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H. Matsumura · T. Takano · G. Takeda · H. Uchimiya

Adh1 is transcriptionally active but its translational product is reduced in a *rad* mutant of rice (*Oryza sativa* L.), which is vulnerable to submergence stress

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Abstract Enzyme activities and transcripts of alcohol dehydrogenase (ADH) were analyzed in a single recessive rice (*Oryza sativa* L.) mutant with reduced ADH activity (*rad*). It has been previously demonstrated that all ADH isozymes are below the level of detection in aerobically grown *rad* seedlings (Matsumura et al. 1995). The present study showed that ADH activity was anaerobically inducible in *rad* seedlings and predominantly localized in the roots, mainly in the root-cap region. The lowest band (ADH2 homodimer) of the ADH isozymes was observed in submerged *rad* seedlings. Although two isozymes (the ADH1 homodimer and heterodimer), abundant in the wild-type, were lost in this mutant, *Adh1* mRNA was produced normally in aerated or submerged *rad* plants. Protein analysis showed that the amount of ADH protein was reduced in *rad* seedlings. Most likely, *Adh1* mRNA was not translated or else its product was unstable in *rad* plants. The viability of submerged *rad* seedlings was evaluated by the growth and greening of the shoots. Our results showed that *rad* seedlings were more vulnerable to a long-term submergence stress than wild-type seedlings.

Key words Alcohol dehydrogenase (ADH) · Mutant · *Oryza sativa* L. · Rice · Submergence stress

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H. Matsumura (✉)
Iwate Biotechnology Research Center, Narita, Kitakami-shi, Iwate 024, Japan
Fax: + 81-197-68-3881; e-mail: hideoma@sv02.sui.pref.iwate.jp

T. Takano
Asian Natural Environmental Science Center, University of Tokyo, Tanashi, Tokyo 188, Japan

G. Takeda
Faculty of Agriculture, Tamagawa University, Machida, Tokyo 194, Japan

H. Uchimiya
Institute of Molecular and Cellular Biosciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Introduction

In higher plants, ADH (alcohol dehydrogenase EC 1.1.1.1) is essential for energy production through alcoholic fermentation in oxygen-deprived (anaerobic) conditions. Anaerobic induction of ADH is known to be regulated at the transcriptional level in maize (Gerlach et al. 1991), *Arabidopsis* (Dolferus et al. 1994), and rice (Xie and Wu 1989). The level of the maize *Adh1* transcript increased 59-fold in the root axis following 24 h anoxia (Andrews et al. 1993). The specific promoter region of maize *Adh1* responsible for anaerobic induction was designated to an anaerobic response element (ARE) (Ellis et al. 1987; Walker et al. 1987; Olive et al. 1991). The ARE contains GC and GT motifs (Olive et al. 1991), where nuclear proteins were bound (Ferl 1990). GT or GT/GC motifs similar to the ARE sequence in maize *Adh1* were also found in the 5′-upstream region of rice *Adh2* (Xie and Wu 1990) and *Arabidopsis Adh* (Ferl and Laughner 1989). Analysis of transgenic *Arabidopsis* possessing the chimeric gene of the *Adh* promoter-beta-glucuronidase gene demonstrated that the ARE region is responsible not only for anaerobic expression but is also required for responses to other forms of environmental stress (Dolferus et al. 1994).

The *Adh* gene is expressed by auxin (Freeling 1973) or ABA treatment (Hwang and Van Toai 1991). However, the induction of *Arabidopsis Adh* by anaerobic or cold stress was not related to ABA (de Bruxelles 1996).

Maize *Adh1* is expressed in the embryo, endosperm, root and pollen, but not in mature photosynthetic tissues (Freeling and Bennett 1985). A similar spatial expression pattern was also reported in the *Arabidopsis Adh* gene (Dolferus et al. 1985). Rice ADH activity is

observed not only in seeds and roots, but also in shoots and green leaves (Xie and Wu 1989). Probably, unknown factors responsible for *Adh* expression in shoots are present in rice, since the maize *Adh1* promoter is functional in transgenic rice shoots (Kyozuka et al. 1994). *Adh* gene expression in shoots is a distinctive characteristic of rice and may contribute to the ability of rice seedlings to tolerate submergence stress.

To study the regulation of *Adh* gene expression, a genetic analysis with mutants was performed in maize (Freeling and Bennett 1985). Since Freeling and Cheng (1978) developed a convenient method for selecting ADH-defective mutants in maize, numerous mutants of maize *Adh1* were obtained. This selection method using allyl alcohol facilitated the isolation of ADH-deficient mutants in other plants, such as *Arabidopsis* (Jacobs et al. 1988), tomato (Wisman et al. 1991) and barley (Harberd and Edwards 1982). In several transposon-inserted mutants of maize, the organ specificity of *Adh1* expression was altered, and DNA sequences responsible for organ specificity were determined (Sutton et al. 1984; Chen et al. 1987; Kloeckner-Gruissem et al. 1992).

Previously, we isolated a single recessive rice mutant (designated as *rad*) with reduced ADH activity (Matsumura et al. 1995). To elucidate *Adh* expression at the molecular level in a *rad* mutant, the accumulation of mRNA, proteins, and enzyme activities were investigated in conjunction with the response to submergence stress.

Materials and methods

Plant materials and methods

Seeds of japonica rice (*Oryza sativa* L. cv Kinmaze; wild-type), and a *rad* mutant derived from the same cultivar, were sterilized in 1% NaOCl and germinated in a 30-ml glass tube filled with sterilized water (submerged germination) or on moist filter paper in a continuously aerated glass tube (aerobic germination). Seedlings were kept in the dark at 28°C. Anaerobic treatment of aerobically grown seedlings was performed by complete submergence in 50 ml of water.

Enzyme extraction

Roots, shoots, and other tissues without endosperm (designated as scutellum) were removed from seedlings. Tissues from 15 seedlings were ground in a mortar with liquid nitrogen. After the addition of 0.5 ml of extraction buffer (0.1 M Tris-HCl pH 8.0, 25% glycerol, 0.8% β-mercaptoethanol, and 5 mM DTT), samples were centrifuged in 1.5-ml microtubes for 15 min at 15 000 g at 4°C. The supernatant was used as a crude extract for enzyme and protein assays. The total soluble-protein content in each extract was determined by a protein-assay kit (Bio Rad) according to the method of Bradford (1976).

Enzyme assay

Alcohol dehydrogenase activities were measured spectrophotometrically by monitoring NADH reduction at 340 nm. ADH activities were assayed by determining the reduction of NAD to NADH in the conversion of ethanol into acetaldehyde at 25°C in a reaction mixture containing 0.15 M Tris-HCl buffer (pH 8.0), 0.3 mM NAD, 30 mM ethanol and 30 µl of enzyme extract, as described by Xie and Wu (1989).

Polyacrylamide-gel electrophoresis

Electrophoresis on a 7.5% polyacrylamide gel was carried out as described by Hoffman et al. (1986), and the gels were stained for ADH activity by the method of Xie and Wu (1989).

Histochemical localization of ADH activity in germinating seeds

Two-day-old germinating seeds in submerged conditions were cut in half using a razor. Bisected seeds were rapidly frozen in liquid nitrogen and then thawed in 0.1 M Tris-HCl pH 8.0 solution. Samples were incubated in ADH activity staining solution (0.1 M Tris-HCl pH 8.0, 1 mM NAD, 1 mM nitroblue tetrazolium, 1 mM phenazine metsulphate, 1% ethanol) for 3 min in the dark. To stop the reaction, treated seeds were transferred to a 0.5% acetic acid solution.

Analysis of mRNA accumulation

Total RNA was isolated from the roots or shoots of wild-type or *rad* seedlings (Umeda and Uchimiya 1994). Each 10-µg of total RNA was separated in a 1.2% agarose gel including formaldehyde and blotted onto a Hybond-N+ (Amersham) membrane using 0.05 M NaOH.

Rice *Adh1* cDNA was kindly provided by the National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fishery. Fragments of 1.0 kb (including the coding region) and 0.3 kb (including the 3′ non-coding region) were prepared as probes by *Sac*I digestion of rice *Adh1* cDNA. The fragments were labelled with alpha-^{32}P dCTP and then added to the hybridization solution (1% SDS, 1 M NaCl, 10% dextran sulphate). The blotted membrane was incubated in the hybridization solution at 65°C for 12 h. The hybridized membrane was washed in 2 × SSC, 0.1% SDS for 5 min and in 0.2 × SSC, 0.1% SDS for 30 min at 65°C. The hybridized signal in the washed membrane was visualized by an Image Analyzer BAS1000 (Fuji).

Immunoblotting

Electrophoresis on a 10% polyacrylamide gel with SDS was done by the method of Laemmli (1970). After electrophoresis, the ge was blotted onto a PVDF membrane (Immobilon, Millipore) in transfer buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol). The membrane was soaked in blocking solution (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5% skim milk), and then incubated with ADH antiserum in a buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X100). After incubation with antiserum for 30 min, the membrane was washed with the same buffer and incubated with anti-rabbit alkaline phosphatase-conjugated antibody for 30 min. Then, the unbound antibody was removed by washing and soaked in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$. The membrane was transferred to the same buffer containing 337.5 µg/ml of nitroblue tetrazolium and 175 µg/ml of 5-bromo-4-chloro-3-indlylphosphate (BCIP) for detection.

Extraction and quantitation of chlorophyll

Shoots of each seedling were weighed and homogenized with liquid nitrogen. For chlorophyll extraction, 1 ml of 80% acetone was added to the homogenized sample and centrifuged at 10000 g for 10 min. The supernatant was collected in another tube and pellets were re-extracted with 80% acetone. Extracts were made up to 5 ml with acetone. The OD_{663} and OD_{645} of extracts were measured and the chlorophyll contents calculated.

Results

rad is an *Adh1* null mutant

Previous studies showed that none of the ADH isozymes were detected in aerobically grown *rad* seedlings (Matsumura et al. 1995) by native PAGE. Consequently, ADH activity in the organs of 7-day-old wild-type or *rad* seedlings was assayed (Fig. 1 A). In wild-type seedlings, the highest ADH activity was observed in the scutellum, when compared with the roots and shoots. In *rad* seedlings, ADH activities were detected in the roots and scutellum, where the activities in the scutellum were much lower than that of the wild-type. No ADH activity was observed in the shoots of *rad* seedlings. To investigate the effects of anaerobic stress, 5-day-old seedlings were submerged for 2 days and ADH activities were assayed. As expected, ADH activities in every organ of wild-type plants were increased and their anaerobic induction was especially notable in the roots. ADH activities were also increased in every organ of *rad* seedlings following submergence. Anaerobically induced ADH activity was highest in the roots of *rad* seedlings and was about 40% of that in the roots of wild-type seedlings.

The isozymes of anaerobically induced ADH were analyzed in *rad* seedlings. Soluble protein from *rad* and wild-type seedlings was subjected to PAGE (Fig. 1 B). The wild-type contained ADH1 homodimer or heterodimer isozymes predominantly (Xie and Wu 1989), whereas only the ADH2 homodimer isozyme was detected in *rad* seedlings, indicating that *rad* is a null mutant in terms of ADH1 expression.

Reduction of ADH proteins in the *rad* mutant

The level of the *Adh1* transcript was investigated in wild-type and *rad* seedlings. Total RNAs were isolated from roots or shoots of 7-day-old seedlings. The coding region of rice *Adh1* cDNA was used as a probe for Northern hybridization. *Adh1* transcripts were also found in *rad* seedlings, and their size was the same as that in the wild-type seedlings (Fig. 2 A). They were greatly increased by submergence in the root or the shoot of wild-type and *rad* seedlings. The level of *Adh1* mRNA accumulation in *rad* seedlings was almost equal to that in wild-type seedlings. Since the coding region of *Adh1* was likely to hybridize to *Adh2* mRNA, the

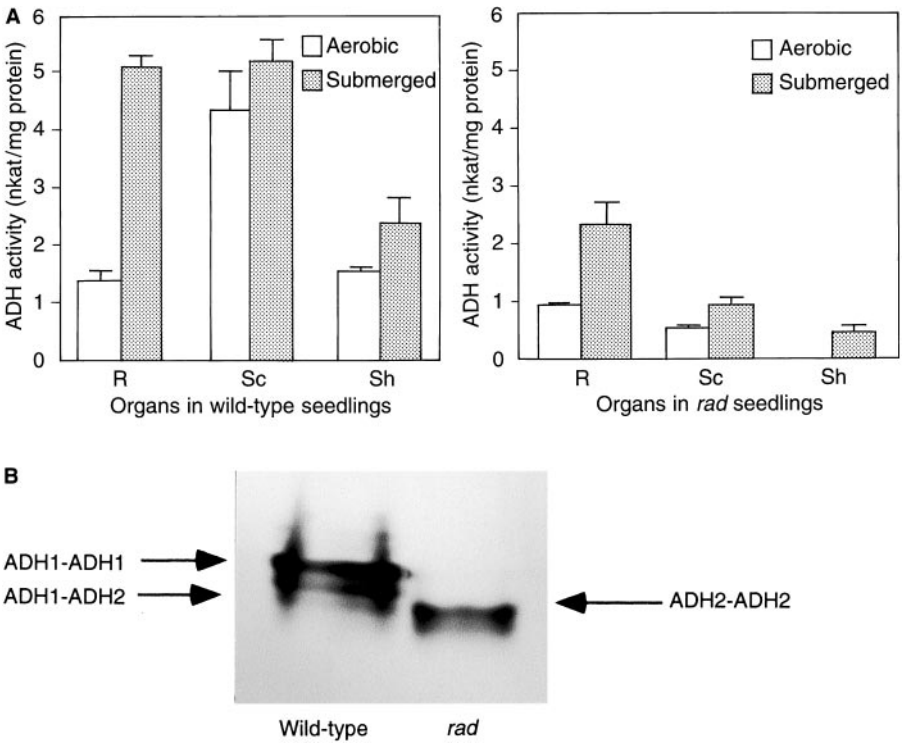

Fig. 1 A, B Comparison of ADH activities and isozymes in wild-type and *rad* seedlings. Seedlings grown either aerobically for 7 days (*open bar*) or for 5 days followed by 2 days submergence (*shaded bar*) were used to measure ADH activities. Panel **A** shows ADH activities in the root (*R*), scutellum (*Sc*) and shoot (*Sh*). Data represent a mean of three independent experiments (± SE). Panel **B** indicates ADH isozymes of 5-day-old seedlings that had germinated and grown in a submerged condition. Soluble proteins extracted from whole seedlings were loaded on a 7.5% non-denaturing PAGE. The amount of total protein in the lane for *rad* was 5-times that of the wild-type. Isozymes of either homo- or hetero-dimers of ADH1 and/or ADH2 are indicated by *arrows*

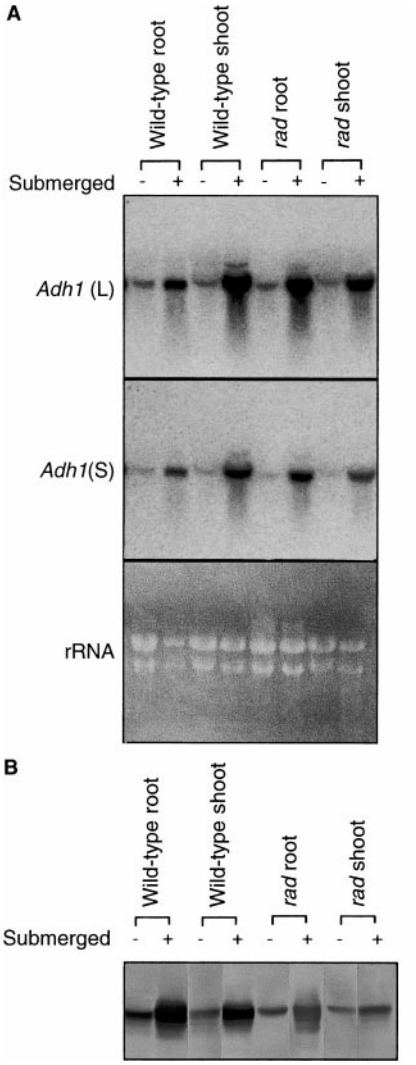

Fig. 2 A, B Analysis of *Adh1* mRNA accumulation and ADH protein in wild-type and *rad* seedlings. Total RNA or proteins were extracted from the roots or shoots of wild-type and *rad* seedlings as described in the legend for Fig. 1. Panel **A** shows a Northern-blot analysis of *Adh1* mRNA accumulation. The 5′-end 1.0-kb (*L*) or the 3′-end 0.3-kb (*S*) fragments of *Sac*I-digested rice *Adh1* cDNA (Xie and Wu 1989) were used as probes. Ribosomal RNA (rRNA) in each lane is equivalent to the total RNA loaded. Panel **B** shows immunoblotting analysis with rabbit antiserum against rice ADH. Proteins (20 μg) of each organ were applied to 10% SDS-PAGE, and transferred to a PVDF membrane. The membrane was incubated with antiserum and washed, followed by reaction with a secondary antibody conjugated to alkaline phosphatase. Bands were detected by the alkaline phosphatase-activity staining

fragment including the 3′ non-coding region of *Adh1* was used as a probe, so that *Adh1*-specific mRNA could be detected. The results of hybridization were the same as those when the coding region was used as a probe. Therefore, *Adh1* was normally transcribed in a *rad* mutant.

Protein analysis was carried out by immunoblotting with antiserum against rice ADH (Fig. 2 B). Two bands reacted with this antiserum, as shown by Kadowaki et al. (1988). In the roots or shoots of *rad* seedlings, although ADH protein was detectable, its amount was much reduced. ADH protein in *rad* roots was induced by submergence and its amount was more than that in the shoots. The result of immunoblotting was almost identical to that of ADH activity.

Tissue-specific localization of ADH activity

Localization of ADH activity was investigated in submerged germinating seeds. Wild-type and *rad* seeds were germinated in water for 2 days, and their embryos were cut in half. ADH was detected by activity staining with NBT (Fig. 3). In the wild-type embryo, roots, shoots and scutellum were stained. Staining in the epithelium was especially very strong. In *rad* seedling, staining was limited to the root area and was not observed in either epithelium or shoots. As a comparison, staining for malate dehydrogenase (MDH) activity was performed in the same way. Localization of MDH activity in the *rad* embryo was identical to that in the wild-type.

rad plants are sensitive to submergence stress

The effects of submergence on *rad* seedlings were evaluated by their shoot growth (Fig. 4 A) and the recovery of greening (Fig. 4 B). Etiolated seedlings were grown aerobically for 7 days. These seedlings were submerged in the water for 7 days, and then transferred to aerobic and light conditions. There was no significant difference in the shoot length of 7-day-old seedlings between wild-type and *rad*. During submergence, the growth of both type of seedlings was suppressed. When transferred to aerobic conditions, wild-type shoots elongated, whereas no growth of *rad* seedlings was noted. The chlorophyll contents in the shoots were measured as an indication of greening. After etiolated seedlings were recovered from submergence, the contents of chlorophyll a and b were increased by continuous light conditions. The increased rate of chlorophyll in wild-type seedlings was twice that in *rad* seedlings. Furthermore, in wild-type seedlings, greening of the leaf sheath and blade was often observed. On the other hand, only a few *rad* seedlings showed greening of the leaf sheath, whereas leaf blades in most of the submerged *rad* seedlings seemed to be killed (data not shown). Consequently, the shoots of *rad* seedlings could not grow and this reduced the ability for greening after submergence. Thus, *rad* seedlings were more heavily damaged by long-term submergence than wild-type seedlings.

Discussion

The results of isozyme analysis suggested that *rad* is a null mutant of *Adh1*. However, *Adh1* was normally

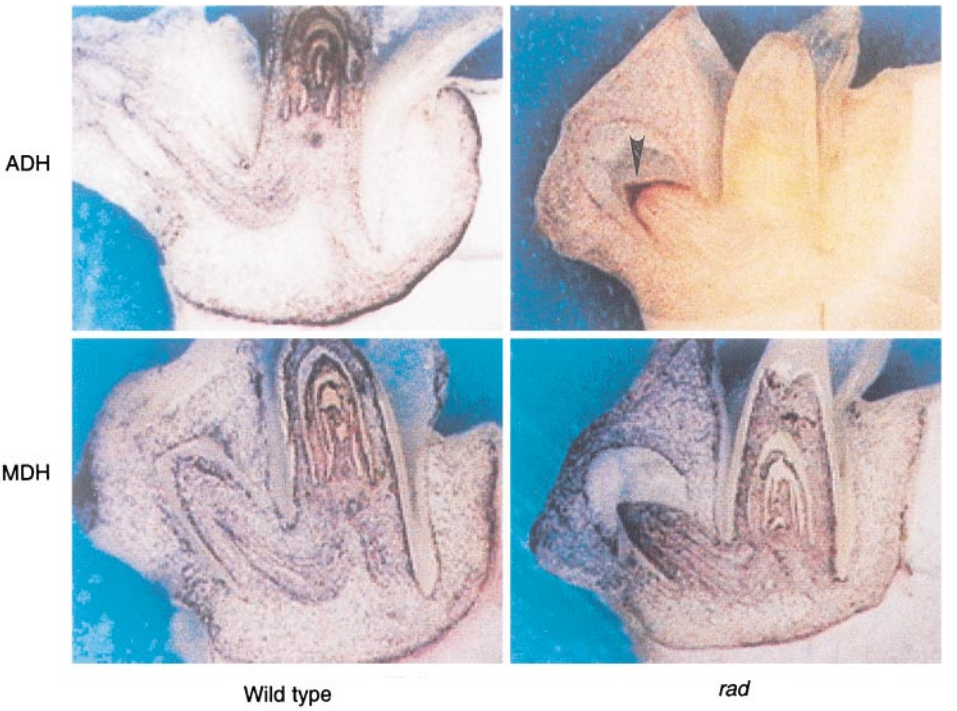

Fig. 3 Localization of enzyme activities in germinating seeds under submerged conditions. Seeds were germinated in water for 2 days, followed by histochemical treatment for the visualization of ADH or MDH activity. Purple color (tetrazolium blue) indicates the presence of ADH or MDH activity. Note the arrow in the *rad* mutant, where ADH is localized in the root-cap region

transcribed in *rad* seedlings and was anaerobically inducible, as in wild-type seedlings, while the amount of total ADH protein decreased in the *rad* seedlings. These results indicated that the promoter of *Adh1* was probably functional and that the mutation has occurred in an open reading frame of the *Adh1* gene in the *rad* mutant.

As previously described (Matsumura et al. 1995) *rad* is a chemically mutagenized mutant and a single base change must be occurred. Many ADH mutants, possessing a single base substitution, are known in *Drosophila* and *Arabidopsis*. Several mutants had a normal size and amount of *Adh* transcript and ADH protein but without enzyme activity (Hollocher and Place 1987 a, b; Jacobs et al. 1988). For one of the *Arabidopsis Adh* mutants, a sequence analysis of *Adh* revealed a base-pair transition which led to an amino-acid substitution at the active site (Dolferus et al. 1990). Amino-acid substitution has been found at a site responsible for the dimerization or activity of ADH protein in a *Drosophila* ADH-less mutant (Chenevert et al. 1995). Several conserved amino acids are known as the sites required for ADH activity by site-directed mutagenesis in *Drosophila* (Chen et al. 1990, 1993).

Another kind of mutant had point mutations, which generated stop codons in the *Adh1* gene. In the nonsense mutant of the *Drosophila Adh* gene, normal mRNA and low-molecular-weight protein were detectable (Martin et al. 1985). However, in the *Arabidopsis* mutant, although a transition to a TAG stop codon in the *Adh* gene was observed, no proteins or transcripts were detected, since the nonsense mutation might enhance the turnover of both the protein and the mRNA (Dolferus et al. 1990). In many *Adh1* mutants of maize, induced with EMS, the size and abundance of *Adh1* transcripts were normal but proteins were not detected (Hake et al. 1984). Similarly in *rad*, ADH1 protein is probably untranslated or else rapidly degraded after translation. Mutational analysis has revealed essential regions, or amino acids, for activity or stability in *Drosophila* ADH (Garvin et al. 1994; Hollocher and Place 1987 a, b). In plant ADH, however, information about functional sites was limited. Thus, nucleotide sequencing of *Adh1* in *rad* should reveal the regions responsible for ADH stability.

Our results demonstrate that total ADH activity was decreased in whole seedlings of the *rad* mutant. In particular, ADH activity dramatically decreased in the scutellum and shoots. Thus, as shown in Fig. 3, ADH was localized in the roots of submerged *rad* seedlings.

Electrophoretic analysis revealed that the lowest band of the three ADH isozymes was present in this mutant. According to the results of Xie and Wu (1989), this isozyme is the ADH2 homodimer. This indicated that the pattern of ADH expression in *rad* seedlings might reflect that of ADH2 expression in wild-type seedlings. Xie and Wu (1989) showed, by starch gel electrophoresis, that ADH2 was expressed predominantly in the roots of 3-week-old rice plants.

The inactive product of an *Adh* allele in *Drosophila* can form an active heterozygous dimer with wild-type ADH. (Hollocher et al. 1987 b). Although the ADH1

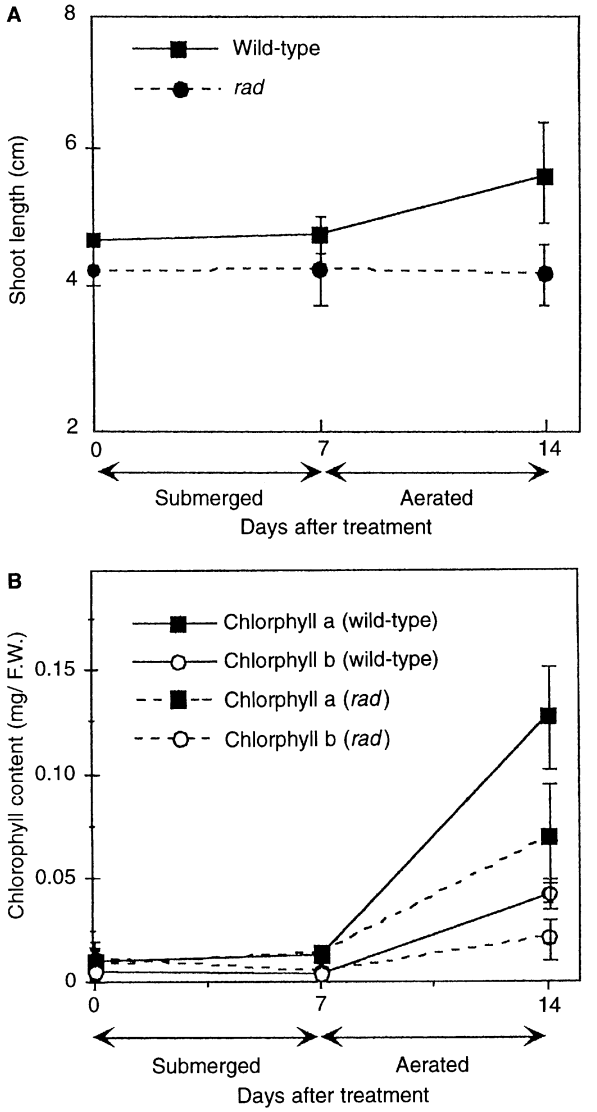

Fig. 4 A, B Analysis of plant growth and chlorophyll content in wild-type and *rad* seedlings-subjected to submergence treatment. Seven-day-old etiolated seedlings were submerged in water for 7 days, and then transferred to aerobic conditions. They were grown under continuous light conditions for an additional 7 days. Shoot length (**A**) and chlorophyll contents (**B**) in the shoots are presented

in *rad* was probably an unstable form, it is possible that heterozygous dimerization with ADH2 made it stable and active. Immunoblotting analysis with antiserum against ADH showed two cross-reactive bands (Kadowaki et al. 1988). Since the size of *Adh1* and *Adh2* transcripts was the same (Ricard et al. 1986; Xie and Wu 1989), Kadowaki et al. (1988) speculated that there was another *Adh* locus that encodes a different molecular-weight protein from ADH1 or ADH2. In barley, a third *Adh* gene was identified as *Adh3* (Hanson and Brown 1984). Therefore, it is possible that there are additional ADH isozymes in rice which have not yet been identified.

Since shoot growth and chlorophyll synthesis of *rad* seedlings were both lower than that of wild-type seedlings after 7 days submergence, ADH activity in the leaves or shoots is essential to the tolerance of long submergence in rice plants. Therefore, the regulatory mechanism of *Adh1* expression in the shoot needs to be resolved. Within the maize *Adh1* promoter, there is a region responsible for expression in the shoot. Although the maize *Adh1* was not expressed in photosynthetic tissues, its promoter was functional in rice shoots and a region responsible for its expression in the shoots was identified (Kyozuka et al. 1991, 1994). Thus, regulatory factors responsible for *Adh1* expression may be present in rice leaves. Screening of rice mutants without ADH in the shoots or leaves would be an effective method for identifying such regulatory factors.

As described above, *rad* is similar to several maize *Adh1* mutants, since *Adh1* mRNA was normally transcribed but the amount of ADH protein was reduced. In maize *Adh1* null mutants, ADH2 enzyme activities were inducible in root tips by hypoxic treatment (Johnson et al. 1994). These characteristics were also similar to those in a *rad* mutant. However, maize ADH2 has only a 5–10% specific ADH1 activity in the roots (Freeling and Bennett 1985), while rice ADH2 shared approximately 40% of total ADH activity in submerged seedlings. Furthermore, *rad* seeds were able to germinate in submerged conditions (Matsumura et al. 1995), while submerged maize kernels containing 5% of wild-type ADH activity were unable to germinate (Chen et al. 1986). Rice ADH2 may have more important roles in submerged seed germination than maize ADH2, although ADH activities in *rad* seeds were localized in the root region.

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