

Expression of a Gene Encoding Mitochondrial Aldehyde Dehydrogenase in Rice Increases under Submerged Conditions¹

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It is known that alcoholic fermentation is important for survival of plants under anaerobic conditions. Acetaldehyde, one of the intermediates of alcoholic fermentation, is not only reduced by alcohol dehydrogenase but also can be oxidized by aldehyde dehydrogenase (ALDH). To determine whether ALDH plays a role in anaerobic metabolism in rice (*Oryza sativa* L. cv Nipponbare), we characterized a cDNA clone encoding mitochondrial ALDH from rice (*Aldh2a*). Analysis of sub-cellular localization of ALDH2a protein using green fluorescent protein and an in vitro ALDH assay using protein extracts from *Escherichia coli* cells that overexpressed ALDH2a indicated that ALDH2a functions in the oxidation of acetaldehyde in mitochondria. A Southern-blot analysis indicated that mitochondrial ALDH is encoded by at least two genes in rice. We found that the *Aldh2a* mRNA was present at high levels in leaves of dark-grown seedlings, mature leaf sheaths, and panicles. It is interesting that expression of the rice *Aldh2a* gene, unlike the expression of the tobacco (*Nicotiana tabacum*) *Aldh2a* gene, was induced in rice seedlings by submergence. Experiments with ruthenium red, which is a blocker of Ca²⁺ fluxes in rice as well as maize (*Zea mays*), suggest that the induction of expression of *Adh1* and *Pdc1* by low oxygen stress is regulated by elevation of the cytosolic Ca²⁺ level. However, the induction of *Aldh2a* gene expression may not be controlled by the cytosolic Ca²⁺ level elevation. A possible involvement of ALDH2a in the submergence tolerance of rice is discussed.

Glycolysis and alcoholic fermentation are important for energy production of plants in anaerobic environments. Alcoholic fermentation is performed by two steps of reactions: the decarboxylation of pyruvate to acetaldehyde, which is catalyzed by pyruvate decarboxylase (PDC), and the following reduction of acetaldehyde to ethanol with the concomitant oxidation of NADH to NAD⁺, which is catalyzed by alcohol dehydrogenase (ADH) (Fig. 1; Perata and Alpi, 1993; Drew, 1997; Vartapetian and Jackson, 1997). This metabolic pathway is recognized as the principal catalytic pathway for recycling NAD⁺ to maintain glycolysis and the ATP level in the absence of oxygen (Perata and Alpi, 1993). It is known that expression of the genes involved in glycolysis and alcoholic fermentation (e.g. glyceraldehyde-3-P dehydrogenase, enolase, ADH, and PDC) are dramatically induced by anaerobiosis (Umeda and Uchimiya, 1994; Sachs et al., 1996). This induction is essential for anaerobic tolerance in plants.

Maize (*Zea mays*), Arabidopsis ADH-null mutants, and rice (*Oryza sativa*) ADH-reduced mutants showed lower tolerance to anaerobic conditions (Schwartz, 1969; Jacobs et al., 1988; Matsumura et al., 1998).

Aldehyde dehydrogenases [ALDHs; aldehyde: NAD(P)⁺ oxidoreductases, EC 1.2.1.3] are a group of enzymes catalyzing the conversion of aldehydes to the corresponding acids. In humans, the *Aldh* genes have been identified and characterized in detail (for review, see Yoshida et al., 1998). There are at least two isozymes of ALDH involved in ethanol metabolism (cytosolic, high-K_m ALDH1 and mitochondrial, low-K_m ALDH2) (Hsu et al., 1988, 1989). The mitochondrial ALDH2 is expressed in various tissues with the highest level in the liver and exhibits a high activity for oxidation of acetaldehyde, which is an intermediate of ethanol metabolism. Therefore, ALDH2 plays an important role in detoxification of acetaldehyde (Yoshida et al., 1998). In 1996, a gene for mitochondrial ALDH was identified for the first time in a plant (maize) (Cui et al., 1996). This gene, the *restorer of fertility 2* (*rf2*) gene, was found to be a nuclear restorer gene of Texas-type cytoplasmic male sterility (Cui et al., 1996; Schnable and Wise, 1998). Two *Aldh* genes (*Aldh2a* and *Aldh2b*) were subsequently identified in tobacco (*Nicotiana tabacum*), and the tobacco *Aldh2a* transcript and the ALDH2a protein were present at high levels in floral tissues,

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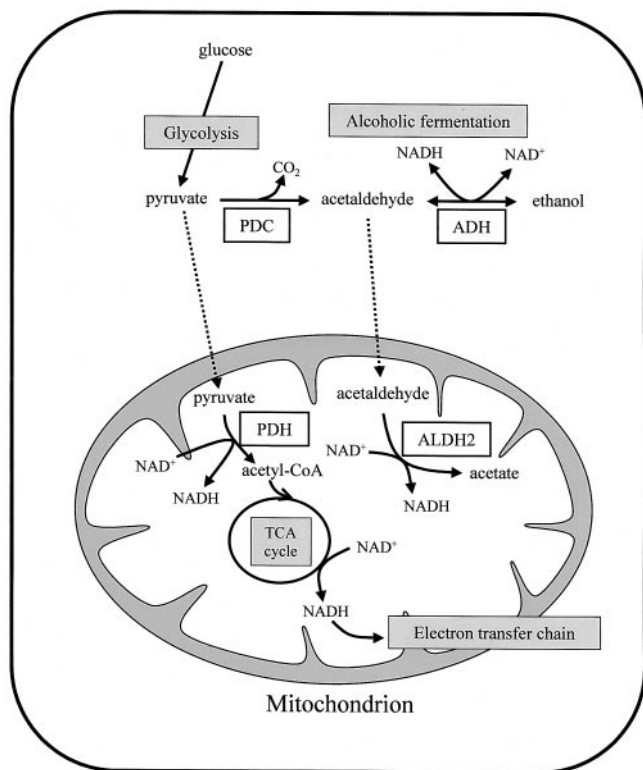


Figure 1. Aerobic and anaerobic metabolic pathways of rice. In the aerobic pathway, pyruvate, which is produced by pathways such as glycolysis, is converted to acetyl-coenzyme A (CoA) by pyruvate dehydrogenase (PDH) and is used for the trichloroacetic acid (TCA) cycle and the electron transfer chain in mitochondria. In the anaerobic pathway, pyruvate is converted to acetaldehyde by PDC. At the same time, acetaldehyde is converted to ethanol by ADH and to acetate by aldehyde dehydrogenase (ALDH).

especially stamens, pistils, and pollen (op den Camp and Kuhlemeier, 1997). Expression of *Adh* and *Pdc* and alcoholic fermentation also increase during pollen development in tobacco even under aerobic conditions, suggesting that alcoholic fermentation and the pathway from acetaldehyde to acetate (catalyzed by ALDH) play a role in biosynthesis and energy production during pollen development (Bucher et al., 1995; Tadege and Kuhlemeier, 1997). Under anaerobic conditions, expression of *Adh*, but not expression of *Aldh2a*, is induced in tobacco leaves. Therefore, tobacco ALDH2a seems not to function in anaerobic environments (op den Camp and Kuhlemeier, 1997).

Rice has a higher tolerance for anaerobic conditions than does tobacco. To determine whether rice ALDH functions under submergence and is involved in tolerance of anaerobic conditions, in the present study we characterized a cDNA clone encoding rice mitochondrial ALDH (*Aldh2a*). In contrast to the tobacco *Aldh2a* gene, the rice *Aldh2a* gene showed increased expression in rice seedlings that were submerged.

RESULTS

Characterization of Rice *Aldh2a* cDNA

As a first step in determining the gene for ALDH in rice, we searched the rice expressed sequence tag (EST) clone database for genes that share sequence identity with the maize *rf2* gene or the tobacco *Aldh2a* gene. As a result, the amino acid sequences of maize RF2 protein and tobacco ALDH2a proteins were found to share sequences with the putative protein encoded by the EST clone C10151 from rice calli. The 1,855-bp insert of the cDNA clone C10151 was completely sequenced (DNA Data Bank of Japan, EMBL, and National Center for Biotechnology Information DNA accession no. AB030939). The clone C10151 contained a complete open reading frame (ORF) encoding a polypeptide of 553 amino acid residues (Fig. 2). The ORF had a significant homology with ALDH proteins of humans (Hsu et al., 1988, 1989) and yeast (Wang et al., 1998), as well as those of maize (Cui et al., 1996), tobacco (op den Camp and Kuhlemeier, 1997), and Arabidopsis (ALDH2a accession no. AB030820; M. Nakazono and A. Hirai, unpublished data) (Fig. 2). Nucleotide sequences of other copies of Arabidopsis ALDH genes (*ALDH2b* and *ALDH1a*), which were determined by the Arabidopsis genome project, have been deposited in the DNA databases (*ALDH2b*, accession no. AC005990; *ALDH1a*, accession no. AB020746). The deduced ALDH protein of rice is also homologous to the Arabidopsis ALDH2b and ALDH1a proteins (Fig. 2).

The amino-terminal portion of the predicted protein contains the typical mitochondrial targeting signal. To clarify whether the rice ALDH protein is imported into the mitochondria, we examined the intracellular localization of the ALDH protein in living cells using the jellyfish green fluorescent protein (GFP). Residues 1 to 95 of the ALDH protein, which contain the predicted mitochondrial targeting signal, were fused in frame to GFP, and the fusion gene was expressed transiently in suspension-cultured tobacco cv Bright Yellow-2 (BY-2) cells. As shown in Figure 3, the GFP fluorescence corresponded to the staining pattern observed using the mitochondrial-specific dye MitoTracker Red (Molecular Probes, Inc.), confirming that the rice ALDH protein is targeted to mitochondria. Therefore, the gene that encodes an ORF in the C10151 clone was designated as the rice mitochondrial *Aldh2* gene (*Aldh2a*).

Expression of Recombinant ALDH2a Protein and ALDH Activity

To express recombinant mature ALDH2a protein in *Escherichia coli* cells, cDNA corresponding to the predicted mature protein was amplified by PCR. The modified cDNA fragment was inserted downstream

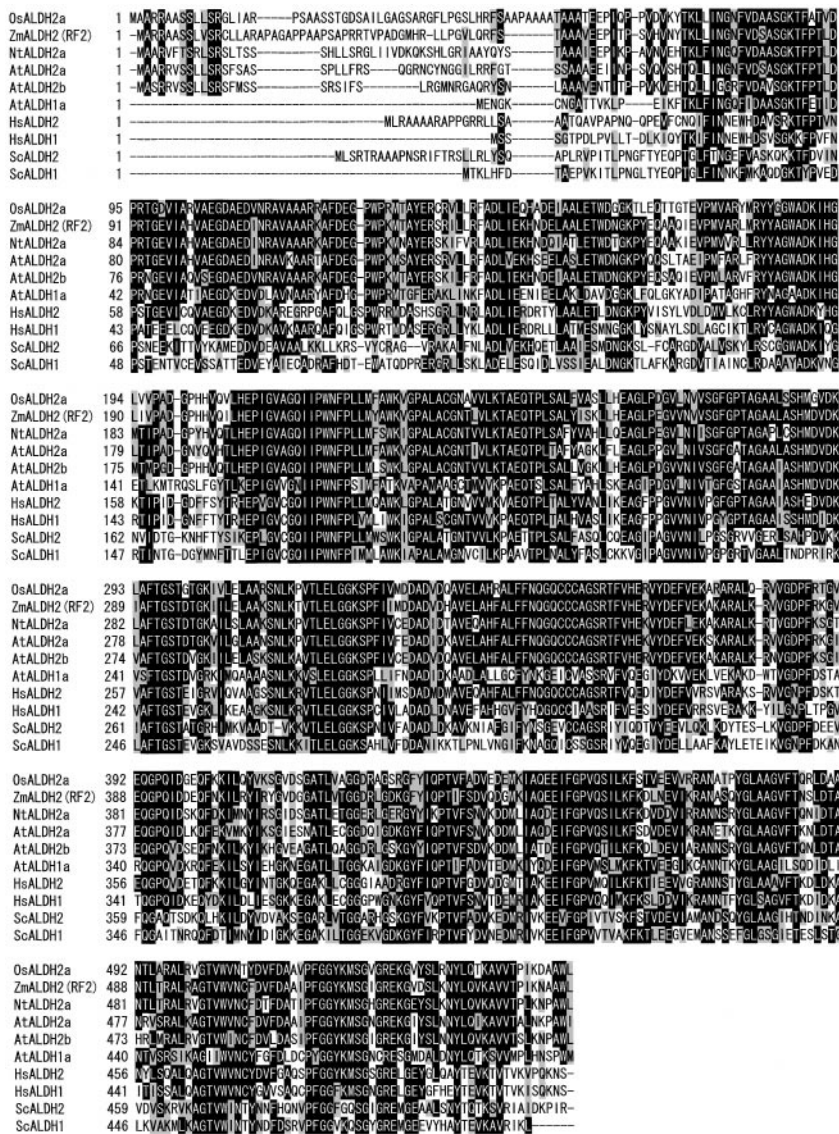


Figure 2. Alignment of the deduced amino acid sequences of ALDH proteins from rice (OsALDH2a; this study), maize (ZmALDH2 RF2; Cui et al., 1996), tobacco (NtALDH2a; op den Camp and Kuhlemeier, 1997), Arabidopsis (AtALDH2a, accession no. AB030820; M. Nakazono and A. Hirai, unpublished data; AtALDH2b, accession no. AC005990; AtALDH1a, accession no. AB020746), human (HsALDH2 and HsALDH1; Hsu et al., 1988, 1989, respectively), and yeast (ScALDH2 [ALD5] and ScALDH1 [ALD1]; Wang et al., 1998). The alignments were generated by the Clustal W algorithm (version 1.74; Thompson et al., 1994). Black boxes indicate identical amino acids and gray boxes indicate homologous amino acids.

of the T7 promoter in the pET-11a plasmid vector (Novagen, Madison, WI), and the resulting plasmid (termed pET-ALDH2a) was introduced into the *E. coli* strain BL21-CodonPlus(DE3)-RP (Stratagene, La Jolla, CA). Transformed cells from a single screened for overexpression of ALDH2a and then one of these colonies was cultured. Total protein extracts were obtained by lysis of the *E. coli* cells that overexpressed the recombinant mature ALDH2a protein and were assayed in vitro for ALDH activity as described in "Materials and Methods." When acetaldehyde was added as a substrate, acetaldehyde dehydrogenase activity was detected in protein extracts from the pET-ALDH2a-introduced *E. coli* cells (Fig. 4). By contrast, we detected no or extremely low ALDH activity in protein extracts from the *E. coli* cells that had been transformed with pET-11a (negative control; Fig. 4). These results indicated that rice ALDH2a protein has an activity for oxidation of acetaldehyde to acetate.

Genomic Southern Hybridization

Genomic Southern hybridization was carried out using probe A, whose sequence corresponds to the coding region of *Aldh2a*. Probe A hybridized to two bands in the *Eco*RI-digest, in the *Eco*RV-digest, and in the *Xba*I-digest of rice total DNA (Fig. 5), indicating that in rice mitochondrial ALDH is actually encoded by at least two genes.

Expression of *Aldh2a* in Various Organs of Rice

To examine the specific expression of the *Aldh2a* gene, we constructed a probe that is specific to the *Aldh2a* gene (probe B, which corresponds to the 3'-untranslated region [UTR] of *Aldh2a*). Genomic Southern hybridization showed that probe B hybridized to only a single band in each lane and the respective signals corresponded to one of the two

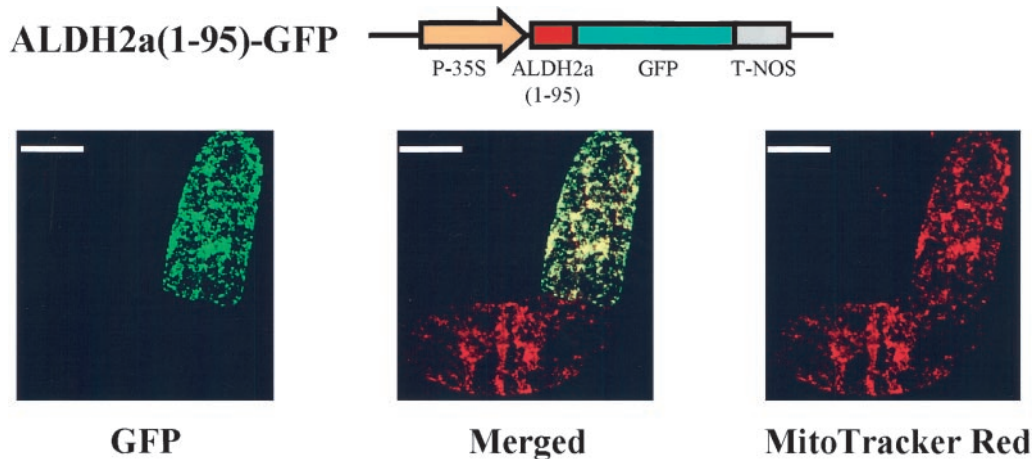


Figure 3. Transient expression of the ALDH2a(1-95)-GFP fusion protein in suspension-cultured tobacco BY-2 cells. *P-35S*, A constitutive promoter from the cauliflower mosaic virus. *GFP*, sGFP(S65T) (Chiu et al., 1996). *T-NOS*, A nopaline synthase terminator. Recombinant plasmid was transformed into BY-2 cells by particle bombardment (PDS-1000, Bio-Rad, Richmond, CA). Mitochondria were stained by the mitochondrial-specific dye MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) at 24 h after bombardment. Left, GFP fluorescence. Right, MitoTracker Red fluorescence. Center, Merging of the two images (GFP fluorescence and MitoTracker Red fluorescence). Each panel shows one of the optical sections through two BY-2 cells, one that is a control and the other that contains the recombinant plasmid and shows expression of the GFP fusion protein. Scale bars = 20 μm .

bands that were detected with probe A (Fig. 5). It was confirmed that probe B is specific for the *Aldh2a* gene. The expression of the *Aldh2a* gene was examined by northern hybridization by determining the relative steady-state mRNA amounts in different organs of rice. Using total RNAs extracted from young leaves and young roots (of 11-d-old seedlings grown under light or dark conditions), mature leaf blades, mature leaf sheaths, young panicles (lengths of 7–8 cm), and panicles after heading, a single transcript of approximately 1.9 kb was observed (Fig. 6). The steady-state levels of the *Aldh2a* transcript in leaves of 11-d-old seedlings were higher than those levels in roots. Furthermore, the amounts of mRNA in the tissues grown under darkness were higher than those amounts in tissues grown in the light. In mature rice plants, high relative amounts of the *Aldh2a* mRNA were detected in young panicles, panicles after heading, and leaf sheaths (Fig. 6).

Increase of the Steady-State Levels of *Aldh2a* mRNA and ALDH2a Protein by Submergence Treatment

To determine whether the expression of *Aldh2a*, like the expression of *Adh1* (Xie and Wu, 1989) and *Pdc1* (Hossain et al., 1996), is induced in an anaerobic environment, 7-d-old seedlings grown under aerobic conditions were submerged for 12, 24, and 36 h and then subjected to a northern hybridization analysis. It is interesting that the steady-state level of *Aldh2a* mRNA dramatically increased by the submergence treatment, as did the expressions of the *Adh1* and *Pdc1* genes (Fig. 7, A–C). When the submerged seed-

lings were transferred to an aerobic environment, the amount of the *Aldh2a* transcript decreased (Fig. 7A).

We examined whether the protein level of ALDH2a also increases under submerged conditions. An immunoblot experiment of mitochondrial total proteins, which were extracted from rice seedlings that were submerged in the dark at 28°C for 12 and 24 h, was performed using an antibody against ALDH2. The data in Figure 8 show that the amount of the ALDH2 protein, which has a molecular mass of about 55 kD, increased more in the seedlings submerged for 12 h than in the aerobically grown seedlings. However, the ALDH2 protein level in the seedlings submerged for 24 h was unexpectedly comparable to that in the control plants (0 h of submergence), suggesting that the amount of the rice ALDH2a protein transiently increases by oxygen deprivation. Although the pattern of increase of the ALDH2a protein was different from that of the *Aldh2a* mRNA, these results indicated that expression of the rice *Aldh2a* gene is induced by low oxygen and that the rice ALDH2a protein is one of the anaerobic proteins such as ADH1 and PDC1.

Ruthenium Red (RR) Does Not Suppress the Increase in the Steady-State Level of *Aldh2a* mRNA under Submerged Conditions

In maize and Arabidopsis, oxygen deprivation elevates the cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$) level through intracellular Ca^{2+} flux, and RR, which is thought to be a blocker of Ca^{2+} fluxes from organelles, suppresses induction of expression of the anaerobic-inducible

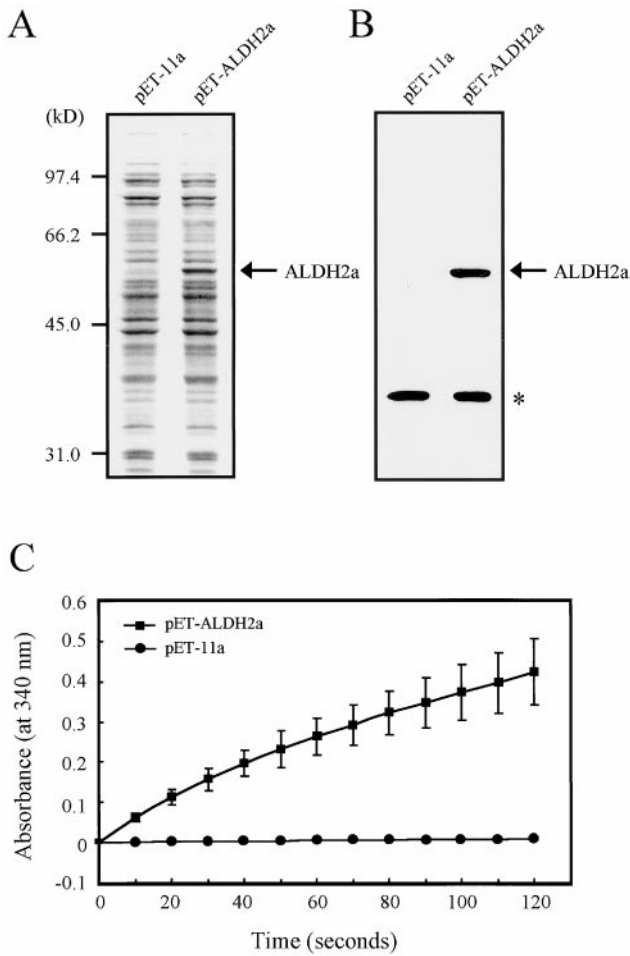


Figure 4. In vitro assay of acetaldehyde dehydrogenase activity of *E. coli* cells overexpressing recombinant ALDH2a protein. A and B, *E. coli* cells transformed with pET-ALDH2a or pET-11a (negative control) were incubated with isopropylthio- β -galactoside to induce gene expression. Total proteins were extracted, denatured, and separated by SDS-PAGE. The gel was stained with SYPRO Orange Protein Stain (Bio-Rad) (A) and was subjected to an immunoblot analysis using an antibody raised against ALDH2 (B). The positions of the ALDH2a protein are indicated by arrows. An asterisk indicates a non-specific band. C, An in vitro ALDH assay. Immediately after acetaldehyde (final concentration 100 μ M) was added to the reaction mixture, the A_{340} was measured at 10-s intervals for up to 120 s. Results are expressed as mean values \pm SE of five separate experiments.

Adh gene (Subbaiah et al., 1994a, 1994b, 1998; Sedbrook et al., 1996). Here it was of interest to examine whether the increase in the amount of mRNA of the *Aldh2a* gene is also regulated by oxygen deprivation-induced $[Ca^{2+}]_c$ elevation. We investigated the effect of RR on the expressions of the *Aldh2a* gene after 12 and 24 h of submergence. Treatment of rice seedlings with 100 μ M RR under submerged conditions showed that the steady-state level of *Adh1* and *Pdc1* mRNAs decreased to 50% of the level induced in seedlings in water (without RR) (Fig. 9), indicating that the RR treatment affects the increase of the *Adh1*

and *Pdc1* mRNAs by oxygen deprivation in rice, as it does in maize. On the other hand, addition of RR to rice seedlings did not affect the expressions of the *Adh1* and *Pdc1* genes under aerobic conditions (data not shown). Under these conditions, we examined whether the expression of the *Aldh2a* gene is influenced by the RR treatment under hypoxia. As shown in Figure 9, the transcript levels of *Aldh2a* with the RR treatment were about the same as the levels without RR. We suggest that, unlike its effect on *Adh1* and *Pdc1*, RR does not affect the expression of *Aldh2a* under hypoxia.

DISCUSSION

The predicted amino acid sequence of the rice ALDH2a protein showed high sequence identities with the sequences of the human and yeast ALDH proteins as well as the maize RF2 protein and the tobacco ALDH2a protein (Fig. 2). In contrast, the rice ALDH2a showed lower sequence conservation with other enzymes that oxidize some aldehydes (such as betaine aldehyde dehydrogenase and methylmalonate semi-aldehyde dehydrogenase; data not shown). As shown in Figure 4, rice ALDH2a has

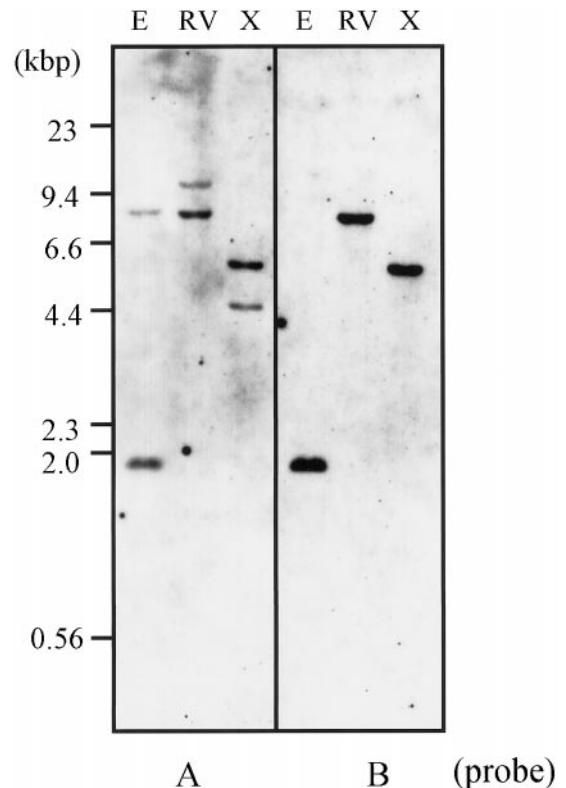


Figure 5. Genomic Southern hybridization analysis of rice total DNA digested with *EcoRI* (E), *EcoRV* (RV), and *XbaI* (X). Hybridization was performed with probe A, which is specific for the coding region of *Aldh2a*, and probe B, which is specific for the 3'-UTR region of *Aldh2a*. The numbers given on the left indicate sizes of fragments in kb (kbp).

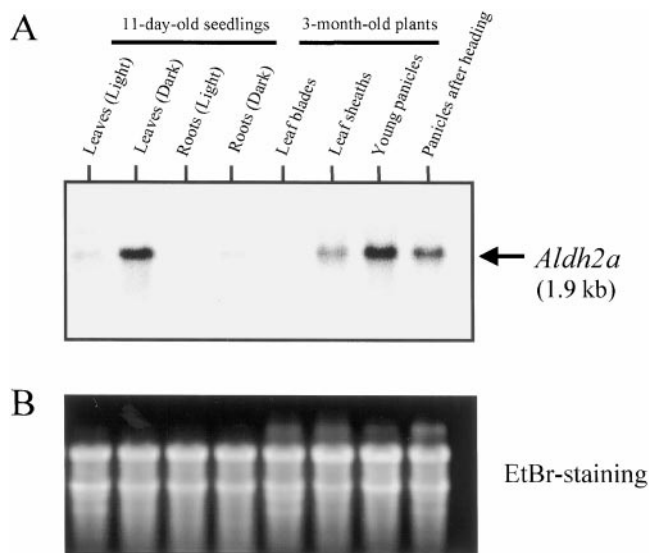


Figure 6. A, Northern hybridization analysis of transcripts of the *Aldh2a* gene in various organs. Each lane was loaded with 5 μ g of total RNA extracted from young leaves and young roots (of 11-d-old seedlings under light or dark conditions), mature leaf blades, mature leaf sheaths, young panicles (whose lengths were 7–8 cm), and panicles after heading. The size of the transcript of *Aldh2a* (1.9 kb) is shown by the arrow at the right. B, Equal loadings of total RNA were checked by ethidium bromide staining (EtBr-staining).

acetaldehyde dehydrogenase activity, although it might also have a role in the oxidation of other aldehydes in rice mitochondria. Analysis of intracellular localization of the rice ALDH2a protein using GFP indicated that ALDH2a functions in mitochondria (Fig. 3). Northern hybridization using total RNA extracted from several organs showed that *Aldh2a* is expressed mainly in leaves of dark-grown seedlings, mature leaf sheaths, and panicles (Fig. 6). This expression pattern was very similar to that of *Adh1* (data not shown). Therefore, we suggest that alcoholic fermentation might occur in these organs and ALDH2a might convert the alcoholic fermentation intermediate, acetaldehyde, to acetate in the mitochondria.

It seems likely that aerobic alcoholic fermentation is a general phenomenon in pollen and developing microspores in plants, and is controlled not by oxygen availability but by substrate (e.g. Glc) availability (Tadege and Kuhlemeier, 1997; Tadege et al., 1999). In rice, we found that *Aldh2a* (Fig. 6) and *Adh1* (data not shown) transcripts accumulate to high levels in young panicles and panicles after heading. Xie and Wu (1989) reported that the ADH1 isozyme, but not the ADH2 isozyme, is present in rice pollen. These findings suggest that, even in an aerobic environment, alcoholic fermentation takes place in the floral tissues (the panicles) in rice as in the case of tobacco (Bucher et al., 1995; Tadege and Kuhlemeier, 1997). In fact, we detected substantial acetaldehyde and etha-

nol production in the developing anthers of rice by gas chromatography (data not shown).

Although the steady-state levels of *Aldh2a* transcripts in the leaves and roots of rice seedlings grown in darkness were higher than those in tissues grown in the light (Fig. 6), the amount of *Aldh2a* mRNA slightly decreased when aerobically light-grown seedlings were transferred to a dark environment under aerobic conditions (Fig. 7, aerobic). Thus it seems unlikely that the expression of *Aldh2a* is simply regulated by light; rather, it might be regulated by some aspect of the metabolic status in etiolated or green seedlings.

One of the findings of this study is that the steady-state levels of the rice *Aldh2a* mRNA dramatically increased in an anaerobic environment (Fig. 7A). When the rice plants were transferred from an anaerobic environment to an aerobic environment, the amounts of the *Aldh2a* transcripts decreased (Fig. 7A). This expression pattern was very similar to those of the anaerobic-inducible genes *Adh1* and *Pdc1* (Fig. 7, B–C). An immunoblot analysis showed that

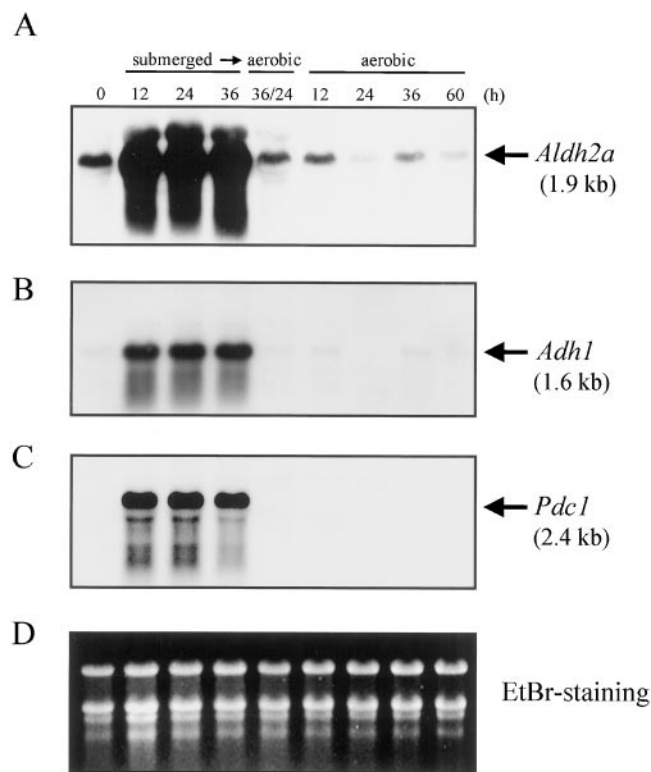


Figure 7. Steady-state transcript levels of *Aldh2a* (A), *Adh1* (B), and *Pdc1* (C) increase under submerged conditions. Seven-day-old rice seedlings grown in an aerobic environment in the light were submerged in the dark for 12, 24, and 36 h. After 36 h, the seedlings were returned to aerobic conditions in the darkness for 24 h (36/24). As a control, 7-d-old seedlings were kept in the dark under aerobic conditions for 12, 24, 36, and 60 h. The sizes of the transcripts of *Aldh2a* (1.9 kb), *Adh1* (1.6 kb), and *Pdc1* (2.4 kb) are shown by the arrows at the right. D, Equal loadings of total RNA were checked by ethidium bromide staining (EtBr-staining).

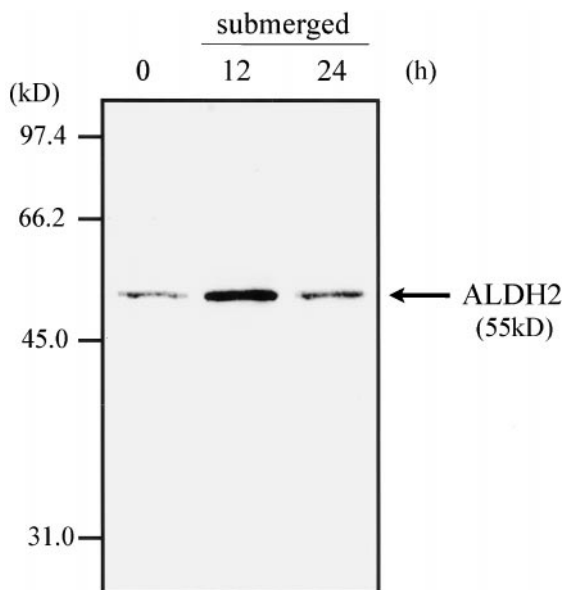


Figure 8. Influence of low oxygen on the rice ALDH2 protein level. Seven-day-old aerobically grown seedlings were submerged for 12 and 24 h. Total mitochondrial proteins were extracted from leaves of the seedlings and then subjected to an immunoblot analysis using an antibody raised against ALDH2. The position of the ALDH2 protein at approximately 55 kD is indicated by an arrow.

the amount of the ALDH2 protein in the mitochondria increased by submergence for 12 h and then decreased by more submergence treatment (for 24 h; Fig. 8). The reason for the difference between the patterns of increase of ALDH2a protein and *Aldh2a* mRNA is not known. One possible explanation for the difference is that the decrease of the ALDH2 protein after 24 h of submergence is due to a shortage of ATP and to a disappearance of the membrane potential, because both ATP and a membrane potential are required for the import of proteins into the mitochondria (for review, see Glaser et al., 1998). Under submerged conditions, efficiencies of respiration and ATP synthesis in the mitochondria are reduced because of a lack of oxygen. Thus it is possible that further submergence (more than 12 h) might cause an ATP shortage and a reduction in the membrane potential in the mitochondria and might reduce the efficiency of importing ALDH2 protein into the mitochondria.

The difference between ALDH2a protein and *Aldh2a* mRNA might alternatively be due to the existence of isozymes. There are at least two isozymes of mitochondrial ALDH2 in rice. Because the antibody against ALDH2 used in this study is able to recognize both mitochondrial ALDH2 isozymes, it is possible that the signals detected in the immunoblot analysis (Fig. 8) originate from both isozymes. We are now investigating the structure and expression of another copy of the rice *Aldh2* gene (termed *Aldh2b*). It is interesting that steady-state levels of *Aldh2b*

mRNA were relatively abundant under aerobic conditions, whereas when rice seedlings were submerged, the *Aldh2b* mRNA levels rapidly decreased (H. Tsuji, M. Nakazono, and A. Hirai, unpublished data). This finding suggests that changes in the expression of the rice *Aldh2a* and *Aldh2b* in response to changes in the oxygen status are antiparallel to each other. Thus the ALDH2 protein signals from rice under aerobic conditions (Fig. 8, 0 h) might have originated mostly from ALDH2b protein, and the ALDH2 signals from rice that had been submerged for 24 h might have originated mostly from an increase in ALDH2a protein. In contrast, the signal from rice that had been submerged for 12 h might have originated from both ALDH2a protein, whose amount is increasing, and ALDH2b protein, whose amount is decreasing. Further investigations will be necessary to test these possibilities.

In contrast to the rice *Aldh2a* transcript levels, the tobacco *Aldh2a* transcript levels did not increase during anaerobiosis in leaf tissue (op den Camp and Kuhlemeier, 1997). The authors proposed that a pathway involving ALDH2 is not important for normal metabolism in tobacco leaves, even in anaerobiosis. Our preliminary results showed that expression of the Arabidopsis *ALDH2a* gene was not enhanced under submerged conditions as in the case of tobacco *Aldh2a* (data not shown). We propose that rice might have a greater ability than tobacco and Arabidopsis to detoxify acetaldehyde, which is produced during alcoholic fermentation in anaerobiosis, both by ALDH and ADH. In other words, higher levels of ALDH could be one of the reasons why rice is more tolerant of submergence than other plant species such as tobacco and Arabidopsis. One means of testing this hypothesis would be to examine whether transgenic tobacco or Arabidopsis plants that overproduce ALDH2 can better tolerate anaerobiosis. In addition, in rice plants during anaerobiosis, pyruvate might be converted to acetyl-CoA by the sequential actions of PDC, ALDH, and acetyl-CoA synthetase (ACS). Kuhlemeier and his colleagues (Tadedge and Kuhlemeier, 1997; op den Camp and Kuhlemeier, 1997) proposed that acetyl-CoA produced through PDC/ALDH/ACS might be supplied as a substrate for the trichloroacetic acid cycle, lipid biosynthesis, and the glyoxylate cycle in tobacco pollen. A similar pathway might operate in rice under submerged conditions, and acetyl-CoA production via the PDC/ALDH/ACS pathway might be important for the biosynthesis of several intermediates in the process of adaptation in anaerobiosis of rice.

Under anaerobic conditions, fermentation regenerates NAD^+ from NADH, allowing plant cells to continue glycolysis and maintain the ATP level (Perata and Alpi, 1993). The conversion of acetaldehyde to acetate by ALDH consumes NAD^+ and this consumption could potentially block glycolysis. However, the hypoxia-inducible ALDH2a protein is local-

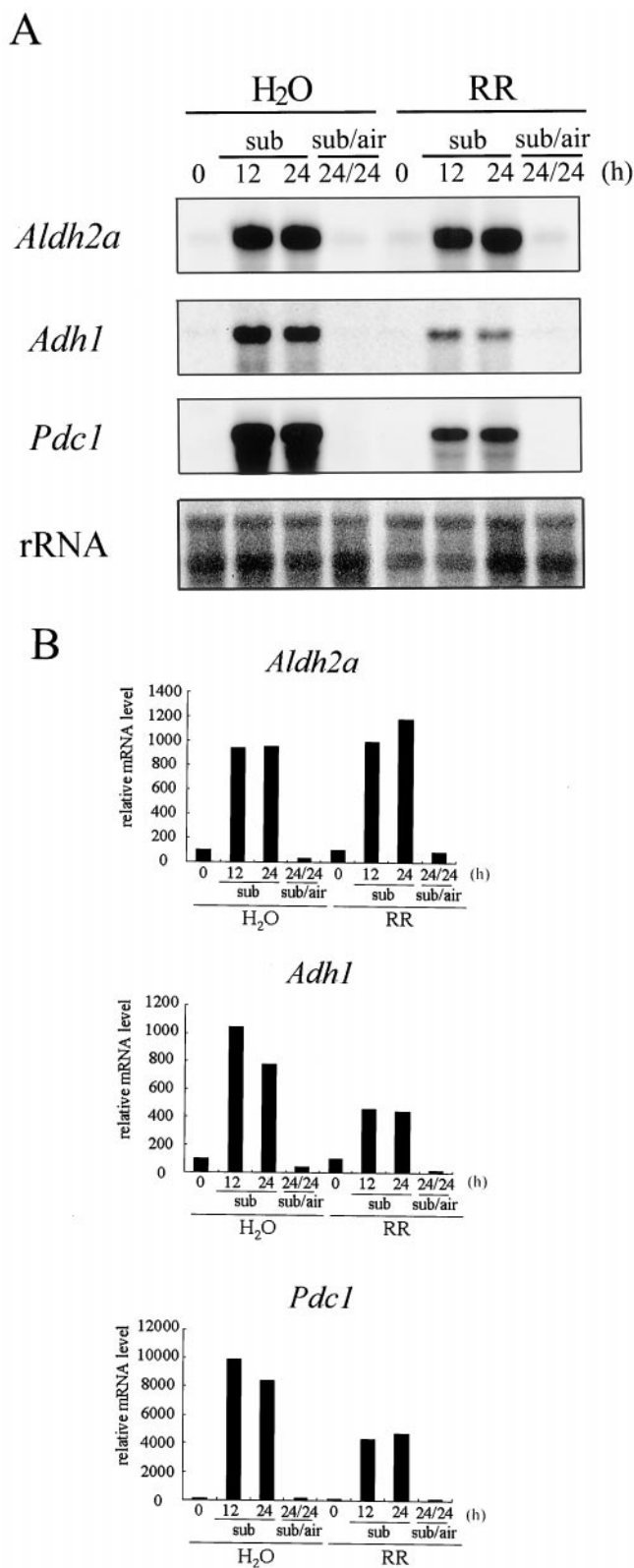


Figure 9. Effect of RR on the expressions of *Aldh2a*, *Adh1*, and *Pdc1* genes of rice under submerged conditions. Seven-day-old aerobically grown rice seedlings were submerged in water or in 100 μ M RR for 12 and 24 h (sub 12 and sub 24), and then 24-h-submerged seedlings were transferred to aerobic conditions where they were

ized in mitochondria and is separated from the cytosolic enzymes involved in glycolysis and alcoholic fermentation (Fig. 1). This fact suggests that reduction of NAD⁺ to NADH by rice ALDH2a does not influence the efficiency of glycolysis. Plants have many ALDH isozymes. We assume that at least two of these isozymes are involved in the oxidation of acetaldehyde. One isozyme is a mitochondrial enzyme (Cui et al., 1996; op den Camp and Kuhlemeier, 1997) and another is a cytosolic enzyme (Li et al., 2000). In contrast to the expression of the mitochondrial *Aldh2a* gene, the expression of the rice cytosolic *Aldh1a* gene is probably not influenced by the oxygen status (Y Li, M. Nakazono, and A. Hirai, unpublished data). Thus it seems reasonable that the mitochondrial ALDH2a protein is induced to ensure a continuation of glycolysis and that the cytosolic ALDH isozymes are not induced for this purpose.

Subbaiah et al. (1994a, 1994b, 1998) proposed a model for maize in which induction of *Adh1* gene expression by oxygen deprivation is preceded by [Ca²⁺]_c elevation, which might be due to Ca²⁺ released from the mitochondria. RR has been shown to block Ca²⁺ fluxes from organelles (Knight et al., 1992) and induction of ADH1 was suppressed by treatment of maize cells with RR under submerged conditions (Subbaiah et al., 1994b). In rice, treatment of seedlings by RR suppressed increase of transcripts of *Adh1* and *Pdc1* under anaerobiosis, as it does in maize (Fig. 9). Furthermore, we found that CaCl₂, when added simultaneously with RR, prevented the effect of RR on the expressions of *Adh1* (Tsuiji et al., 2000) and *Pdc1* (data not shown). This prevention of the effects of RR by Ca²⁺ supports the hypothesis that Ca²⁺ is involved in the expressions of the rice *Adh1* and *Pdc1* genes under anaerobic conditions. In contrast, treatment with RR (Fig. 9) and/or CaCl₂ (data not shown) had no inhibitory effect on the anaerobic induction of the rice *Aldh2a* gene, suggesting that elevation of [Ca²⁺]_c might not trigger an increase in the *Aldh2a* mRNA. These findings indicate that there are at least two pathways of oxygen signaling in rice: One is a signaling pathway regulated by a release of Ca²⁺ from intracellular stores, which is sensitive to RR, and another is a signaling pathway that is independent of [Ca²⁺]_c elevation (Tsuiji et al., 2000). In rice, it is likely that the former pathway controls the increase of the *Adh1* and *Pdc1* mRNAs and the latter pathway controls the increase of the *Aldh2a* mRNA.

In conclusion, we have demonstrated that, unlike expression of the tobacco *Aldh2a* gene, expression of

kept for 24 h (sub/air 24/24). A, Total RNA (5 μ g) from each treatment was used to determine the transcript levels by northern hybridization. As a control, a probe based on the 25S/17S rRNA genes was used. B, Quantification of relative mRNA levels of *Aldh2a*, *Adh1*, and *Pdc1* shown in A. All mRNA levels were normalized to the rRNA level and are presented as a percent of that observed at 0 h in water.

the rice mitochondrial *Aldh2a* gene is induced under anaerobic conditions and that, as shown by experiments with RR, the induction of *Aldh2a* gene expression is not regulated by an elevation of $[Ca^{2+}]_c$. We propose that a high level of mitochondrial ALDH2 protein under anaerobiosis (at least for the first 12 h) might confer more tolerance to submergence in rice than in tobacco or Arabidopsis. To further test this hypothesis, we are currently attempting to produce a transgenic Arabidopsis plant that overproduces ALDH2.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Treatments

Rice (*Oryza sativa* L. cv Nipponbare) was grown in the light at 28°C for 10 d for extraction of total DNA. For extraction of total RNA, leaves and roots of seedlings that were grown in the light or in the dark at 28°C for 11 d were used. Mature leaf blades, mature leaf sheaths, and young panicles were prepared from 3-month-old plants and panicles after headings were prepared from 3.5-month-old plants.

For treatment of oxygen deprivation, 7-d-old aerobically grown seedlings were submerged in the dark at 28°C for 12, 24, and 36 h. After 36 h, the submerged seedlings were transferred to aerobic conditions in the dark at 28°C for 24 h. For RR treatment, seedlings were submerged in water that contained 100 μ M RR (Sigma Chemical, St. Louis) in the dark at 28°C for 12 and 24 h. Leaves of the seedlings were collected after the indicated time intervals. After harvesting, the leaves were frozen in liquid nitrogen.

For isolation of mitochondrial proteins for immunoblotting, 7-d-old aerobically grown seedlings were submerged in the dark at 28°C for 12 and 24 h. Leaves of the seedlings were collected after the indicated time intervals.

EST Clone

The EST clone C10151 from rice calli was provided by the Rice Genome Research Program of the National Institute of Agrobiological Resources (Tsukuba, Japan).

Sequence Analysis

The cDNA clone was completely sequenced with an automatic DNA sequencer (model 373S; Perkin-Elmer Applied Biosystems, Foster City, CA). DNA sequencing data were analyzed with GENETYX-WIN Software (version 3, Software Development, Tokyo).

Oligonucleotides

The following 12 oligonucleotides were synthesized: ALDH-P1, 5'-TACAAGATGAGCGCGTTGGCA-3'; ALDH-P2, 5'-ATTACTACAGCTACAACCAGGC-3'; ALDH-P3, 5'-TAGCTGTAGTAATCGATCCT-3'; ALDH-P4,

5'-TGTACAAAAGATTGCCCGAT-3'; ALDH-P5, 5'-TCA-CCATGGGATCCACCGTCGCGAACGTCTTC-3'; ALDH-P6, 5'-ATACATATGAGCGCTGCACCGGCCGCCGCTGCCA-3'; ALDH-P7, 5'-ATATGATCATTATTCATCGCAATCTCTGGCGTTG-3'; ADH-P1, 5'-ATTATGGTGTGGGTAATAAGATT-3'; ADH-P2, 5'-AACTGAAACTCGTATAAATATATG-3'; PDC-P1, 5'-ATCGTCTGTGAATTAATTGT-3'; PDC-P2, 5'-CCTTCAAATCGTCCATGTTA-3'; and M13, 5'-CAGTCACGACGTTGTAACGACGGC-CAGT-3'.

Construction and Visualization of a GFP Fusion Protein

The ALDH2a(1–95)-GFP recombinant plasmid was constructed as follows: The sequence corresponding to residues 1 to 95 of the predicted ALDH2a precursor protein was amplified by PCR from the cDNA clone using primers M13 and ALDH-P5. The primer ALDH-P5 contained an *NcoI* site near its 5'-end. Because the amplified fragment included an *SalI* site, which originated from the multicloning site of the pBluescript vector, it was digested with both *SalI* and *NcoI* and cloned in-frame into the *SalI* and *NcoI* sites of the enhanced synthetic GFP vector [CaMV35Spro::sGFP(S65T)::NOSter; a gift from Dr. Y. Niwa (University of Shizuoka, Japan); Chiu et al., 1996]. This plasmid was termed ALDH2a(1–95)-GFP (Fig. 3). Its sequence was checked before proceeding.

Ten micrograms of plasmid was precipitated onto 1.0- μ m spherical gold beads. Suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells were bombarded using a PDS-1000 particle delivery system (Bio-Rad) as described previously (Nakazono et al., 2000). After bombardment, the samples were placed on a benchtop for 24 h. Transformed BY-2 cells were treated with 500 nM MitoTracker Red CMXRos (Molecular Probes), a mitochondrial-specific dye, for 30 min. These cells were examined with a confocal laser-scanning microscope (Micro-Radiance MR/AG-2; Bio-Rad). The samples were illuminated with an argon ion laser (488 nm wavelength) for GFP or a green HeNe laser (543 nm) for MitoTracker Red fluorescence.

Expression of the ALDH2a Protein in *Escherichia coli*

The cDNA clone C10151 was used as a template for PCR of a 1.68-kb fragment encoding the mature ALDH2a protein. The oligonucleotides used were the 5'-end primer ALDH-P6 and the 3'-end primer ALDH-P7. An ATG initiation codon was introduced by changing Phe-47 (the last amino acid of the mitochondrial targeting signal; Fig. 2) to Met in the 5'-end primer ALDH-P6. The primers ALDH-P6 and ALDH-P7 contained an *NdeI* site and a *BclI* site, respectively, near their 5'-ends. PCR was performed using KOD-plus, a high-fidelity thermophilic DNA polymerase (Toyobo, Tokyo). The amplified fragment was digested with both *NdeI* and *BclI* and cloned into the *NdeI* and *BamHI* sites of the pET-11a vector (Novagen). This plasmid, termed pET-ALDH2a, and pET-11a, which was used as a control, were introduced into the *E. coli* strain BL21-

CodonPlus(DE3)-RP (Stratagene). Transformed *E. coli* cells were incubated at 37°C overnight in Luria-Bertani medium with 50 µg/mL ampicillin. The overnight cultures (1 mL) were added into 50 mL of fresh Luria-Bertani-ampicillin medium and incubated with shaking at 37°C until OD₆₀₀ reached 0.6. Isopropyl β-D-thiogalactopyranoside, an inducer of gene expression, was added to a final concentration of 1 mM and the incubation was continued at 30°C for 3 h. Cells were washed with 100 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-NaOH (pH 7.4) and then sonicated in 100 mM HEPES-NaOH (pH 7.4), 1 mM EDTA, 10% (v/v) glycerol, and 0.1% (v/v) Triton X-100 on ice. The lysate was centrifuged at 15,000g for 5 min. The supernatant was used as a crude enzyme extract for the assay of ALDH activity.

Assay of ALDH Activity against Acetaldehyde

ALDH activity was assayed according to the method of op den Camp and Kuhlemeier (1997). The reaction mixture contained 100 mM sodium-pyrophosphate (pH 9.5), 1.3 mM NAD⁺, 100 µM acetaldehyde, and 1 volume of *E. coli* extract containing 200 µg of protein in a total volume of 100 µL. The reaction was initiated by addition of acetaldehyde. ALDH activity was evaluated from the rate of increase in A₃₄₀ due to the conversion of NAD⁺ to NADH.

Probe Labeling

The fragments that correspond to the coding region and the 3'-UTR of *Aldh2a* and the 3'-UTRs of *Adh1* and *Pdc1* were amplified from cDNA clones by PCR using the primer sets ALDH-P1/ALDH-P2, ALDH-P3/ALDH-P4, ADH-P1/ADH-P2, and PDC-P1/PDC-P2, respectively. A 7.4-kb *EcoRI*-fragment containing the rice 25S/17S rRNA genes was obtained from the rRNA genomic clone pRR217. These fragments were used for probes by labeling them with the DIG DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany).

Extraction of Total DNA and Genomic Southern Hybridization

Total DNA was extracted using the method of Shure et al. (1983) from 10-d-old plants. Total DNA (5 µg) was digested by *EcoRI*, *EcoRV*, and *XbaI*, and then subjected to electrophoresis on a 0.7% (w/v) agarose gel. Southern hybridization was carried out using the method of Ohtsu et al. (1999).

Extraction of Total RNA and Northern Hybridization

Rice total RNA was extracted by the standard guanidine thiocyanate/CsCl method (Kingston, 1991). Total RNA was denatured by treatment with formaldehyde and fractionated in a 1% (w/v) denaturing agarose gel. The gel was subsequently stained with ethidium bromide and blotted

onto a nylon membrane (Magna Pure Nylon, Micron Separations, Westborough, MA). Northern-blot hybridization was performed with the DIG DNA Labeling and Detection Kit (Roche Diagnostics).

Antibody Preparation

Two oligopeptides corresponding to internal sequences of rice ALDH2a and Arabidopsis ALDH2a were synthesized and purified by Biologica (Nagoya, Japan). The sequences of the synthetic peptides were as follows: NH₄-(G) CAGSRTFVHERVYDEFVEK-COOH (rice ALDH2a) and NH₄-(G) CAGSRTFVHEKVYDEFVEK-COOH (Arabidopsis ALDH2a). A mixture of the two oligopeptides was injected into rabbits to raise ALDH2-specific antiserum by Biologica. To purify the ALDH2-specific antibody, the antiserum was loaded onto a synthetic peptide-coupled *N*-hydroxysuccinimide-activated Sepharose 4 Fast Flow column (Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Antibody was eluted from the column using 100 mM Gly-HCl (pH 2.5) and 150 mM NaCl and was neutralized with 1 M Tris [tris(hydroxymethyl)aminomethane]-HCl (pH 8.0). The purified antibody was stored at -80°C.

Preparation of Mitochondrial Protein Extracts

Mitochondria were isolated from leaves of aerobically grown rice seedlings or submergence-treated seedlings by Suc step-gradient centrifugation according to the method of Newton (1994). Mitochondrial protein extracts were obtained by lysis of isolated mitochondria with the lysis buffer (5% [w/v] sodium *N*-lauroyl sarcosinate, 0.25% [v/v] Triton X-100, 25 mM Tris-HCl [pH 7.5], 1 M KCl, 20 mM EDTA, and 40% [v/v] glycerol).

Immunoblotting

E. coli protein extract or mitochondrial protein extract was denatured and separated by SDS-PAGE. Each lane was loaded with 20 µg of protein. Acrylamide concentrations were 4.75% (w/v) and 10% (w/v) in the stacking and separation gels, respectively. Proteins were electroblotted onto a nitrocellulose membrane (Hybond-C extra; Amersham, Buckinghamshire, UK). The membrane was initially incubated for 1 h in 5% (w/v) skim milk (in 20 mM Tris-buffered saline plus Tween 20 [TBST: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% {v/v} Tween 20]), and reacted with the ALDH2-specific antibody (1:200 dilution) at 4°C for 12 h. The blot was washed three times for 10 min each in 20 mM TBST, and then was incubated with 1:5,000 diluted donkey antirabbit antibody coupled to peroxidase (Amersham) at 25°C for 1 h. After three washes in 20 mM TBST for 10 min, the reaction was visualized using LumiLight (Roche Diagnostics) and a Lumino-image analyzer LAS-1000 (Fuji Photo Film, Tokyo).

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