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Effects of Isocitrate Lyase Inhibitors on Spore Germination and Appressorium Development in *Magnaporthe grisea*

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Abstract The glyoxylate cycle can conserve carbons and adequately supply tricarboxylic acid (TCA) cycle intermediates for biosynthesis when microorganisms grow on C_2 carbon sources. It has been reported that isocitrate lyase (ICL1), a key enzyme of the glyoxylate cycle, is highly induced when *Magnaporthe grisea*, the causal agent of rice blast, infects its host. Therefore, the glyoxylate cycle is considered as a new target for antifungal agents. A 1.6-kb DNA fragment encoding the *ICL1* from *M. grisea* KJ201 was amplified by PCR, cloned into a vector providing His-tag at the *N*-terminus, expressed in *Escherichia coli*, and purified using Ni-NTA affinity chromatography. The molecular mass of the purified ICL1 was approximately 60 kDa, as determined by SDS-PAGE. The ICL1 inhibitory effects of TCA cycle intermediates and their analogs were investigated. Among them, 3-nitropropionate was found to be the strongest inhibitor with an IC_{50} value of 11.0 μg/ml. 3-Nitropropionate inhibited the appressorium development in *M. grisea* at the μM level, whereas conidia germination remained unaffected. This compound also inhibited the mycelial growth of the fungus on minimal medium containing acetate as a C_2 carbon source. These results suggest that ICL1 plays a crucial role in appressorium formation of *M. grisea* and is a new target for the control of phytopathogenic fungal infection.

Key words: *Magnaporthe grisea*, glyoxylate cycle, isocitrate lyase, gene expression, inhibitors, appressorium

In microorganisms, the glyoxylate bypass of the tricarboxylic acid (TCA) cycle provides the means to grow on C_2 compounds by converting them into C_4 dicarboxylic acids [8, 11]. This is achieved through the activity of two unique enzymes, isocitrate lyase (ICL) and malate synthase (MLS). The oxaloacetate supplied by the bypass maintains the TCA cycle by replacing intermediates that are removed for biosynthesis. This is an archetypal anaplerotic reaction. Bacterial and fungal mutants lacking ICL are unable to grow on acetate as the sole carbon source [16, 17].

Magnaporthe grisea (Hebert) Barr (anamorph: *Pyricularia grisea*) is a typical heterothallic Ascomycete and the causal agent of rice blast, one of the most destructive diseases of cultivated rice worldwide [25, 26]. *M. grisea* causes plant infection by means of a specialized infection structure called an appressorium. Appressoria are dome-shaped cells, which develop enormous turgor in order to generate an invasive force to rupture the rice leaf cuticle [24]. During conidial germination and appressorium formation in *M. grisea*, lipid bodies are known to be mobilized from the conidium to the germ tube apex. At the onset of tugor generation, lipid bodies coalesce and are taken up by vacuoles before rapid lipolysis. Thus, *M. grisea* uses lipid metabolism extensively during appressorium formation. A consequence of reliance upon lipid metabolism for tugor generation during the prepenetration stage of development may be induction of the glyoxylate cycle to provide a mechanism of generating glucose [2].

Recently, it has been reported that the genes of the glyoxylate cycle are highly induced, when *M. grisea* infects rice [25]. *Δicl1* mutants are less virulent than an isogenic wild-type strain of *M. grisea* and impaired in virulence-associated functions such as germ tube emergence, appressorium development, and cuticle penetration [25]. Therefore, ICL1 could be a promising target for the control of fungal infection and development of antifungal agents. In this study, the *ICL1* gene was cloned from *M. grisea* KJ201 and expressed in *Escherichia coli*. The inhibitory activities of itaconate-related compounds and TCA cycle metabolites were investigated. We also present evidence that the ICL1 inhibitor inhibits appressorium formation in *M. grisea*.

cDNA of *M. grisea* KJ201 was used as a template for the amplification of the *ICL1* gene by PCR [12, 14]. Highly purified mRNA from *M. grisea* KJ201 was prepared using

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an Oligotex mRNA kit (Qiagen), and cDNA synthesis was carried out by PCR using a cDNA synthesis kit (Amersham Biosciences) according to the manufacturer's instructions [12]. The *M. grisea* KJ201 homolog of *ICL1* was identified by searching currently available *M. grisea* 70-15 cDNA sequence data from NCBI GenBank (accession number AF540383). Based on the nucleotide sequence of *ICL1*, two synthetic primers (5'-GCG**GAATTC**ATGGCTTCTA-AAAACATGGTG-3', forward primer; 5'-AT**GCGGCCGC**-TCAGTGGAATTGGTCTTCTG-3', reverse primer) were designed to carry the EcoRI and NotI recognition sites, respectively (bold). PCR was performed under the following conditions on a thermal cycler: an initial denaturation at 94°C for 5 min, then 30 cycles consisting of 94°C for 20 sec, 55°C for 2 min, and 72°C for 2 min, followed by a final extension at 72°C for 10 min. The resulting PCR product (1.6 kb) was purified on agarose gel (iNtRON Biotech, Korea), subcloned into the pLitmus 28i vector (NewEngland Lab.), and subsequently transformed into *E. coli* TOP10 (Invitrogen). The plasmid DNA was extracted using a Wizard plasmid DNA purification kit (Promega), and the sequence was analyzed using the ABIPrism BigDye Terminator version 3.1 Cycle Sequencing Kit in the ABIPRISM 377 Stretch Laser-induced Fluorescence automatic sequencer (Applied Biosystems). The analysis of the nucleotide sequences and confirmation were performed by comparison with those nucleotide sequences registered in the GenBank database, using the BLAST Network Service provided by the NCBI (National Center for Biotechnology Information, Bethesda, MD, U.S.A.). The sequence of the *M. grisea* KJ201 *ICL1* subclone (pMICL1) showed 100% identity with the *M. grisea* 70-15 *ICL1* registered (accession no. AF540383) at the amino acid level (data not shown).

To obtain and investigate the recombinant ICL1, the subcloned plasmid was subsequently digested with EcoRI and NotI restriction enzymes and inserted into the pET32a expression vector (Novagen) (pMICL1). The constructed plasmid pEICL1 was transformed into *E. coli* BLR (DE3) (Stratagene), and ICL1 expression was induced by the addition of IPTG (Sigma) into Luria-Bertani (LB) broth at a final concentration of 0.5 mM. After incubation for 4 h at 25°C with shaking at 200 rpm, cells were harvested by centrifugation at 6,000 ×*g* for 10 min, resuspended in 10 ml of distilled water, and lysed by sonicating in six short bursts of 5 sec with a rest period of 60 sec between bursts [10, 15]. The homogenate was then centrifuged at 10,000 ×*g* for 30 min and the supernatant was collected. The supernatant containing *N*-terminal His-tagged ICL1 protein was purified using Ni-NTA column chromatography (Qiagen) according to the manufacturer's instructions and the *N*-terminal His-tag was cleaved with EnterokinaseMax (Invitrogen) [20]. By SDS-PAGE analysis, the molecular mass of the enterokinase-treated ICL1 was calculated to be 60 kDa (547 amino acids protein) (Fig. 1). The *ICL1* gene

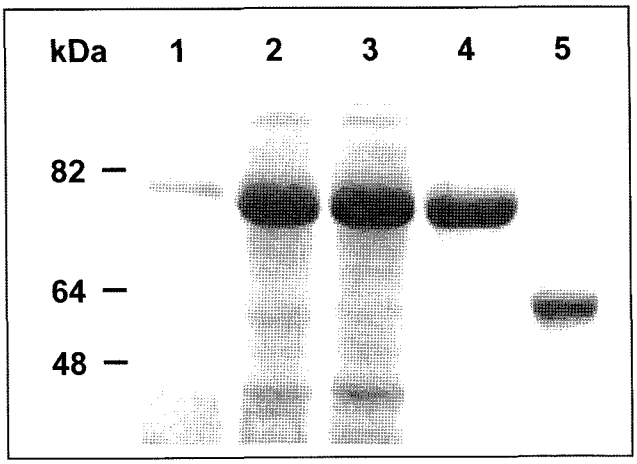

Fig. 1. SDS-PAGE analysis of ICL1 expressed in *E. coli* BLR (DE3).
The protein samples were loaded on a 12% polyacrylamide gel. Lane 1, total proteins of host without induction of pMICL1; lane 2, total proteins of host with induction of pMICL1; lane 3, supernatant containing the fused ICL1; 4, ICL1 fusion protein purified using Ni-NTA column chromatography (70 kDa); 5, enterokinase-treated recombinant ICL1 (60 kDa).

product showed 85.5% amino acid identity to the isocitrate lyase gene *acu3* of *Neurospora crassa* and 76.3% identity to *acuD* of *Aspergillus nidulans* [5].

The enzyme activity of the purified ICL1 was determined by the method of Dixon and Kornberg [6, 18]. A basic concept of this method is to spectrophotometrically measure the formation of glyoxylate phenylhydrazone at 324 nm in the presence of phenylhydrazine and isocitrate. One ml of the reaction mixture contained 20 mM sodium phosphate buffer (pH 7.0), 1.27 mM *threo*-DL (+) isocitrate, 3.75 mM $MgCl_2$, 4.1 mM phenylhydrazine, and various amounts of the ICL1 enzyme. The reaction was carried out at 37°C

Table 1. Inhibition of ICL1 activity by several metabolites and related compounds.[a]

Compound		IC_{50} (μg/ml)
Known inhibitors		
	Itaconate	12.6
	Itaconic anhydride	18.5
	3-Nitropropionate	11.0
Related metabolites		
	Citrate	>100
	Succinate	>100
	Malate	>100
	Glycolate	>100
	Malonate	>100

[a]Reactions were carried out in 1 ml of incubation mixture containing 20 mM sodium phosphate buffer (pH 7.0), 1.27 mM *threo*-DL-isocitrate, 3.75 mM $MgCl_2$, 4.1 mM phenylhydrazine, and 2.5 μg/ml of purified ICL1 at 37°C for 15 min. Appropriate blank contained all of the above-mentioned compounds, except test sample. The IC_{50} values represent the concentration giving 50% inhibition relative to the blank.

for 15 min. Protein concentration was determined by the method of Bradford [1] using a Bio-Rad protein assay kit (Bio-Rad) and bovine serum albumin as a standard. Since glycolysis and TCA cycle intermediates have frequently been reported to inhibit the ICL1 activity [6, 16, 17, 23], the inhibitory effects of the related metabolites and their analogs were examined (Table 1). Among the compounds tested, 3-nitropropionate was found to be the strongest inhibitor with an IC_{50} value of 11.0 μg/ml. However, the TCA cycle intermediates did not show inhibitory activity at concentrations up to 100 μg/ml. To determine the type of inhibition, kinetic studies were also performed with 3-nitropropionate as a representative ICL1 inhibitor. The Lineweaver-Burk plot obtained for the cleavage of *threo*-DL (+) isocitrate under standard assay conditions showed that the K_m value for *threo*-DL (+) isocitrate was 1.42 mM (Fig. 2), which is comparable to the values for the enzymes from *Candida brassicae* and *C. tropicalis* with K_m values of 1.5 and 1.53 mM [19], respectively, but much higher than the values reported for *Neurospora crassa* [9] and *Ashbya gossypii* [22] ICLs, both at 0.05 mM. Kinetics analysis of the inhibition of the cleavage reaction showed that 3-nitropropionate was a noncompetitive inhibitor of ICL1 with a K_i value of 118 μM (Fig. 2). 3-Nitropropionate and bromopyruvate were previously shown to inhibit *Mycobacterium avium* ICL (K_i values of 3 μM and 120 μM, respectively) [21]. 3-Nitropropionate has been reported to be a potent inhibitor of the ICL as an analog of the carbanion intermediate [21]. 3-Nitropropionate binds to *Pseudomonas indigofera* ICL in a two-step fashion, forming an initial rapid complex with an apparent K_i of 250 μM at pH 8 (23 μM for the fully ionized form), followed by conversion to a tighter complex [21]. Currently, we cannot fully appreciate the present results, and further studies on the inhibition of *M. grisea* ICL1 by 3-nitropropionate are needed.

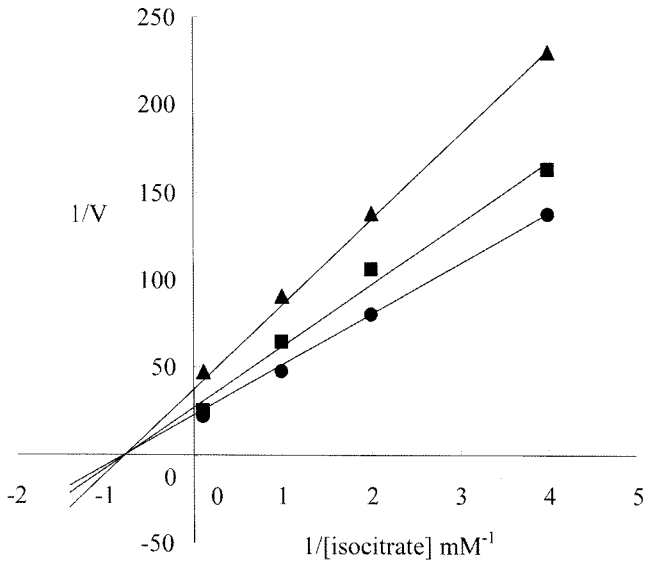

Fig. 2. Lineweaver-Burk plot of ICL1 inhibition.
Inhibition by 3-nitropropionate (●, 0 μM; ■, 0.3 μM; ▲, 0.8 μM) using 2.5 μg of purified ICL1 protein per assay.

To investigate the influence of the ICL1 inhibitor on appressorium formation in *M. grisea* KJ201, the fungus was grown on oatmeal agar medium (Difco) at 24°C under fluorescent light to promote conidiation [13]. Conidia were collected from 14-day-old cultures and washed twice with distilled water. Development of appressoria in germinating conidia was monitored on the hydrophobic surface of GelBond film (Amersham Biosciences). Thus, GelBond sheets were washed in sterile distilled water for 30 min, and the GelBond assays [3, 4, 12, 13] were performed by placing pieces of the sheet, hydrophobic side up, in a 24-well microtiter plate, containing 0.4 ml of 0.9% agarose. A 40-μl sample of the conidia suspension (1.25×10^5 spores/ml) was added to the hydrophobic side of the GelBond sheet, and conidia were allowed to attach to the surface for 1 h before sterile distilled water (0.96 ml) was carefully added. 3-Nitropropionate dissolved in dimethyl sulfoxide was added to a final solvent concentration of less than 1%. At this concentration of the solvent, no effect on germination, mycelial growth, or appressoria development was observed. The plates were incubated for 12 h at 24°C. As shown in Fig. 3, in the presence of 20 μg/ml 3-nitropropionate, conidia germinated and mycelial growth continued without the formation of infectious structure, whereas 95% of the conidia formed appressoria in the control. In this study, 3-nitropropionate was found to inhibit appressorium formation in *M. grisea* at concentrations above 20 μg/ml. These data suggest that the enzyme activity of ICL1 is important for appressorium development in *M. grisea*.

As a representative ICL1 inhibitor, we then investigated the effect of 3-nitropropionate on fungal growth and survival, when *M. grisea* was grown on C_2 carbon source. Thus, *M. grisea* KJ201 (2.5×10^5 spores/ml) was plated on a minimal growth medium [25] [carbon source 10 g/l,

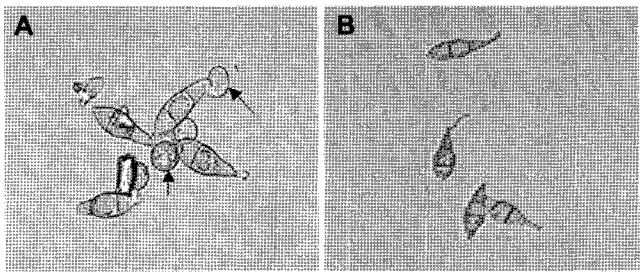

Fig. 3. Effects of 3-nitropropionate on appressorium formation by *M. grisea* KJ201 on a hydrophobic surface.
A. On the hydrophobic side of the GelBond sheet, 95% of the conidia formed appressoria. **B**. Twenty μg/ml of 3-nitropropionate inhibited appressorium formation. Pictures were taken after incubation for 14 h at 24°C. The arrow indicates appressorium formation.

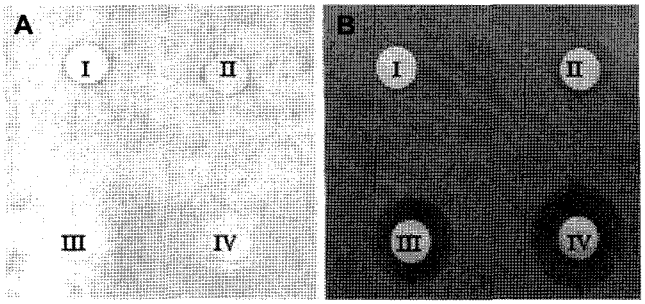

Fig. 4. *M. grisea* is unable to use acetate (C_2) as the sole carbon source in the presence of ICL1 inhibitor, 3-nitropropionate.
M. grisea KJ201 was cultured on minimal growth medium containing either 1% glucose (**A**) or 1% acetate (**B**) as the sole carbon source. Discs of filter paper soaked with 20 (I), 40 (II), 60 (III), and 80 μg/ml (IV) of 3-nitropropionate were added to each plate. Pictures were taken after incubation for 10 days at 24°C.

$Ca(NO_3)_2 \cdot 4H_2O$ 1 g/l, KH_2PO_4 0.2 g/l, $MgSO_4 \cdot 7H_2O$ 0.25 g/l, NaCl 0.15 g/l, agar 20 g/l] containing either sodium acetate or glucose as the sole carbon source. Discs of filter paper soaked with various concentrations of 3-nitropropionate were added to the plates. Fungal growth inhibition was graded, based on the radius of the inhibition zone after incubation for 10 days at 24°C. As shown in Fig. 4, 3-nitropropionate had no effect on the *M. grisea* grown on glucose, but was inhibitory to *M. grisea* grown on acetate, albeit at high concentration. These data strongly suggest that specific inhibition of ICL1 is a new way to protect against rice blast fungal infection and 3-nitropropionate is a good starting candidate for a structure-based antifungal agent design.

In conclusion, the results obtained for the metabolite inhibition indicate that the *M. grisea* ICL1 may be a regulatory enzyme playing a crucial role in fungal growth, in good agreement with the recent finding that ICL1 plays a pivotal role in the coupling system of the TCA and glyoxylate cycles in oxalate biosynthesis [14, 15, 19]. In addition, since the enzymes of the glyoxylate cycle are not found in mammals, they are prime targets for antifungal agents. Further research is underway to control the *M. grisea* infection of rice with specific ICL1 inhibitors that were synthesized based on the present investigation.

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