Isocitrate Dehydrogenase and Isocitrate Lyase are Essential Enzymes for Riboflavin Production in *Ashbya gossypii*

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Abstract For this study, we hypothesized that mitochondrial NAD^+-dependent isocitrate dehydrogenase 1 (ICDH1) and isocitrate lyase (ICL1) were important enzymes for riboflavin synthesis in the fungus *Ashbya gossypii*. Here, the genes encoding ICDH1 and ICL1 were disrupted in order to analyze the enzymes' functions on riboflavin production by the fungus. The riboflavin production resulting from these disruptants was markedly decreased compared to the concentration produced by its parental strain when cultured in a rich nutrient medium used to optimize riboflavin production. Furthermore, when comparing the transcription levels of the genes encoding ICDH1 and ICL1, between wild-type *A. gossypii* and an itaconate resistant mutant of *A. gossypii* obtained by UV irradiation, the mRNA levels in the mutant were 1.8- and 2.0-fold higher than those in the wild-type strain, respectively. These results indicate that ICDH1 and ICL1 are key enzymes for riboflavin synthesis in *A. gossypii*. © KSBB

Keywords: Ashbya gossypii, *NAD^+-dependent isocitrate dehydrogenase 1, isocitrate lyase, riboflavin production, gene disruption*

INTRODUCTION

Riboflavin is a precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), and is an indispensable nutrient for humans and animals. A deficiency in riboflavin can cause diseases such as dermatitis, particularly dermatitis around the mouth in humans. Previously, riboflavin was only produced by chemical synthesis methods. Today, however, it is mainly produced by fermentation using microorganisms such as *Ashbya gossypii* and *Bacillus subtilis* [1]. The filamentous hemiascomycete *A. gossypii* is a producer of riboflavin [2], and has a completely sequenced and annotated genome [3,4]. More than 40% of the riboflavin produced in the world is produced by *A. gossypii*, which is commercially available worldwide [1,5,6]. Attempts to enhance its riboflavin production have been carried out by introducing mutation [1] and by genetic engineering, to achieve the overexpression or disruption of genes [7-10].

Among the enzymes involved in riboflavin synthesis in *A. gossypii*, mitochondrial NAD^+-dependent isocitrate dehydrogenase (ICDH) and isocitrate lyase (ICL1) are believed to be key, based on the study results to date (Fig. 1). The former catalyzes the oxidation of isocitrate to α-ketoglutarate in the tricarboxylic acid (TCA) cycle [1], and the latter catalyzes the formations of succinate in the glyoxylate cycle, and glyoxylate from isocitrate that is provided from the TCA cycle [11], which is an important branching point between the TCA and glyoxylate cycles. In *Saccharomyces cerevisiae*, a yeast and close genetic relative to *A. gossypii*, mature ICDH is formed from the two hetero-subunits ICDH1 and ICDH2, and the enzyme's function is lost by disrupting one of the subunits [12,13]. In the case of *A. gossypii*, homologous genes encoding ICDH1 and ICDH2 were previously found [4]. In addition, genes encoding ICL1 [14] and ICL2 [15] were identified in *S. cerevisiae*, and their homologous

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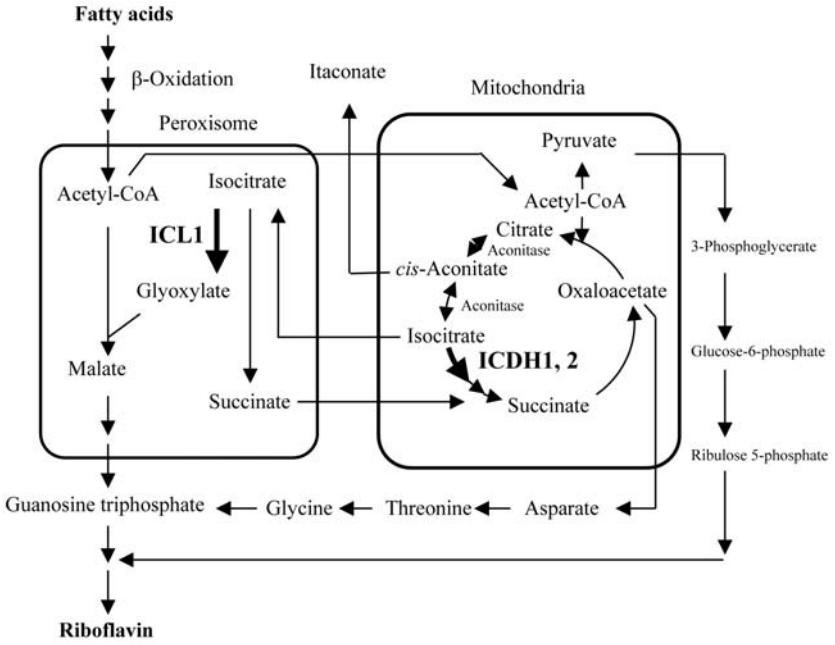

Fig. 1. A hypothetical outline of the metabolic pathway for riboflavin synthesis in *A. gossypii.*

genes *ICL1* and *ICL2* were found in *A. gossypii*, respectively [4,16]. In *S. cerevisiae*, ICL1 functions in the glyoxylate cycle [14] and ICL2 functions in the methylcitrate cycle [15].

In this study, to clarify the effects of these enzymes on riboflavin production, we disrupted the genes encoding ICDH1 and ICL1 in wild-type *A. gossypii*, as well as in the itaconate resistant mutant *A. gossypii* strain ZP4. Furthermore, transcriptional analyses of *ICDH1* and *ICL1* in the wild-type and ZP4 strains were performed.

MATERIALS AND METHODS

Strains, Media, Plasmids, and Cultivation

For this study we used the fungi *A. gossypii* ATCC10895 (wild-type) and *A. gossypii* strain ZP4, which is an itaconate-resistant mutant obtained by UV irradiation [17]. *Escherichia coli* DH5α was used for genetic manipulation. Each strain of *A. gossypii* was grown at pH 6.0 and 28°C on YD medium containing (per liter) 10 g of glucose and 10 g of yeast extract (Oriental Yeast Co., Ltd., Tokyo, Japan) for genetic manipulation, and on YR medium containing 10 g of rapeseed oil and 10 g of yeast extract for total RNA isolation and riboflavin production. The production medium consisted of (per liter) 50 g of rapeseed oil, 30 g of gelatin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 60 g of corn steep liquor (CSL) (Sigma Chem. Co., St. Louis, MO, USA), 1.5 g of glycine, 1.5 g KH_2PO_4, and minerals (2 mg of Co^{2+}, 5 mg of Mn^{2+}, 20 mg of Zn^{2+}, and 1 mg of Mg^{2+}) (pH 6.8). For the solid-state cultivations, 20 g L^{-1} of agar was added to prepare the agar medium plates. Soft agar YD medium containing 250 μg mL^{-1} of geneticin (Wako Pure Chemical Industries) in 5 g L^{-1} of agar was added onto YD plates for the transformant selections. A YD medium plate containing 200 μg mL^{-1} of geneticin was used for further transformant selections. LB medium containing (per liter) 10 g of polypepton, 5 g of yeast extract, and 8 g of sodium chloride was used for the cultivation of *E. coli*. The plasmid pPKT [10], which contained an *E. coli* kanamycin-resistance-gene-expressing fragment for *A. gossypii* was used as template DNA in PCR. Finally, pT7Blue (Merck KGaA, Darmstadt, Germany) was used for cloning *ICDH1* and *ICL1* of *A. gossypii*.

Gene Disruptions of *A. gossypii*

The DNA manipulation of *E. coli* followed standard procedures [18-20]. The gene disruptions of the wild-type *A. gossypii* and ZP4 strains were essentially carried out according to the method of Wendland *et al.* [21], except in this study the promoter and terminator of the translation elongation factor EF-1α gene (*TEF1*) from *A. gossypii* were used. The nucleotide sequence data of the *A. gossypii* chromosome were obtained from the Ashbya Genome Database [4]. In the database, *ICDH1* corresponds to ADL223W and *ICL1* to ADL066C. The primers Dicdh1-F1 and Dicdh1-R1 were used for the PCR-based gene disruption of *ICDH1*, and the primers Dicl1-F1 and Dicl1-R1 were used for that of *ICL1* (Table 1). In each reaction, KOD-plus-DNA polymerase (Toyobo Co., Ltd., Osaka, Japan) was used with pPKT as the template DNA, and a 50-μL aliquot of the reaction mixture for PCR was prepared according to the manufacturer's protocol. The PCR conditions were 95°C for 3 min, 30 cycles of 95°C for 30 sec, 55°C for 50 sec, and 68°C for 2 min. After the reactions, the PCR products were separated by 1% agarose gel electrophoresis and the objective bands were col-

Table 1. Primers used in this study

Name	Sequence (5' to 3')
Primers used for construction of the gene targeting cassettes	
Dicdh1-F1*	tcatccgagtgtcgggtccgaaaagtgctcgaggtggaaaagccgagacacggaggtCTGCAGACATGGAGGCCCAGAATAC
Dicdh1-R1*	accaaagtactgggacagcgtaccaagcctggtcttactttatcatgttagtcgtcgataaataGAATTCTTTCTGCGCACTTAACTTCGCA
Dicl1-F1*	gagcacattagtcgtttagtgactgtgcgcatgtgtgcggacgtgatgcactcgaacCTGCAGACATGGAGGCCCAGAATAC
Dicl1-R1*	gtccatctccctctgctggatgccttgtgcatacgcacgcattccgaacctgctGAATTCTTTCTGCGCACTTAACTTCGCA
Primers used for confirmation of the gene disruptants	
Dicdh1-F2	GTACCTGGTGTGCTGTGGCACGAACC
Dicdh1-R2	CAGTGTTAAGAAGCGGTGTTGTGGCCATGATCC
Dicl1-F2	CCGGTATCCACATGGAGGACCAGTCC
Dicl1-R2	GGTTACACCCTTACCCATCGAGGCTGTCG
Kan-F	GCTCTAGAATGAGCCATATTCAACGGGAAACG
Kan-R	GGGGTACCTTAGAAAAACTCATCGAGCATC
Primers used for Q-PCR	
Q-ACT-F	ACGGTGTTACCCACGTTGTTCC
Q-ACT-R	TCATATCTCTGCCGGCCAAGTC
Q-ICDH1-F	CTTTGCGCTCAAGAACAACCGGAA
Q-ICDH1-R	TGAAGAGACCATCGCCCAGTTTCA
Q-ICL1-F	CAATGCCTTGGCCATCGACAAC
Q-ICL1-R	CCTCTGCTGGATGCCTTGTG
Primers used for the sequencing analysis of the *ICL1* gene	
ICL1-Seq-F	TTGAGCCGATCAGCGCAAGAACGCGCA
ICL1-Seq-R	TAGGATGTTCTTGGGGCGCAGTGGACT
ICL1-Seq1	CCGTCGAGTCACCATTAGGAGTAGAGCA
ICL1-Seq2	CGTAATGGCGGACAAGCTCGTGGAGACA
ICL1-Seq3	TTGGGGCCTGAGATTGACTACTTGAGG
ICL1-Seq4	GATCGAAGTTTTGGACGCCGCGCAGCA
ICL1-Seq5	GACCTGGTCTGGTTCGAATCCAACTTC
ICL1-Seq6	GTTGACAGCATTCTCAAGCTTGCCCAGG

*Homologous sequences to the targeted genes for gene disruption are represented in lower case.

lected and purified. The structures of the fragments constructed for gene disruption are shown in Fig. 2.

Fragments for *ICDH1* and *ICL1* disruption were introduced according to the method reported by Monschau [7]. In brief, 24-h–cultured mycelia of *A. gossypii* were harvested and transformed using a Gene Pulser Xcell system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in 10 mM Tris-HCl buffer containing 270 mM sucrose and 1 mM $MgCl_2$. The exponential decay pulse was provided in a 0.2-cm cuvette (Bio-Rad Laboratories) with the following conditions: 1.5 kV cm^{-1}, 200 Ω, and 25 μF. The transformants that were resistant to geneticin were selected on YD medium plates containing geneticin. The spore solutions of these transformants were prepared as described previously [17], and were diluted 500-fold and spread onto YD medium plates containing geneticin to obtain separated colonies.

Confirmation of *ICDH1* and *ICL1* Disruptants of *A. gossypii* by PCR Methods

The gene disruptions of the purified transformants were confirmed by colony-directed PCR as follows. The primer set of Dicdh1-F2 and Kan-R was used to select for *ICDH1* disruptants, and the primer set of Dicl1-F2 and Kan-R was used to select for *ICL1* disruptants (Table 1, Fig. 2). Each primer set was designed to form PCR products only in the case where the target gene was replaced with a disrupting cassette. Blend Taq-plus (Toyobo Co.) was used in the reaction, and a 15-μL aliquot of the reaction mixture for PCR was prepared according to the manufacturer's protocol, except the template DNAs were added by suspending colonies in the reaction mixture via transfer with sterile toothpicks. The PCR conditions were 95°C for 3 min, 32 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec. Then, the reactants were loaded onto a 1% agarose gel and electrophoresed. The chromosomes of the positive transformants from the PCR screening were extracted using a DNeasy Plant Mini kit (Qiagen GmbH, Düsseldorf, Germany), and used for further confirmation. The primers Kan-F and Dicdh1-R2 were used to select for *ICDH1* disruptants, and the primers Kan-F and Dicl1-R2 were used to select for *ICL1* disruptants (Table 1, Fig. 2). The PCR products were amplified with these primer sets when target genes had been replaced. The reaction mixtures were prepared in the same manner as those

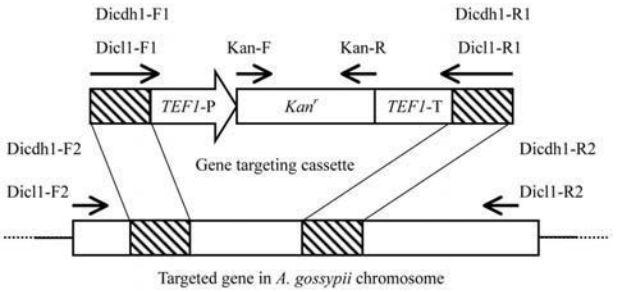

Fig. 2. Schematic drawing of the gene-targeting cassettes for gene disruption. Arrows indicate the positions of primers used in the PCRs. Abbreviations used in the gene-targeting cassettes are as follows: *Kan*^{r}, kanamycin resistance gene of *E. coli*; *TEF1*-P, promoter of *TEF1* gene; *TEF1*-T, terminator of *TEF1* gene.

in the colony-direct PCR described above, with the exception of the primers and templates. The reaction was performed using the same program used in the colony-direct PCR. Moreover, the chromosomes of the positive transformants in this PCR were used for examination by another round of PCR. The primer set of Dicdh1-F2 and Dicdh1-R2 was used to select for *ICDH1* disruptants, and the primer set of Dicl1-F2 and Dcil1-R2 was used to select for *ICL1* disruptants (Table 1, Fig. 2). By using these primer sets, a difference in PCR products size could be detected between the parental strains and gene disruptants. Again the reaction mixtures were prepared in the same manner as those in the colony-direct PCR described above, except for the primers and templates. The PCR conditions were 95°C for 2 min, 32 cycles of 95°C for 20 sec, 55°C for 30 sec, and 72°C for 100 sec.

Confirmation of *ICDH1* and *ICL1* Disruptants of *A. gossypii* by Southern Blotting

The chromosomes of the positive transformants from the three PCR patterns were confirmed by Southern blotting to estimate the copy numbers of gene-targeting cassettes in each transformant. A DNA fragment of an *E. coli* kanamycin resistance gene was used as a specific probe, and was prepared by PCR with the primers Kan-F and Kan-R and with pPKT as the template. A reaction mixture containing *Premix Taq* (Takara Bio Inc., Shiga, Japan) was prepared according to the manufacturer's protocol. The PCR conditions were 94°C for 2 min, 32 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min. The amplified fragment was non-RI labeled using an AlkPhos Direct Labelling and Detection system (GE Healthcare Bio-Sciences Corp., Buckinghamshire, UK) according to the manufacturer's protocol. Signals were visualized using Fluor-S/MAX (Bio-Rad Laboratories). Each transformant containing a single copy of the gene-targeting cassette was determined to be a gene disruptant.

Riboflavin Concentration Measurement

To produce riboflavin, each *A. gossypii* fungus was grown at 28°C on a rotary shaker at 220 rpm for 6 days in a 500-mL flask containing either 50 mL of YR or production medium. After the cultivation, the hydrophobic fraction containing oil in the cultured broth was removed, and 0.5 mL of the broth was diluted with 4.5 mL of water and centrifuged (10 min, 1,500 × g). Next, 1.6 mL of the supernatant was mixed with 0.4 mL of 1 N NaOH and 5 mL of 50 mM potassium phosphate buffer (pH 7.0). The optical density at 444 nm was measured, and the riboflavin concentration was calculated with a conversion factor of 127.294 mg/(optical density/L) [22]. In the cultivation using YR medium, the dry mycelial weight of a strain was also measured.

Estimation of mRNA Levels of *ICDH1* and *ICL1*

Wild-type *A. gossypii* and ZP4 were precultured at 28°C on a reciprocating shaker at 80 rpm for 24 h in a 500 mL-flask containing 100 mL of YD medium, after which the mycelia were harvested on filter paper. The mycelia were washed three times with sterile water, and 1 g (wet weight) of each mycelium was transferred into a 300 mL-flask containing 50 mL of YR medium and cultured for 10 h. Then, the mycelia were harvested on filter paper and washed with sterile water. The total RNA was extracted from each mycelium using ISOGEN (Wako Pure Chemical Industries) according to the attached protocol. A first-strand cDNA was synthesized from the total RNA using an oligo $(dT)_{16}$ primer and reverse transcriptase (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol.

The transcript levels of *ICDH1* and *ICL1* in the synthesized cDNAs were estimated using a real-time quantitative PCR (Q-PCR) Mx3000P system (Stratagene, La Jolla, CA, USA). The transcript level amount of an actin gene (*ACT1*), a constitutively expressed gene that can be used as an internal standard of transcript levels, was also measured. To prepare the reaction mixture we mixed a FullVelocity SYBR Green QPCR Master mix (Stratagene) with each primer set designed for Q-PCR and each synthesized cDNA according to the attached protocol. The transcript levels were measured with the following primers: *ACT1* was measured with the primers Q-ACT-F and Q-ACT-R, *ICDH1* with the primers Q-ICDH1-F and Q-ICDH1-R, and *ICL1* with the primers Q-ICL1-F and Q-ICL1-R (Table 1).

Cloning and Sequencing Analyses of *ICL1s* from Wild-type *A. gossypii* and ZP4

The primers ICL1-Seq-F and ICL1-Seq-R (Table 1), along with chromosomes of wild-type *A. gossypii* and ZP4 were used to amplify each fragment by PCR, including the open reading frame (ORF) of *ICL1*. The Takara LA Taq hot start version (Takara Bio Inc.) was used in the reaction, and a 40-μL aliquot of the reaction mixture for PCR was prepared according to the manufacturer's protocol. The PCR conditions were 95°C for 4 min, 30 cycles of 95°C for 25 sec, 57°C for 40 sec, and 72°C for 3 min. The amplified fragments were inserted into pT7Blue for the sequencing analyses.

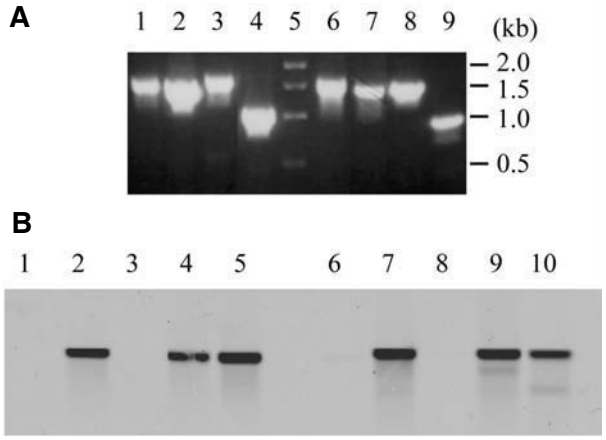

Fig. 3. Genomic analyses for the confirmation of gene disruptions by PCR (A) and the Southern blotting (B). (A) PCR products amplified from chromosomes of the following loaded strains: Lane 1, W-*icdh*; Lane 2, wild-type; Lane 3, W-*icl*; Lane 4, wild-type; Lane 6, ZP4-*icdh*; Lane 7, ZP4; Lane 8, ZP4-*icl*; Lane 9, ZP4. A size marker was loaded on Lane 5. The primer sets used in the reactions were Dicdh1-F2 and Dicdh1-R2 (Lanes 1, 2, 6, and 7) or primers Dicl1-F2 and Dicl1-R2 (Lanes 3, 4, 8, and 9). (B) Aliquots (5 μg) of *Xho*I (from Lanes 1 to 5) or *Pst*I (from Lanes 6 to 10) digested chromosomes were loaded on agarose gel for the Southern blotting. Samples from following strains were loaded: Lane 1, wild-type; Lane 2, W-*icdh*; Lane 3, ZP4; Lanes 4 and 5, ZP4-*icdh*; Lane 6, wild-type; Lane 7, W-*icl*; Lane 8, ZP4; Lanes 9 and 10, ZP4-*icl*.

The sequencing reaction for each nucleotide sequence of *ICL1* cloned in pT7Blue was carried out using a DTCS Quick Start Master mix (Beckman Coulter Inc., Fullerton, CA, USA) with the primers ICL1-Seq1, ICL1-Seq2, ICL1-Seq3, ICL1-Seq4, ICL1-Seq5, and ICL1-Seq6 (Table 1) according to the attached protocol. The prepared sequencing samples were analyzed using a capillary DNA sequencer CEQ2000 (Beckman Coulter Inc.), and each *ICL1* was analyzed using three independent clones.

RESULTS AND DISCUSSION

Construction of *ICDH1*- and *ICL1*-Disrupted Strains

Among the metabolic enzymes that are related to riboflavin synthesis in *A. gossypii*, we chose ICDH1 and ICL1 to perform the functional analyses of this study, because these enzymes were thought to play important roles in riboflavin production. The fragments used for *ICDH1* and *ICL1* disruption were prepared by PCR and introduced into *A. gossypii* cells by electroporation. Each gene disruption was performed for both the wild-type *A. gossypii* and ZP4 strains, and the transformants were selected on YD medium plates containing geneticin. The transformation efficiency with these cassettes was approximately 0.1 colony forming units/μg of DNA.

The second screening for gene disruptants was carried out using colony direct PCR with the primers Dicdh1-F2 and Kan-R or Dicl1-F2 and Kan-R, and showed that homologous recombination between the gene-targeting cassette and the genome occurred at the *ICDH1* or *ICL1* locus of the genome, respectively (data not shown). Positive strains in this PCR were analyzed by genomic PCR using either the primers Kan-F and Dicdh1-R2 or Kan-F and Dicl1-R2 (data not shown), and finally these strains were confirmed by genomic PCR with either the primers Dicdh1-F2 and Dicdh1-R2 or Dicl1-F2 and Dicl1-R2 (Fig. 3A). The copy number of the gene-targeting cassette in each transformant was estimated as one by Southern blotting (Fig. 3B). These results indicate that each targeted gene was replaced with the gene-targeting cassette, and there was no recombination at any unexpected locus in the genome. Positive strains were determined to be gene disruptants, and the *ICDH1* and *ICL1* disruptants of the wild-type *A. gossypii* strains were named W-*icdh* and W-*icl*, respectively, and the *ICDH1* and *ICL1* disruptants of ZP4 were named ZP4-*icdh* and ZP4-*icl*, respectively.

Effects of *ICDH1* and *ICL1* Disruptions on Riboflavin Production

The riboflavin production of each gene disruptant was measured and compared with that of the parental strain to clarify the importance of ICDH1 and ICL1 in riboflavin synthesis in both the YR and production media. The maximum riboflavin concentrations produced by the gene disruptants compared with those of the parental strains after 6 days of growth in YR medium were as follows: 30% in W-*icdh*, 163% in W-*icl*, 14% in ZP4-*icdh*, and 135% in ZP4-*icl* (Fig. 4A). The maximum dry mycelial weights of the disruptants compared with those of their parental strains after 6 days growth in YR medium were the following: 30% in W-*icdh*, 54% in W-*icl*, 16% in ZP4-*icdh*, and 52% in ZP4-*icl* (Fig. 4B). In the production medium, the maximum riboflavin concentrations produced by the gene disruptants compared with those of the parental strains after 6 days growth were: 19% in W-*icdh*, 31% in W-*icl*, 23% in ZP4-*icdh*, and 18% in ZP4-*icl* (Fig. 5). The growth rates of these strains in the production medium could not be accurately measured due to solid bodies that were contained in the medium. However, those growth rates of W-*icdh* and ZP4-*icdh* were low, and those of W-*icl* and ZP4-*icl* were almost the same as their parental strains (data not shown). The growth rates of the ICDH1 null strains in both media indicated that the enzyme was crucial for the growth of *A. gossypii*, and that low riboflavin production by these strains resulted in a low growth rate. *ICL1* disruption enhanced riboflavin production in YR medium, however, their growth rates were about half of those of the parental strain. On the other hand, riboflavin production decreased with the disruption of *ICL1* in the production medium, and the growth rates of those disruptants were almost the same as those of the parental strains (data not shown). From these results one can conclude that ICDH1

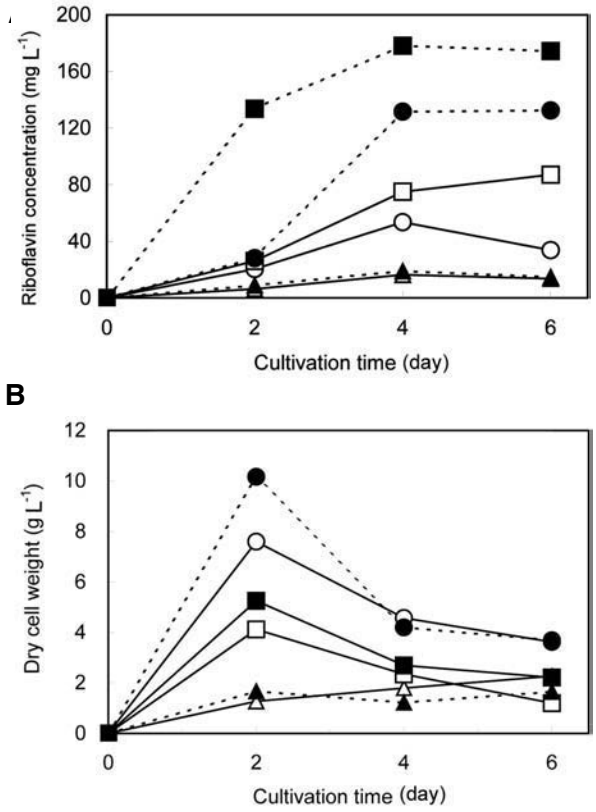

Fig. 4. Riboflavin concentrations produced by *A. gossypii* strains (A) and dry mycelial weight of the strains (B) cultivated in YR medium. Symbols: open circle, wild-type; open triangle, W-*icdh*; open square, W-*icl*; closed circle, ZP4; closed triangle, ZP4-*icdh*; closed square, ZP4-*icl*. The riboflavin concentrations produced by each strain were measured as described in the Materials and Methods.

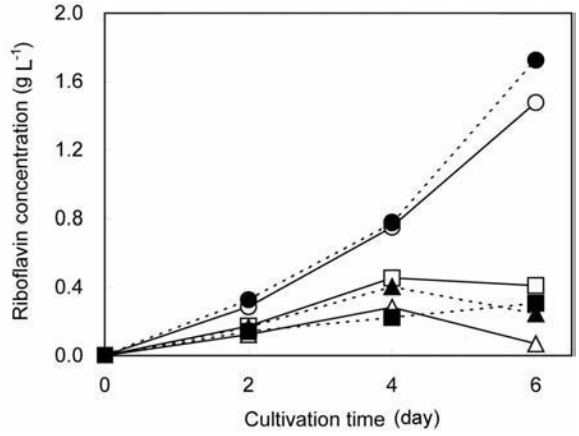

Fig. 5. Riboflavin concentrations produced by *A. gossypii* strains cultivated in production medium. Symbols are the same as those in Fig. 4. The riboflavin concentrations produced by each strain were measured as described in the Materials and Methods.

is an essential enzyme for riboflavin production during cultivation in whichever medium, while ICL1 plays a different role depending on the medium composition.

Multiple pathways for riboflavin synthesis in *A. gossypii* have been reported, as shown in Fig. 1. In *ICDH1* disrupted cells isocitrate accumulates, which may contribute to an increased carbon flux from the TCA cycle into the glyoxylate cycle. However, the riboflavin concentrations in W-*icdh* and ZP4-*icdh* were markedly low (Fig. 4A and 4B). We presume that in *ICDH1*-disrupted cells, *cis*-aconitate is formed from the accumulation of isocitrate, as indicated by the balanced reaction of aconitase (Fig. 1). *cis*-Aconitate is a substrate for *cis*-aconitic acid decarboxylase, whose product is itaconate, which inhibits ICL1. This may cause a reduction of carbon flux from the TCA cycle into the glyoxylate cycle. On the other hand, in *ICDH1*-disrupted cells, the production of adenosine triphosphate (ATP) by the TCA cycle was interrupted, resulting in a low capacity of the ATP pool. This may explain the poor growth rates of the strains and their low riboflavin productions. In the case of ZP4-*icdh*, its riboflavin concentration was 2-fold that produced by W-*icdh* (Fig. 4A). Since ZP4, the parental strain of ZP4-*icdh*, was obtained based on itaconate resistance [17], the pathway for riboflavin synthesis in ZP4-*icdh* may be resistant toward itaconate with highly expressed ICL1, compared with that in the wild-type *A. gossypii* (Fig. 6). In the *ICL1*-disrupted cells, a pathway from isocitrate to glyoxylate was blocked (Fig. 1), resulting in a low carbon flux in the glyoxylate cycle. However, the riboflavin concentrations produced by W-*icl* and ZP4-*icl* were increased in the YR medium (Fig. 4A), yet were obviously decreased in the production medium (Fig. 5). These results suggest that the TCA cycle and/or gluconeogenesis is the major pathway for riboflavin synthesis in *A. gossypii* during cultivation with YR medium, while the glyoxylate cycle is the major pathway during cultivation with production medium. As described in the Materials and Methods, the major difference between the YR and production media used in this study was their amino acid compositions, including the nitrogen source, thus the carbon flux for riboflavin synthesis in *A. gossypii* may have depended on the composition of the media ingredients.

Transcription Analyses of *ICDH1* and *ICL1* of Wild-type *A. gossypii* and ZP4

Since ZP4 was obtained by UV irradiation [17], the mutated locus (or loci) that improved itaconate resistance remained unknown. Our previous work showed that the crude enzyme activity of ICL1 in ZP4 was higher than that in wild-type *A. gossypii* [17]. In this study, the transcription levels of *ICL1* in wild-type *A. gossypii* and in ZP4 were compared by Q-PCR. We also compared the *ICDH1* mRNA levels of these strains. The total RNAs were extracted from mycelia that were cultivated in YR medium, and first-strand cDNAs were synthesized. The analyses by Q-PCR showed that the mRNA levels of *ICDH1* and *ICL1* in ZP4 were 1.8- and 2.0-fold greater than those of the wild-type strain (Fig. 6), and

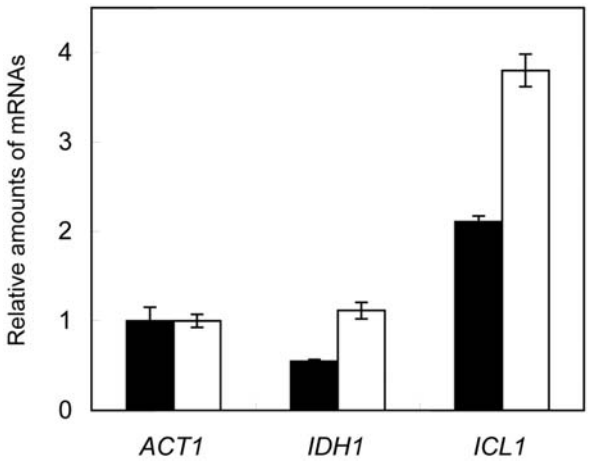

Fig. 6. Comparison of transcription levels of *ICDH1* and *ICL1* of wild-type strain (closed bar) and ZP4 strain (open bar). The cDNA of each strain was prepared as described in the Materials and Methods. Each transcript amount was measured by Q-PCR and expressed as a relative amount to the transcript of *ACT1*.

that the higher enzyme activities of ZP4 observed in a previous study were due to higher transcription levels, as compared to wild-type *A. gossypii*. This suggests that the mutated loci produced by UV irradiation were in the promoters of these ZP4 genes, which enhanced the activity of the promoters. Another hypothesis is that mutations occurred in the metabolic enzyme genes, or within the promoters that act upstream to ICDH1 or ICL1 in whatever pathways, which led to an accumulation of substances that induced transcription.

Sequencing Analysis of *ICL1* from ZP4

As shown above, ICL1 is a key enzyme for itaconate resistance when *A. gossypii* is cultured with vegetable oil as a carbon source. Schmidt *et al.* [23] reported that ICL1 activity is inhibited by itaconate, an antimetabolite of ICL1. It is possible that higher ICL1 activity was due to a change in its amino acid sequence, as well as an increase in its transcription, since ZP4 was selected using itaconate resistance and the ICL1 of ZP4 might have resistance to itaconate. From this aspect we cloned *ICL1s* from wild-type *A. gossypii* and ZP4 strains, and sequenced them. The sequencing analysis showed that the nucleotide sequence of *ICL1* from ZP4 was exactly the same as that of wild-type *A. gossypii* (data not shown). These sequences agreed with the *ICL* sequence that was registered by Stahmann in the DNA Data Bank of Japan (accession number, AJ010727). This result indicates that the higher ICL1 activity observed in ZP4 was not brought by a change in its amino acid sequence, but rather by an increase in its expression level. The overexpression of these genes improves the riboflavin production of *A. gossypii*; nevertheless, for riboflavin synthesis in ZP4 there may be uncharacterized mutations related to carbon flux. Via genetic engineering we are now trying to obtain an advanced riboflavin-overproducing strain by developing an improved ICL1 with higher activity than that of the wild-type enzyme.

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