbon source, and showed reduced inhibition of *Botrytis cinerea* growth (Dubey et al. 2013).

Pyricularia oryzae (syn. *Magnaporthe oryzae*) is a filamentous ascomycete fungus that causes rice and wheat blast, diseases leading to serious crop loss around the world (Dean et al. 2012). Carbon metabolism plays important roles in the development and pathogenicity of *P. oryzae*. Lipid catabolism, the glyoxylate cycle, gluconeogenesis, and their regulatory pathways such as the PMK1 mitogen-activated protein kinase pathway, the cAMP-dependent protein kinase A signaling pathway, and the carbon catabolite repression pathway are required for the production of appressorium turgor, appressorium-mediated penetration, and infection (Badaruddin et al. 2013; Cao et al. 2018; Fernandez et al. 2012; Thines et al. 2000; Wang et al. 2007). Deletion of many enzyme genes in carbohydrate metabolism and lipid metabolism, including a transketolase, *Tkl1* (Fernandez et al. 2014); a glyoxylate aminotransferase, *Agt1* (Bhadoria et al. 2012); an isocitrate lyase, *Icl1* (Wang et al. 2003); a methylmalonate-semialdehyde dehydrogenase, *Msdh* (Norvienyeku et al. 2017); and glycerol-3-phosphate dehydrogenases *Gpd1* and *Gpd2* (Shi et al. 2018) all led to defects in fungal development and virulence. However, the role of the methylcitrate cycle, an important pathway involved in propionyl-CoA metabolism, in

the development and pathogenicity of *P. oryzae* still remains unclear.

In this study, we characterized the functions of the methylcitrate cycle in *P. oryzae* by deleting a 2-methylcitrate synthase gene, *MCS1*, and a 2-methylisocitrate lyase gene, *MCL1*. Using genetic and molecular analyses, we showed that the methylcitrate cycle is necessary for propionate metabolism, cellular redox state, nitric oxide (NO) production, growth, conidiation, appressorium turgor production, and virulence in the rice blast fungus.

RESULTS

Methylcitrate cycle in *P. oryzae*.

Propionate inhibited the growth of a *P. oryzae* wild-type strain cultured on complete medium (CM) and minimal medium (MM) containing 1% glucose (Fig. 1B). The depressing effect of propionate was dose dependent, and 1% propionate completely blocked the growth of the wild type on CM and MM, suggesting that propionate is deleterious to this fungus. Propionate is metabolized via the methylcitrate cycle (Fig. 1A) and homologous proteins of the five enzymes in the methylcitrate cycle were found in *P. oryzae* by searching its genome against homologous proteins identified in other organisms (Fig. 1A; Supplementary Table S1). *MCS1* (MGG_02617) encodes a 2-methylcitrate synthase and *MCL1* (MGG_02616) encodes a 2-methylisocitrate lyase (Fig. 1A). *MCS1* and *MCL1* are adjacent genes on the genome. We measured the changes in transcript levels of *MCS1* and *MCL1* in response to propionate addition in MM. The expression of both *MCS1* and *MCL1* was significantly upregulated in MM with 0.1% propionate, whereas only the expression of *MCL1* continued to be upregulated in MM with 1.0% propionate (Fig. 1C). We then examined *MCS1* and *MCL1* expression levels in the hyphae cultured in CM, resting conidia, and developing appressoria, and found that the transcript levels of *MCS1* and *MCL1* increased during conidium germination and appressorium formation, reaching the highest in the appressoria at 6 h post-inoculation (hpi) (Fig. 1D).

To identify the roles of the methylcitrate cycle in development and virulence of *P. oryzae*, we deleted *MCL1* and *MCS1* in the wild-type strain 70-15 (Supplementary Fig. S1; Supplementary Table S2) and measured the 2-methylcitrate synthase and 2-methylisocitrate lyase activities in the hyphae of the wild type, $\Delta mcs1$, and $\Delta mcl1$. Deletion of *MCS1* or *MCL1* led to greatly decreased 2-methylcitrate synthase or 2-methylisocitrate lyase activities (Fig. 2A and B), implying that *MCS1* encodes a 2-methylcitrate synthase and *MCL1* encodes a 2-methylisocitrate lyase in *P. oryzae*. To determine cellular organelles where the methylcitrate cycle works, we colocalized enhanced green fluorescent protein (GFP)-tagged *Mcl1* (GFP-*Mcl1*) and DsRED2-tagged *Atp1* (*Atp1*-RED), a mitochondrial inner membrane marker protein (Shi et al. 2018), in *P. oryzae*. The signals of GFP-*Mcl1* and *Atp1*-RED were well overlapped (Fig. 2C), suggesting that *Mcl1* is localized in the mitochondria.

MCL1 and *MCS1* are involved in propionate metabolism in *P. oryzae*.

The $\Delta mcl1$ mutant was sensitive to a low concentration of propionate (0.002%) but the wild type, $\Delta mcs1$, and the double-deletion mutant $\Delta mcs1\Delta mcl1$ were not (Fig. 3A). Isoleucine, valine, and methionine are converted into propionyl-CoA; glutamic acid, however, does not produce propionyl-CoA during catabolism. On MM containing NaNO_3 , $\Delta mcl1$ grew slower than the wild type, whereas $\Delta mcs1$ had growth similar to that of the wild type. Relative to those on medium containing NaNO_3

Fig. 2. Enzyme activity and subcellular localization assays. **A**, 2-Methylcitrate synthase activity in the hyphae of $\Delta mcs1$. **B**, 2-Methylisocitrate lyase activity in the hyphae of $\Delta mcl1$. Enzyme activity was shown in microunits per milligram of protein ($\mu\text{U mg protein}^{-1}$). Error bars represent standard deviation. Asterisks indicate significant differences between two columns as estimated by Tukey's honestly significant difference test, $P < 0.05$. **C**, Subcellular localization of *Mcl1* in the mitochondria of *Pyricularia oryzae* hyphae. Green fluorescent protein (GFP)-*Mcl1* fusion proteins were colocalized with an α subunit of mitochondrial F1F0 ATP synthase *Atp1*. Bar = 10 μm .

Fig. 3. The role of *MCL1* and *MCS1* in propionate metabolism. **A**, A low concentration of propionate inhibits the growth of $\Delta mcl1$ but not of $\Delta mcs1$ or $\Delta mcs1\Delta mcl1$. Strains were cultured on minimal medium (MM) (1% glucose) with or without 0.002% propionate for 10 days. **B**, Propionyl-CoA-producing amino acids inhibit the growth of $\Delta mcl1$ and $\Delta mcs1$. The wild type, $\Delta mcs1$, $\Delta mcl1$, and their complementation strains *mcs1-c* and *mcl1-c* were cultured on MM (NaNO_3) and MM in which NaNO_3 was replaced by 25 mM isoleucine, valine, methionine (producing propionyl-CoA), or glutamic acid (without producing propionyl-CoA) as the sole nitrogen source for 10 days. In A and B, numbers (mean $\pm$ standard deviation) are relative growth rate of substrate mycelia in treatment groups, compared with those in MM. Asterisks indicate significant differences between the mutant and the wild type as estimated by Tukey's honestly significant difference test, $P < 0.05$.

or glutamic acid, the $\Delta mcl1$ and $\Delta mcs1$ mutants, especially $\Delta mcl1$, grew more slowly and had fewer aerial hyphae than the wild type when using isoleucine, valine, and methionine as the sole nitrogen source (Fig. 3B). The $\Delta mcl1$ mutant could not grow on CM with propionate as the sole carbon source, whereas the $\Delta mcs1$ mutant had a strongly reduced growth rate relative to the wild type on such medium (Fig. 4A). On MM with propionate or the C5 fatty acid valerate as the sole carbon source, both $\Delta mcl1$ and $\Delta mcs1$ could not grow (Fig. 4A). The growth of $\Delta mcl1$ and $\Delta mcs1$ was recovered in the complementation strains *mcl1-c* and *mcs1-c* on MM with isoleucine, valine, or methionine as the sole nitrogen source or with propionate as the sole carbon source (Figs. 3B and 4A). The *MCS1* and *MCL1* expression levels in the hyphae cultured in different carbon sources were examined by quantitative real-time PCR (qRT-PCR) (Fig. 4B and C). Compared with glucose (MM), *MCS1* and *MCL1* transcript levels were significantly upregulated in the media with isoleucine, valine, heptadecanoic acid ($C_{17}H_{34}O_2$), acetate, or oleic acid ($C_{18}H_{34}O_2$) but not glutamic acid as the sole carbon source ($P < 0.05$). These results suggested that *MCL1* and *MCS1* are required for propionate utilization, and deletion of *MCS1* in $\Delta mcl1$ eliminates the sensitivity of $\Delta mcl1$ to a low concentration of propionate, suggesting that *MCL1* and *MCS1* play diverse roles in fungal growth.

***MCL1* and *MCS1* are required for growth and conidiation in *P. oryzae*.**

Both $\Delta mcs1$ and $\Delta mcl1$ grew more slowly than the wild type on CM (Fig. 5A and B). Mutant $\Delta mcl1$ produced fewer conidia, approximately one-sixth of the wild type (Fig. 5C). However, on CM, $\Delta mcs1$ had sparse and short aerial hyphae (Fig. 5A) and did not produce any conidia (Fig. 5C). Mutant $\Delta mcs1$ had dense aerial hyphae on MM, similar to the wild type (Fig. 3A). Because hydrophobins are necessary for the formation of aerial hyphae (Elliot and Talbot 2004; Shi et al. 2018), we measured the expression level of hydrophobin genes in mycelia of $\Delta mcs1$ cultured on CM or MM. Compared with the wild type, the transcript levels of three hydrophobin genes (*MGG_09134*, *MGG_10105*, and *MPG1*) in $\Delta mcs1$ were downregulated on both CM and MM, whereas *MHP1* was upregulated on MM (Fig. 5D). However, $\Delta mcs1$ had higher transcript levels of four hydrophobin genes (*MHP1*, *MGG_09134*, *MGG_10105*, and *MPG1*) on MM compared with CM. These results were consistent with the phenotype of aerial mycelia of $\Delta mcs1$: sparse aerial hyphae on CM and dense but easily collapsed hyphae on MM (Fig. 5A and E). The conidial germination and appressorium formation of $\Delta mcl1$ is comparable with that of the wild type. However, the conidial germination of $\Delta mcl1$ was easily impaired by propionate addition, relative to the wild type (Fig. 5F and G). In the presence of 1% propionate, the spores of $\Delta mcl1$ had lower germination rates and shorter germ tubes than in H_2O (Fig. 5F and G).

***MCL1* is involved in virulence of *P. oryzae*.**

Mutant $\Delta mcl1$ produced conidia on CM but $\Delta mcs1$ did not; therefore, we assayed the virulence of $\Delta mcl1$ on rice by spraying a conidial suspension onto rice seedlings. Compared with the wild type and the complemented strain *mcl1-c*, the disease severity caused by $\Delta mcl1$ on rice was reduced (Fig. 6A and B). Nevertheless, $\Delta mcl1$ still produced a few typical, spindle-like blast lesions with gray centers on rice leaves. We then observed the infection process of $\Delta mcl1$ on barley leaves using droplet assays. At 24 hpi, 40.0% of the wild-type appressoria had produced infection hyphae, while no appressoria of $\Delta mcl1$ had formed penetration pegs. At 48 hpi, 38.2% of $\Delta mcl1$ appressoria finished penetration and produced short infection hyphae which were limited to the initially penetrated cells, while the wild-type

infection hyphae had completed cross-wall penetration and spread among cells (Fig. 6C and D). The wild type also produced secondary aerial, spore-forming hyphae on lesions earlier than $\Delta mcl1$. An appressorium penetrates the plant cuticle primarily mediated by its huge intracellular turgor pressure in *P. oryzae* (de Jong et al. 1997). The appressorium turgor of $\Delta mcl1$ was evaluated by an incipient cytorrhysis assay (Liu et al. 2007). Compared with the wild type and the complemented strain, more collapsed appressoria were observed in $\Delta mcl1$ under hyperosmotic glycerol solutions (Fig. 6E), suggesting that $\Delta mcl1$ had lower appressorium turgor than the wild type. The mycelial blocks of $\Delta mcl1$ were inoculated on intact rice leaves and caused weak lesions; however, they caused severe lesions on abraded rice leaves, similar to the wild type (Fig. 7), also suggesting decreased penetration ability of $\Delta mcl1$.

Icl1, an isocitrate lyase, is homologous to Mcl1 in protein sequence (57% identity) and is involved in the glyoxylate cycle in *P. oryzae* (Wang et al. 2003). We deleted *ICL1* in the wild-type strain 70-15 and $\Delta mcl1$, generating $\Delta icl1$ and the *ICL1* and *MCL1* double deletion mutant $\Delta icl1\Delta mcl1$. In virulence assays, $\Delta icl1$ displayed similar virulence to $\Delta mcl1$, and its virulence was weaker than the wild type. Mutant $\Delta icl1\Delta mcl1$, however, had much weaker virulence than both $\Delta icl1$ and $\Delta mcl1$, and only caused pinpoint-like brown specks on rice leaves (Fig. 6A), implying that Icl1 and Mcl1 have a synergistic effect in the infection process of *P. oryzae*. Because Icl1 is essential for lipid utilization (Wang et al. 2003), we tested the growth of $\Delta icl1\Delta mcl1$ on the media using olive oil or glucose as the sole carbon source. On MM without glucose (MM-C) supplemented with 1% olive oil as the sole carbon source, $\Delta icl1\Delta mcl1$ grew very slowly, similar to $\Delta icl1$, and could not utilize lipids efficiently, whereas $\Delta mcl1$ grew similarly to the wild type. On MM containing glucose, $\Delta icl1\Delta mcl1$ grew similarly to $\Delta mcl1$ and slower than the wild type, whereas $\Delta icl1$ grew similarly to the wild type (Fig. 8A). On CM, $\Delta icl1\Delta mcl1$ grew similarly to $\Delta mcl1$ and slower than the wild type and $\Delta icl1$. On CM without glucose (CM-C) supplemented with 1% olive oil, $\Delta icl1\Delta mcl1$ produced fewer aerial mycelia than $\Delta icl1$, $\Delta mcl1$, and the wild type (Fig. 8B). In liquid media, however, $\Delta icl1\Delta mcl1$ did not show significant differences in growth from $\Delta icl1$ and $\Delta mcl1$ (Fig. 8C). These results suggested that Icl1 is essential for lipid utilization, whereas Mcl1 is likely involved in utilization of glucose during fungal growth.

Acetate relieves the toxicity of propionate.

On MM, 0.08% sodium propionate hindered the growth of the wild type, $\Delta mcs1$, and $\Delta mcl1$; addition of sodium acetate recovered the growth of tested strains, especially the wild type and $\Delta mcs1$, which was severely inhibited by propionate (Fig. 9A). The growth recovery was dependent on the acetate concentrations. One molecule each of acetyl-CoA and propionyl-CoA were produced from valerate after β -oxidation. Valerate, however, inhibited the growth of the wild type, $\Delta mcs1$, and $\Delta mcl1$ (Fig. 9B). On CM, acetate also recovered conidiation, which was defective in $\Delta mcs1$ and $\Delta mcl1$ (Fig. 9B). We used the conidia produced by $\Delta mcs1$ on CM after adding 0.10% sodium acetate to test its conidial germination rate, appressorium formation rate, and virulence by droplet assays. The conidial germination and appressorium formation rate of $\Delta mcs1$ was comparable with that of the wild type. The conidia of $\Delta mcs1$ caused disease lesions on barley leaves that were similar to those caused by the wild type (Fig. 9C).

***MCS1* affects the $NAD^+/NADH$ ratio and NO production in *P. oryzae*.**

Cellular redox state and NO signaling affect the differentiation of aerial hyphae and conidia (Shi et al. 2018). Because

Fig. 4. *MCS1* and *MCL1* are required for propionate utilization. **A**, Growth of the wild type, $\Delta mcl1$, $\Delta mcs1$, and their complementation strains *mcs1-c* and *mcl1-c* on the media with propionyl-CoA-producing carbon sources. Strains were cultured on complete medium (CM) in which 1% glucose was replaced by 1% (= 121.9 mM) propionate for 7 days (CM-C), and on minimal medium (MM) in which 1% glucose was replaced by 121.9 mM propionate or valerate (producing propionyl-CoA and acetyl-CoA) as the sole carbon source for 25 days (MM-C). Bar = 5 mm. **B** and **C**, Relative expression levels of *MCS1* and *MCL1* in the wild-type hyphae cultured in MM (glucose) and MM in which glucose was replaced by 25 mM isoleucine, 25 mM valine, 1% heptadecanoic acid (producing propionyl-CoA), 1% sodium acetate, 0.01% oleic acid (producing acetyl-CoA), or 25 mM glutamic acid (without producing propionyl-CoA and acetyl-CoA) as the sole carbon source. β -TUBULIN and α -ACTIN were used as reference genes. Asterisks indicate significant differences between two columns as estimated by Tukey's honestly significant difference test, $P < 0.05$.

Fig. 5. Phenotypic assays of $\Delta mcs1$ and $\Delta mcl1$. **A**, Colony morphology of $\Delta mcs1$ and $\Delta mcl1$. Strains were cultured on complete medium (CM) for 8 days. Bar = 5 mm. **B**, Growth of $\Delta mcs1$ and $\Delta mcl1$. The diameter of mycelial colonies on CM was measured after 8 days. **C**, Conidiation of the wild type, $\Delta mcs1$, $\Delta mcl1$, and their complemented strains *mcs1-c* and *mcl1-c* cultured on CM for 8 days. **D**, Relative transcript level of hydrophobin genes in the aerial mycelia of $\Delta mcs1$ cultured on CM or minimal medium (MM) compared with the wild type. β -TUBULIN and α -ACTIN were used as reference genes in quantitative real-time PCR. **E**, Collapsed aerial mycelia of $\Delta mcs1$ on MM incubated at 25°C under a cycle of 16 h of light and 8 h of darkness for 12 days. Bar = 10 mm. **F**, Conidial germination of $\Delta mcl1$ was impaired by propionate. Germination of spores in H₂O or 1% propionate was counted at 24 h postinoculation (hpi). **G**, Morphology of germinated spores in propionate at 24 hpi. Bar = 20 μ m. Asterisks indicate significant differences between columns in figures or between the mutant and the wild type as estimated by Tukey's honestly significant difference test ($P < 0.05$). Error bars represent standard deviation.

Fig. 6. Virulence assays of $\Delta mcl1$. **A**, Virulence tests. The conidial suspension (1×10^5 spores ml^{-1}) of the wild type, $\Delta mcl1$, $\Delta icl1$, $\Delta icl1\Delta mcl1$, *mcl1-c* (*MCL1* was complemented in $\Delta mcl1$), and $\Delta icl1_mcl1-c$ (*MCL1* was complemented in $\Delta icl1\Delta mcl1$) was sprayed on rice seedlings and incubated for 7 days. **B**, Disease lesion severity assays for tests in **A**. **C**, Images of invasive hyphae of the wild type and $\Delta mcl1$ on barley epidermal cells. White arrows = invasive hyphae and black arrow = invasive hyphae which penetrated into the second cell. Bar = 10 μm . **D**, Penetration rate by appressoria of the wild type and $\Delta mcl1$ on barley cuticle. **E**, Assessment of appressorium turgor pressure by incipient cytorrhysis assay. Appressoria at 48 h postinoculation were immersed in glycerol solutions (0.5, 1.0, and 1.5 M), and the collapsed appressoria were counted. Error bars represent standard deviation. Asterisks indicate significant differences as estimated by Tukey's honestly significant difference test ($P < 0.05$).

$\Delta mcs1$ grown on CM produced sparse and short aerial hyphae and failed to differentiate into conidia, we measured the contents of NAD and NO in the mycelia of $\Delta mcs1$ cultured on CM under a light-dark cycle. Compared with the wild type, $\Delta mcs1$ had a lower NAD^+/NADH ratio (Fig. 10A), suggesting that the hyphal cells of $\Delta mcs1$ have stronger reducing power. Mutant $\Delta mcs1$ had an NO content of $1.61 \pm 0.25 \mu\text{mol g protein}^{-1}$, much lower than that in the wild type (Fig. 10B).

DISCUSSION

In fungi, several amino acids (valine, isoleucine, methionine, and threonine), odd-chain fatty acids, and propionate are metabolized via the methylcitrate cycle. In this study, we found the homologous genes encoding the five enzymes involved in the methylcitrate cycle in *P. oryzae*, and functionally identified Mcs1 and Mcl1 as a 2-methylcitrate synthase and a 2-methylisocitrate lyase, respectively, by genetic and phenotypic assays. Furthermore, we evaluated the roles of the methylcitrate cycle in fungal development and virulence of *P. oryzae*.

The methylcitrate cycle is required for fungal growth, conidiation, appressorium turgor, plant penetration, virulence, cellular redox state, and NO production (Figs. 1A and 10) by enabling detoxification of propionyl-CoA and 2-methylisocitrate in *P. oryzae*. Deletion of *MCS1* led to slowed mycelial growth, deficient aerial hyphal differentiation, and blocked conidiation in *P. oryzae* (Fig. 5). Deletion of *MCL1* led to slowed mycelial growth, decreased conidiation, and reduced virulence (Figs. 5 and 6). Hydrophobin proteins, cellular redox state (NAD^+/NADH ratio), and NO signaling play important roles in aerial hyphae differentiation and conidiation in fungi (Shi et al. 2018; Zhang et al. 2015). Deficiencies in aerial hyphal differentiation and conidiation in $\Delta mcs1$ were partly caused by downregulation of hydrophobin genes, lowered NAD^+/NADH ratio, and decreased NO content in the mycelia of *P. oryzae* (Figs. 5D and 10). In humans, 2-methylcitrate affects the TCA cycle by inhibiting citrate synthase, aconitase, NAD^+ - and NADP^+ -linked isocitrate dehydrogenase, phosphofructokinase, and the tricarboxylase carrier (Cheema-Dhadli et al. 1975). In the yeast *Yarrowia lipolytica*, the methylcitrate cycle is important for glycerol metabolism

(Papanikolaou et al. 2013). Produced by a high concentration of glycerol, the appressorium turgor is a main force for *P. oryzae* to penetrate the plant cuticle and initiate infection (de Jong et al. 1997). The reduced virulence of $\Delta mcl1$ could be due to its lowered appressorium turgor and slowed cuticle penetration (Fig. 6C, D, and E). Compared with intact rice leaves, the recovered virulence of $\Delta mcl1$ on abraded rice leaves also suggested that the deficient virulence of the mutant was partly caused by its impaired penetration ability (Fig. 7).

The metabolic reactions catalyzed by Mcs1 and Mcl1 are the first and last step, respectively, in the conversion from propionyl-CoA to pyruvate (Fig. 1). Although both Mcs1 and Mcl1 are required for propionyl-CoA metabolism and fungal development, the phenotypic differences in development and virulence between $\Delta mcs1$ and $\Delta mcl1$ were obvious. Mutant $\Delta mcs1$ displayed more severe defects in aerial hyphal differentiation and conidiation than $\Delta mcl1$ (Fig. 5A and C). The conidia of $\Delta mcl1$ showed reduced virulence, while $\Delta mcs1$ did not (Figs. 6A and 9C). Differences were also found between $\Delta mcs1$ and $\Delta mcl1$ in the utilization of propionate as the sole carbon source (Fig. 4A) or isoleucine, valine, methionine, and threonine as the sole nitrogen source (Fig. 3B; Supplementary Fig. S2), as well as in the resistance to the growth inhibition caused by propionate in the presence of glucose (Figs. 3A and 9A). Mutant $\Delta mcl1$ was more sensitive than $\Delta mcs1$ to propionate, isoleucine, and methionine during growth (Fig. 3A and B). The phenotypic differences between $\Delta mcs1$ and $\Delta mcl1$ are likely caused by a discrepancy in the accumulation of different types of intermediate metabolites. The toxicity of different metabolites to different target proteins varies. In *A. fumigatus* and *A. nidulans*, propionyl-CoA accumulated in $\Delta mcsA$, a 2-methylcitrate synthase deletion mutant (Ibrahim-Granet et al. 2008; Zhang et al. 2004). In *A. nidulans*, 2-methylisocitrate was secreted to the medium by $\Delta mclA$, a 2-methylisocitrate lyase deletion mutant, but not by the wild type when cultured with propionate. This methylisocitrate excretion, however, was competitively inhibited by acetate (Brock 2005). In $\Delta mcl1$, the metabolites methylcitrate and 2-methyl-cis-aconitate are also likely accumulated in cells (Fig. 1A). In *A. nidulans*, propionyl-CoA inhibits the activity of CoA-dependent enzymes (such as pyruvate dehydrogenase, succinyl-CoA synthetase, and ATP-citrate lyase) and interferes with the balance of carbon metabolism (Brock and Buckel 2004), while 2-methylisocitrate competitively inhibits the activity of the NADP-dependent isocitrate dehydrogenase (Brock 2005). In humans, propionyl-CoA uncompetitively inhibits the pyruvate dehydrogenase complex (Schwab et al. 2006), while 2-methylcitrate inhibits citrate synthase, aconitase, and the NAD^+ - and NADP^+ -linked isocitrate dehydrogenase (Cheema-Dhadli et al. 1975). Propionyl-CoA, 2-methylisocitrate, and 2-methylisocitrate seem to function on different enzymes and have different physiological roles, leading to diverse and distinct phenotypes between mutants $\Delta mcs1$ and $\Delta mcl1$. In *P. oryzae*, $\Delta mcs1\Delta mcl1$ showed sensitivity to 0.002% propionate similar to that of $\Delta mcs1$ but lower sensitivity than $\Delta mcl1$ (Fig. 3A), implying that 2-methylisocitrate possibly has more severe cytotoxicity in $\Delta mcl1$ than propionyl-CoA in $\Delta mcs1$ and $\Delta mcs1\Delta mcl1$. On MM containing 0.08% propionate, 0.10% acetate completely eliminated the toxicity of propionate on the wild type and $\Delta mcs1$ but not on $\Delta mcl1$ (Fig. 9A). In *A. fumigatus* and *A. nidulans*, acetate also relieved the growth inhibition of propionate on medium containing glucose (Brock et al. 2000; Maerker et al. 2005). Acetate detoxifies propionyl-CoA by competitively binding CoA to form acetyl-CoA and thereby decreases the production of propionyl-CoA (Fleck and Brock 2008; Limenitakis et al. 2013). Lesser relief on the growth of $\Delta mcl1$ by adding acetate may be due to the accumulation of

Fig. 7. Mycelial virulence of $\Delta mcl1$ on abraded rice leaves. Mycelial blocks were inoculated onto intact and abraded rice leaves for 4 days.

2-methylisocitrate, which could not be substantially removed by acetate in *P. oryzae*.

Fungal 2-methylisocitrate lyases evolved from an ancient isocitrate lyase of the basidiomycete lineage by gene duplication

(Müller et al. 2011). The deletion of *ICL1*, an isocitrate lyase gene in the glyoxylate cycle, in the *P. oryzae* wild-type strain 70-15 or Guy11 led to reduced virulence and defects in lipid utilization (Fig. 6A and B) (Wang et al. 2003). Similar to $\Delta icl1$,

Fig. 8. Role of *Icl1* and *Mcl1* in utilization of lipids and glucose with and without amino acids present. **A**, Strains (wild type, $\Delta icl1$, $\Delta mcl1$, and $\Delta icl1\Delta mcl1$) were cultured on minimal medium (MM) and MM without glucose (MM-C) containing 1% olive oil for 10 days. **B**, Strains were cultured on complete medium (CM) and CM without glucose (CM-C) containing 1% olive oil for 7 days. Bar = 5 mm. **C**, Mycelial biomass (wet weight) of the strains cultured in liquid CM, CM, or CM-C containing 1% olive oil for 24 h. Error bars represent standard deviation. Different capital letters indicate significant differences between the strains in the same medium as estimated by Tukey's honestly significant difference test ($P < 0.01$).

$\Delta mcl1$ also displayed reduced virulence (Fig. 6A and B). After both *ICL1* and *MCL1* were deleted, the $\Delta icl1\Delta mcl1$ mutant nearly completely lost its virulence in rice (Fig. 6A). *Icl1* and *Mcl1* appear to have a synergistic effect on the virulence of

P. oryzae. In *G. zeae*, both $\Delta icl1$ and $\Delta mcl1$ did not show defects in virulence but $\Delta icl1\Delta mcl1$ did, indicating that the impairment of either the glyoxylate cycle or the methylcitrate cycle can be compensated by each other but, in the defects,

Fig. 9. Inhibition of propionate on fungal growth and conidiation relieved by acetate. **A**, Colonies of the wild type, $\Delta mcs1$, and $\Delta mcl1$ grown on minimal medium (MM) containing 0.08% sodium propionate and different concentrations of sodium acetate (NaOAc) for 10 days (upper) or sodium valerate for 13 days (lower). Numbers (mean ± standard deviation [SD]) are diameters (in millimeters) of colonies. Bar = 5 mm. **B**, Sodium acetate recovered conidiation on complete medium (CM); conidiation on CM was defective in $\Delta mcs1$ and $\Delta mcl1$. Error bars represent SD. Asterisks indicate significant differences as estimated by Tukey's honestly significant difference test ($P < 0.05$). **C**, Virulence tests of $\Delta mcs1$. Conidial suspension (20 μ l; 1×10^5 spores ml⁻¹) was dropped onto barley leaves and incubated for 4 days.

cannot be compensated if both pathways are impaired (Lee et al. 2009). In *P. oryzae*, the greatly reduced virulence in $\Delta icl1\Delta mcl1$ possibly occurred because $\Delta icl1\Delta mcl1$ combined both defects of $\Delta icl1$ and $\Delta mcl1$ in utilizing different carbon sources: $\Delta icl1$ displayed defects in utilizing lipids and $\Delta mcl1$ displayed defects in utilizing glucose and amino acids (valine, isoleucine, and methionine) (Figs. 8A and 3B). In *Saccharomyces cerevisiae*, 2-methylisocitrate lyase is located in the mitochondrial matrix (Luttik et al. 2000). In *Y. lipolytica*, three enzymes (2-methylcitrate synthase, 2-methylcitrate dehydratase, and 2-methylisocitrate dehydratase) are localized in the mitochondria; 2-methylisocitrate lyase, however, is located in both the mitochondria and cytoplasm (Uchiyama et al. 1982). In *Toxoplasma gondii*, a one-celled eukaryotic parasite, the 2-methylcitrate cycle is split between the mitochondria and cytoplasm, in which 2-methylcitrate synthase is localized in the mitochondria, 2-methylcitrate dehydratase is in the cytoplasm, and 2-methylisocitrate lyase is in the proximity of the mitochondria but in the cytoplasm (Limenitakis et al. 2013). In *P. oryzae*, 2-methylisocitrate lyase (Mcl1) is localized in the mitochondria (Fig. 2C). Disruption of the 2-methylcitrate cycle and the glyoxylate cycle impairs nutrient utilization coupling with various cellular metabolic pathways in the mitochondria and peroxisome (lipid breakdown, glycogen catabolism, and TCA) during fungal growth and appressorial formation. The expression levels of *MCL1* and *MCS1* were increased not only

by propionyl-CoA-producing carbon sources but also by acetyl-CoA-producing carbon sources (Fig. 4A and B), suggesting the metabolic connections between the methylcitrate cycle and lipid breakdown, the glyoxylate cycle, or TCA cycles. Therefore, the defects of $\Delta icl1\Delta mcl1$ in metabolizing several carbon sources and amino acids possibly led to a large decrease in virulence.

In summary, we demonstrated that deletion of *MCS1* and *MCL1* resulted in slowed growth on several carbon or nitrogen sources (propionate, glucose, valine, isoleucine, methionine, and threonine), decreased conidiation, reduced appressorium turgor, and severely delayed appressorial penetration and virulence by interfering with carbon metabolism, cellular redox state ($NAD^+/NADH$), and NO signaling in *P. oryzae*. Our findings show that the methylcitrate cycle is essential for fungal development and virulence through detoxification of propionyl-CoA, methylcitrate, and 2-methylisocitrate, and plays an important role in maintaining carbon metabolism in *P. oryzae*.

MATERIALS AND METHODS

Fungal strains, media, culture conditions, PCR, and statistical tests.

P. oryzae wild-type strain 70-15 and its derivatives used in this study are listed in Table 1. Growth tests were performed on CM (Talbot et al. 1993); MM (CM without peptone140, case-amino acid, and yeast extract); CM-C; MM-C; MM without $NaNO_3$ (MM-N); MM-C and MM-N supplemented with 25 mM isoleucine, valine, methionine, or glutamic acid; CM-C and MM-C supplemented with 1% (vol/vol or wt/vol) olive oil, sodium propionate, sodium acetate, heptadecanoic acid, oleic acid, or 121.9 mM sodium valerate; and CM and MM supplemented with different concentrations of sodium propionate, sodium acetate, or sodium valerate at 25°C under a cycle of 16 h of light and 8 h of darkness. qRT-PCR analyses were performed with five biological replicates and quantification was standardized to β -TUBULIN and α -ACTIN mRNA levels. The PCR primers used are listed in Supplementary Table S3. Statistical differences among strains or treatments were analyzed using Tukey's honestly significant difference tests in the Data Processing System software package (Tang and Zhang 2013).

Targeted gene deletion and complementation.

The deletion mutants were generated using a revised method based on a high-throughput gene knockout method described previously (Cao et al. 2016; Lu et al. 2014). A binary vector and a yeast-*Escherichia-Agrobacterium* shuttle vector, pKO3A and pKO3B, were constructed by replacing GFP in pKO1 or pKO1B (Lu et al. 2014) with a 1,131-bp synthesized *HSVtk* gene with codons optimized for *P. oryzae* using a ClonExpress MultiS One Step Cloning Kit in an in-fusion assembly method (Vazyme Biotech). *HSVtk* is a suicide gene encoding the herpes

Fig. 10. $NAD^+/NADH$ ratio and nitric oxide (NO) content in mycelia grown on complete medium (CM). **A**, Intracellular $NAD^+/NADH$ ratio in the aerial mycelia of the wild type and $\Delta mcs1$. **B**, NO content in the aerial mycelia of the wild type and $\Delta mcs1$. Error bars represent standard deviation. Asterisks indicate significant differences as estimated by Tukey's honestly significant difference test ($P < 0.05$).

Table 1. *Pyricularia oryzae* strains used in this study

Strain	Genotype description	Reference
70-15	Wild-type	Chao and Ellingboe 1991
$\Delta mcl1$	<i>MCL1</i> deletion mutant of 70-15	This study
$\Delta mcs1$	<i>MCS1</i> deletion mutant of 70-15	This study
$\Delta mcs1\Delta mcl1$	<i>MCS1</i> and <i>MCL1</i> deletion mutant of 70-15	This study
$\Delta icl1$	<i>ICL1</i> deletion mutant of 70-15	This study
$\Delta icl1\Delta mcl1$	<i>ICL1</i> and <i>MCL1</i> deletion mutant of 70-15	This study
<i>mcl1-c</i>	<i>MCL1</i> rescued strain of $\Delta mcl1$	This study
<i>mcs1-c</i>	<i>MCS1</i> rescued strain of $\Delta mcs1$	This study
$\Delta icl1_mcl1-c$	<i>MCL1</i> rescued strain of $\Delta icl1\Delta mcl1$	This study

simplex virus type 1 thymidine kinase. The construction of gene-deletion cassettes in pKO3B was performed following a previously reported method (Lu et al. 2014). *Agrobacterium tumefaciens*-mediated transformation (ATMT) was carried out according to a previously reported method (Lu et al. 2014), with a modification. The nitrocellulose membrane strips containing the germinated conidia cocultivated with *A. tumefaciens* were transferred onto the selection plates supplemented with 0.5 μM 5-fluoro-2'-deoxyuridine (F2dU) and chlorimuron-ethyl at 100 $\mu\text{g ml}^{-1}$ (for *SUR*, a sulfonylurea resistance gene), hygromycin B at 200 $\mu\text{g ml}^{-1}$ (for *HPH*, a hygromycin B phosphotransferase gene), or glufosinate-ammonium at 350 $\mu\text{g ml}^{-1}$ (for *BAR*, a baialaphos resistance gene). Ectopic transformants containing *HSVtk* could not grow on the selection media supplemented with F2dU (Khang et al. 2005). Transformants grown on the selection plates were individually transferred to the new selection plates using sterile toothpicks. Genomic DNAs of transformants were isolated and the mutants were identified and confirmed using previously reported methods (Lu et al. 2014). The mutants were complemented with their respective native copy of genes amplified from the wild type. The complementation strains were identified on the selection plates supplemented with G418 at 800 $\mu\text{g ml}^{-1}$ (for *NEO*, a neomycin phosphotransferase II gene) and confirmed at the mRNA level by RT-PCR.

Observation of fluorescence fusion proteins.

The coding sequence of *MCL1* was cloned from the wild-type genomic DNA and inserted into the *XbaI-SalI* site of pKD5-GFP containing *SUR* (Li et al. 2012) to obtain fusion gene *GFP-MCL1*. *ATP1-RED*, a fusion gene in a plasmid pKD8-RED containing *NEO*, encodes a mitochondrion marker protein (Shi et al. 2018). Both *GFP-MCL1* and *ATP1-RED* were cotransformed into the *P. oryzae* wild-type strain via ATMT. Samples were observed under a fluorescence microscope (filter: FITC and TRITC; Nikon eclipse 80i).

Phenotypic analysis of mutants.

The growth of strains on CM, MM, and all other media was assayed at 7 to 10 days postinoculation (dpi). Conidiation on CM was analyzed at 8 dpi. The conidial germination rate was counted at 4 or 24 hpi. Appressorium turgor was evaluated through incipient cytorrhysis (cell collapse) assays, as described previously (Liu et al. 2007). The experiments were performed three times with three (for turgor test) or five (for other tests) replicates each time.

Plant infections.

Conidial virulence experiments were performed by spraying conidial suspensions (1×10^5 conidia ml^{-1}) onto 10- to 14-day-old rice seedlings (*Oryza sativa* 'CO39') using an artist's airbrush (Cao et al. 2016). The disease severity was estimated by the proportion of the surface area that was diseased in a 5-cm section of the infected leaf at 7 dpi. Mycelial virulence experiments were performed by inoculating 5-mm mycelium blocks on the leaf explants of rice or barley (*Hordeum vulgare* 'ZJ-8') and culturing them at 25°C for 4 days. Cuticle penetration experiments by appressoria on barley leaf explants and, therefore, infection growth observation were carried out according to a previously reported protocol (Liu et al. 2007).

Quantification of intracellular NAD⁺, NADH, and NO contents.

Intracellular NAD⁺ and NADH contents in aerial mycelia were quantified using the NAD/NADH quantification kit (Sigma-Aldrich) and NO was quantified with the NO assay kit (S0021; Beyotime) according to previously reported methods (Shi et al. 2018). The concentrations of total NAD and NADH

were shown in picomoles per milligram of protein and intracellular NO in micromoles per gram of protein.

Enzyme activity assays.

The 2-methylcitrate synthase activity was measured with a citrate synthase assay kit (Sigma-Aldrich) following the manufacturer's instructions, except that acetyl-CoA from the kit was replaced with 0.3 mM propionyl-CoA (Sigma-Aldrich). The 2-methylisocitrate lyase activity was measured using an isocitrate lyase assay kit (Solarbio) following the manufacturer's guidelines, except that isocitrate was replaced with sodium DL-threo-2-methylisocitrate (MedChem Express) at 2.5 mg ml^{-1} . *P. oryzae* was inoculated in liquid CM in a rotator (150 rpm) at 28°C for 2 days, then cultured in CM containing 0.002% sodium propionate for 16 h. The mycelia were collected and ground in liquid nitrogen; then, whole-cell extracts were prepared according to the corresponding procedure in the kits. The enzyme activities were determined following the manufacturer's instructions of the kits. The 2-methylcitrate synthase and 2-methylisocitrate lyase activities were shown in microunits (nanomoles per minute) per milligram of protein.

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