A Point Mutation of *Adh1* Gene is Involved in the Repression of Coleoptile Elongation under Submergence in Rice

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In higher plants, alcoholic fermentation is required to supply NAD⁺ to the glycolytic pathway, which is responsible for ATP synthesis under anaerobic conditions. Matsumura *et al.* (1995) previously isolated the *reduced adh activity* (*rad*) mutant in rice, in which elongation of the coleoptile is repressed under submergence. However, the *rad* gene had not been characterized. In the present study, we observed that, in the coleoptile, the *Adh1* mRNA levels were comparable between the *rad* mutant and the wild type cultivar Kinmaze, while the amount of ADH1 protein was much lower in the *rad* mutant than in the wild type. Sequencing showed that G106 of the *Adh1* gene of Kinmaze was replaced with A in the *rad* mutant, resulting in an E36K substitution in the deduced amino acid sequence. A genotyping experiment using F₂ plants from a cross of the *rad* mutant with the *indica* cultivar Kasalath indicated that the point mutation was involved in the repression of coleoptile elongation. Furthermore, since the reduced ADH activity appeared to cause an ATP deficiency, elongation of the coleoptile was repressed in the submerged *rad* mutant.

Key Words: alcohol dehydrogenase, submergence, rice, coleoptile, ATP deficiency.

Introduction

Rice (*Oryza sativa* L.) is one of the few crops that can germinate under complete submergence. The morphology of submerged rice seedlings is markedly different from that of aerobic seedlings. In rice seedlings, that germinate aerobically, the coleoptile emerges from imbibed seeds and elongation stops within a few days. In contrast, leaves and roots continue to grow even after the cessation of coleoptile elongation. On the other hand, in rice seedlings that germinate anaerobically, the elongation of the coleoptile is efficient, unlike that of the leaves and roots. When the elongated coleoptile reaches the water surface, it acts as a snorkel, supplying oxygen to leaves and roots. Therefore, coleoptile elongation is essential for further growth of submerged rice seedlings, including the growth of roots and photosynthetic leaves. In Japan, direct sowing of rice seeds in paddy fields has increasingly replaced the traditional transplanting method because of the reduced costs and labor. Coleoptile growth is necessary for direct sowing to be successful in a submerged field (Ogiwara and Terashima 2001, Miura *et al.* 2002). Thus, elucidation of the mechanisms of coleoptile elongation might enable to enhance coleoptile growth in rice cultivars that are directly seeded under submerged condi-

tions (Miura *et al.* 2004).

In many higher plants, alcoholic fermentation is necessary for germination and survival under anaerobic conditions caused by heavy rain and flooding (Tadege *et al.* 1999). Alcoholic fermentation occurs in two reaction steps (Fig. 1): the decarboxylation of pyruvate to acetaldehyde by pyruvate decarboxylase (PDC) and the subsequent reduction of acetaldehyde to ethanol by alcohol dehydrogenase (ADH). This metabolic pathway supports glycolysis and ATP synthesis by recycling NAD⁺. Analyses of ADH-deficient mutants in maize (Schwartz 1969, Freeling and Bennett 1985, Johnson *et al.* 1994), barley (Harberd and Edwards 1982), *Arabidopsis* (Jacobs *et al.* 1988, Ellis *et al.* 1999) and rice (Matsumura *et al.* 1998) have shown that ADH is required for anaerobic tolerance in plants.

When rice germinates under water, ADH activity is required for coleoptile elongation (Setter and Ella 1994, Kato-Noguchi 2001, Kato-Noguchi and Kugimiya 2003). Matsumura *et al.* (1995) isolated a single recessive rice mutant, whose ADH activity was markedly reduced. Thus, the mutant was designated as *reduced adh activity* (*rad*) mutant. Elongation of the coleoptile was strongly repressed in the submerged *rad* mutant (Matsumura *et al.* 1995, see Fig. 2A). Subsequently, the amount of ADH1 protein, but not the amount of ADH2 protein, in shoots and roots in the *rad* mutant was found to be lower than that in the wild type, while the *Adh1* mRNA levels in the *rad* mutant were comparable to those in the wild type (Matsumura *et al.* 1998). However,

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Fig. 1. Diagram of alcoholic fermentation pathway in plants. Alcoholic fermentation occurs in two reaction steps: the decarboxylation of pyruvate to acetaldehyde by PDC and the subsequent reduction of acetaldehyde to ethanol by ADH.

it remains to be determined whether the *Adh1* gene showed a similar expression pattern in the rice coleoptile. Furthermore, the gene responsible for the *rad* mutation was not identified, although it was assumed to encode ADH1 itself or a protein that regulates ADH1 protein stability (Matsumura *et al.* 1998).

In the present study, we examined the *Adh1* mRNA levels and the ADH protein levels in the coleoptiles in submerged *rad* mutants. We found that the *rad Adh1* gene displayed a point mutation linked to the phenotype of repressed coleoptile elongation. Thus, we identified *Adh1* as the *rad* gene. We also found that the reduced ADH activity decreased the ATP content in the coleoptile, suggesting that the reduced ATP amount repressed coleoptile elongation in the submerged *rad* mutant.

Materials and Methods

Plant materials, growth conditions and treatments

Rice (*Oryza sativa* L., cv. Kinmaze) coleoptiles were grown under complete submergence in darkness at 28°C. After 5 days, the coleoptiles were harvested, frozen in liquid nitrogen and stored at -80°C.

For the genotyping experiment, we used an F₂ population consisting of 112 plants derived from the cross *indica* cultivar 'Kasalath' × *rad* mutant. These seeds were germinated under water in darkness at 28°C for 4 days. After the length of 4-day-old coleoptiles was measured, the seedlings were transplanted to soil and grown over a one-month period. Total DNA was extracted from 1-month-old leaves and the nucleotide sequences for the *Adh1* gene were determined in all the F₂ lines.

DNA extraction, PCR and sequence analysis

Total DNA was extracted from frozen leaves by the benzyl-chloride method (Zhu *et al.* 1993). The sequence of the *Adh1* gene of the *rad* mutant, from just upstream of the initiation codon to just downstream of the termination codon (referred to as the *Adh1* fragment), was first amplified from total DNA by PCR using the Adh1-f1 (f1) and Adh1-r7 (r7) primers (Table 1). The Adh1 fragment was used as the template for direct sequencing with f1 and r7 and 12 other forward and reverse primers (Table 1) whose target sequences lay between f1 and r7. The sequences were read with an ABI3100 sequencer (Perkin Elmer ABD, Foster City, CA, USA) and were assembled using SEQMAN Software (DNASTAR Inc., Madison, WI, USA).

The *Adh1* genotype was based on a 496 bp sequence between the f2 and r2 target sequences. This sequence was obtained by directly sequencing the Adh1 fragment with the f2 and r2 primers.

Fig. 2. Comparison of coleoptiles of wild type rice and the *rad* mutant. (A) Seeds without glumes after imbibition under complete submergence at 28°C in darkness for 7 days. Bar = 1 cm. White arrowheads indicate the tips of each coleoptile. (B) Growth of coleoptiles. The length of the coleoptiles of Kinmaze (wild type) and the *rad* mutant was measured. Values are mean ± SD for 10 seedlings.

Table 1. Primers used in the present study

	Primer	Sequence (5' to 3')
For sequencing	Adh1-f1	CTAATAATCCACGCGTGGTG
	Adh1-f2	TGGATTGGGCGAACTTTTAG
	Adh1-f3	CCATGAAGCTGGAGGGTATG
	Adh1-f4	ATGCTGTTGCCTGTGTCAAA
	Adh1-f5	ACAGGGGTGTGATGATTGGT
	Adh1-f6	GACCTGAACGCCAACAGATT
	Adh1-f7	TCTCAAGGGAACCTTCTTCG
	Adh1-r1	CTAAAAGTTCGCCCAATCCA
	Adh1-r2	CATACCCTCCAGCTTCATGG
	Adh1-r3	TTTGACACAGGCAACAGCAT
	Adh1-r4	ACCAATCATCACACCCCTGT
	Adh1-r5	AATCTGTTGGCGTTCAGGTC
	Adh1-r6	CGAAGAAGGTTCCTTGAGA
	Adh1-r7	AACTCGAGCGCACAAATCTC
For RT-PCR	Adh1-RT-f	TCATCCGCATGGAGAACTA
	Adh1-RT-r	CCGTATATCATCATTACCCC
	Pdc1-RT-f	GCAACTGCTGGACAAAGAAG
	Pdc1-RT-r	AGAAGCTCTTTGCTCGTGTC
	17SrRNA-RT-f	TCCTACCGATTGAATGGTCC
	17SrRNA-RT-r	CTTGTTACGACTTCTCCTTCCTC

RNA extraction and quantitative RT-PCR

Total RNA was extracted from the frozen coleoptiles using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Transcript levels of *Adh1* and *Pdc1* were measured by quantitative RT-PCR using a LightCycler (Roche Diagnostics, Mannheim, Germany) and the QuantiTect SYBR Green RT-PCR kit (Qiagen), according to the manufacturers' protocols. Primers used for RT-PCR are shown in Table 1. A cDNA clone of each gene was used to draw standard curves for quantification. Experiments were repeated twice and the same results were obtained.

Protein extraction and immunoblotting

Total protein was extracted from 5-day-old coleoptiles by the method of Xie and Wu (1989).

Polyclonal antibodies against rice ADH (Kadowaki *et al.* 1988) were provided by Dr. K. Kadowaki (National Institute of Agrobiological Sciences, Tsukuba, Japan).

Polyclonal antibodies against rice PDC were prepared as follows. An oligopeptide corresponding to an internal sequence of rice PDC (NH₂-(GC)EVIAHKDDTSKELLE-COOH) was synthesized, purified and injected into rabbits, at Biologica Co. (Nagoya, Japan). The PDC-specific antibodies were purified at Biologica Co. by the following method. The antiserum was loaded onto a synthetic peptide-coupled NHS-activated Sepharose 4 Fast Flow column (Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer's instructions. Antibodies were eluted with 100 mM glycine-HCl (pH 2.5) and 150 mM NaCl, and were neutralized with 1 M Tris-HCl (pH 8.0). The purified antibodies were stored at -80°C.

Total protein was denatured and separated by SDS-PAGE. Each lane was loaded with 20 µg of protein. Acryl-

amide concentrations were 4.75% and 10% (w/v) in the stacking and separation gels, respectively. Immunoblotting was performed with rice ADH-specific antibodies (Kadowaki *et al.* 1988) and rice PDC-specific antibodies (this study) according to the method of Nakazono *et al.* (2000). Signal intensities were quantified using LAS-1000 (FUJIFILM, Tokyo, Japan).

Extraction and measurement of ATP

ATP was extracted according to the method of Saglio and Pradet (1980). Ten frozen coleoptiles were homogenized and mixed with 70 µl of 0.6 M trichloroacetic acid (TCA) in diethylether and 700 µl of 0.6 M TCA in H₂O. The homogenate was centrifuged at 13,000 × g for 10 min at 4°C. TCA was removed three times from the supernatant with 350 µl diethylether. The supernatant was then neutralized with 300 µl of 0.5 M Tris-acetate (pH 7.75). ATP content was measured using an ATP assay system LL100-1 (TOYO B-net, Tokyo, Japan), according to the supplier's instructions.

Results and Discussion

Expression of *Adh1* gene in the coleoptiles of the *rad* mutant

Under submerged conditions, the coleoptiles of the wild type Kinmaze began to elongate one day after imbibition and reached an average length of 52.2 mm after 5 days (Fig. 2B). However, the coleoptiles of the *rad* mutant hardly elongated even after 5 days (Fig. 2A and 2B). This result was consistent with the findings of Matsumura *et al.* (1995).

The relative transcript levels of *Adh1* in the coleoptile were comparable between the *rad* mutant and Kinmaze (Fig. 3A), while the amount of ADH protein in the coleoptiles in the *rad* mutant was 5-fold lower than that in Kinmaze

Fig. 3. Gene expression of *Adh* and *Pdc* in submerged coleoptiles. (A) Quantitative RT-PCR of *Adh1* and *Pdc1* genes. Total RNA was extracted from 5-day-old coleoptiles grown under submergence. For amplification, 0.1 µg of total RNA was used as a template. All the mRNA levels were normalized to the 17S rRNA level. (B) Immunoblotting using ADH-specific and PDC-specific antibodies. Total protein was extracted from 5-day-old coleoptiles grown under submergence. For each treatment, total protein (20 µg) was used to determine the enzyme protein levels. Equal loading of total protein was confirmed by SDS-PAGE.

Fig. 4. Analysis of *Adh1* gene in the *rad* mutant. (A) Structure of the *Adh1* gene. Black boxes indicate exons. G106 in the wild type was replaced with A in the *rad* mutant. Bar=1 kb. (B) Alignment of the deduced amino acid sequences of ADH1 proteins from various plant and animal species. Black boxes denote identical amino acids and gray boxes denote similar amino acids. ADH1 proteins were derived *Oryza sativa* (cultivar Kinmaze, this study), *Zea mays* (Paul and Ferl 1993, accession no. CAA27681), *Arabidopsis thaliana* (Innan *et al.* 1996, accession no. BAA19622), *Caenorhabditis elegans* (accession no. AAF39899), *Drosophila melanogaster* (Danielsson *et al.* 1994, accession no. NM_000668) and *Homo sapiens* (accession no. AAD37446).

(Fig. 3B). These results were similar to those observed in the leaves and roots of the *rad* mutant (Matsumura *et al.* 1998). On the other hand, the *Pdc1* mRNA levels and the PDC protein levels were comparable between the *rad* mutant and Kinmaze (Fig. 3A and 3B), suggesting that the expression of the *Pdc1* gene was not affected by the reduction of the ADH protein level.

Characterization of *rad* gene

Matsumura *et al.* (1995, 1998) reported that the activation of the ADH1 isozyme was markedly reduced in the *rad* mutant and suggested that either *Adh1* mRNA was not translated or else its product was unstable in the *rad* plants. A

comparison of the sequences of the *Adh1* genes of the *rad* mutant and Kinmaze showed that in the *rad* mutant G was replaced with A at the 106th nucleotide from the initiation codon (Fig. 4A), resulting in a predicted replacement of Glu with Lys at position 36 (E36K) (Fig. 4B). We could not find any other mutation in the *Adh1* gene in the *rad* mutant (data not shown). Glu36 in ADH1 is conserved among diverse plant and animal species such as maize (Paul and Ferl 1993), *Arabidopsis thaliana* (Innan *et al.* 1996), nematoda, fly (Danielsson *et al.* 1994) and human (Fig. 4B). This glutamate residue does not appear to be related to the regions that are essential for catalysis [e.g., zinc-binding domains and the pyridine nucleotide-binding domain (Dennis *et al.*

Fig. 5. Genotyping of the *Adh1* gene. (A) Average length of 4-day-old coleoptiles. Each value is the mean of the length of coleoptiles $\pm$ SD from 27 (G/G), 62 (G/A) and 23 (A/A) seedlings, respectively. (B) Length distribution of 4-day-old coleoptiles of G/G, G/A and A/A seedlings.

1984, Yoshida *et al.* 2004)]. The structure of the ADH1 proteins of the *rad* mutant and wild-type, as predicted by the SWISS-MODEL (<http://www.expasy.ch/swissmod>; Guex and Peitsch 1997) was slightly different (data not shown). If the structure of the ADH1 protein of the *rad* mutant is different, the change in structure might reduce the protein stability or translational efficiency rather than decrease the enzyme activity. Labeling of the ADH1 protein with a tracer may enable to determine whether the protein stability or translational efficiency is reduced.

Hake *et al.* (1984) described maize *Adh1* mutants, in which the ADH1 protein was not detectable, although the *Adh1* mRNA level was normal, as in the case of the *rad* mutant. Since the mutations in these mutants have not been characterized, it remains to be determined whether these mutants have the same defects. Nucleotide sequencing of the *Adh1* gene of the maize *Adh1* mutant may enable to identify the regions responsible for the stability or translational efficiency of the ADH1 protein.

To confirm that the point mutation (G106A) of the *Adh1* gene was correlated with the repressed elongation of the coleoptile in the *rad* mutant, we genotyped an F_2 population consisting of 112 plants generated from a cross of the *rad* mutant and the *indica* line 'Kasalath'. The 106th nucleotide from the initiation codon in the *Adh1* gene of the *rad* mutant segregated as G/G (wild type): G/A : A/A (*rad* mutant)=27:62:23, which fitted to a 1:2:1 ratio ($\chi^2=1.57$; $P>0.05$). The length of the coleoptile was much shorter in the A/A lines than in the G/G and G/A lines (Fig. 5A and 5B). Therefore, these results indicate that the substitution at position 36 (E36K) affected the amount of ADH1 protein and thus the elongation of the coleoptile in the *rad* mutant. This further showed that the *rad* gene was identical with the *Adh1* gene.

Rahman *et al.* (2001) reported that coleoptile elongation was repressed in an *Adh1*-antisense transgenic rice plant under anoxic conditions. Moreover, coleoptile elongation was found to be strongly correlated with the ADH activity

among Japanese cultivars (Kato-Noguchi 2001). The present findings are consistent with these reports. Therefore, our results confirmed that the ADH activity is essential for coleoptile elongation in submerged rice.

ATP content in the coleoptiles of the *rad* mutant

Alcoholic fermentation supplies NAD^+ for glycolysis in order to generate ATP under anaerobic conditions (Tadege *et al.* 1999). The ATP contents of the coleoptiles in the *rad* mutant and Kinmaze were 70.1 ± 11.8 pmol/coleoptile and 619.8 ± 327.7 pmol/coleoptile, respectively. Because ADH is required to generate ATP (Fig. 1), this result suggests that since the reduced ADH activity in the *rad* mutant caused the ATP-deficiency in the coleoptiles, the elongation of submerged coleoptiles was repressed.

Conclusion

In the *rad* mutant, a point mutation (G106A) in the *Adh1* gene was identified, indicating that Glu36 of the deduced amino acid sequence was replaced with Lys. Our results suggest that this single-base mutation caused a decrease in the amount of ADH protein and a reduction of the ADH activity. As expected, the ATP level in the *rad* coleoptile was considerably reduced, because alcoholic fermentation is essential for ATP synthesis by glycolysis under anaerobic conditions. Therefore, ATP deficiency may result in the repression of coleoptile elongation in the submerged *rad* mutant.

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