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ORIGINAL ARTICLE

# Rice *RHC* Encoding a Putative Cellulase is Essential for Normal Root Hair Elongation

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**Abstract** Root hairs are tubular shaped protuberances of root epidermal cells and are found in nearly all vascular plants. Co-ordinate expression of a number of root hair morphogenesis genes involved in cytoskeleton reorganization, changes in homeostasis and distribution of ion gradients, and cell wall reassembly are required during root hair cell elongation. In this report, we have characterized a root hair-specific putative cellulase gene in rice, *OsRHC*. *OsRHC* is specifically expressed in elongating root hairs and OsRHC is targeted to the plasma membrane. The mutation of the *OsRHC* gene by a T-DNA knock-out and CRISPR-Cas9 system causes a severe reduction in root hair length. Bimolecular fluorescence complementation analysis demonstrated that the OsRHC protein interacts with a root hair-specific cellulose synthase protein (OsCSLD1) in the plasma membrane. Furthermore, we observed a moderate reduction of cellulose content in the *osrhc* mutant. Our results suggest that the plasma membrane-localized OsRHC plays a critical role in cell wall remodeling during root hair extension.

**Keywords:** Cellulase, Cellulose, Rice, Rice T-DNA, Root hairs

## Introduction

Root hairs are tubular shaped protuberances of root epidermal cells that are found in nearly all vascular plants (Dolan and Costa 2001; Jungk 2001). Root hairs play an important role in plant growth and development, particularly in water and mineral nutrient uptake as well as interactions with the soil microbiome. An early study showed that the root system of an individual rye (*Secale cereal*) plant can produce up to 14 billion root hairs (Dittmer 1937). Physiological and genetic investigations using root hairless mutants clearly demonstrated that root hairs are critical in maintaining a sufficient level of water and mineral uptake during drought or nutrient deficiency (Gahoonia et al. 2001; 2003). The root hair length and density are modulated by environmental conditions, suggesting a high developmental plasticity in root hairs to cope with external environmental factors (Muller et al. 2004).

Since the root hair cell is a unicellular extension, it has been recognized as a useful model system for understanding cell specification, differentiation, and development. Accordingly, the creation and diversification of cell type-specific differentiation programs have been intensively studied in the model plant Arabidopsis (Won et al. 2009; Bruex et al. 2012; Becker et al. 2014). Molecular genetic analysis coupled with transcriptome analysis has led to the identification of a number of root hair defective mutants and numerous root hair-specific genes in Arabidopsis. Thus, transcriptional regulators involved in root hair cell specification and root hair initiation, as well as genes that function in root hair tip growth, have been well elucidated in Arabidopsis (Gilroy et al. 2000). The MYB-bHLH-WD40 transcriptional complex functions as an early cell patterning determinant and specifies the distribution of root hair cells (trichoblasts) and nonhair cells (atrichoblasts) (Kim et al. 2006). Once determined, trichoblast-specific activation of two bHLH transcription factors *ROOT HAIR DEFECTIVE 6* (*RHD6*) and *ROOT HAIR DEFECTIVE6 LIKE 1* (*RSL1*) results in the outgrowth of the root hair by directly or indirectly mediating expression of several downstream root hair morphogenesis genes, which are involved in cytoskeleton reorganization, changes in homeostasis and distribution of ion gradients, and cell wall reassembly (Slabaugh et al. 2011). Cytological features, endogenous phytohormones, and environmental cues that modulate root hair development have also been identified

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(Salazar-Henao et al. 2016).

Although molecular mechanisms underlying the coordinated expression of genes during root hair development have been intensively studied in Arabidopsis, our knowledge about how root hair cells are specified and formed in the monocot model plant rice (*Oryza sativa*) is fragmentary. Different from Arabidopsis and other crop plants, most rice cultivars are grown in flooded arable fields during the vegetative and early reproductive stages (Kawata et al. 1959). Several studies showed that the proliferation of rice root hairs greatly increases the surface area of the roots, thereby serving as an important site for water and nutrient uptake, especially during drought and when levels of nutrients are low (Kawata et al. 1964; Ma et al. 2001; Haling et al. 2013). Recently, a molecular genetic study demonstrated that the initiation of rice root hairs is regulated by three rice genes encoding bHLH transcription factors, which are orthologous to *AtRHD1* and *AtRSL1*, implicating the presence of a conserved mechanism for early root hair formation between Arabidopsis and rice (Kim et al. 2017). Rice *CELLULOSE SYNTHASE-LIKE 1* (*OsCSLD1*) is specifically expressed in root hairs and demonstrated to be required for root hair elongation (Kim et al. 2007). Similar mutant phenotypes and root hair-specific expression were observed in rice *EXPANSIN 17* (*OsEXP17*) and rice *XYLOGLUCAN 6-XYLOSYLTRANSFERASE* (*OsXXT1*), which are likely to function in cell wall reconstitution during root hair growth (Yu et al. 2011; Wang et al. 2014). Recently, Huang et al. (2017) reported a large-scale transcriptome analysis of root hair-specific genes in diverse vascular plants, including rice, and provided the first comprehensive information of conservation and diversification of root hair genes across multiple species. However, the precise cellular and biochemical function of genes found in transcriptome analysis remain to be determined.

Previously, we reported the functional module of the Carbohydrate-Active Enzyme (CAZY) family in rice using meta-expression data and found that 10 genes were preferentially expressed in the roots, two of which encode glycosyltransferase (GT) and eight of which encode glycosyl hydrolase (GH) (Chandran et al. 2016). Given the fact that polar tip outgrowth during root hair formation and elongation is accompanied by dynamic cell wall reconstitution including partial wall digestion and reassembly, it is plausible that many GTs and GHs participate in this process. Although two genes (*OsCSLD1* and *OsXXT1*) encoding GTs have been known to be involved in rice root hair growth, a GH responsible for root hair development has not been reported in Arabidopsis and rice.

In this report, we characterized a member of the GH family 9, *OsRHC*. To the best of our knowledge, this is the first report to systematically evaluate the functional role of a root hair-specific cellulase and its importance during root hair elongation. Our results, along with previous findings, suggest that concomitant enzymatic actions including cell wall digestion and biosynthesis are prerequisite for normal root hair growth.

## Results

### *OsRHC* is Preferentially Expressed in Root Hairs

Root hairs grow out of root epidermal cells and have been used as a model for identifying genes that participate in cell specification and differentiation (Dolan and Costa. 2001; Jungk 2001). To find candidate genes responsible for root hair differentiation and elongation, we performed microarray analysis and identified a number of genes that were specifically induced in rice root hairs (Moon et al. 2018). In addition, we also combined our functional module data of the rice root-specific CAZY family. In this report, we studied the function of a putative cellulase gene, *Os04g57860,* which was highly induced during root hair development. The *Os04g57860* gene is a member of the GH9 encoding endo-1,4-*β*-glucanases and named rice Root Hair Cellulase (*OsRHC*). There are 25 members of the GH9 family in the rice genome and 26 in the *Arabidopsis* genome (CAZY database; http://www.cazy.org/). Phylogenetic analysis indicated that *OsRHC*

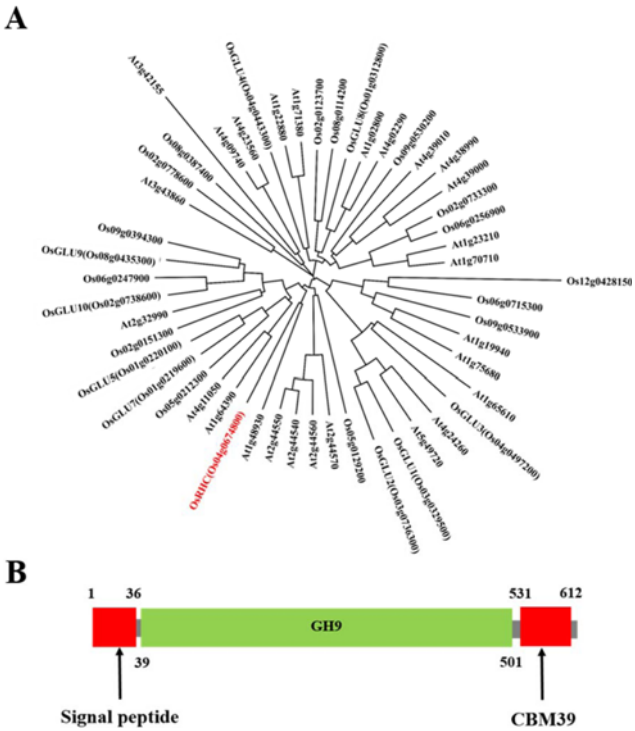


**Fig. 1.** Sequence analysis of OsRHC. (A) A phylogenetic tree of GH9 family members in rice and Arabidopsis genome. The 0.1 scale denotes 10% change. OsRHC is highlighted in red. (B) Diagram of OsRHC protein structure.

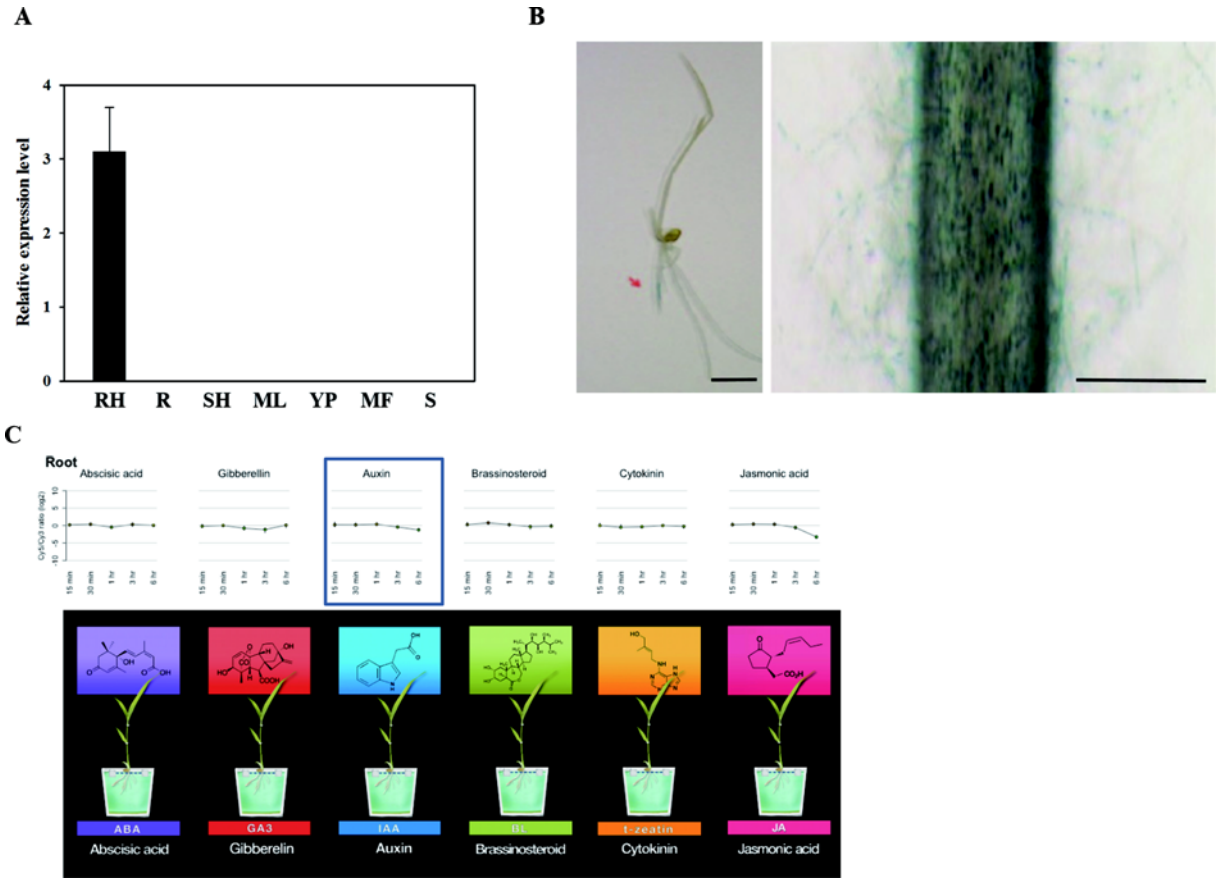


**Fig. 2.** Expression analysis of the *OsRHC* gene by qRT-PCR, the GUS reporter gene, and meta-expression profiles. (A) Real-Time PCR analysis of the *OsRHC* gene in different tissues. RH, root hairs; R, root without root hairs; SH, shoot; ML, mature leaf; YP, young panicle; MF, mature flower; S, seed. Error bars represent the standard errors (SE) of three replicates. (B) Histochemical GUS analysis of transgenic plant carrying the p*OsRHC*::GUS vector. Right panel is an enlarged picture of the red arrow in the left panel. Bars in right panel = 1 cm; bar in right panel = 1 mm. (C) In-silico analysis of *OsRHC* expression in roots by different phytohormones in RiceXPro (ricexpro.dna.affrc.go.jp).

is an ortholog of *At1g48930* (Fig. 1A). Sequence analysis indicated that an open reading frame of 1875 nucleotides encodes 625 amino acid residues to produce a ~68.45 kDa mature protein (Fig. 1B). OsRHC contains a putative signal peptide of 36 amino acid residues at the N terminus with a GH9 domain spanning residues 39 to 501. It also contains a CBM39 domain at the C terminus. To validate our microarray data and functional module data, we first used quantitative RT-PCR to examine the expression pattern of the *OsRHC* gene. We found that the *OsRHC* gene was specifically expressed in root hairs, but its transcript was not detected in the other organs (root, shoot, mature leaf, young panicle, mature flower, and developing seed) tested (Fig. 2A). Next, the GUS reporter system was used to visualize the tissue-level expression pattern of the *OsRHC* gene. In transgenic plants harboring the GUS reporter gene under the control of the *OsRHC* promoter and second exon region, GUS staining was evident in the lower part of the roots, especially in root epidermis (root hair-bearing trichoblasts) and root hairs in the early seedling stage (Fig. 2B). We performed in silico analysis of *OsRHC* expression by different phytohormones based on the microarray data from the RiceXPro Rice expression Profile Database (http://ricexpro.dna.affrc.go.jp/RXP_1000/index.php). It revealed that its expression was slightly but not signifianctly downregulated in response to Indole-3-acetic acid (IAA), and jasmonic acid (JA), and was not altered in response to abscisic acid (ABA), gibberellic acid (GA), brassinosteroid (BR), and trans-zeatin (tZ)/cytokinin (CK) (Fig. 2C). This result indicates that expression of *OsRHC* may not be directly regulated by IAA and JA. Our findings indicate that *OsRHC* is specifically expressed in trichoblasts and root hairs and likely to play a substantial role in root hair development, independently with phytohormones.

## Mutations of *OsRHC* by T-DNA Insertion and CRISPR/Cas9 System Have Defects in Root Hair Elongation

To investigate the potential role of *OsRHC* in root hair

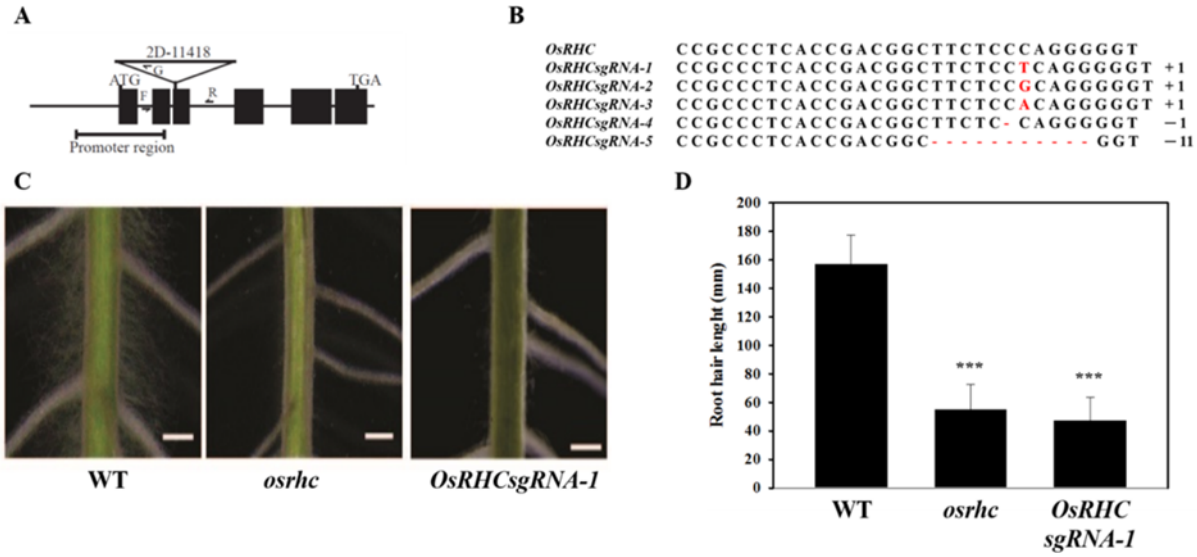


**Fig. 3.** Identification of the *osrhc* T-DNA mutant and its effect on root hair elongation. (A) A diagram showing the site of T-DNA insertion in the *OsRHC* gene. Black boxes in the diagram indicate exons and lines between exons indicate introns. Primers used for endogenous *OsRHC* (F and R) and T-DNA insertion (F and G) are indicated. (B) A sequencing analysis of the gRNA target sites on the genomic regions of *OsRHC*. Insertions are highlighted in red, and dashes indicate deletions. (C) Root hair morphology of one-week-old plants of WT, *osrhc* T-DNA mutant, and the *OsRHC sgRNA-1* transgenic plant. (D) Root hair length of WT, *osrhc* T-DNA mutant, and the *OsRHC sgRNA-1* transgenic plant. Data are means ± SE of three independent assays. ***, *p*-value <0.001, based on *t-test*, bar, 1 mm.

development, we searched our rice T-DNA insertion collection (Rice Functional Genomic Express Database, http://signal.salk.edu/cgi-bin/RiceGE) (Jeon et al. 2000). As a result, we found a line, 2D-11418, in which T-DNA was inserted into the third exon of *OsRHC* (Fig. 3A). A homozygous insertion line was identified by DNA genotyping and RT-PCR (Fig. S1A, B). Microscopic observation of one-week-old roots clearly showed that *osrhc* exhibited shorter root hair length compared to that of WT (Fig. 3C). Except for the shortened root hair phenotype, no morphological differences were observed even in mature plants (data not shown). To confirm that the T-DNA insertion in the third exon of *OsRHC* was responsible for the root hair phenotype, we designed sgRNAs targeting the first and second exon of *OsRHC* using the CRISPR/Cas9 system and generated transgenic plants via *Agrobacterium*-mediated transformation. Mutations of target regions were detected in five independent transgenic plants (named *OsRHC-sgRNA-1* to *5*) by sequencing analysis (Fig. 3B). In accordance with previous plant CRISPR/Cas9 studies, small deletions or one nucleotide insertions were found in the transgenic lines (Fig. 3B). An identical phenotype was observed in all confirmed CRISPR/Cas lines (Fig. 3C). For quantitative analysis, we measured the length of the root hairs located 2-3 mm from the apex at 3 DAG. Root hairs were much shorter by 35% and 30% in both T-DNA insertional mutants (*osrhc*) and CRISPR/Cas9 generated point-mutated lines (*OsRHC-sgRNA*), respectively, compared to those of the wild type (Fig. 3D). These findings strongly suggest that *OsRHC* plays a critical role during root hair elongation.

In addition, we investigated whether the phenotype of the *osrhc* mutants is related to phosphate use efficiency. WT, *osrhc* mutant and *OsRHCsgRNA-1* line were grown under phosphate sufficient (MS medium with 0.323 mM KHPO4) and phosphate deficient conditions (MS medium with 0.012 mM KHPO4) for 2 weeks. The length of seminal and lateral roots increased significantly in WT plants under phosphate deficient concentration. Whereas mutants of *OsRHC* exhibited severe defect in seminal and lateral roots elongation under phosphate deficient conditions, any difference was not observed in root length between mutants of *OsRHC* and WT under phosphate sufficient condition (Fig. S2). Our result suggested that *OsRHC* plays an important role in regulating root hair growth by cellulose synthesis as well as in controlling primary and lateral root elongation in response to decreased phosphate concentration.

## OsRHC and OsCSLD1 were Co-localized in the Plasma Membrane

Since OsRHC encodes a putative endo-1,4-*β*-glucanase and is likely to exert its enzymatic activity on cellulose, we next set out co-expression pattern analysis to find cellulose synthase (*CesA*) genes functionally associated with the *OsRHC* during root hair elongation. Among 47 *CesA*/*Csl* genes, expression of 41 *CesA*/*Csl* genes can be detected in Agilent 44K microarray data (data not shown). To check the degree of co-expression between *OsRHC* and these genes, we used the Pearson Correlation Coefficient (PCC). As a result, *OsCSLD1*

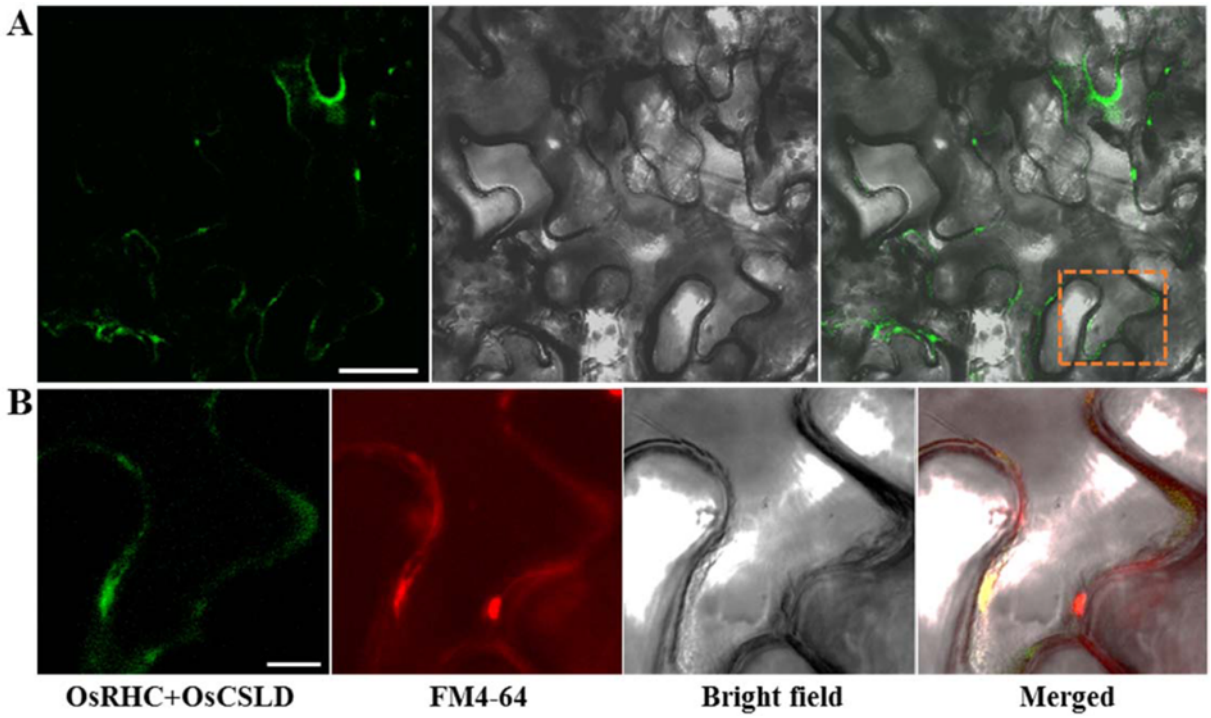


**Fig. 4.** BiFC analysis for interaction between OsRHC and OsCSLD1 in *Agrobacterium*-infiltrated tobacco leaves. (A) OsRHC-CGFP and OsCSLD-NGFP were cotransformed and transiently expressed in tobacco epidermal leaf cells and BiFC (GFP) signal was visualized by Confocal microscopy. (B) Co-localization of BiFC signal with plasma membrane marked by FM4-64 (red channel). Bars = 40 μm in A and 10 μm in B.

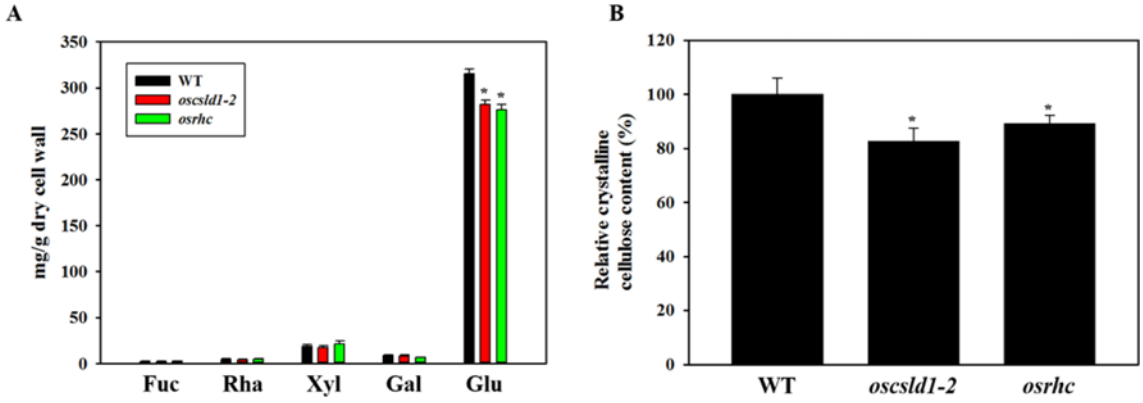


**Fig. 5.** Analysis of cell wall materials from roots of WT, *osrhc,* and *oscsld1* mutant plants. (A) Comparison of the neutral cell wall monosaccharide composition of WT, *osrhc,* and *oscsld1*. (B) Crystalline cellulose analysis. Cell wall residues were prepared from roots of 10-day-old plants. Data are means (mg/g dry cell wall) ± SE of three independent assays. *, *p*-value <0.05, based on *t-test*. The amount of cellulose in the WT was taken as 100, and the amount in the mutants was expressed as a percentage of the WT.

exhibited the highest PCC value out of *CSL* genes in rice (0.92; Table S1). OsCSLD1 belongs to a cellulose synthase subfamily D (CSLD). A previous investigation demonstrated that *OsCSLD1* is specifically expressed in rice root hairs and an *oscsld1* mutant induced by a gene trap system caused shorter root hair length, an identical phenotype to the *osrhc* mutant (Kim et al. 2007). Considering that OsRHC encodes a putative endo-1,4-*β*-glucanase and is likely to exert its enzymatic activity on cellulose, and *OsCSLD1* encodes a cellulose synthase, it is highly plausible that the OsRHC and OsCSLD1 proteins are targeted to the same subcellular organelle, which is likely to be the plasma membrane where cellulose is polymerized. To directly demonstrate this possibility, we conducted BiFC analysis through tobacco infiltration experiments. *Agrobacterium* harboring OsRHC-CGFP or OsCSLD1-NGFP plasmids were cotransformed into the tobacco. Fluorescence signal of OsRHC-OsCSLD1-GFP was detected at plasma membrane of tobacco leaf epidermis cells

and BiFC signal overlaps with FM4-64, plasma membrane marker (Fig. 4), supporting that OsRHC and OsCSLD work together at plasma membrane. In addition, we confirmed that both OsRHC-GFP and OsCSLD1-RFP are localized at plasma membrane (Fig. S3).

### Genetic Mutation of *OsRHC* Causes a Moderate Reduction of Cellulose Content

Next, we reasoned that the enzymatic activity of OsCSLD1 and OsRHC may contribute to the biosynthesis of cellulose, and mutations of both genes may cause a cellulose-deficient phenotype. We first examined the total neutral sugar composition of destarched cell walls isolated from WT, *oscsld1-2*, and *osrhc* roots. *oscsld1-2* is a new mutant allele that was found in our rice T-DNA lines. It revealed that the levels of glucose, which is the main component of cellulose, was slightly reduced in both *oscsld1-2* and *osrhc* (Fig. 5A). By contrast, the amount of several other neutral sugars was not changed. Next, we determined the crystalline cellulose content of WT, *oscsld1-2*, and *osrhc* roots using the Updegraff method. As with our neutral sugar compositional analysis, cellulose contents in *osrhc* and *oscsld1-2* mutants were reduced to 89% and 83%, respectively, compared to that of wild type (Fig. 5B).

## Discussion

Root hair cells develop from trichoblasts that are formed by the division of a subset of root epidermal cells. Understanding how root hair cells are specified and differentiated can contribute to many critical aspects of cell biology. Once specified by early patterning genes, auxin plays a critical role in modulating the endogenous levels of reactive oxygen species (ROS) by class III peroxidases and NADPH oxidases for polar tip growth (Mangano et al. 2017). During root hair elongation, dynamic cellular events, including cell wall remodeling, accompany root hair emergence and elongation. The rapid disassembly by diverse GHs and subsequent reassembly by biosynthetic GTs are required for cell wall remodeling (Marzec et al. 2015). Accordingly, recent RNA-seq analysis has shown that a number of GTs and GHs, as well as expansin proteins, were highly expressed in rice root hairs (Huang et al. 2017).

Sequence analyses have shown that the *OsRHC* gene encodes a member of the GH9 family, previously known as the cellulase family (Fig. 1B). Two members in this family, *PttCel9A1* from *Populus* and *CEL16* from Brassica, exhibited endo-hydrolytic activity toward noncrystalline cellulose, but not toward other cell wall polysaccharides (Mølhøj et al. 2001; Takahashi et al. 2009). Thus, OsRHC might have a biochemical function similar to those GH9 members. qRT-PCR and the GUS reporter system clearly revealed that the *OsRHC* gene was specifically expressed in root hairs (Fig. 2A, B). Consistent with its expression pattern, mutations of the *OsRHC* gene resulted in a severe defect in root hair elongation (Fig. 3C). We also utilized a CRISPR/Cas9 system to confirm that T-DNA insertion in *OsRHC* was responsible for the phenotype (Fig. 3B, C). Transgenic plants generated by CRISPR/Cas9 displayed the same phenotype, unequivocally demonstrating that *OsRHC* is a causal gene for the phenotype. Cell wall composition analysis and measurement of crystalline cellulose content showed that the *osrhc* mutation caused a moderate reduction in the level of cellulose in cell walls isolated from roots (Fig. 5). These chemical studies indicated that *OsRHC* is most likely involved in cellulose remodeling during root hair elongation. Furthermore, our co-localization experiment suggested that OsRHC works coordinately with a root hair-specific cellulose synthase, OsCSLD1 (Fig. 4). Cellulose microfibrils are synthesized from the plasma membrane by cellulose synthases and cellulose synthase-like enzyme complexes (Kumur et al. 2015). In bacteria, at least three proteins are encoded in the Bacterial cellulose synthesis (Bcs) operon; two genes (*BcsA* and *BcsB*) have been shown to encode cellulose synthases and BcsZ is a cellulase (Römling et al. 2015). Apart from cellulose synthases, other accessory proteins, including cellulase, sucrose synthase, and cytoskeleton-related proteins, are attributed to cellulose biosynthesis (Kumur et al. 2015; Römling et al. 2015). In Arabidopsis, genetic studies have identified an endoglucanase in the GH9 family, commonly referred to as KORRIGAN, which appears to be essential for cellulose biosynthesis in primary and secondary cell walls (Lane et al. 2001; Mansoori et al. 2014). In addition, down-regulation of a KORRIGAN ortholog in *Populus*, *PtrKOR1*, caused brittle stems, collapsed vessel elements, and a reduction in cellulose content (Takahashi et al. 2009). Although it is difficult to provide experimental evidence for a definitive function of cellulase in cellulose remodeling during root hair elongation, our mutant analysis and previous studies suggest that it probably exerts its effects by cleaving glucosyl residues during cellulose biosynthesis.

Recently, we found 409 root hair-specific genes including *OsRHC*, and twelve genes among them are involved in auxin-mediated signaling pathway (Moon et al. 2018). Several studies previously demonstrated that higher level of endogenous auxin is required for root hair formation, architecture, and elongation (Grieneisen et al. 2007; Bhosale et al. 2018; Giri et al. 2018). Thus, our findings and previous studies support the critical role of auxin-dependent root hair development and elongation in Arabidopsis and rice. Elevated levels of auxin in trichoblasts induce Auxin Responsive Factor *(ARF19)*, which likely binds to the promoters of root hair-specific transcription factors (*OsRSL2* and *OsRSL4*). Then, RSL genes would

induce expression of lots of downstream genes. However, our in-silico analysis of *OsRHC* expression revealed that its expression is not directly regulated by IAA. To demonstrate the relationship between *OsRHC* and auxin signaling pathway, more intensive further studies will be required.

Comparative transcriptome analysis of root hair-specific genes in seven diverse vascular plant species, including rice, revealed that a core set of genes are conserved among vascular plants (Huang et al. 2017). However, it was also found that one-third of genes highly expressed in root hairs are diverged and not conserved in those species. Since most cultivated rice is grown in paddy fields from the early vegetative to early reproductive stages, rice roots and root hairs grow in soil under flooded conditions, which is a very different environmental condition to that of other plant species (Kawata et al. 1964; Haling et al. 2013). Thus, a strong selection for rice with a high yield and better rhizoid system may be the underlying mechanism for the preferential divergence of root hair genes and may be responsible for the substantial degree of root hair gene diversification.

*Cellulose synthase like* (*CSL*) genes are multigene family; 30 *CSL* genes are classified into six distinct groups in Arabidopsis and more than 40 *CSL* genes into eight distinct groups in rice (Hazen et al. 2002; Yin et al. 2011). It is generally believed that CSL proteins catalyze the biosynthesis of β-linked polysaccharides including cellulose and mannan. Only a few of them have been biochemically characterized. Yin et al. (2011) reported that different combination of double and triple mutant analysis of *AtCSLD2*, *AtCSLD3*, and *AtCSLD5* resulted in dwarfism and reduced viability as well as the reduction in root hair elongation (Yin et al. 2011). Biochemical characterization using tobacco transient system suggested that AtCSLD5 exhibited mannan synthase activity. Furthermore, immunostaining using LM21 monoclonal antibody, which specifically binds to mannan, clearly showed the presence of mannan at the tip of the Arabidopsis root hairs. Li et al. (2009) showed that rice golgi-localized CSDL4 (OsCSLD4) mutation caused the significant reduction in plant viability, retarded cell division, and reduced levels of arabinoxylan and cellulose. Therefore, it cannot be ruled out of the possible involvement of OsCSLD1 in other polysaccharide biosynthesis. However, our two observations argue against this. First, our cell wall analysis of *oscsld1* mutant indicated a moderate reduction in the glucose content, suggesting that cellulose biosynthesis was impaired in the mutant. Second, OsCSLD1 was targeted to the plasma membrane, but not in the Golgi. Considering cellulose and callose are only two polysaccharides synthesized in the plasma membrane and other β-linked polymers are expected to be synthesized in the Golgi, it is still possible that *OsCSLD1* is responsible for cellulose biosynthesis during root hair elongation.

In conclusion, we have found that a root hair-specific cellulase, OsRHC, functions in a critical role during root hair elongation. Its coexpression and colocalization with OsCSLD1 indicate that dynamic cell wall remodeling is required for the tip growth of rice root hairs.

## Materials and Methods

### Plant Materials and Growth Conditions

Rice seeds of wild type (*Oryza sativa* japonica cv. Dongjin), T-DNA insertional mutant lines for *OsRHC* and *OsCSLD1*, and CRISPR/Cas9 mutant lines for *OsRHC* (*e.g.,* OsRHC sgRNA-1 to 5), were germinated at 28ºC under a cycle of 16 h light/8 h dark for 3 days and then transferred to soil. Rice plants were grown in a temperature-controlled glasshouse with natural lighting conditions. Leaves of 4-week-old plants were used for genotyping. Tissue samples for RNA analysis were collected at the indicated developmental stages, frozen in liquid nitrogen, and stored at -80ºC until analysis. To explore whether the phenotype of the *osrhc* mutants is related to phosphate use efficiency, phosphate limited experiments were conducted using a modified Murashige & Skoog (MS) media without phosphate (MSP11-50LT) containing in phosphate-sufficient condition (0.320 mM KH2PO4) or phosphate-deficient condition (0.012 mM KH2PO4). The pH of the MS media was adjusted to 6 using 0.1 M NaOH. Rice seedlings were grown in growth chambers with a 16 h light/8 h dark and a day/night temperature of 28°C for 2 weeks.

### Phylogenetic Analysis

For phylogenetic analysis, we used glycosyl hydrolase (GH) family 9 from rice and Arabidopsis. All amino acid sequences for rice and Arabidopsis were extracted from the Carbohydrate-Active Enzyme Database (CAZY, http://www.cazy.org/). We used ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) for drawing a comparative phylogenetic tree.

### RNA Sample Preparation and Quantitative Real Time PCR (qRT-PCR)

Seminal roots were detached from the 3 days after germination (DAG) seedlings and immediately submerged in liquid nitrogen. To collect the root-hair tissues, we gently rubbed each root surface with a brush. Those seminal roots without root hairs were used as a root sample for RNA isolation. Five more tissues (7-day-old shoot, two-month-old mature leaf, young panicle, mature flower, and seed) were also prepared. Four biological replicates for RNA isolation were prepared and each RNA replicate was analyzed independently. Total RNA was extracted with TRIzol and purified with an RNeasy plant mini kit (Qiagen, Hilden, Germany). For synthesis of complementary DNA (cDNA), 1 mg of total RNA was reacted with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA), 2.5 mM deoxyribonucleotide triphosphate, and 10 ng of oligo (dT). Real-time PCR analysis was performed using the first-strand cDNA as template with the QuantiTect SyBR Green PCR kit (Clontech, Mountain View, CA, USA). For each analysis, three technical replicates were performed. The PCR threshold cycle number of each gene was normalized with expression level of the rice *Ubiquitin5* (*OsUbi5*) as a reference gene.

### Generation of Transgenic Plants with *OsRHC* Promoter-GUS Expression and Histochemical GUS assay

The 1.7 kb promoter region from the translation start site of *OsRHC*

was PCR amplified with gene-specific primer set (Table S2) and inserted into the pGEM-T Easy Cloning Kit (Promega, USA). After transformation into *Escherichia coli* Top10, colonies were selected on LB plates supplemented with 50 μg $mL^{-1}$ ampicillin. Plasmid DNA was isolated with a plasmid DNA extraction kit (Geneall, Seoul, South Korea). After sequencing, the inserts were digested by two restriction enzymes (*Kpn1* and *BamH1*), followed by ligation into the pGA3519 vector. The construct was transformed into *Agrobacterium tumefaciens* LBA4404. *Agrobacterium*-mediated transformation into embryogenic callus from mature seeds were performed and regenerated plants were obtained as described previously (Jeon et al. 2000). Seedlings of transgenic plants were incubated overnight at 37°C in GUS staining solution (100 mM sodium phosphate, pH 7, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.5% Triton X-100, 10 mM EDTA, pH 8, 0.1% 5-bromo-4-chloro-3-indolyl-β-d-GlcA/cyclohexylammonium salt, 2% dimethyl sulfoxide, and 5% methanol). Following washing by 70% ethanol, the tissues were observed under a light microscope (Olympus, Tokyo, Japan) for GUS staining.

Isolation of the *osrhc* and *oscsld1-2* T-DNA Insertional Mutants

The *osrhc* and *oscsld1-2* mutants were isolated from our rice T-DNA insertion collection (Rice Functional Genomic Express Database, http://signal.salk.edu/cgi-bin/RiceGE). Homozygous lines for the T-DNA insertion were confirmed by genotyping using gene-specific primers (Table S2).

Generation of Transgenic Plants using the CRISPR/Cas9 System

To generate single guide (sg) RNA-Cas plant expression vectors, three sets of oligomers targeting the first and second exon of *OsRHC* were synthesized using an ABI 394 Synthesizer (Macrogen, Daejeon, South Korea). The sequence information of three sets of oligomers are listed in Table S2. A Cas9 expression backbone vector pRGEB32 (Addgene plasmid ID: 63142) was used in this study. The oligos contain GGCA/AAAC overhangs. The synthesized 24 bp oligos were annealed and inserted into *Bsa I* sites of the RGEB32 binary vector. The expression of Cas9 was driven by promoter of *OsUbiquitin* and the gRNA was expressed under the control of U3 snRNA promoter from rice (*OsU3* promoter). Ligation products were transformed into *E. coli*. The pRGEB32 vector containing the sgRNA and Cas9 expression cassette was transformed into *Agrobacterium tumefaciens* LBA4404. *Agrobacterium*-mediated transformation into embryogenic callus via mature seeds was performed and regenerated plants were obtained as described previously (Jeon et al. 2000). Genomic DNAs were isolated from the leaves of two-week-old transgenic plants and PCRs were performed using *OsRHC*-specific primers. PCR products were purified and sequenced.

Bimolecular Fluorescence Complementation (BiFC) Analysis of OsRHC and OsCSLD1 in Tobacco Leaves

OsRHC, OsCSLD1, N-terminal fragment of GFP (NGFP) and C-terminal fragment of GFP (CGFP) were amplified by PCR using gene-specific primers (Table S2). After digestion of pGreen vector with *EcoRI* and *SpeI*, fragments infused into digested vector to generate OsCSLD1-NGFP and OsRHC-CGFP vectors, using In-Fusion HD Cloning Kit (Clontech). The constructs were transfected into *Agrobacteria* GV3101 and used for tobacco (*Nicotiana benthamiana*) infiltration experiments. At 48 h after infiltrations, the leaves were detached and stained with 8.2 μm FM4-64 (Molecular Probes, Leiden, Netherlands) for 15 min. Although FM4-64 are used to stain vacuolar membrane, it selectively stains the plasma membrane within the first 15 to 30 min of incorporation (Speth, et al. 2009). After washing with distilled water, leaves were observed immediately with a confocal laser scanning microscopy (LSM 510 META; Carl Zeiss, Thornwood, NY, USA). GFP and RFP were detected using 488-/505- to 530-, and 543-/560- to 615-nm excitation/emission filter sets, respectively. Fluorescence images were digitized with the Zeiss LSM image browser.

Extraction of Alcohol-insoluble Cell Wall Residue (AIR) and Starch Removal

Root tissues of ten-day-old rice were ground to a fine powder. The ground material (~1 gram) was washed in 15 mL of 70% ethanol and heated for 15 min at 70°C to inactivate the endogenous enzymes and remove the cell contents. Samples were centrifuged for 10 min at 4000*g* and then washed twice with 100% ethanol. Finally, samples were washed with 100% acetone. The remaining pellet was considered to be alcohol-insoluble cell wall residues (AIRs) and was dried at 70°C in an oven. Ten mg of AIRs were resuspended in a 0.1 M sodium acetate buffer pH 5.0 and incubated at 80°C for 20 min. Two starch-specific hydrolases (amylase from Bacillus species and pullulanse from Bacillus acidopullulyticus; Sigma-Aldrich, St Louis, MO, USA) were added and incubated overnight at 37°C. Enzymatic removal of starch were stopped by incubating samples at 100°C for 10 min. Pellets were collected by centrifugation and washed three times by adding sterile water followed by 100% acetone twice, and finally dried at 70°C in an oven.

Neutral Sugar Composition Analysis

Cell wall sugars (as alditol acetates) were determined following a procedure described previously (Lee et al. 2007). AIRs (5 mg) were pre-hydrolyzed with 72% sulfuric acid at room temperature for 30 min. After the dilution of sulfuric acid to a final concentration of 6% and the addition of a standard control (0.4 mg/mL of inositol), samples were incubated at 105°C for two hours. The solution was then reduced with sodium borohydride in DMSO and heated for 90 min at 40°C, followed by sequential treatment with glacial acetic acid, acetic anhydride, 1-methylimidazole, dichloromethane, and water. The organic layer containing the alditol acetates of the hydrolyzed cell wall sugars was washed three times with water, and sugars were analyzed using a gas–liquid chromatograph (model 6890; Hewlett-Packard, http://www.hp.com) equipped with a 30 m × 0.25 mm (i.d.) silica capillary column DB 225 (Alltech Assoc., Deerfield, IL, USA).

Cellulose Content Analysis

Cellulose was quantified according to the method of Updegraff (Updegraff 1969). First, 10 mg of AIR was hydrolyzed in acetic/nitric reagent (acetic acid:nitric acid:water, 8:1:2) in a boiling water bath for 30 min and then washed with 100% acetone. Hydrolyzed samples were swelled by the addition of 1 mL of 67% sulfuric acid for 60 min at RT and a 0.3% anthrone solution was added. Cellulose content was measured by a spectrophotometer at 620 nm. At least five replicates for each sample were measured.

Co-expression Analysis

Using the microarray datasets, we identified the most highly co-expressed *OsCSL* gene of RHC gene by the PCC method. The result of PCC analysis between RHC and CSLD genes is prepared Table S1.

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## Author's Contributions

SM, GA, CL, and KHJ designed the research; SM, YJK, CL, and YG performed experiments; SM, CL, AKNC, and WJH analyzed data; SM, CL, and KHJ wrote the manuscript. All the authors agreed on the contents of the paper and post no conflicting interest.

## Supporting Information

**Fig. S1.** Identification of the T-DNA mutant of *osrhc*.
**Fig. S2.** Phenotype analysis among WT, *osrhc* mutant, and *OsRHC-sgRNA-1* line under sufficient or deficient phosphate conditions.
**Fig. S3.** Co-localization analysis of OsRHC and OsCSLD1
**Table S1.** PCC analysis for anatomical expression patterns between *RHC* and rice *CSLD* genes.
**Table S2.** Gene accession numbers used in this study and primers used for qRT-PCR.

## References

Becker JD, Takeda S, Borges F, Dolan L, Feijo JA (2014). Transcriptional profiling of Arabidopsis root hair and pollen defines an apical cell growth signature. BMC Plant Biol 14:197

Bhosale R, Giri J, Pandey BK, Giehl RFH, Hartmann A, Traini R et al (2018). A mechanistic framework for auxin dependent Arabidopsis root hair elongation in response to low external phosphate. Nat Comm 9:1409

Bruex A, Kainkaryam RM, Wieckowski Y, Kang YH, Bernhardt C, Xia Y, Zheng X, Wang JY, Lee MM, Benfey P, Woolf PJ, Schiefelbein J (2012). A gene regulatory network for root epidermis cell differentiation in Arabidopsis. PLoS Genetic 8:e1002446

Chandran AKN, Jeong HY, Jung KH, Lee C (2016). Development of functional modules based on co-expression patterns for cell-wall biosynthesis related genes in rice. J Plant Biol 59:1–15

Dittmer HJ (1937). A quantitative study of the roots and root hairs of a winter rye plant (Secale cereale). Am J Bot 24:417–420

Dolan L, Costa S. Evolution and genetics of root hair stripes in the root epidermis (2001). J Exp Bot 52:413–417

Gahoonia TS, Nielsen NE (2003). Phosphorus (P) uptake and growth of a root hairless barley mutant (bald root barley, brb) and wild type in low- and high-P soils. Plant Cell Environ 26:1759–1766

Gahoonia TS, Nielsen NE, Joshi PA, Jahoor A (2001). A root hairless barley mutant for elucidating genetics of root hairs and phosphorus uptake. Plant and Soil 235:211–219

Gilroy S, Jones DL (2000). Through form to function: root hair development and nutrient uptake. Trends Plant Sci 5:56–60

Giri J, Bhosale R, Huang G, Pandey B, Parker H, Zappala S. et al (2018). The rice auxin influx carrier OsAUX1 facilitates root hair elongation in response to low external phosphate. Nat Comm 9:1408

Grieneisen V, Xu J, Marée A, Hogeweg P, Scheres B (2007). Auxin transport is sufficient to generate a maximum and gradient guiding root growth. Nature 449:1008–1013

Haling RE, Brown LK, Bengough AG, Young IM, Hallett PD, White PJ, George TS (2013). Root hairs improve root penetration, root-soil contact, and phosphorus acquisition in soils of different strength. J Exp Bot 64:3711–3721

Hazen SP, Scott-Craig JS, Walton JD (2002). Cellulose synthase-like genes of rice. Plant Physiol 128:336–340

Huang L, Shi X, Wang W, Ryu KH, Schiefelbein J (2017). Diversification of root hair development genes in vascular plants. Plant Physiol 174:1697–1712

Jeon JS, Lee S, Jung KH, Jun SH, Jeong DH, Lee J, Kim C, Jang S, Yang K, Nam J, An K, Han MJ, Sung RJ, Choi HS, Yu JH, Choi JH, Cho SY, Cha SS, Kim SI, An G. T-DNA insertional mutagenesis for functional genomics in rice (2000). Plant J 22:561–570

Jungk A. Root hair and the acquisition of plant nutrients from soil (2001). J Plant Nutr Soil Sci 164:121–129

Kawata S, Ishihara K. Studies on the root hairs in rice plant (1959). Proc Crop Sci Soc Jpn 27:341–348

Kawata S, Ishihara K, ShioyaT (1964). Studies on the root hairs of lowland rice plants in the upland fields. Proc Crop Sci Soc Jpn 32:250–253

Kim CM, Han CD, Dolan L (2017). RSL class I genes positively regulate root hair development in Oryza sativa. New Phytol 213:314–323

Kim CM, Park SH, Je BI, Park SH, Park SJ, Piao HL, Eun MY, Dolan L, Han CD (2007). OsCSLD1, a cellulose synthase-like D1 gene, is required for root hair morphogenesis in rice. Plant Physiol 143:1220–1230

Kim DW, Lee SH, Choi SB, Won SK, Heo YK, Cho M, Park YI, Cho HT (2006). Functional conservation of a root hair cell-specific cis-element in angiosperms with different root hair distribution patterns. Plant Cell 18:2958–2570

Kumar M, Turner S (2015). Plant cellulose synthesis: CESA proteins crossing kingdoms. Phytochemistry 112:91–99

Lane DR, Wiedemeier A, Peng L, Höfte H, Vernhettes S, Desprez T, Hocart CH, Birch RJ, Baskin TI, Burn JE, Arioli T, Betzner AS, Williamson RE (2001). Temperature-sensitive alleles of RSW2 link the KORRIGAN endo-1,4-beta-glucanase to cellulose synthesis and cytokinesis in Arabidopsis. Plant Physiol 126:278–288

Lee C, Zhong R, Richardson EA, Himmelsbach DS, McPhail BT, Ye ZH (2007). The PARVUS gene is expressed in cells undergoing secondary wall thickening and is essential for glucuronoxylan biosynthesis. Plant Cell Physiol 48:1659–1672

Li M, Xiong G, Li R, Cui J, Tang D, Zhang B, Pauly M, Cheng Z, Zhou Y (2009). Rice cellulose synthase-like D4 is essential for normal cell-wall biosynthesis and plant growth. Plant J 60:1055–69

Ma JF, Goto S, Tamai K, Ichii M (2001). Role of root hair and lateral roots in silicon uptake by rice. Plant Physiol 127:1773–1780

Mangano S, Denita-Juarez SP, Choi HS, Marzol E, Hwang Y, Ranocha P, Velasquez SM, Borassi C, Barberini ML, Aptekmann AA, Muschietti JP, Nadra AD, Dunand C, Cho HT, Estevez JM (2017). Molecular link between auxin and ROS-mediated polar growth. Proc Natl Acad Sci USA 114:5289–5294

Mansoori N, Timmers J, Desprez T, Kamei CL, Dees DC, Vincken JP, Visser RG, Hofte H, Vernhettes S, Trindade LM2014;. KORRIGAN1 interacts specifically with integral components of the cellulose synthase machinery. Plos One 9:e112387

Marzec M, Melzer M, Szarejko I (2015). Root hair development in the grasses: what we already know and what we still need to know. Plant Physiol 168:407–414

Mølhøj M, Ulvskov P, Dal Degan F (2001). Characterization of a functional soluble form of a Brassica napus membrane-anchored endo-1,4-beta-glucanase heterologously expressed in Pichia pastoris. Plant Physiol 127:674–684

Moon S, Chandran AKN, An G, Lee C, Jung KH (2018). Genome-wide analysis of root hair-preferential genes in rice. Rice (N Y) 11(1):48

Muller M, Schmidt W (2004). Environmentally induced plasticity of

root hair development in Arabidopsis. Plant Physiol 134:409–419.

Römling U, Galperin MY (2015). Bacterial cellulose biosynthesis: diversity of operons, subunits, products, and functions. Trends Microbiol 23:545–457

Salazar-Henao JE, Vélez-Bermúdez IC, Schmidt W (2016). The regulation and plasticity of root hair patterning and morphogenesis. Development 143:1848–1858

Slabaugh E, Held M, Brandizzi F (2011). Control of root hair development in Arabidopsis thaliana by an endoplasmic reticulum anchored member of the R2R3-MYB transcription factor family. Plant J 67:395–405

Speth EB, Imboden L, Hauck P, He SY (2009). Subcellular localization and functional analysis of the Arabidopsis GTPase RabE. Plant Physiol 149:1824–1837

Takahashi J, Rudsander UJ, Hedenström M, Banasiak A, Harholt J, Amelot N, Immerzeel P, Ryden P, Endo S, Ibatullin FM, Brumer H, del Campillo E, Master ER, Scheller HV, Sundberg B, Teeri TT, Mellerowicz EJ (2009). KORRIGAN1 and its aspen homolog PttCel9A1 decrease cellulose crystallinity in Arabidopsis stems. Plant Cell Physiol 50:1099–1115

Updegraff DM. Semimicro determination of cellulose in biological materials (1969). Anal Biochem 32:420–424

Wang C, Li S, Ng S, Zhang B, Zhou Y, Whelan J, Wu P, Shou H (2014). Mutation in xyloglucan 6-xylosytransferase results in abnormal root hair development in Oryza sativa. J Exp Bot 65:4149–4157

Won SK, Lee YJ, Lee HY, Heo YK, Cho M, Cho HT (2009). Cis-element- and transcriptome-based screening of root hair-specific genes and their functional characterization in Arabidopsis. Plant Physiol 150:1459–1473

Xie K, Minkenberg B, Yang Y (2015). Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc Natl Acad Sci USA 112:3570–3575

Yin L, Verhertbruggen Y, Oikawa A, Manisseri C, Knierim B, Prak L, Jensen JK, Knox JP, Auer M, Willats WG, Scheller HV (2011). The cooperative activities of CSLD2, CSLD3, and CSLD5 are required for normal Arabidopsis development. Mol Plant 4:1024–1037

Yu ZM, Kang B, He XW, Lv SL, Bai YH, Ding WN, Chen M, Cho HT, Wu P (2011). Root hair-specific expansins modulate root hair elongation in rice. Plant J 66:725–734