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Molecular cloning of a root hairless gene *rth1* in rice

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Root hairs, projections from the epidermal cells of the roots, are contributing to water and nutrient uptake and anchorage to the soil. To better understand genetic control of root hair formation in rice, we analyzed *root hairless 1* (*rth1*) mutant that was induced by NaN_3 treatment. SEM observation showed that in *rth1* plants, root hair elongation was abolished after the formation of bulge. High-resolution mapping using 2,088 segregants revealed three predicted genes in a 38-kb candidate interval on chromosome 7. Sequences comparison of the three genes between wild-type Oochikara and *rth1* detected a nucleotide substitution only in apyrase (*OsAPY*). This nucleotide substitution (G → A) lies in the junction between the third intron and the forth exon, and results in the splicing anomaly to the *rth1* cDNA sequence. Transgenic plants with introduced *OsAPY* allele restored normal root hairs and plant growth, showing a complementation of *rth1* phenotype. We concluded that the root hairless phenotype of *rth1* is caused by a mutation of *OsAPY*. *OsAPY* appears to be an important gene for root hair elongation and plant growth in rice.

Key Words: root hair, bulge, rice, positional cloning, apyrase.

Introduction

The root epidermal cells are composed of hair-forming cells (trichoblasts) and non-hair-forming cells (atrichoblasts), and root hairs are formed as specialized projections from modified trichoblasts in the elongation zone of a root. Root hairs are estimated to constitute about 80% of the surface area in crop roots, and they are considered to play important roles in water and nutrient uptake, anchorage to the soil and microbe interactions (Hofer 1991). Thus, understanding of root hair formation and its genetic control could greatly contribute to improvement of crop productivity and stability.

Root hair development occurs in four phases; cell fate specification, initiation, subsequent tip growth, and maturation (Gilroy and Jones 2000). The mode of root hair development may be categorized into three types on the basis of root epidermal cell development. In Type 1, all root epidermal cells appear capable of producing a root hair (Leavitt 1904), and most plant species are included in this category. In Type 2, root hairs form from the smaller cells produced by an asymmetric cell division in the meristem (Cormack 1937). Kawata and Ishihara (1959) reported that rice (*Oryza sativa* L.), the monocot model plant, forms root hairs from short epidermal cells produced by an asymmetrical cell division in root tips. In Type 3, root epidermal cells arranged in files composed of only one cell type, either trichoblasts or atrichoblasts (Cormack 1935). The dicot model plant *Arabidopsis thaliana* belongs to Type 3. In *Arabidopsis*, epidermal cells that are located over the intercellular space between underlying cortical cells develop into trichoblasts, whereas epidermal cells that are located over a single cortical cell develop into atrichoblasts (Schiefelbein 2003). Thus, the pattern of epidermal cell development in *Arabidopsis* is position-dependant (Dolan *et al.* 1994, Galway *et al.* 1994).

The structure of the *Arabidopsis* root hair is simple and invariant, and provides a useful system for studying root hair development. Moreover, in *Arabidopsis*, a lot of root hair mutants are available, and many genes involved in the root hair morphogenesis have been isolated (Grierson and Schiefelbein 2002). On the other hand, rice has a more complicated pattern of root hair formation (Kawata and Ishihara 1959). Only two root hair mutants in rice have been reported (Suzuki *et al.* 2003, Kim *et al.* 2007), and, causal gene was isolated in only one mutant (Kim *et al.* 2007). A mutant gene, *root hairless 1* (*rth1*) reported by Suzuki *et al.* (2003), remains unidentified. Thus, genetic studies on root hairs in rice lag behind compared to *Arabidopsis*.

In the present study, we have identified a new root hair mutant gene, *rth1* in rice by means of positional cloning. We describe morphological and physiological characteristics, and gene expression pattern in *rth1* mutant. We revealed that *RTH1* encodes an enzyme apyrase that can hydrolyze NTPs and/or diphosphates (Shibata *et al.* 1999) and that apyrase is a key gene for root hair elongation and plant growth in rice.

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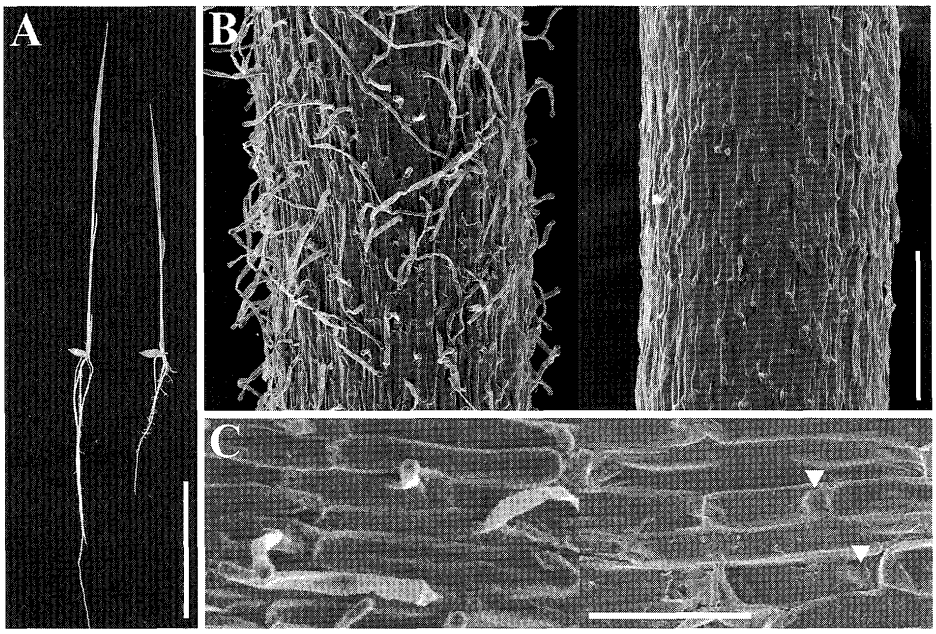

Fig. 1. Comparison of the morphology of wild type Oochikara and mutant *rth1*. (A) 14-day-old seedling of Oochikara (left) and *rth1* (right); Scale bars indicate 5 cm. (B) SEM images of root hair zones of about 5 mm from the root tip of seminal roots in Oochikara (left) and *rth1* (right) from 3-day-old seedlings. Scale bars indicate 1 mm. (C) Close-ups of the root hair zone in Oochikara (left) and *rth1* (right). Arrowheads indicate bulge. Scale bars indicate 100 μm.

Materials and Methods

Plant growth conditions

rth1 (Fig. 1A right) is an induced mutant from a japonica cv. Oochikara (Fig. 1A left), and its root hairless phenotype is controlled by a single recessive gene; *rth1* seedlings show reduced lengths of root (50% of wild-type Oochikara) and shoot (80% of wild type), as reported in Suzuki *et al.* (2003). Germinated seeds were sown on a net float in a plastic container with Kimura B nutrient solution [0.18 mM $(NH_4)_2SO_4$, 0.27 mM $MgSO_4 \cdot 7H_2O$, 0.09 mM KNO_3, 0.09 mM KH_2PO_4, 0.05 mM K_2SO_4, 0.18 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.04 mM NaEDTA-Fe·$3H_2O$ and 0.08 mM Na_2SiO_3, pH 5.0]. Until 25th day, plants were grown in a growth chamber with a constant temperature at 25°C and continuous illumination. Some plants were transferred to 1/5000 a Wagner pots containing the nutrient solution and grown to maturity in a greenhouse.

Scanning Electron Microscopy (SEM)

Plants were grown for 3 days in a solution under the conditions described above. Then, we sampled approximately 1-cm tip of seminal roots. The root tips were fixed in solution (2% glutaraldehyde and 2% paraformaldehyde) for 1 h and dehydrated in a graded ethanol series. Dehydrated samples were critically point dried with liquid carbon dioxide. The dried root samples were glued onto the metallic stubs coated with silver, conductive carbon tape. Mounted samples were coated with platinum-palladium in a vacuum evaporator. We investigated the number and length of root epidermal cells within an area of 0.5 mm (width) × 2.0 mm (length) that was 5-mm distant from the root tip. The experiment was carried out with 5 replications in each genotype. T-test was used for statistical analysis.

Mapping of rth1

Mutant *rth1* were crossed with an *indica* var. Kasalath, to generate an F_2 mapping population of 2,088 individuals. For mapping of *rth1*, we compared genomic sequences between *japonica* cv. Nipponbare (http://rgp.dna.affrc.go.jp) and *indica* var. 93-11 (http://rise.genomics.org.cn), and searched for indels to develop polymorphic markers. Primer sequences of flanking markers were RK30 (forward 5′-CCA GCCTCTAGCTCCATCAC-3′ and reverse 5′-TCGTCTCG CAATTTTCATGT-3′) and RK20 (forward 5′-CATGCATG GTTCATCTTTCG-3′ and reverse 5′-GGACGTACGACA AAACAATTAACA-3′). Candidate genes for *rth1* were searched from the region delimited by mapping. We used the web sites of The Institute for Genomic Research (http://www.tigr.org/tdb/e2k1/osa1/), Rice Genome Automated Annotation system (http://ricegaas.dna.affrc.go.jp/), Rice annotation project database (http://rapdb.dna.affrc.go.jp/) and plant promoter db (http://ppdb.gene.nagoya-u.ac.jp/cgi-bin/index.cgi).

Complementation test

For complementation test, we used a 5970-bp genomic DNA fragment of Nipponbare containing the entire *OsAPY* coding region, the 1515-bp upstream sequence, and the 978-bp downstream sequence. This fragment was PCR amplified using primers (forward 5′-CACCGGGCAGCAACAAACT GATTT-3′ and reverse 5′-CAGCTCCTGGAGAGAGGAGA-3′) and cloned into pENTR/D-TOPO vector (Invitrogen). The CACC nucleotide sequence was added to upstream

of the forward primer to allow the directional cloning of Nipponbare *OsAPY* allele into the pENTR/D-TOPO vector. Cloned fragment was recombined to pEASY-genomic SK (NIAS; National Institute of Agrobiological Sciences) by LR Clonase Enzyme (Invitrogen). Then, the construct was transformed into *Agrobacterium tumefaciens* strain EHA101. Because *rth1* is in the genetic background of Oochikara, which is a recalcitrant variety for transformation, we backcrossed *rth1* twice with Nipponbare. In the BC_2F_2 generation, recessive homozygous lines for *rth1* were selected, and the next generation was used for *Agrobacterium*-mediated transformation according to a method described in Hiei *et al.* (1994). We generated transgenic lines transformed with *OsAPY* allele or control empty vector, and observed their root hair morphology in the T_0 and T_1. Transgenic plants were selected by PCR using primers specific to the streptomycin-resistance gene (forward 5′-AGCCGAAG TTTCCAAAAGGT-3′ and reverse 5′-TAACGATGACAG AGCGTTGC-3′).

Phylogenetic analyses

For phylogenetic analysis, apyrase amino acid sequences of rice and other plants were obtained from DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/index-j.html). Multiple sequence alignments were conducted using CLUSTAL W (http://clustalw.ddbj.nig.ac.jp/top-j.html). A neighbor-joining tree was constructed using MEGA version 3.1. Statistical support for the tree obtained was assessed using the bootstrap method. The number of bootstrap replicates was 1000.

RNA extraction and quantitative RT-PCR (qRT-PCR) analysis

Total RNAs were extracted from various tissues of plants sampled at three stages (7-day-old roots, 25-day-old seedlings, and adult plants 1 week after anthesis) using an Isogen kit (Nippon Gene). To compare the *OsAPY* expression in different parts of seminal root, the 7-day-old seminal roots were dissected into three parts, namely, apical meristem zone without root hair (0–5 mm from the tip), root hair elongating zone (6–10 mm from the tip), and mature root hair zone (11–15 mm from the tip). This dissection length was determined on the basis of optical microscopic observation of root hairs on seminal roots of wild type (WT) grown in distilled water. RNA samples were treated with DNase I (Promega). For qRT-PCR, the first strand cDNA was synthesized with High-capacity cDNA reverse transcription kits (Applied Biosystems). Quantitative analyses were carried out on Thermal cycler dice TP800 (TaKaRa) using a SYBR green detection kit (TaKaRa) according to the manufacturer's instructions. Primers used for expression analyses of genes were as follows; for *OsAPY*, forward 5′-ATCTGC ATGGATCTCGTTTACC-3′ and reverse 5′-CGTTGCTGT AAGGGACTTTCTT-3′; for *OsAPYL*, forward 5′- GGTGC ATTCCAGTGGGTTAC-3′ and reverse 5′-AGCCATTTGG ACAGAACCAC-3′. We used glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) as an internal control according to Kim *et al.* (2003). Relative expression levels of the *OsAPY* and *OsAPYL* in each cDNA sample were obtained by normalization to the *GAPDH* gene. Average data from three to five independent experiments are presented.

Extraction and measurement of ATP content

ATP extraction and measurement were performed according to the method of Saika *et al.* (2006). The samples measured were kernel, and shoot and root of 7- and 14-day-old seedlings. The experiment was carried out with 5 replications. T-test was used for statistical analysis.

Results

Root epidermal cell morphology

In a previous study (Suzuki *et al.* 2003), epidermal cells of root hair zone in WT and *rth1* were observed with an optical microscope. In this study, we observed root hair morphology in detail with SEM (Fig. 1B). At a low magnification, *rth1* mutant seemed complete root hairless (Fig. 1B right), however at a higher magnification, bulges were observed in some epidermal cells of *rth1* (Fig. 1C right). A bulge is an initiation site of root hair in epidermal cells (Fig. 1C). To test the relationship between cell size and bulge formation, lengths of epidermal cells in the root hair zone were measured. The means (±SD) of all epidermal cell lengths in the root hair zone were 92.6±5.0 μm for WT and 97.0±3.4 μm for *rth1*, without significant difference. The frequency distribution of epidermal cell lengths in WT was similar to that in *rth1* (Fig. 2). Epidermal cells were classified into trichoblast and atrichoblast in each genotype. The average lengths of trichoblasts and atrichoblasts in WT were 67.4±11.0 μm and 109.2±15.3 μm, respectively, and those in *rth1* were 62.4±10.8 μm and 104.9±15.6 μm, respectively. Thus, in both WT and *rth1*, the lengths of trichoblasts were about 60% of those of atrichoblasts, showing that in rice, root hairs tend to originate from smaller cells, as previously reported by Kawata and Ishihara (1959). The frequencies of trichoblasts were similar between WT (39.4±5.3%) and *rth1* (38.6±8.2%), indicating that *rth1* did not affect the density of trichoblasts. Thus, in *rth1* mutant, root hair formation appears to proceed normally until the formation of bulges, but subsequent elongation of bulges is inhibited.

Positional cloning of rth1

F_1 progeny derived from a cross between *rth1* and Kasalath showed normal root hairs and plant growth. F_2 progeny segregated 1561 plants with normal phenotype and 527 root-hairless plants with short root phenotype, fitting to a 3 : 1 ratio ($\chi^2=0.80$, $0.3<p<0.4$). Therefore, *rth1* is considered a recessive mutation. In a population of 116 F_2 plants, the *rth1* gene mapped roughly to the distal region of the long arm of chromosome 7. For fine mapping, a total of 2,088 F_2 plants were studied. The *rth1* gene was flanked by STS markers, RK30 and RK20, with the distances of 0.26 cM and 0.05 cM, respectively (Fig. 3A). The physical distance

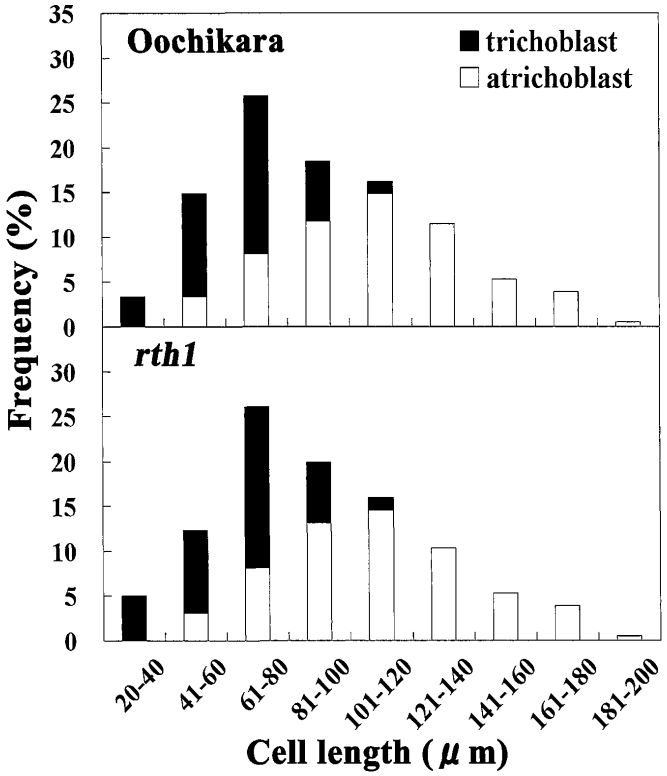

Fig. 2. Frequency distribution of lengths of root epidermal cells in the root hair zone on the seminal root of Oochikara and *rth1*. Root tips of 3-day-old seedlings were used for the measurement by SEM according to the methods described in materials and methods. Cells were classified as either trichoblast (marked in black) or atrichoblast (marked in white). In *rth1*, bulge-forming cells were classified as trichoblasts. Both genotypes included about 120 cells within an area used for cell length measurement (1 mm^2), and the data represent averages of five independent areas.

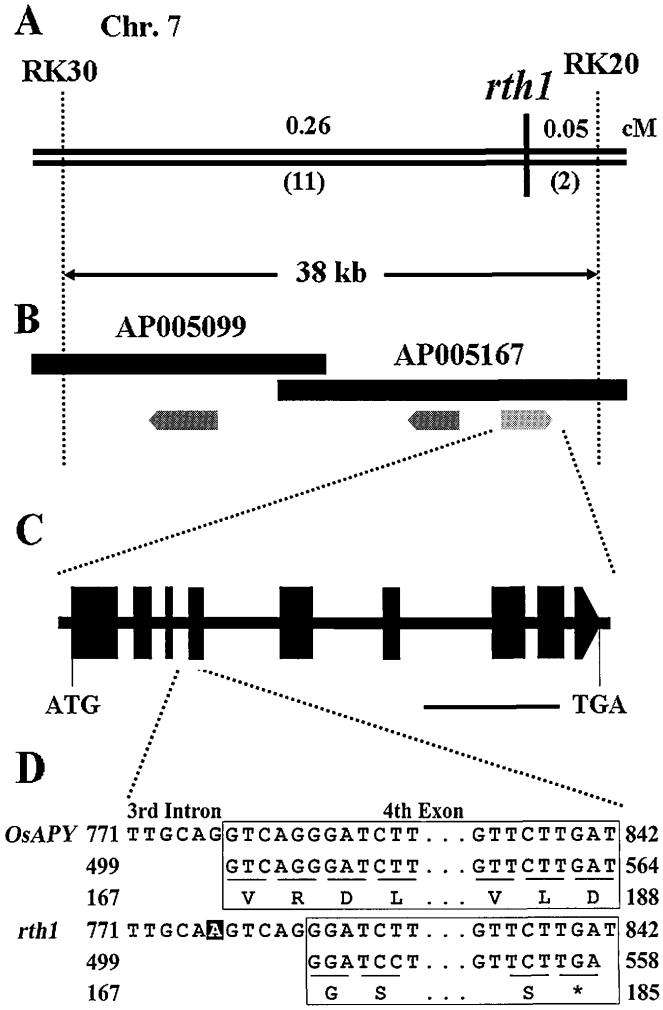

Fig. 3. Map based cloning of *RTH1* gene. (A) Fine mapping of the *rth1* locus. The numerals and numbers in parentheses indicate genetic distance and the number of recombinants identified from 2,088 F2 plants, respectively. (B) Predicted genes in the candidate region harboring the *rth1* locus. (C) *RTH1* gene structure. An arrow shows the mutated site in *rth1*. The start codon (ATG) and the stop codon (TGA) are indicated. Closed boxes indicate the coding sequence and lines between boxes indicate intron. Scale bar indicates 500 bp. (D) Alignment of the *OsAPY* genomic DNA (upper lane), cDNA (middle lane) and predicted amino acid sequences (bottom lane) that differed between wild-type Oochikara and *rth1*. Mutation site in *rth1* is highlighted by dark box. Forth exon is boxed. In the investigated region, Oochikara had a genomic DNA sequence identical to that of Nipponbare.

between them is about 38 kb. In the *rth1* candidate region, three genes are predicted in public databases. Those include nucleic acid binding protein (AK109592), expressed protein (AK065098) and putative apyrase protein (AK066262). The entire coding region of all these genes was sequenced. Comparison of the DNA sequences between WT and *rth1* detected a single nucleotide change only in the putative apyrase (*OsAPY*; ATP-diphosphohydrolase EC.3.6.1.5) gene, and the sequences of two other genes are identical between WT and *rth1*. Compared to the *OsAPY* gene of WT, *rth1* carries a nucleotide substitution (G→A) in the junction between the third intron and the forth exon (Fig. 3C, D). This substitution caused the splicing anomaly to the *rth1* cDNA sequence that shifted the splicing site 5-bp downstream and produced a premature stop codon, resulting in 185-amino acid peptide instead of normal 467-amino acid protein. We confirmed alternation of splicing in *rth1* by cDNA sequencing.

The identity of *rth1* was confirmed by complementation test. The BC_2F_3 *rth1* homozygous lines used for complementation test exhibited short-root and semi-dwarf phenotype in addition to root hairless trait (data not shown). Eight independent transgenic lines were obtained and all were normal in root hairs and plant growth (Fig. 4), showing a complementation of *rth1* phenotype by the introduced *OsAPY* gene. Transformation with empty vector did not complement the root hairless, short root, and semi-dwarf phenotype of *rth1* (Fig. 4A right, D). Therefore, we conclude that the mutation of *OsAPY* is responsible for the *rth1* phenotype.

Structure and expression analyses of OsAPY

Comparison between genomic DNA (3477 bp) and cDNA (1404 bp) showed that *OsAPY* is composed of 9 exons that encode a 467-amino acid protein (Fig. 3C). Basic Local Alignment Search Tool (BLAST) analysis revealed that the rice OsAPY protein shares the highest identity (about 74% of the entire length) with rice apyrase-like protein

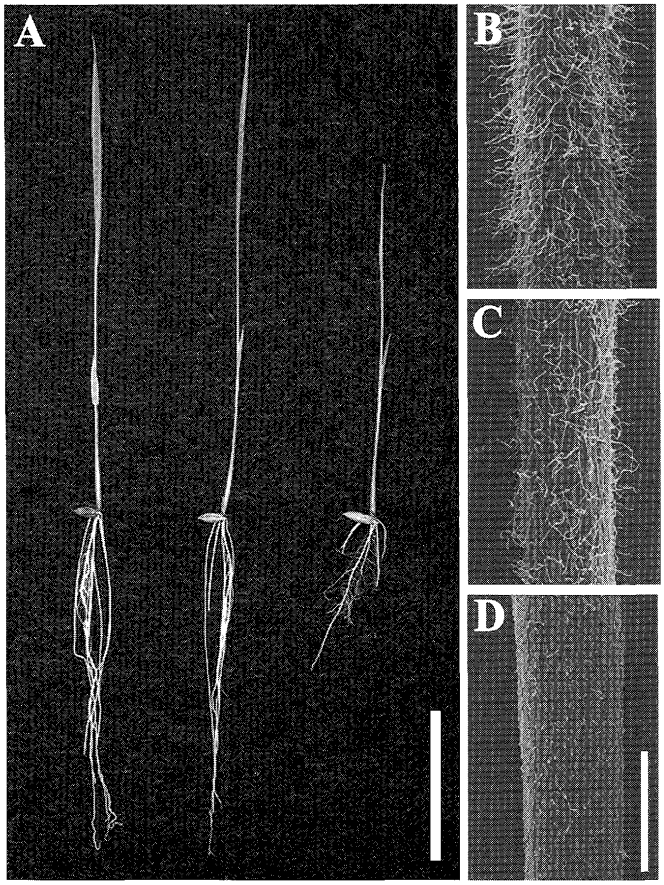

Fig. 4. Complementation test of the *rth1* mutant lines (BC_2F_3) with the Nipponbare *OsAPY* allele. (A) 12-day-old seedlings of Nipponbare (left), a transgenic plant containing the Nipponbare *OsAPY* gene (middle) and a control transgenic plant containing the vector DNA (right). Transgenic plants are all T_1 generation. Scale bars indicate 5 cm. (B to D) SEM images of seminal roots of 3-day-old seedlings corresponding to the three genotypes shown in the order of (A). Scale bars indicate 500 μm.

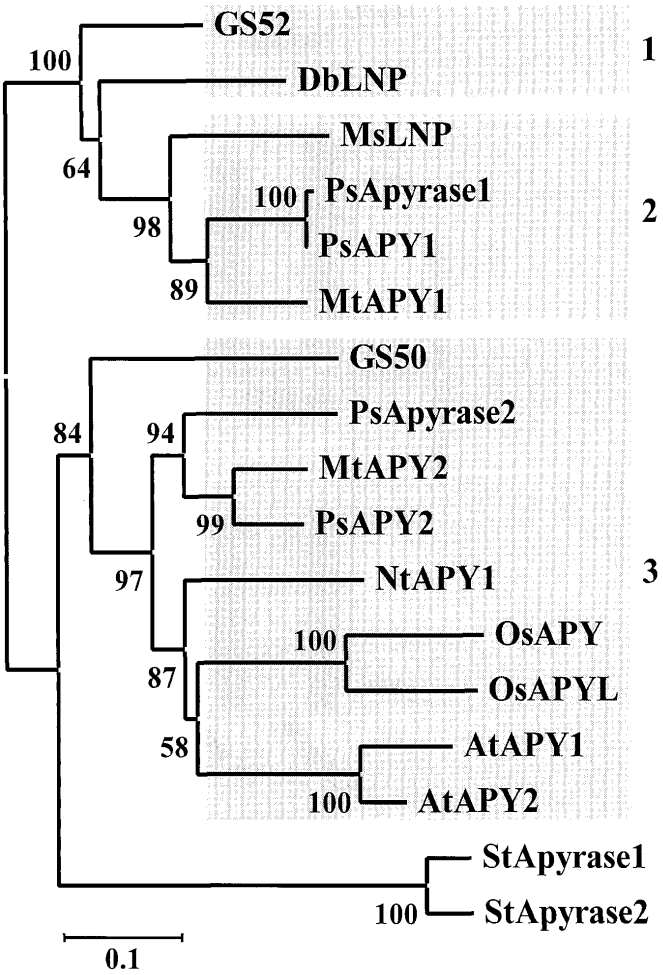

Fig. 5. Phylogenetic analysis of amino acid sequences of plant apyrases. The tree was constructed by the neighbor-joining method using MEGA version 3.1. Three major subfamilies are labeled with shaded boxes. Sequences (DDBJ accession no.): *Glycine soja* apyrase (GS52: AF207688), *Dolichos biflorus* nod factor binding lectin-nucleotide phosphohydrolase (DbLNP: AF139807), *Medicago sativa* nod factor binding lectin-nucleotide phosphohydrolase (MsLNP: AF156782), *Medicago truncatula* putative apyrase (MtAPY1: AF288132), *Pisum sativum* apyrase 1 (PsAPY1: AB071369), *Pisum sativum* ATP diphosphohydrolase 1 (PsApyrase1: AB098123), *Glycine soja* apyrase (GS50: AF207687), *Pisum sativum* ATP diphosphohydrolase 2 (PsApyrase2: AF305783), *Pisum sativum* apyrase 2 (PsAPY2: AB071370), *Medicago truncatula* apyrase-like (MtAPY2: AY180382), NtAPY1, AtAPY1, AtAPY2, OsAPY and OsAPYL.

(OsAPYL: AF358764). *Nicotiana tabacum* carries apyrase-like protein (NtAPY1: EF051589). *Arabidopsis thaliana* carries APYRASE 1 (AtAPY1: AF093604) and APYRASE 2 (AtAPY2: AF156783). Apyrase proteins have "apyrase-conserved region (ACR)" motifs (Handa and Guidotti 1996). Phylogenetic analysis showed that plant apyrases are divided into three major groups (Fig. 5). Apyrase proteins of rice (OsAPY and OsAPYL) and *Arabidopsis* (AtAPY1 and AtAPY2) were included in the same group together with those of tobacco and leguminous species.

Expression of two rice apyrase genes (*OsAPY* and *OsAPYL*) was compared between WT and *rth1* by qRT-PCR analysis (Fig. 6). Compared to WT, *rth1* had significantly reduced *OsAPY* expression in all tissues and stages studied, whereas, *OsAPYL* expression was not altered in *rth1* mutant. Furthermore, in 7 day-old-roots, *OsAPY* expression was stronger in apical meristem zone compared to the other parts.

Comparison of ATP content

Apyrases are enzymes that can hydrolyze NTPs and/or diphosphates (Shibata *et al.* 1999). To confirm whether *OsAPY* functioned as apyrase, ATP content in the shoot and root of 7- and 14-day-old seedling was investigated (Fig. 7). In 7-day-old seedlings, the ATP content in the shoot was 15.2 ng/mg for WT and 16.6 ng/mg for *rth1*. However, the ATP content in the root was 12.1 ng/mg for WT and 22.8 ng/mg for *rth1*. Similarly, in 14-day-old seedlings, the ATP content in the *rth1* root was almost two folds of that in WT, however, there was no significant difference in shoot ATP content between WT and *rth1*.

To know the cause of the ATP content difference in the root between WT and *rth1*, we measured ATP content in the non-germinated kernel. The ATP content of WT and *rth1*

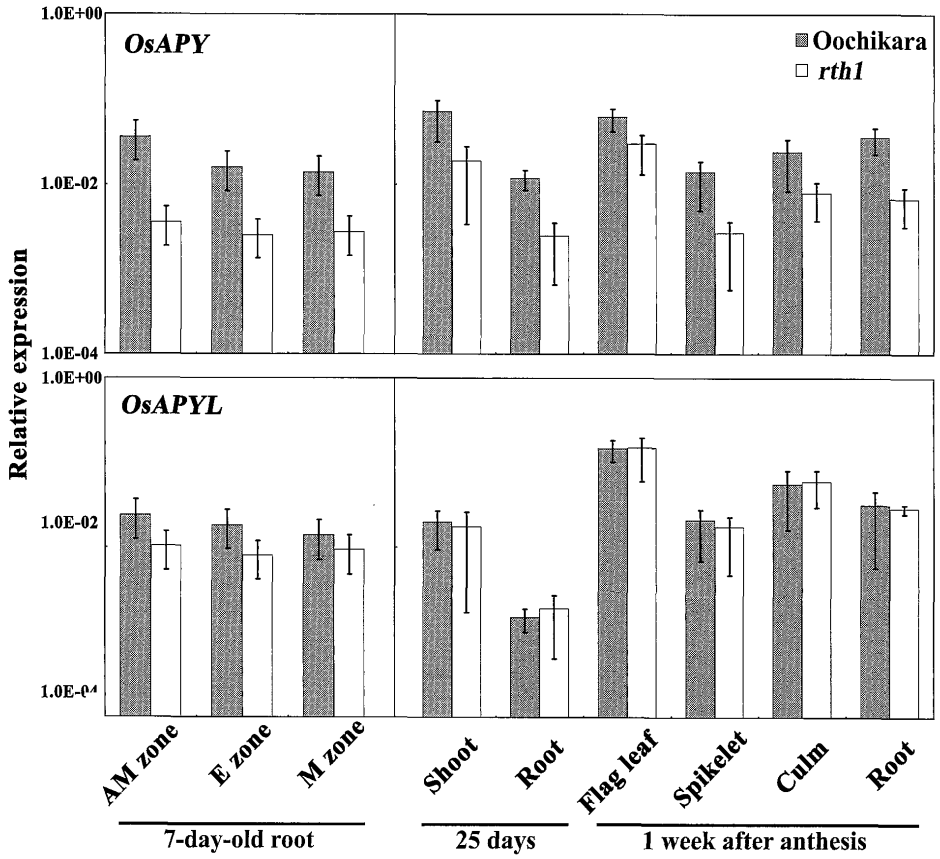

Fig. 6. Relative expression of rice apyrase genes in Oochikara and *rth1*. Apical meristem zone without root hair (AM zone, 0–5 mm from the tip), root hair elongating zone (E zone, 6-10 mm) and mature root hair zone (M zone, 11–15 mm) of seminal root of 7-day-old seedlings, shoot and root of 25-day-old seedlings, and flag leaf, spikelet, culm and root of adult plants 1 week after anthesis were studied. Transcript levels of each gene were normalized by use of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference. Error bars indicate standard deviation. The numbers of replications for 7-day-old roots and other stages were 3 and 5, respectively.

was 32.0±6.0 ng/kernel and 31.5±1.3 ng/kernel, respectively, and there were no significant differences in initial ATP content of kernel between WT and *rth1*. Thus, the higher ATP content in the root of *rth1* seedlings is likely to be ascribed to reduced apyrase activity.

Discussion

By means of positional cloning and complementation test, we demonstrated that the root hairless phenotype of *rth1* is caused by a mutation of *OsAPY* on chromosome 7. Also, our SEM observation showed that, in *rth1*, tip growth is abolished after the formation of bulges. Because *rth1* homozygous plants segregated in crosses with other genotypes always showed short root and semi-dwarf phenotype (see Fig. 4A right), such dwarfism appears to be pleiotropic effects of the *rth1* gene. In rice, another root hair mutant gene was isolated: mutation of cellulose synthase-like D1 gene (*OsCSLD1*) on chromosome 10, showed short root hair phenotype (Kim *et al.* 2007). Role of *OsCSLD1* in root hair elongation was identified based on homology to an *Arabidopsis* cellulose synthase-like D3 gene *KOJAK/AtCSLD3*, whose mutations result in short root hairs that rupture at the tip immediately after initiation (Wang *et al.* 2001, Favery *et al.* 2001). *OsCSLD1* and *KOJAK/AtCSLD3* show root-specific expression, therefore, *OsCSLD1* may be the functional ortholog of *KOJAK/AtCSLD3*, although no rupture in root hair tips was reported for *OsCSLD1* (Kim *et al.* 2007).

We performed phylogenetic analysis of plant apyrases incorporating those of rice (Fig. 6). The results were basically similar to those reported in Kawahara *et al.* (2003). Two apyrase proteins are present in both rice and *Arabidopsis*. OsAPY and OsAPYL, and AtAPY1 and AtAPY2, were all included in the same group, and each species formed different subgroups. We studied expression of *OsAPY* and *OsAPYL* by qRT-PCR. Both genes were expressed in any tissues or stages examined, but expression of *OsAPY* was significantly reduced in *rth1* compared to WT in any tissues or stages studied. Moreover, in seminal root, *OsAPY* is strongly expressed in apical meristem zone. Therefore, it appears that *OsAPY* plays important roles in cell division and/or elongation of seminal root. In *Arabidopsis*, promoter:GUS analysis showed that *AtAPY1* and *AtAPY2* were expressed in a variety of tissues; in roots, *AtAPY1* was expressed in apical meristem zone, but *AtAPY2* was expressed in both apical meristem zone and elongation zone (Wu *et al.* 2007). In *Arabidopsis*, single knockout lines of apyrase genes lacked a discernible phenotype, but double knockout plants showed severe short root and dwarf phenotype

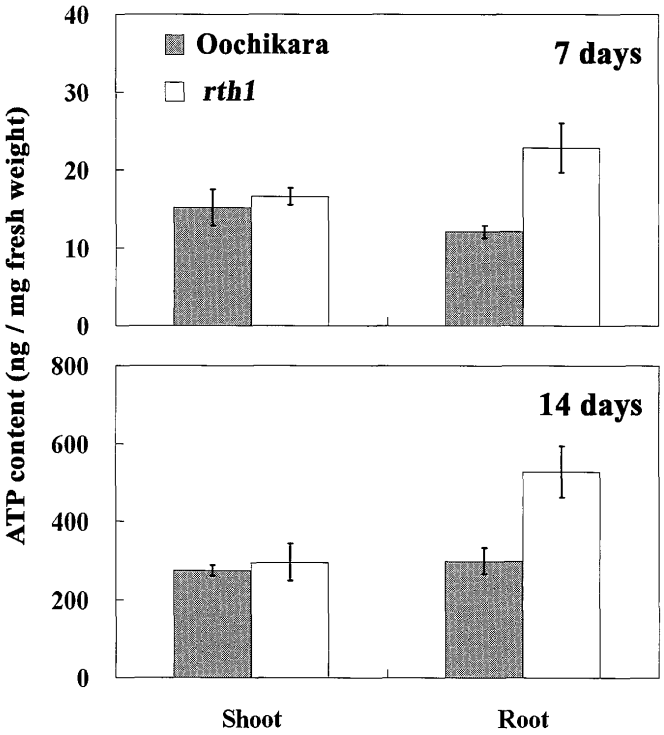

Fig. 7. ATP content in shoot and root from 7- and 14-day-old seedlings of WT and *rth1*. Seedling was cultured with Kimura B nutrient solution. ATP contents were expressed as per unit fresh weight. Bars indicate the standard deviation (n=5).

(Steinebrunner *et al.* 2003, Wu *et al.* 2007). In rice, however, mutation of *OsAPY* alone caused root hairless, short root and semi-dwarf phenotype. Although we have not tested mutant of *OsAPYL*, available results might suggest that apyrases in rice and *Arabidopsis* are functionally differentiated. It is noteworthy that the level of homology between the two apyrase proteins in rice (74%) is much lower than that in *Arabidopsis* (88%).

Apyrase catalyzes the hydrolysis of phosphoanhydride bonds of nucleoside tri- and di-phosphates into monophosphates (Shibata *et al.* 1999). Our genetic experiment showed that, in rice, mutation in *OsAPY* results in the root hairless phenotype, accompanied by reduced root and shoot growth. To obtain indirect estimate of apyrase activity, we quantified ATP content (Fig. 7). Our results showed that the roots of *rth1* contained twice as much ATP as those of WT, suggesting that *rth1* roots have reduced apyrase activity. The arrested root hair elongation and short root phenotype in *rth1* may be interpreted due to its inability to use ATP as energy source in roots. Slightly reduced shoot length in *rth1* might be caused by impaired root growth, because shoot ATP content did not differ between WT and *rth1*. Importance of the apyrase gene family in tuber growth of potato was suggested from apyrase gene silencing experiments (Riewe *et al.* 2008). On the other hand, Wu *et al.* (2007) implied that apyrases also control extracellular ATP (eATP) concentration. eATP is shown to function as signal molecules for growth control (Roux and Steinebrunner 2007). Furthermore, Kim *et al.* (2006) visualized eATP by using a luciferase reporter fused to cellulose-binding domain peptide and showed localization of eATP in regions of active growth and cell expansion, such as root hair tip. Application of such analytical techniques to *rth1* and WT might give a clue to understand the roles of apyrases in control of eATP level, and root and root hair elongation.

Our previous study using *rth1* mutant (Suzuki *et al.* 2003) did not detect significant differences from WT in uptake of phosphate or water. However, it was reported that, in *Arabidopsis*, overexpression of an introduced apyrase gene from pea, showed enhanced phosphate transport (Thomas *et al.* 1999) and increased resistance to herbicides (Windsor *et al.* 2003). Thus, in rice, apyrase overexpression lines may deserve examination in their effects on nutrient uptake, herbicide resistance and root hair morphology.

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