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Anaerobic Induction of Isocitrate Lyase and Malate Synthase in Submerged Rice Seedlings Indicates the Important Metabolic Role of the Glyoxylate Cycle

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Abstract The glyoxylate cycle is a modified form of the tricarboxylic acid cycle that converts C2 compounds into C4 dicarboxylic acids at plant developmental stages. By studying submerged rice seedlings, we revealed the activation of the glyoxylate cycle by identifying the increased transcripts of mRNAs of the genes of isocitrate lyase (ICL) and malate synthase (MS), two characteristic enzymes of the glyoxylate cycle. Northern blot analysis showed that ICL and MS were activated in the prolonged anaerobic environment. The activity assay of pyruvate decarboxylase and ICL in the submerged seedlings indicated an 8.8-fold and 3.5-fold increase over that in the unsubmerged seedlings, respectively. The activity assay of acetyl-coenzyme A synthetase in the submerged seedlings indicated a 3-fold increase over that in the unsubmerged seedlings, which is important for initiating acetate metabolism. Consequently, we concluded that the glyoxylate cycle was involved in acetate metabolism under anaerobic conditions.

Key words *Oryza sativa*; glyoxylate cycle; isocitrate lyase; malate synthase; acetyl-coenzyme A synthetase; anoxia

Plants are continually confronted with challenges by environmental stresses, such as drought, salinity, low temperature and flooding. Ethanolic fermentation is the principal process of anaerobic carbohydrate catabolism in plants [1]. Under anaerobic conditions, respiration is blocked and ethanolic fermentation substantially contributes to NAD^+ regeneration. Ethanolic fermentation produces acetaldehyde and ethanol by the catalysis of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). With continuing oxygen deficiency, activation of ethanolic fermentation would lead to the accumulation of acetaldehyde and acetate. An experiment involving carrot cells has shown that the observed toxic effects of ethanol are not ascribed to ethanol itself, but to acetaldehyde [2]. In other instances, post-anoxia injury may be a result of the rapid metabolism of ethanol without coordinated oxidation of acetaldehyde [3]. Rice acetaldehyde dehydrogenases (ALDHs) are involved in the conversion of acetaldehyde to acetate in the mitochondria and cytoplasm [4–8]. Increased transcripts of rice *Aldh2a* have been identified in low-oxygen environments [9]. These results suggest that the oxidation of acetaldehyde generated by ethanolic fermentation is necessary to mitigate acetaldehyde accumulation and ensure survival during anoxia.

The glyoxylate cycle is a modified form of the tricarboxylic acid (TCA) cycle that occurs in bacteria, fungi and plants [10]. Acetyl-coenzyme A synthetase (ACS) catalyzes the conversion of acetate into acetyl-coenzyme A (acetyl-CoA). The acetyl-CoA is metabolized in the glyoxylate cycle for the synthesis of succinate and malate [6]. Bacterial and fungal mutants are unable to grow with acetate as the sole carbon source because of the absence of the glyoxylate cycle [11,12]. In oilseed plants, the glyoxylate cycle plays a key role in converting acetyl-

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CoA produced by fatty acid β-oxidation into oxaloacetate, and subsequently into sugar [12]. The contribution of the glyoxylate cycle to malate synthesis has been studied in anaerobic rice coleoptiles using a ^{13}C NMR spectrometer [13]. The glyoxylate cycle presumably has an anaplerotic role that provides the intermediate for gluconeogenesis [14]. The glyoxylate cycle plays a potential role in acetate metabolism when acetate is converted into acetyl-CoA under anaerobic conditions.

Isocitrate lyase (ICL) and malate synthase (MS) are the two characteristic enzymes of the glyoxylate cycle which enable cells to use acetyl-CoA to generate increased levels of TCA cycle intermediates for biosynthetic pathways, such as gluconeogenesis [12]. Studies of the *Arabidopsis icl*-1 and *icl*-2 mutants, which are absent in the glyoxylate cycle, have shown that an additional anaplerotic source of carbon provided by the glyoxylate cycle is required for lipid breakdown and seedling establishment after prolonged dark conditions [15]. The gene expression and enzyme activities in the glyoxylate cycle of *Arabidopsis* have been studied [16]. ICL and MS have been revealed to play a role in various developmental stages [17,18] and senescent tissues [19,20]. Usually, the glyoxylate cycle is involved in the bypassing the decarboxylation steps of the TCA cycle.

In this study, we used ICL and MS to investigate the mechanism of the glyoxylate cycle in rice under hypoxic conditions. We found that rice ICL and MS can be induced by low-oxygen stress. We also detected the activation of ACS in the prolonged period of oxygen deficiency, which initiates acetate metabolism.

Materials and Methods

Plant materials and treatment

Rice (*Oryza sative* ssp. *indica* cv Guangluai 4) was grown in natural light at 24–35 °C in a water culture (pH is about 6.1). The leaves of at least 10-day-old aerobically seedlings were used for the extraction of total RNA. Ten-day-old seedlings were entirely submerged in water for 12, 24, 48 and 72 h to deprive them of oxygen. The seedlings that were submerged for 48 hours were then transferred to an aerobic environment for 24 h (designated as 48/24 h). The seedling leaves were collected after each time interval. The leaves were immediately frozen in liquid nitrogen for RNA isolation. Total RNA was extracted by a Trizol method using RNAex reagent & systems IV (Cat. No. W6061, Watson, Shanghai, China).

Oligonucleotides

The following oligonucleotides were synthesized: ICL-f, 5′-gacatggccaagaagctgt-3′; ICL-r, 5′-gatgaactccgacatgtc-3′; MS-f, 5′-atggccaccaacgccgcagc-3′; Ms-r, 5′-tcagagcttgcacggcgacg-3′; Adh1-f, 5′-attatggtgttgggtaataagatt-3′; Adh1-r, 5′-aactgaaactcgtataaatatatg-3′; Pdc1-f, 5′-ccgcctaatccccagtgatc-3′; Pdc1-r, 5′-ccttcaaatcgtccatgttattg-3′; rRNA-f, 5′-cgcaaattacccaatcctgac-3′; rRNA-r, 5′-aaatacgaatgcccccgac-3′; Actin-f, 5′-tatggtcaaggctgggttcg-3′; Actin-r, 5′-cgcacttcatgatggagttg-3′; ICL-5out, 5′-acagcttcttggccatgtc-3′; ICL-5in, 5′-acgtcgcgcgccgtgtag-3′; ICL-3out, 5′-tacaacctctcgccgtcc-3′; ICL-3in, 5′-gacatgtcggagttcatc-3′; MS-5out, 5′-agtcagccatgaagaccttg-3′; MS-5in, 5′-ggcgttgatgaccatcttgc-3′; MS-3out, 5′-gtcgtggaggtggagatg-3′; MS-3in, 5′-acgacttcctcacgctcgac-3′; ALDH1a-f, 5′-agagatcaagagcctattcg-3′; ALDH1a-r, 5′-gtacactctagctatagctg-3′; ALDH2a-f, 5′-cagcttatcatggcggcaag-3′; ALDH2a-r, 5′-ccttggtctggaggtagttgc-3′; ALDH2b-f, 5′-aggtatcggtcatggctgc-3′; ALDH2b-r, 5′-ttacaaccacgcggcgttc-3′.

Amplification of full-length cDNAs and sequencing

Full-length cDNAs of the rice ICL and MS genes were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA end (RACE). The primers were designed on the basis of genomic sequence and the expressed sequence tags (ESTs). The open reading frames (ORFs) of ICL and MS were amplified by RT-PCR and gene-specific primers. The 5′-untranslated regions (5′-UTRs) of ICL and MS were amplified by using SMART™ (Switching mechanism at 5′ end of the RNA transcript) cDNA technology (Clontech, CA, USA) with nested primers of ICL-5out/ICL-5in and MS-5out/MS-5in, respectively. The amplification of the 3′ regions of ICL and MS was carried out with an RNA PCR™ kit (AMV, ver. 1.1) according to the manufacturer's instructions (TaKaRa Biotechnology, Dalian, China), using nested primers of ICL-3out/ICL-3in and MS-3out/MS-3in, respectively. The cDNA of *Pdc1* and *Adh1* genes in their 3′-UTRs were amplified and used as the probes in the Northern blot analysis, using the primer pairs of Adh1-f/Adh1-r and Pdc1-f/Pdc1-r, respectively. The cDNA of the rice actin 1 gene was amplified using Actin-f/Actin-r, and was used to normalize the RNA level in the RT-PCR. All the PCR products were sequenced by the ABI377 sequencing system (Applied Biosystems, CA, USA). The sequences of coding regions and untranslated regions, including 3′-UTRs and 5′-UTRs, were assembled to form the full-length cDNA sequences of the ICL and

MS genes. The amplified fragments were identified by sequencing and searching the relevant databases.

Probe labeling and Northern hybridization

Four cDNA probes amplified by RT-PCR were used to detect *Pdc1*, *Adh1* and the genes of ICL and MS. The primer pairs of Pdc1-f/Pdc1-r, Adh1-f/Adh1-r, ICL-f/ICL-r and MS-f/MS-r were used for the synthesis of double-strand cDNAs of *Pdc1*, *Adh1* and the genes of ICL and MS, respectively. A 1.8 kb fragment of the rice 18S rRNA gene was amplified from rRNA-f/rRNA-r. These probes were ^{32}P labeled with the Rediprime™ II random prime labeling system (Amersham Biosciences, NJ, USA).

Total RNA was denatured by treatment with formaldehyde and separated in a 1% (*W*/*V*) denaturing agarose gel. The gel was subsequently stained with ethidium bormide and blotted onto a Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech, NJ, USA). Northern blot hybridization was performed with ExpressHyb™ hybridization solution (Clontech).

Preparation of glyoxysomes and assay of ICL activity

The treated seedling leaves were harvested, washed with cold water at 4 °C, suspended and homogenized in a minimal volume of buffer A [10 mM HEPES-KOH (pH 7.5), 0.4 M sucrose, 0.1% (*W*/*V*) bovine serum albumin (BSA), 1% (*V*/*V*) polyvinylpolypyrrolidone (PVP), 1 mM EDTA and 10 mM β-mercaptoethanol]. The homogenate was squeezed through four layers of gauze. The filtrate was centrifuged at 1500 *g* for 15 min at 4 °C to remove plastids and cell debris. The supernatant was subjected to a second centrifugation at 10,000 *g* for 15 min. The pellet was resuspended in 5 ml of buffer B [HEPES-KOH (pH 7.5), 0.4 M sucrose, 0.1% (*W*/*V*) BSA and 1 mM EDTA]. The suspension was then centrifuged at 10,000 *g* for 15 min. The resuspended pellet in 1 ml of buffer B was layered directly on top of 30 ml of a 28% (*V*/*V*) solution of Percoll in buffer B and was centrifuged at 40,000 *g* for 45 min with slow acceleration and deceleration. Glyoxysomes sedimented near the bottom of the self-generated gradient were collected. To remove the Percoll, the glyoxysomes were washed by centrifugation at 5000 *g* for 10 min after the addition of 4 volumes of buffer C [HEPES-KOH (pH 7.5), 1 mM EDTA and 0.3 mM raffinose]. The pellet was resuspended in 1 ml of buffer C and used as purified glyoxysome and for subsequent activity analysis.

The ICL activity was determined using the method described by Dixon and Kornberg [21] and modified by Ranaldi *et al*. [22]. The assay cocktail consisted of 4 mM *D*,*L*-isocitric acid, 5 mM $MgCl_2$, 4 mM cysteine, 6 mM phenylhydrazine and 50 mM triethanolamine (pH 7.5). The reaction was started with the substrate, 4 mM *D*,*L*-isocitric acid. The ICL activity was assayed at 25 °C and the absorbance was monitored at 324 nm for 2 min using a spectrophotometer (UV-2450, Shimadzu, Japan). Under these conditions, the absorbance increased linearly for at least 5 min. All of the proteins were quantified by the method described by Bradford [23] with BSA as the standard.

Preparation of crude extracts and assay of PDC activity

The PDC activity was assayed according to the method described by Rivoal *et al*. [24] with some modification. The treated seedling leaves were used in the preparation of crude enzyme extracts. The leaves were harvested, washed with cold water at 4 °C, suspended and homogenized in a minimal volume of buffer D [50 mM MES-NaOH (pH 6.2), 5 mM dithiothreitol (DTT), 1 mM $MgCl_2$ and 1 mM thiamine pyrophosphate (TPP)]. The homogenate was squeezed through four layers of gauze. The extract was clarified by centrifugation at 25,000 *g* at 4 °C for 15 min. The supernatant was directly assayed for enzyme activity. The protein in each tissue was also quantified by Bradford's method.

The PDC activity was assayed at 25 °C in 50 mM MES-NaOH (pH 6.2), 0.1 mM TPP, 0.5 mM $MgCl_2$, 0.2 mM NADH, 3.3 mM sodium pyruvate and 120 U/ml ADH (Worthington, NJ, USA). The reaction started with 3.3 mM sodium pyruvate and was monitored at 340 nm for 2 min, using a spectrophotometer. In all our assays, the absorbance decreased linearly for at least 3 min.

Preparation of crude protein extracts and assay of ACS activity

The ACS activity was essentially assayed by following the methods described by Roughan and Ohlrogge [25] and Ke *et al*. [26] with some modification. The treated seedling leaves were harvested, washed with cold water at 4 °C, suspended and homogenized in a minimal volume of buffer E (25 mM Tris-HCl and 50 mM KCl, pH 8.5) supplemented with 10 mM mercaptoethanol, 1 mM bezamidine, 0.1 mM PMSF, 0.1% (*W*/*V*) defatted BSA, 1 mM EDTA and 1% (*V*/*V*) PVP. The homogenate was filtered through four layers of gauze. The extract was clarified by centrifugation at 25,000 *g* for 15 min at 4 °C. The supernatant was subjected to 50% ammonium sulfate precipitation. Subsequently, it was subjected to precipitation by centrifugation at 15,000 *g* for 10 min at 4 °C. The precipitate was resuspended in 1.5 ml of buffer F (25

mM Tris-HCl and 150 mM KCl, pH 8.5). The suspension was applied to the HiTrap™ desalting column (Cat. No. 17-1408-01, Amersham Biosciences) equilibrated in buffer F. Fractions were collected and characterized by enzyme assay.

Aliquots (10 μl) of crude protein were diluted with buffer E to a final volume of 90 μl. A 10 μl volume of 10×assay mix [2.5 mM CoASH, 25 mM ATP, 50 mM $MgCl_2$, 10 mM DTT, and 0.1 mM sodium [1-^{14}C]acetate (1.4 Ci/mol)] was added to each reaction tube to start the reaction. After incubation for 5 min at room temperature, 80 μl of the reaction mix was spotted onto a 2.5 cm DE-81 filter circle, which was washed 3 times for 5 min each time in 2% (*V*/*V*) acetic acid, rinsed in acetone, dried and subjected to liquid scintillation counting in the presence of 1 ml of scintillation fluid (LS5000, Beckman, USA).

Transcriptional investigation of ALDH family under anaerobic conditions using RT-PCR

The first-strand cDNA of the genes was amplified by reverse transcription with the TaKaRa RNA PCR™ kit (AMV, ver. 1.1), using the oligo dT-adaptor primer. The fragments of the *Aldh1a*, *Aldh2a*, *Aldh2b* and rice actin 1 genes were amplified by PCR, using the primer pairs of Aldh1a-f/Aldh1a-r, Aldh2a-f/Aldh2b-r, Aldh2b-f/Aldh2b-r and actin-f/actin-r, respectively. The primers were designed on the basis of the annotation of the rice genome and ESTs. All the PCR products were sequenced by the ABI3730 sequencing system (Applied Biosystems and Hitachi). The PCR process was carried out for 30 cycles, each consisting of 30 s at 95 °C for denaturation, 30 s at 56 °C for annealing and 2 min at 72 °C for extension. All of the mRNA levels were normalized to the actin 1 levels.

Results

Cloning and identification of MS and ICL genes in rice

Since the ICL and MS genes have not been cloned from rice, we used the known yeast ICL and MS amino acid sequences to search for homologous rice genes among the nucleotide sequences in publicly available rice databases (http://ricegaas.dna.affrc.go.jp/rgadb/) *in silico*. Either the ICL or MS gene was the single copy gene in the rice genome. We cloned and sequenced both transcripts of the ICL and MS genes of rice through RT-PCR. The rice ICL and MS exhibited significant alignment with known proteins from plants and yeast.

ICL had a 2.1 kb transcript encoding a polypeptide of 572 residues with a 74 bp 5′-UTR and an approximately 300 bp 3′-UTR. The rice ICL amino acid sequence had over 75% identity with those from other plants and over 50% identity with that from yeast. Multiple alignments of the ICL amino acid sequences show a conserved domain in these species [**Fig. 1(A)**]. A heptapeptide of Cys-Gly-His-Met-Gly(Ala)-Gly-Lys in the domain has been identified to be important to the enzyme's catalytic activity [27]. The plant ICLs show a characteristic sequence of the putative glyoxysomal targeting signal Ser(Ala)-Arg-Met in the C-terminal region [**Fig. 1(B)**] [28,29].

The MS gene encoded a polypeptide of 567 amino acid residues. The amino acid level identity of rice MS protein to yeast (*Saccharomyces cerevisiae*), cucumber, soybean and *Neurospora* (*Neurospora crassa*) was 47%, 74%, 74%, and 45%, respectively. The MS protein carried an amino acid sequence of Cys-Lys-Leu at its C-terminal, which was similar to the identified and widely distributed peroxisomal targeting signal Ser-Lys-Leu-COOH [**Fig. 1 (C)**] [29,30].

Increase of the transcripts of *Pdc1*, *Adh1*, MS and ICL mRNAs under submergence

To investigate the metabolic activation of glyoxylate cycle under submergent conditions, two cDNA probes corresponding to the genes of ICL and MS were used to detect the ICL and MS expressions in unsubmerged and submerged rice through the Northern blotting approach. The expression of the ICL and MS genes was too low to be detected in aerobic conditions. The Northern blot analysis showed that the transcripts of ICL mRNA were hardly detected in the unsubmerged rice seedlings. The level of ICL mRNA dramatically increased in the period from 24 h to 72 h after treatment under anaerobic conditions [**Fig. 2(A)**], while it decreased when the seedlings were transferred to aerobic conditions again (48/24 h). Similarly, the MS gene expression increased in submerged rice seedlings for more than 24 h and decreased after re-aeration . The Northern blot hybridization analysis indicated that rice ICL and MS were induced by oxygen deprivation for approximately 24 h. The glyoxylate cycle was activated during severe hypoxia.

To examine the alteration of known ethanolic fermentation in the submerged seedlings, Northern blotting was performed to detect the mRNA levels of the identified rice *Pdc1* and *Adh1* genes, using two cDNA probes corresponding to *Pdc1* and *Adh1*. The induction of the mRNAs of *Pdc1* and *Adh1* was dramatically increased in

(A)	yeast	179	DADAGHGGLTAVFKLTKMFIERGAAGIHMEDQTSTNKK**CGHMAG**RCVIPVQEHVNRLVTI	238
	Neurospora	177	DADTGHGGLTAVMKLTKLFIEKGAAGIHIEDQAPGTKK**CGHMAGK**VLVPIQEHINRLVAI	236
	cotton	175	DGDTGFGGTTATVKLCKLFVERGAAGVHIEDQSSVTKK**CGHMAGK**VLVAVSEHINRLVAA	234
	bean	175	DGDTGFGGTTATVKLCKLFVERGAAGVHIEDQSSVTKK**CGHMAGK**VLVAISEHINRLVAA	234
	squash	175	DGDTGFGGTTATVKLCKLFVERGAAGVHIEDQSSVTKK**CGHMAGK**VLVAVSEHINRLVAA	234
	cucumber	175	DGDTGFGGTTATVKLCKLFVERGAAGVHIEDQSSVTKK**CGHMAGK**VLVAVSEHINRLVAA	234
	tomato	175	DGDTGFGGATATVKLCKLFVERGAAGVHIEDQSSVTKK**CGHMAGK**VLVAVSEHINRLVAA	234
	Arabidopsis	175	DGDTGFGGTTATVKLCKLFVERGAAGVHIEDQSSVTKK**CGHMAGK**VLVAVSEHINRLVAA	234
	soybean	157	DGDTGFGGTTATVKLCKLFVERGAAGIHIEDQSSVTKK**CGHMAGK**VLVAISEHINRLVAA	216
	Oryza	175	DGDTGFGGATATVKLCKLFVERGAAGVHLEDQSSVTKK**CGHMAGK**VLVAVSEHVNRLVAA	234
	Escherichia	157	DAEAGFGGVLNAFELMKAMIEAGAAAVHFEDQLASVKK**CGHMGGK**VLVPTQEAIQKLVAA	216
	Photorhabdus	158	DAEAGFGGVLNAFELMKAMIEAGAAAVHFEDQLAAVKK**CGHMGGK**VLVPTQEAIQKLVAA	217

(B)	yeast	530	LAQGGVSATAAMGTGVTEDQFKENGVKK----------	557
	Neurospora	527	MVTGGVSSTAAMGKGVTEDQFH----------	548
	cotton	531	TVQGGISSTAAMGKGVTEEQFKETWTRPGAGNIGSEGNLVVAK**ARM**	576
	bean	531	TVQGGISSTAAMGKGVTEEQFKETWTRPGAMEMGSAGSEVVAK**ARM**	576
	squash	531	TVQGGISSTAAMGKGVTEEQFKESWTRAGAGNLGEEGSVVVAK**SRM**	576
	cucumber	531	TVQGGISSTAAMGKGVTEEQFKESWTREGAVNLGEEGNVVVAK**SRM**	576
	tomato	513	TVQGGITSTAAMGKGVTEEQFEEKWTRTGATNLG-DGSVVIAK**ARM**	575
	Arabidopsis	513	TVQGGISSTAAMGKGVTEEQFKESWTRPGADGMGEGTSLVVAK**SRM**	576
	soybean	513	TVQGGVASTAAMGKGVTEEQFKESWTRPGAVEID-RGSIVVAK**ARM**	557
	Oryza	531	TVQGGISSTAAMGKGVTEEQFKGSWTGPGS----ESSSHVLAK**SRM**	572
	Escherichia	514	IIQGGTSSVTALTGSTEESQF----------	434
	Photorhabdus	515	IIQGGTSSVTALTGSTEEQQF----------	435

(C)	cucumber	523	RFKKGMYKEACKMFTRQCTAPNLDDFLTLDAYNYIVIHHPREL--**SKL**	568
	soybean	519	KFKEGMYKEACKIFTRQCTSPMLDDFLTLDAYNYIVVHHPRET--**SKL**	564
	Oryza	520	RFRRGRYAEAGRIFSRQCTAPELDDFLTLDAYNLIVVHHPGASSP**CKL**	567
	yeast	508	GDK-NKFALAAKYFLPEIRGEKFSEFLTTLLYDEIVSTKATPTDL**SKL**	554
	Neurospora	499	G---NKFNLAAQYFASQVTGEDYADFLTCLLYNEITSAGNS-LPA**SKL**	542

Fig. 1 Alignment of amino acid sequences of ICL and MS proteins

(A) Part of the multiple sequence alignments for ICL protein from yeast (accession No. P28240), *Neurospora* (CAC18302), cotton (P17069), bean (P15479), squash (P93110), cucumber (P49296), tomato (P49297), *Arabidopsis* (P28297), soybean (P45457), *Oryza* (*Oryza sativa*, this study), *Escherichia* (NP_418439) and *Photorhabdus* (CAE16767). Residues in bold show conserved function domains in which a cysteine is important to the enzyme's catalytic activity. (B) Multiple alignments at the C-terminus for ICL proteins. The residues in bold show the putative tracking signal Ala(Ser)-Arg-Met for the peroxisome (glyoxysome) protein. (C) Multiple alignments at the C-terminus for MS protein from cucumber (accession No. SYKVMA), soybean (P45458), *Oryza* (*Oryza sativa* in this study, CAD79703), yeast (S26645), and *Neurospora* (P28345). The residues in bold represent the putative glyoxysomal tracking signal Ser(Cys)-Lys-Leu at the C-terminus of MS.

the seedlings that were submerged for 12 h, indicating that ethanolic fermentation was rapidly activated in the tissues. In addition, the decrease in transcripts of *Pdc1* and *Adh1* mRNAs in the seedlings submerged for more than 24 h is different to the continuous increase in transcripts of ICL and MS mRNAs. These results are in agreement with those of previous studies [31,32].

Histograms of gene expressions in seedlings submerged for 12, 24, 48 and 72 h were drawn according to the scanned signals from outputs of the Northern blotting and presented as that observed at 0 h in water [**Fig. 2(B)**]. The expression levels of *Pdc1* and *Adh1* dramatically increased after the short period under anaerobic conditions (12 h), while those of the MS and ICL genes increased

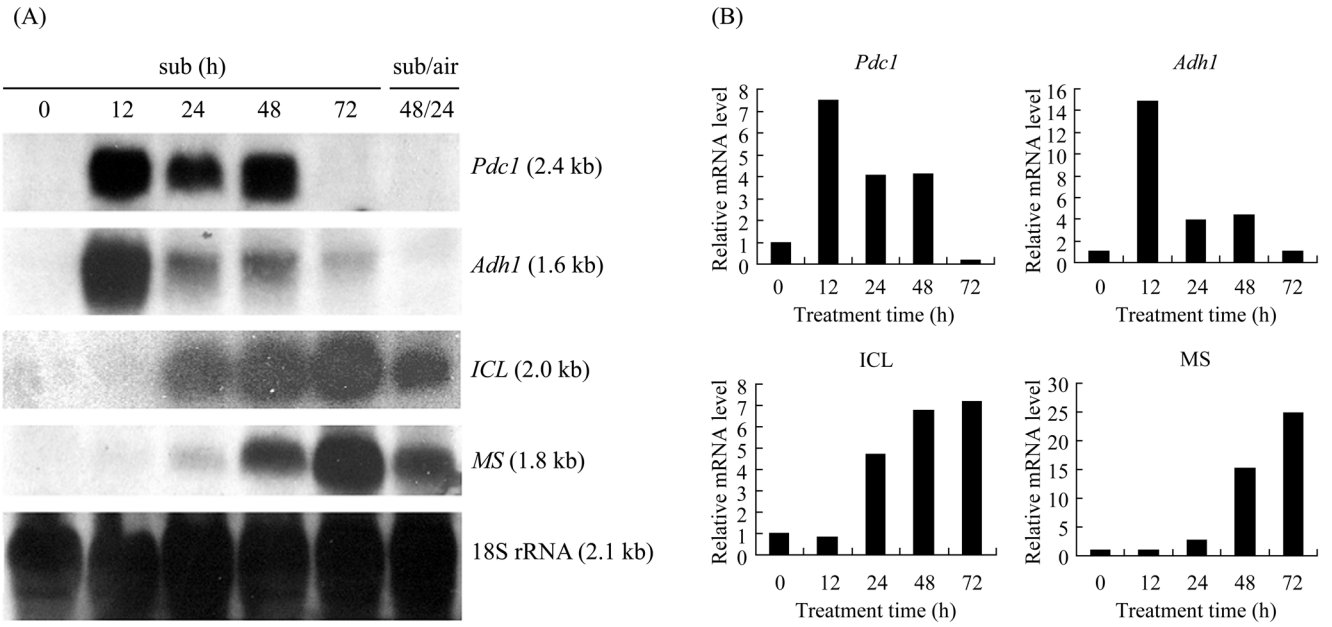

Fig. 2 Northern blot analysis of transcript of *Pdc1*, *Adh1*, ICL and MS mRNAs

(A) Transcript levels of *Pdc1*, *Adh1*, ICL and MS increase under anaerobic conditions. Ten-day-old rice seedling leaves were submerged for 12, 24, 48 and 72 h (sub 12, sub 24, sub 48 and sub 72). The seedlings submerged for 48 h were returned to aerobic conditions for an additional 24 h (48 /12 h). To determine the transcript levels by Northern hybridization, 15 μg of total RNA from each category of seedling was used. The levels of the transcripts of the *pdc1* (2.4 kb), *Adh1* (1.6 kb), ICL (2.1 kb) and MS (2.0 kb) genes are shown on the left. The rice *18S rRNA* gene was used as a control. (B) Quantification of the relative mRNA levels of *pdc1*, *Adh1*, ICL, and MS shown in (A). The levels of mRNA from seedlings submerged for 12, 24, 48 and 72 h were normalized to the rRNA level and are presented as the fold of that was observed at 0 h in water.

continuously under the same treatment. The transcripts of ICL and MS mRNAs in 72 h submerged tissues showed 7-fold and 25-fold increases over those in the unsubmerged tissues.

Activity assay of ICL and PDC

The ICL activity for purified glyoxysomes from the submerged rice seedling leaves (12, 24, 48 and 72 h, as well as 48/24 h) and the control (0 h) was assayed. A unit of ICL activity was defined as the amount of enzyme required to produce 1.0 μmol of glyoxylate in 1 min. The ICL activity increased in the seedlings submerged for more than 12 h. The seedlings submerged for 24, 48 and 72 h showed consistently higher activity than the control. The ICL activity was approximately increased by 3.5-fold in the seedlings submerged for 72 h [**Fig. 3(A)**]. The re-aerated seedlings (48/24 h) showed a decreased in enzyme activity, even though there was a 3.0-fold increase at 0.18±0.00 U/mg protein over the control tissues. The increase in ICL activity indicated the activation of the glyoxylate cycle under low oxygen conditions.

The PDC activity was measured in the crude cell extracts of rice seedlings exposed to aerobic conditions and during the course of a 72 h anoxic treatment [**Fig. 3 (B)**]. One unit (U) of PDC activity was defined as the amount of enzyme required to convert 1.0 μmol of NADH to NAD^+ in 1 min. An induction of PDC activity was detected after 12 h of anoxia that continuously increased with the duration of submergence. The enzyme activity in the 72 h anaerobic tissues showed an 8.8-fold increase over that in the 0 h control tissues. The seedlings subjected to 48 h of submergence followed by 24 h of re-aeration (48/24 h) exhibited a remarkable decrease in PDC activity at 0.56±0.04 U/mg protein, and only a 2.2-fold increase over the control. The PDC activity of seedlings, 48/24 h, was much less than the 48 h anaerobic seedlings at 1.09±0.11 U/mg protein and the 72 h anaerobic seedlings at 2.21±0.22 U/mg protein. It was obvious that both the ICL and PDC activities increased in the seedlings subjected to hypoxia and anoxia, although the activation of ICL was less intensive than that of PDC.

Assay of ACS activity

Acetyl-CoA is used in the glyoxylate cycle for synthesizing succinate and malate [6]. ACS catalyzes the conversion of acetate into acetyl-CoA. To examine the

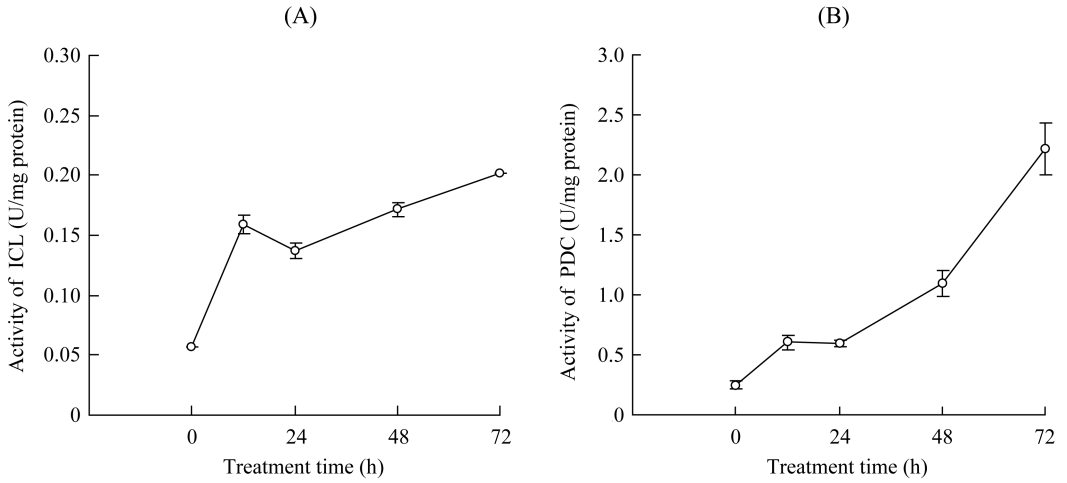

Fig. 3 Activity assays of ICL and PDC

(A) Assay of ICL activity. Rice ICL activity was measured in the isolated glyoxysomes from the seedling leaves submerged for 0, 12, 24, 48 and 72 h. Results are shown as mean±SE of three separate experiments. The units of ICL were calculated from the increase of glyoxylate during the assay. (B) Assay of PDC activity. Rice PDC activity was measured in the crude extracts of seedling leaves submerged for each indicated interval. Data are expressed as mean ± SE of three separate experiments. Units of PDC per mg protein were calculated from the decrease of NADH during the assay.

synthesis of acetyl-CoA during submergence, we assayed the activity of rice ACS in the stressed tissues.

The ACS activity was measured in the crude cell extracts of the rice seedlings grown for 10 days under aerobic conditions and during the course of a 72 h anaerobic treatment. We used sodium [1-^{14}C]acetate as the substrate and measured the ACS activity on the basis of radioactive concentration in the products containing [1-^{14}C]acetyl-CoA. It was found that the ACS activity dramatically increased in the seedlings submerged for 48 h and 72 h (**Fig. 4**). The activity reached a 3-fold increase over the initial level for seedlings grown in aerobic conditions. The 48 h/24 h (without air/with air) tissues showed 64% of the enzyme activity of the 72 h seedlings at 1580 cpm, but with a 1.8-fold increase over the 0 h control tissues. This indicated that an induction in ACS activity took place in the course of the anoxic treatments and the activity decreased in the re-aerated tissues.

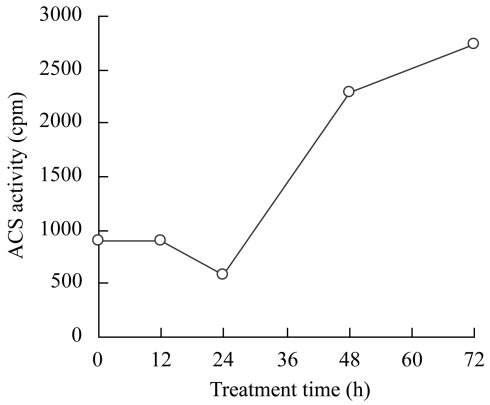

Fig. 4 Assay of ACS activity

Rice ACS activity was measured in desalted 50% ammonium sulfate precipitate from the seedling leaves submerged for 0, 12, 24, 48 and 72 h. Sodium [1-^{14}C]acetate was used as the substrate. The activity of ACS was measured on the basis of radioactive concentration in the products containing [1-^{14}C]acetyl-CoA.

Discussion

Rice can survive for up to 7 days if the shoots are completely submerged [4,33]. The Northern blot analysis and activity assay showed that ICL and MS were consistently active in the seedlings submerged from 12 to 72 h, suggesting that the glyoxylate cycle might play a metabolic role during the absence of oxygen. The increase in ACS activity in the tissues subjected to stress for over 48 h implies that there exists a pathway to convert acetate to acetyl-CoA, which is subsequently metabolized in the glyoxylate cycle. The Northern blot analysis and activity assay showed that induction of the *Pdc1* and *Adh1* genes took place at the early stage of submergence, indicating that rapid initiation of ethanolic fermentation was propitious for relieving the accumulation of pyruvate and

regenerating NAD^+. The regulation of ACS is still unclear. In comparison, activation of ACS activity occurred in the seedlings treated for 48 h or more, implying that the accumulation of substrate was one of the regulatory elements. The activity assays of PDC, ICL and ACS showed a slight decrease during the period of submergence from 12 h to 24 h. The accumulation of acetaldehyde and ethanol occurred in the early period of anoxia because of the activation of fermentation. The product accumulation may result in toxicity and inhibit the fermentation so as to reduce the energy production [33]. The enzyme activities decreased under these conditions. The induction of ICL and MS in seedlings subjected to more than 24 h of stress reduced the accumulation of acetaldehyde, while the PDC, ICL and ACS activities increased continuously. As the transcripts of ICL and MS mRNAs were increased in the prolonged period of submergence, we presume that activation of ACS and the glyoxylate cycle is correlated to the metabolism of accumulated acetate during anoxia.

ICL and MS play a role in various plant processes during pollen development [17], germination of oilseed rape (*Brassica napus*) [18], and senescence of barley (*Hordeum vulgare*) [19] and rice (*Oryza sativa*) [20]. The function performed by ICL and MS is necessary for plants' utilizing lipid or fatty acid as the carbon source for growth [34]. It was similar that the activation of ICL and MS acted as the modified routine of TCA cycle. The study of micro-organisms has shown that the glyoxylate cycle is the bypass of the TCA cycle, which makes it possible for the micro-organisms to grow on C2 compounds by converting them into C4 dicarboxylic acids [6]. The study of rice coleoptile during submergence has shown the high rate of ^{13}C enrichment of C3 compared to C2 in malate, suggesting that the glyoxylate cycle contributes to malate synthesis [13]. This evidence shows that the glyoxylate cycle is probably activated to maintain the fundamental metabolism and replenish the intermediates.

Regulation of metabolism during anoxia presumably involves changes in not only the rate of processes supplying energy, but also the direction of energy flow to processes essential to survival [33]. With continuing oxygen deficiency, the acetaldehyde and acetate from the dehydrogenation of acetaldehyde in mitochondria are unable to be effectively metabolized by the TCA cycle. The RT-PCR analysis of most rice ALDH genes in the submerged rice seedlings showed that their transcripts were dramatically increased under anaerobic conditions (**Fig. 5**), indicating the possibility that acetaldehyde was converted to acetate. The activation of ACS and the glyoxylate cycle might decrease the sum of HA (undissociated form of acetate) and A^- (anions of acetate) [33,35]. Such a decrease is related to the increase of vacuolar pH. During anoxia that lasts for days, the increase in vacuolar pH may reduce energy demands by reducing the energy required to maintain ΔpH across the tonoplast [36]. The most convincing evidence for the increase in vacuolar pH during anoxia has been found for rice shoots [3,33]. It has been confirmed that reducing energy requirements for maintenance is very important for survival during anoxia [33,35]. The activity of PDC in the submerged seedlings showed an 8-fold increase over that in the unsubmerged seedlings, while the activity of ICL showed a 3-fold increase on average, suggesting that the increase of PDC activity was more rapid and intensive than that of ICL activity. The regeneration of NAD^+ by the activation of fermentation was enough to keep several reactions going for a long time in anaerobiosis, including the glyoxylate cycle. The activation of ACS and the glyoxylate cycle might finally result in a net decrease in energy demands.

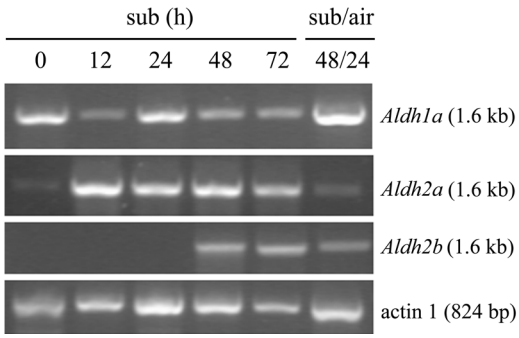

Fig. 5 Transcripts of ALDH family genes, *Aldh1a*, *Aldh2a* and *Aldh2b*, in rice seedlings detected by RT-PCR

The rice seedlings were submerged for 0, 12, 24, 48, 72 h and 48/24 h (submerged for 48 h and re-aerated for 24 h). The length of each amplified fragment is shown on the left. Actin 1 was used as control.

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