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Cell division and cell elongation in the coleoptile of rice *alcohol dehydrogenase 1*-deficient mutant are reduced under complete submergence

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• *Background and Aims* When rice seeds germinate under complete submergence, only the coleoptile elongates efficiently. It has been reported previously that coleoptile elongation is reduced in the rice *alcohol dehydrogenase 1* (*ADH1*)-deficient mutant, *reduced adh activity* (*rad*). The aim of this study was to elucidate how expressions of genes responsible for coleoptile elongation are affected by the *ADH1* deficiency in the *rad* mutant under submergence.
• *Methods* To identify genes whose expressions are changed in the *rad* coleoptile at an early stage in germination (i.e. 1 d after imbibition), coleoptiles of the *rad* mutant and its wild type (WT) were isolated by laser microdissection, and their mRNA levels were examined with a microarray.
• *Key Results* The microarray analysis identified 431 genes whose transcript levels were different between *rad* and WT. Interestingly, among the down-regulated genes in the *rad* coleoptile, 17·5 % were cell division-related genes and 5·1 % were cell elongation-related genes. It was found that cell division started at 1 d after imbibition and then gradually ceased, whereas in the WT coleoptile cell elongation started between 1 d and 2 d after imbibition and then continued. However, neither cell division nor cell elongation actively occurred in the *rad* coleoptile, in which the amounts of ATP were reduced.
• *Conclusions* These results indicate that cell division, as well as cell elongation, occur during coleoptile elongation in rice under complete submergence, and that the reduced ATP levels caused by the *ADH1* deficiency repress both of them, thereby impairing coleoptile elongation in the *rad* mutant under submerged conditions.

Key words: Cell division, cell elongation, rice, *Oryza sativa*, laser microdissection, coleoptile, microarray, *alcohol dehydrogenase 1*, *ADH1*, *reduced adh activity*, *rad*.

INTRODUCTION

Rice (*Oryza sativa*) can germinate under complete submergence, whereas other cereal crops such as wheat, barley, sorghum and maize cannot. In anaerobically germinated rice seedlings, the coleoptile and mesocotyl rapidly elongate, whereas the root does not (Magneschi and Perata, 2009). When the elongated coleoptile reaches the water surface, aerenchyma is formed in the coleoptile, thereby supplying oxygen to leaves and root (Alpi and Beevers, 1983; Kawai and Uchimiya, 2000). Coleoptile elongation is thus a survival strategy under submergence.

When a rice seed germinates under water, sugars derived from starch hydrolysis in the endosperm move to the embryo where they are metabolized through glycolysis to produce energy (i.e. ATP) and other important metabolites (Perata and Alpi, 1993; Gibbs and Greenway, 2003; Magneschi and Perata, 2009). Anaerobic metabolic pathways such as glycolysis and fermentation are required for coleoptile elongation (Setter and Ella, 1994; Kato-Noguchi, 2001; Kato-Noguchi and Kugimiya, 2003). Recently, Lee *et al.* (2009) reported that rice CIPK15 (calcineurin B-like-interacting protein kinase 15) regulated carbohydrate metabolism and fermentation through a sugar signalling pathway during germination under submergence. Thus, coleoptile elongation was suppressed in a *cipk15* knockout mutant under submergence, and was rescued by adding external sucrose (Lee *et al.*, 2009).

Alcoholic fermentation [catalysed by pyruvate decarboxylase and alcohol dehydrogenase (ADH)] and lactate fermentation (catalysed by lactate dehydrogenase) support glycolysis and ATP synthesis by recycling NAD^+ (Bailey-Serres and Voesenek, 2008). In a study of hypoxic rice coleoptiles, about 92 % of the carbon flux from pyruvate went to ethanol, 7 % went to alanine and 1 % went to lactate (Kato-Noguchi, 2006), suggesting that alcoholic fermentation is a more important source of NAD^+ for glycolysis than lactate fermentation (Kato-Noguchi, 2006; Magneschi and Perata, 2009). Indeed, reduction of ADH activity in rice

coleoptiles slows down their elongation under submergence (Matsumura *et al.*, 1995, 1998; Rahman *et al.*, 2001; Saika *et al.*, 2006; see Fig. 1).

Coleoptile length is mainly increased by cell elongation (Wada, 1961; Opik, 1973; Atwell *et al.*, 1982; Magneschi and Perata, 2009). Expansins probably have a major role in coleoptile cell elongation under anaerobic conditions (Huang *et al.*, 2000; Choi *et al.*, 2003; Magneschi *et al.*, 2009). Expansins are classified into two groups, α-expansins and β-expansins, both of which are encoded by large gene families (Lee and Kende, 2001, 2002; Shin *et al.*, 2005). Huang *et al.* (2000) reported that rice α-expansin genes, *Os-EXP2* (*EXPA2*) and *Os-EXP4* (*EXPA4*), were highly expressed in the submerged coleoptile, whereas their mRNAs were weakly detected in the coleoptile under aerobic or anoxic conditions. Additionally, coleoptile and mesocotyl lengths depended on the transcription level of *Os-EXP4*. Elongations of the coleoptile and mesocotyl were enhanced in *Os-EXP4* over-expressing plants and repressed in *Os-EXP4*-antisense plants (Choi *et al.*, 2003). Transcriptome analysis of an anoxic rice coleoptile recently revealed that *EXPA7* and *EXPB12* were up-regulated in the rice coleoptile under anoxic conditions (Lasanthi-Kudahettige *et al.*, 2007). These results suggest that some expansins contribute to coleoptile elongation in rice under hypoxia or anoxia. Genes involved in carbohydrate and lipid metabolism and genes encoding transcription factors and heat-shock proteins have also been found to be up-regulated or down-regulated in rice embryos and coleoptiles under anaerobic conditions (Lasanthi-Kudahettige *et al.*, 2007; Narsai *et al.*, 2009), suggesting that their gene products are involved in anaerobic growth (i.e. coleoptile elongation) of rice seedlings. Coleoptile length also appears to also be increased partly by cell division because the number of cells in the coleoptile increased during germination under submerged conditions (Wada, 1961; Opik, 1973; Atwell *et al.*, 1982). However, the results of these earlier studies have not yet been confirmed by modern molecular methods.

It has been reported previously that in the rice *reduced adh activity* (*rad*) mutant, the amount of ADH protein was greatly reduced because of a point mutation in the *ADH1* gene (Matsumura *et al.*, 1995, 1998; Saika *et al.*, 2006). Coleoptile elongation in the *rad* mutant was reduced under submergence (Matsumura *et al.*, 1995), but so far it remains unclear how the *ADH1* deficiency affects coleoptile elongation in the *rad* mutant under submergence. Answering this question would help to elucidate the elongation mechanism in wild-type (WT) rice.

In this study, coleoptiles were isolated from *rad* and WT embryos at an early stage in germination under submergence (before the start of coleoptile elongation) using laser microdissection (LM) (Nakazono *et al.*, 2003; Nelson *et al.*, 2006), and then their mRNA levels were examined with a microarray and semi-quantitative RT-PCR. As a result, many down-regulated genes related to cell division as well as cell elongation in the *rad* coleoptile were identified. To test the idea that cell division-related genes are at least partly responsible for the reduced elongation of *rad* coleoptile, the expressions of several of these genes, the mitotic index and 5′-bromo-2′-deoxyuridine (BrdU) incorporation in the WT and *rad* coleoptiles were investigated during germination and coleoptile elongation under submergence. The results show that cell division in the WT is active at the early stage of germination (i.e. 1–2 d after imbibition) and that during this period cell division in the *rad* coleoptile is much reduced.

MATERIALS AND METHODS

Plant materials and growth conditions

The rice (*Oryza sativa* L.) *rad* mutant and its WT ('Kinmaze') were used in this study. Dehulled caryopses (seeds) of rice were sterilized in a 0·6 % (v/v) sodium hypochlorite solution for 30 min. After washing with deionized water five times, 15 seeds were placed on the bottom of 1-L glass bottle filled with 1 L of deionized water. The seeds germinated and were grown under complete submerged or aerobic conditions in darkness at 28 °C. For the time-course experiments, seeds were placed in separate glass bottles for each time point and then harvested as seedlings. Dry seeds were used at 0 d after imbibition.

Extraction and quantification of ATP

ATP was extracted from freeze-dried rice coleoptiles using the luciferin–luciferase ATP assay system (Toyo Ink, Tokyo, Japan) according to the manufacturer's protocol. ATP amounts were determined by recording the relative light intensity for 10 s using a Junior LB9509 luminometer (Berthold Technologies, Bertech, Germany).

Preparation for frozen tissue sections

Frozen tissue sections were prepared following Nakazono *et al.* (2003) with minor modifications. Rice seeds or seedlings were fixed by a 5-min infiltration of 75 % (v/v) ethanol/25 % (v/v) acetic acid into the tissues under vacuum on ice. This fixation was repeated three times by replacing with new fixative solution and then the tissues were kept in fixative at 4 °C overnight. The fixed tissues were transferred to 10 % sucrose solution, which was prepared with PBS buffer (137 mM NaCl, 8·01 mM Na_2HPO_4, 2·68 mM KCl and 1·47 mM KH_2PO_4, pH 7·3). The sucrose solution infiltrated to the tissues under vacuum on ice for 5 min and the vials were kept at 4 °C overnight. The fixed tissues were embedded in RNase-free water, frozen in hexane cooled with dry ice, and stored at −80 °C. The tissues were sectioned into 8-μm-thick slices in a cryostat (Leica CM1850, Leica Microsystems, Wetzlar, Germany) by the method of Ishimaru *et al.* (2007). The frozen sections were dehydrated in 100 % ethanol for 10 s twice and dried at room temperature for 10 min.

Laser microdissection

The coleoptiles were cut from the frozen sections by UV laser (wavelength of 337 nm) using Leica AS LMD (Leica Microsystems), and then the cut coleoptile sections were dropped by gravity and were collected in a 0·5-mL tube cap containing mineral oil.

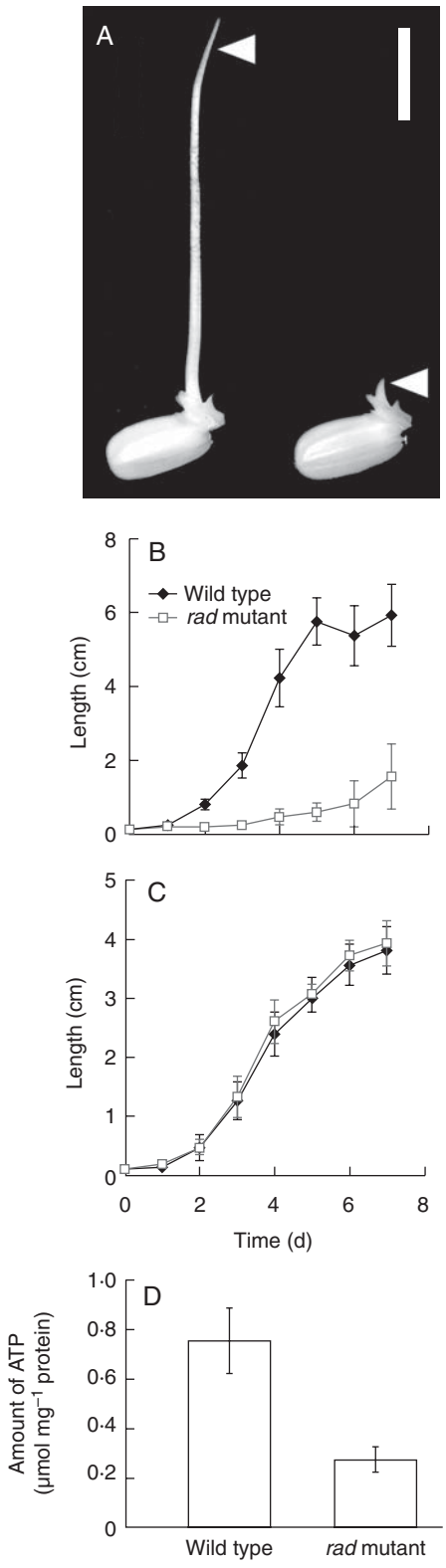

FIG. 1. Comparison of WT and *rad* mutant rice coleoptiles. (A) WT and *rad* mutant seeds were germinated for 5 d under complete submergence. Left: WT; right: *rad* mutant. Arrowheads indicate the coleoptiles. Scale bar = 1 cm. (B, C) Growth of coleoptiles under submerged (B) and aerobic (C) conditions. Data are means of 15 replicates ± s.d. (D) ATP concentrations in coleoptiles under submerged conditions at 1 d after imbibition. Data are means of three replicates.

Extraction and quantification of total RNA and assessment of RNA quality

Total RNA was extracted from LM-isolated coleoptile sections (10–20 sections for each experiment) with a PicoPure™ RNA isolation kit (Molecular Devices, Toronto, Ontario, Canada) according to the manufacturer's protocol. The extracted total RNA was quantified with a Quant-iT™ RiboGreen RNA reagent and kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quality of total RNA extracted from LM-collected tissues was assessed using an RNA 6000 Pico kit on the Agilent 2100 bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). RNA integrity was judged by RNA Integrity Number (RIN), which was calculated with 2100 Expert Software (Agilent, version B.02·02, eukaryote total RNA pico mode), and total RNA, whose RIN is $>6{\cdot}0$, was used for further analyses.

Microarray experiment

Aliquots of total RNAs (100 ng each) were labelled with a Quick Amp labelling kit (Agilent Technologies) according to the manufacturer's instructions. Aliquots of Cy5-labelled and Cy3-labelled cRNAs were used for hybridization in a rice 22K oligo-DNA microarray (Agilent Technologies). The array has 21 938 60-mer oligo probes to rice genes. Three biological replicates and a colour swap for each replicate were analysed. The hybridized slides were scanned using a DNA microarray scanner G2505B (Agilent Technologies), and signal intensities were extracted by Feature Extraction software (Version 8·5·1·1; Agilent Technologies). A complete set of microarray data was deposited with the Gene Expression Omnibus (GEO) repository under accession number GSE26632.

Microarray data analysis

Microarray signal intensities were digitized and log ratio or *P*-values were obtained by Feature Extraction software version 8·5·1·1 (Agilent Technologies). *rad* mutant genes that had $>2{\cdot}5$- or $<0{\cdot}4$-fold change in signal intensity and *P*-values

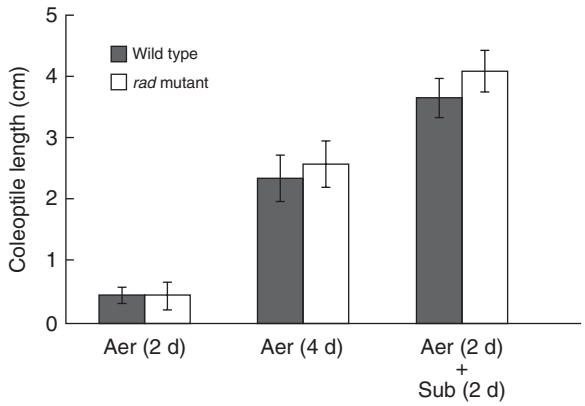

FIG. 2. Effect of switching from aerobic to submerged conditions on coleoptile elongation in WT and *rad* mutant. Aerobic growth for 2 d followed by submerged growth for 2 d. Control seedlings were grown under aerobic conditions for 4 d. Data are means of eight replicates ± s.d.

$<0{\cdot}01$ in all three replications and in each colour swap were identified. The fold change of each probe was calculated using the average of three replications and each colour swap. Gene descriptions in the 22K microarray were replaced with new descriptions based on RAP-DB (Build 5, http://rapdb.dna.affrc.go.jp/).

cDNA synthesis and amplification

A 1-ng aliquot of total RNA was used for cDNA synthesis and amplification. Reactions were performed by using a WT-Ovation™ RNA amplification system (NuGEN Technologies, San Carlos, CA, USA) according to the manufacturer's instructions.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR analysis was performed to examine the expression pattern of selected genes identified by the microarray analysis during germination. AmpliTaq® 360 (Applied Biosystems, Foster City, CA, USA) was used for subsequent PCR amplification with appropriate primers (Supplementary Data Table S3, available online): initial denaturation (95 °C for 10 min) and 22–37 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30 s), extension (72 °C for 30 s), and final extension (72 °C for 10 min).

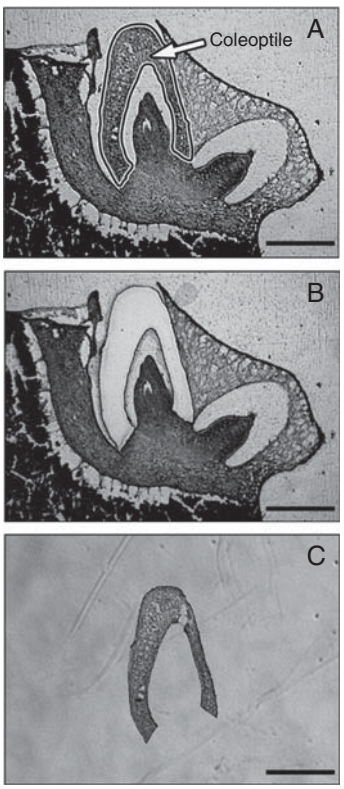

FIG. 3. Collection of a vertical coleoptile section from a rice embryo using LM. (A) An 8-μm-thick frozen section of a rice embryo before LM. The area enclosed with a black line (i.e. the coleoptile) was cut by LM and the cut coleoptile was collected. (B) The same frozen section after LM. (C) LM-isolated coleoptile. Scale bars = 500 μm.

Measurement of longitudinal cell length and counting cells in mitotic phase

Rice seeds or seedlings were fixed by a 5-min infiltration of 4 % (w/v) paraformaldehyde in PBS buffer (pH 7·4) into the tissues under vacuum on ice. This fixation was repeated three times by replacing with new fixative solution and then the tissues were kept in fixative at 4 °C overnight. After fixation, embryos were separated from seeds and were embedded in paraffin by the microwave method reported by Takahashi *et al.* (2010)

Serial longitudinal sections of the paraffin-embedded embryos, 6 μm thick, were cut, floated on water on glass slides, and incubated at 42 °C for 10 min. Subsequently, the solution was removed with a micropipette, and then sections were dried at 42 °C overnight. To remove paraffin, slides were gently immersed in xylene for 15 min twice, and then immersed in 50 % xylene/50 % ethanol for 15 min. The deparaffinized slides were rinsed with 100 % ethanol for 1 min, followed by acclimation steps of 1 min each in 90 % ethanol, 80 % ethanol, 70 % ethanol, 50 % ethanol and water for 1 min. Finally, slides were washed with PBS buffer three times. DNA in the nuclei on the sections were stained with 2·5 μg mL^{-1} DAPI (4',6-diamidino-2-phenylindole) solution [containing 1 % (v/v) Triton X-100 in PBS buffer] for 20 min. After staining, slides were washed with PBS buffer for 5 min.

Longitudinal cell length was measured and the numbers of cells in the mitotic phase were counted under a microscope. The results were expressed as the fraction of cells in the mitotic phase (the mitotic index).

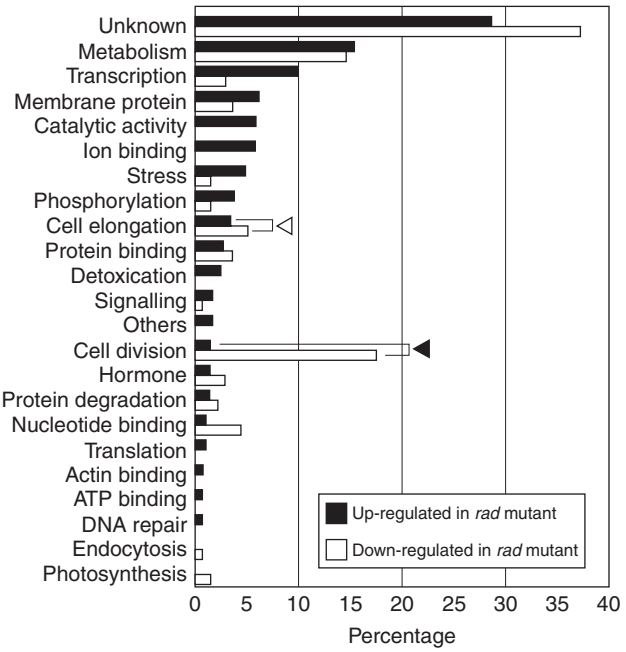

FIG. 4. Percentages of up-regulated and down-regulated genes in the *rad* mutant that are involved in different cellular functions. Up-regulated genes were those whose expressions were >2·5-fold greater in the *rad* mutant than in the WT; down-regulated genes were those whose expressions were >2·5 fold greater in the WT than in the *rad* mutant ($P < 0{\cdot}01$). The white arrowhead indicates cell elongation-related genes, the black arrowhead cell division-related genes.

TABLE 1. *List of genes related to cell division, whose expression was up-regulated or down-regulated in the* rad *coleoptiles*

Fold change (*rad*/WT) *	Locus ID†	Full-length cDNA accession no.†	Description†	Functional classification
4·77	Os03g0107700	AK063743	Similar to EL2 protein	Cell cycle
3·14	Os12g0147800	AK059860	Similar to phytosulfokines 5 precursor (secretory protein SH27A)	Cell proliferation
3·04	Os07g0596600	AK067238	Similar to Cdc2MsC protein	Cell cycle
2·66	Os03g0285800	AK067339	MAP kinase. (OsMAPK2)	Phosphorylation
0·18	Os01g0191800	AK102360	Similar to serine/threonine-protein kinase 12 (EC 2·7·1·37) (Aurora-B) (fragment)	Cell cycle
0·20	Os04g0563700	AK070211	Cyclin (cycB2;1)	Cell cycle
0·22	Os06g0726800	AK070518	G_2/mitotic-specific cyclin 2 (B-like cyclin) (CycOs2) (cycB2;2)	Cell cycle
0·24	Os04g0375900	AK101769	Kinesin, motor region domain-containing protein	Motor protein
0·25	Os02g0699700	AK072471	Similar to DNA topoisomerase II	DNA replication
0·26	Os08g0421800	AK103087	Similar to mitogen-activated protein kinase kinase kinase 1 (EC 2·7·1.–) (arabidospsis NPK1-related protein kinase 1); splice isoform 1S	Cell cycle
0·26	Os03g0114000	AK063381	Similar to kinesin	Motor protein
0·27	Os03g0712100	AK106258	Cell division cycle-associated protein domain-containing protein	Cell cycle
0·29	Os01g0685900	AK064024	Similar to 65-kD microtubule-associated protein	Cell cycle
0·29	Os02g0739700	AK111537	E2F Family domain-containing protein	Cell cycle
0·31	Os03g0162200	AK061605	Similar to histone H2A	DNA replication
0·32	Os07g0659500	AK073537	Non-SMC condensin subunit, XCAP-D2/Cnd1 family protein	Chromosome condensation
0·32	Os05g0497100	AK064293	Similar to SMC4 protein	Chromosome segregation
0·33	Os08g0512600	AK059682	Protein cdc2 kinase (cdc2Os3)	Cell cycle
0·33	Os02g0193600	AK060499	Mitotic checkpoint serine/threonine protein kinase, Bub1 domain-containing protein	Cell cycle
0·34	Os07g0507200	AK102722	Targeting for Xklp2 family protein	Spindle assembly
0·35	Os03g0119900	AK058741	Similar to histone H4	DNA replication
0·35	Os02g0699700	AK063335	Similar to DNA topoisomerase II	DNA replication
0·37	Os04g0583600	AK059019	Similar to histone H4	DNA replication
0·37	Os05g0459400	AK073413	Kinesin, motor region domain-containing protein	Motor protein
0·38	Os05g0113900	AK074018	Similar to histone H2A	DNA replication
0·39	Os01g0748000	AK103720	Similar to dynamin family protein	GTPase activity
0·39	Os01g0243100	AK064212	Similar to kinesin	Motor protein
0·40	Os03g0214100	AK060582	Replication protein A1	DNA replication

* Fold change shows average expression ratio of three biological replicates and a colour swap for each replicate.
† Locus ID, full-length cDNA accession number and description in Rice Annotation Project Database (RAP-DB; http://rapdb.dna.affrc.go.jp/).

Incorporation of BrdU and immunostaining

Labelling and immunostaining of BrdU were performed using a 5′-bromo-2′-deoxyuridine labelling and detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol with minor modifications. WT and *rad* mutant seeds germinated in 10 μM BrdU and 1 μM 5-fluorodeoxyuridine solution under darkness at 28 °C for 1 d, and then were fixed with 4 % (v/v) paraformaldehyde solution. After fixation, embryos were separated from seeds. The fixed embryos were embedded in paraffin and sections were prepared by the microwave method reported by Takahashi *et al.* (2010).

The immunostained sections were further stained with DAPI solution [containing 1 % (v/v) Triton X-100 in PBS buffer] for 20 min. After staining, slides were washed with PBS buffer for 5 min. BrdU and DAPI signals were observed under a confocal laser scanning microscope (Nikon, Tokyo, Japan).

RESULTS

When seeds of the *rad* mutant and its WT ('Kinmaze') germinated completely submerged, the WT coleoptiles began to elongate between 1 d and 2 d after imbibition and reached 51·2–67·4 mm at 5–7 d after imbibition (Fig. 1A, B). On the other hand, coleoptiles of the *rad* mutant elongated more slowly, reaching only 15·4 ± 8·9 mm at 7 d after imbibition (Fig. 1B). Under aerobic conditions, the elongation patterns of WT and *rad* coleoptiles were comparable (Fig. 1C). Under submerged conditions, the amount of ATP in the *rad* coleoptiles at 1 d after imbibition was only about 37 % of that in the WT (Fig. 1D), which is probably the reason for its slow elongation.

Next, we examined whether *rad* coleoptiles can actively elongate under submergence once coleoptile elongation, which occurs by both cell division and cell elongation, starts under aerobic conditions. Seeds were germinated and seedlings grown under aerobic conditions for 2 d. Then the seedlings, whose coleoptiles started elongation (Fig. 1C), were completely submerged for 2 d. Interestingly, the WT and *rad* coleoptiles elongated equally (Fig. 2).

To identify which genes are involved in coleoptile elongation during submergence, RNA samples were prepared on day 1 (1 d after imbibition) when the coleoptile lengths of the *rad* mutant (1·7 ± 0·3 mm) and the WT (2·3 ± 0·3 mm) were just beginning to diverge (Fig. 1B). The seeds which imbibed under submergence for 1 d were fixed, and frozen tissue sections were prepared for LM. Coleoptile cross-sections were collected from the tissue sections via LM

(Fig. 3). The RNA samples extracted from the LM-isolated coleoptiles were labelled with Cy3 or Cy5 dye, and the labelled cDNA from each of three biological replications was hybridized to rice oligo-microarrays. Genes were selected whose intensities differed by a factor of at least 2·5 in the *rad* and WT coleoptiles. After submergence, 294 genes were up-regulated in the *rad* coleoptile compared with the WT (Supplementary Data Table S1, available online) and 137 genes were down-regulated (Supplementary Data Table S2).

These genes were classified by function in Fig. 4. What stands out in this figure is that most of the genes involved in cell division and some of the genes involved in cell elongation (arrowheads in Fig. 4) were less expressed in the *rad* mutant, which suggests that the down-regulation of these genes is responsible for the slow elongation of the *rad* coleoptile under submergence. Thus, we examined whether cell division, as well as cell elongation, occurred during coleoptile growth in rice when it is submerged.

To confirm that the cell division-related genes are less expressed in the *rad* coleoptile than in the WT coleoptile, transcript levels of some cell division-related genes in the WT and *rad* coleoptiles were measured during germination and early growth of seedlings under submergence by semi-quantitative RT-PCR. The cell-division- and cell-elongation-related genes making up the bars in Fig. 4 and their *rad*/WT expression ratios are listed in Table 1. Six of these genes were selected for the semi-quantitative RT-PCR analyses: three G_2- and M-phase-expressed genes (*cycB2;1*, *cycB2;2* and *cdc2Os3*) (Umeda *et al.*, 1999*a*, *b*; Lee *et al.*, 2003), one M-phase-expressed gene (*Aurora-B*), one S-phase-expressed gene (*Histone H2A*) and one G_1- and S-phase-expressed gene (*E2F family domain-containing protein*). In the WT coleoptile, the transcript levels dramatically increased at 1 d after imbibition and then gradually decreased, whereas in the *rad* coleoptile, the induction levels were much lower (Fig. 5).

In the WT coleoptile, the mitotic index was highest (5·5 ± 1·0 %) at 1 d after imbibition and then gradually decreased to 3·2 ± 1·2 % at 2 d and to 1·4 ± 1·1 % at 3 d (Fig. 6). In contrast, in the *rad* coleoptile, the mitotic index was zero at 1 d, 0·8 ± 0·5 % at 2 d and 1·2 ± 0·6 % at 3 d (Fig. 6), indicating a much lower rate of cell division.

Proliferating cells were identified by incorporation of BrdU into newly synthesized DNA of replicating cells. At 1 d after imbibition, many BrdU-labelled nuclei were observed in the WT coleoptiles, but only a few were observed in the *rad* coleoptiles (Fig. 7). These results suggest that cell division occurs in the WT coleoptile at the early stages (i.e. 1–2 d) of seedling growth under submergence and does not actively occur in the *rad* coleoptile.

The expressions of three cell elongation-related genes identified by the LM-microarray analysis (the expansins *OsEXPA1*, *OsEXPB11* and *OsEXPB17*) were measured by semi-quantitative RT-PCR. Their mRNA levels dramatically increased in the WT coleoptile during germination and early growth, peaking at 2–4 d after imbibition, but were much lower in the *rad* coleoptile (Fig. 5). However, no significant differences were observed between the WT and *rad* coleoptiles in the transcript levels of other expansins (*OsEXPA2*, *OsEXPA4*, *OsEXPA7* and *OsEXPB12*) (data not shown), which were previously suggested to be involved in coleoptile elongation (Huang *et al.*, 2000; Choi *et al.*, 2003; Lasanthi-Kudahettige *et al.*, 2007). This suggests that the expressions of these genes are not affected by the *ADH1* deficiency in the *rad* mutant at 1 d after imbibition.

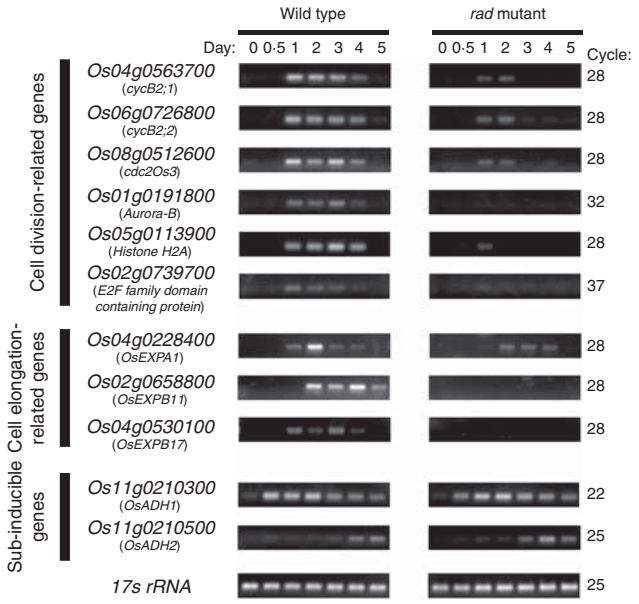

FIG. 5. Changes in the expressions of cell division- and cell elongation-related genes. Coleoptiles of seedlings grown under complete submergence for the indicated number of days after imbibition were isolated by LM. Semi-quantitative RT-PCR analysis of the selected genes was performed with appropriate primers (Supplementary Data Table S3, available online). Six cell division-related genes and three cell elongation-related genes were selected from the microarray result. Submergence-inducible *OsADH1* and *OsADH2* genes and *17s rRNA* were used as controls.

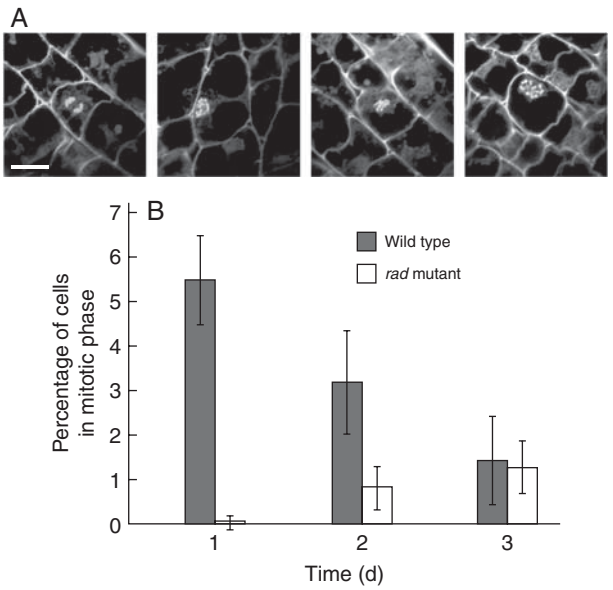

FIG. 6. Frequency of occurrence of mitotic-phase cells. Sections of rice embryos at 1–3 d after imbibition were embedded in paraffin. (A) Nuclei of cells of the WT coleoptiles in mitotic phase. Nuclei were stained with DAPI. Scale bar = 20 μm. (B) Percentage of cells in the mitotic phase. Data are means of three replicates ± s.d.

TABLE 2. *List of genes related to cell elongation, whose expression was up-regulated or down-regulated in the* rad *coleoptiles*

Fold change (*rad*/WT)*	Locus ID†	Full-length cDNA accession no.†	Description†	Functional classification
10·05	Os02g0275200	AK071664	Xyloglucan fucosyltransferase family protein (FUT)	Cell wall biogenesis
7·38	Os05g0542800	AK106049	Pectin lyase fold/virulence factor domain-containing protein	Cell wall loosening
6·45	Os05g0542800	AK105858	Similar to polygalacturonase	Cell wall loosening
6·05	Os04g0604300	AK070734	Similar to xyloglucan endotransglucosylase/hydrolase protein 24 precursor (EC 2·4·1·207) (At-XTH24) (XTH-24) (meristem protein 5) (MERI-5 protein) (MERI5 protein) (endo-xyloglucan transferase) (xyloglucan endo-1,4-beta-D-glucanas e) (OsXTH6)	Cell wall loosening
5·73	Os10g0450900	AK064310	Similar to glycine-rich cell wall structural protein 2 precursor	Cell wall component
4·82	Os10g0521100	AK064063	Similar to actin-depolymerizing factor 6 (ADF-6) (AtADF6)	Actin binding
4·59	Os10g0450900	AK064065	Similar to glycine-rich cell wall structural protein 2 precursor	Cell wall component
4·09	Os03g0133400	AK073032	Peptidoglycan-binding LysM domain-containing protein	Cell wall catabolic process
4·08	Os06g0621900	AK101940	Similar to alpha-expansin OsEXPA 16 (fragment)	Cell wall loosening
2·71	Os10g0407000	AK101494	Pectin lyase fold/virulence factor domain-containing protein	Cell wall loosening
0·14	Os06g0199000	AK106381	Similar to glycine-rich cell wall structural protein 2 precursor	Cell wall component
0·24	Os10g0498900	AK108732	Similar to microtubule-associated protein EB1-like protein	Actin binding
0·32	Os02g0658800	AK059638	Beta-expansin (OsEXPB11)	Cell wall loosening
0·34	Os04g0228400	AK069548	Expansin precursor (alpha-expansin OsEXPA1)	Cell wall loosening
0·35	Os04g0530100	AK107184	Similar to beta-expansin 1 precursor (AtEXPB1) (At-EXPB1) (Ath-ExpBeta-1·5) (OsEXPB17)	Cell wall loosening
0·39	Os07g0551700	AK067424	Similar to cellulose synthase (fragment)	Cell wall synthesis
0·39	Os04g0602500	AK058634	Similar to pectin acetylesterase	Cell wall loosening

* Fold change shows average expression ratio of three biological replicates and a colour swap for each replicate.
† Locus ID, full-length cDNA accession number and description in Rice Annotation Project Database (RAP-DB; http://rapdb.dna.affrc.go.jp/).

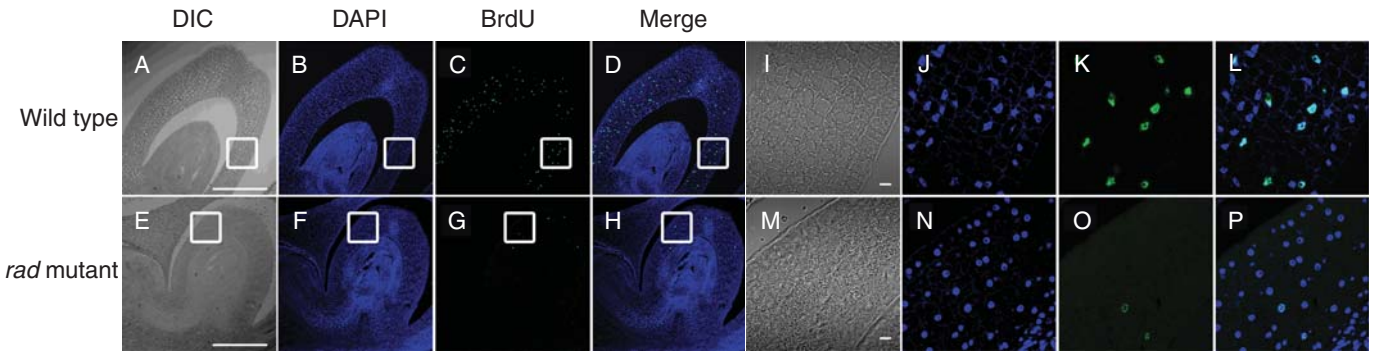

FIG. 7. Immunostaining of BrdU in rice coleoptiles. All photographs were obtained with a confocal laser scanning microscopy: (A–H) low magnification: (I–P) high magnification of square areas in A–H; (A, E, I, M) differential interference contrast images; (B, F, J, N) nuclei stained with DAPI; (C, G, K, O) fluorescent immunostaining of BrdU-incorporated nuclei; (D, H, L, P) merged images of the DAPI and BrdU signals. Scale bars = 300 μm. (A and E), 10 μm (I and M).

Next, longitudinal cell lengths were measured in the WT and *rad* coleoptiles during germination and early growth under submergence. The longitudinal cell lengths at 0 d and 1 d after imbibition were comparable between the WT and the *rad* mutant (Fig. 8). Cell elongation started at between 1 d and 2 d and was enhanced at 3 d in the WT coleoptile, but not in the *rad* coleoptile.

DISCUSSION

It was found that the *rad* coleoptiles, unlike the WT coleoptiles, slowly elongated under submergence (Fig. 1B), whereas the *rad* and WT coleoptiles were equally elongated under aerobic conditions (Fig. 1C). The slow elongation of the *rad* coleoptile may be due to the reduced ATP level in the coleoptile under submerged conditions (Fig. 1D). The finding that *rad* coleoptiles grown under aerobic conditions for 2 d elongated at a normal rate following submergence treatment (Fig. 2) may be due to aerobic ATP production, which compensates for the reduction of ATP levels in the *rad* coleoptile and allows the *rad* coleoptile active cell division and cell elongation under submergence.

The LM-microarray analysis identified 294 genes whose mRNA levels were higher in *rad* than WT (Supplementary Data Table S1) and 137 genes whose mRNA levels were lower in *rad* than WT (Supplementary Data Table S2) under submerged conditions. Whereas we compared the expressions of genes in the WT and *rad* mutant after submergence, Narsai *et al.* (2009) compared the expressions of rice genes in the WT under aerobic and anaerobic conditions. Most of the genes that were expressed more strongly in the *rad* coleoptiles than in the WT coleoptiles during submergence were found by Narsai

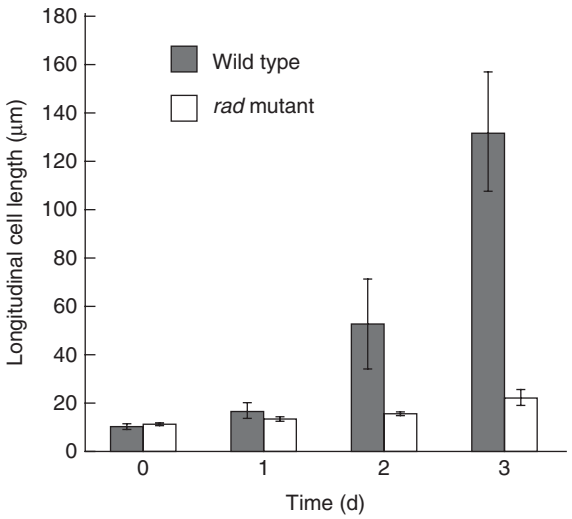

FIG. 8. Changes in longitudinal length of coleoptile epidermal cells. Measurements were made with paraffin-embedded sections of rice embryos under a microscope. Lengths of 50–80 epidermal cells were measured for one replicate. Data are means of three replicates ± s.d.

et al. (2009) to be up-regulated in the WT under anaerobic conditions (Supplementary Data Fig. S1). On the other hand, among genes that were expressed more strongly in the WT coleoptiles than in the *rad* coleoptiles during submergence, most were down-regulated in the WT under anaerobic conditions (Supplementary Data Fig. S1). Expressions of these genes may rather be regulated by ATP levels because more ATP can be produced under aerobic conditions than under anaerobic conditions (Bailey-Serres and Voesenek, 2008) and ATP levels in the coleoptile were lower in the *rad* mutant than in WT under submerged conditions (Fig. 1D). It is noteworthy that most of cell division-related genes that are less expressed in the *rad* mutant (Table 1) were down-regulated in the WT under anaerobic conditions (Supplementary Data Fig. S1 and Table S2). This result suggests that the expressions of these cell division-related genes are regulated by ATP levels.

In rice, coleoptile elongation during submergence is thought to mainly occur by cell elongation (Wada, 1961; Opik, 1973; Magneschi and Perata, 2009), although cell division may contribute to coleoptile elongation at an early stage in germination (Wada, 1961; Opik, 1973; Atwell *et al.*, 1982). In this study, the occurrence of cell division in WT coleoptiles under submergence was confirmed by the expressions of cell division-related genes (Fig. 5), a relatively high mitotic index (Fig. 6) and BrdU incorporation (Fig. 7). Also a shift from cell division to cell elongation was found in the coleoptile. This shift may be important for anaerobic coleoptile growth and survival, because it is likely that cell elongation requires less energy (i.e. ATP) than does cell division (Atwell *et al.*, 1982; Magneschi and Perata, 2009).

In the natural environment, coleoptile elongation during flooding is needed to obtain oxygen from the air. Flooding (e.g. 5–10 cm water depth) would thus be expected to select for rice cultivars that grow longer coleoptiles under submergence. The final length of a coleoptile is determined by both the number of cells and the length of each cell. Thus, some flood-tolerant rice cultivars may increase both cell length and the number of cells in the coleoptile under submergence. However, this strategy may be costly and thus the flooding water depth may determine whether the strategy is useful. In submerged rice, coleoptile lengths vary widely among different cultivars (Setter and Ella, 1994; Magneschi *et al.*, 2009). It would be interesting to investigate the cell numbers and cell lengths of coleoptiles of these cultivars and to examine how cell division contributes to the difference in coleoptile lengths.

In conclusion, the present results indicate that cell division starts within 1 d after imbibition and then gradually ceases, and subsequently cell elongation starts between 1 d and 2 d after imbibition and continues at the later stages of the coleoptile growth under submergence. Deficiency of the *ADH1* gene in the *rad* mutant, which results in reduction of ATP synthesis in the submerged coleoptile (Fig. 1D; Saika *et al.*, 2006), represses both cell division and cell elongation, thereby impairing efficient coleoptile elongation under submergence. Some transcription-factor genes identified by the LM-microarray analysis may also have a role in coleoptile elongation and are currently being examined.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following files. Table S1: up-regulated genes in the *rad* mutant. Table S2: down-regulated genes in the *rad* mutant. Table S3: sequences for primers used in the study. Figure S1: relationship between up-/down-regulated genes in the *rad* mutant and aerobic-/anaerobic-expressed genes.

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