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ORIGINAL ARTICLE

Genome-wide Analysis of Root Hair Preferred *RBOH* Genes Suggests that Three *RBOH* Genes are Associated with Auxin-mediated Root Hair Development in Rice

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Abstract Plant homologs of mammalian NADPH oxidase, respiratory burst oxidase homologs (RBOH), mainly oxidize NADPH to $NADP^+$ and transfer electrons to water. The membrane residing RBOHs thus produce reactive oxygen species (ROS), which allows plants to withstand abiotic and biotic environmental stresses. To understand the spatial and temporal function of rice (*Oryza sativa*) *RBOH* genes, we performed expression analysis of nine *RBOH* genes using qRT-PCR and microarray data. The expression profiling data suggest that *RBOH* genes have diverse roles in various tissues and organs as well as responses to hormonal treatments. Among them, *OsRBOH2, OsRBOH3,* and *OsRBOH5* are preferentially expressed in root hairs. Exogenous auxin upregulates the expression of *OsRBOH1, OsRBOH2, OsRBOH3, OsRBOH4*, and *OsRBOH8* in root hairs, while the expression of *OsRBOH7* and *OsRBOH9* is downregulated. In roots, treatment with an RBOH inhibitor, diphenyleneiodonium (DPI), suppressed the accumulation of ROS in trichoblast cells which initiate root hairs, suggesting that *RBOH*-mediated ROS could play an important role in root hair initiation in the trichoblast cells of rice roots. Promoter analysis revealed that *OsRBOH3* and *OsRBOH5* contain many known *cis*-acting regulatory elements (CREs) associated with root hair development such as the root hair *cis*-acting element, RHE. *OsRBOH2* and *OsRBOH3*, which are upregulated after treatment with indole-3-acetic acid (IAA) with significant expression in root hairs, might be key players in root hair elongation via an auxin-dependent pathway in rice.

Keywords: Auxin, RBOH, Reactive oxygen species, Rice, Root hairs

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Introduction

Root hairs protrude from the surface of roots, helping to consume nutrients and water and to fix plants to the soil (Peterson and Farquhar 1996). Root hairs also increase the surface area of the roots and increase the efficiency of water and nutrient absorption (Gilroy and Jones 2000; Parker et al. 2000). It has been shown that the density and length of root hairs increase when plants are grown in a nutrient stress environment (Bates and Lynch 1996; Ma et al. 2001). Thus, the growth of the root hair which influences the growth of the whole plant is very important. The entire root of the plant is divided into three zones of cell division, elongation, and maturation. However, root hairs develop mostly in the zone of maturation. The epidermal cell of the zone of elongation is divided into trichoblasts and atrichoblasts, and the asymmetric division of the trichoblast cell produces the root hair (Gilroy and Jones 2000). The growth stages of root hairs are very systematic and determine cell fate, positional information, and localized growth. The mechanisms of the initiation and elongation of root hairs have been studied extensively. First, when a root hair is initiated, the pH outside the cell wall was found to be much lower than the cytoplasmic pH of the trichoblast cell (Gilroy and Jones 2000). In addition, auxin was found to be associated with root hair growth, based on the results that auxin treatment affects the frequency and length of the root hair (Rahman et al. 2002).

The NADPH oxidase, respiratory burst oxidase homolog (RBOH), was first discovered in mammalian NADPH oxidase catalytic subunit $gp91^{phox}$ (Torres et al. 1998) and was found to be present in multicellular eukaryotes but not prokaryotic and unicellular eukaryotic organisms (Bedard et al. 2007). RBOH is a heme-containing transmembrane protein that mainly oxidizes NADPH to $NADP^+$ and transfers electrons to H_2O to form reactive oxygen species (ROS) (Segal and Abo 1993). When a plant recognizes pathogenic interactions,

it proceeds with an oxidative burst by increasing the concentration of ROS to resist it. Through loss of function studies by RNAi technology, it has been demonstrated that *OsRBOHA (OsRBOH2/LOC_Os01g53294)* and *OsRBOHE (OsRBOH3/LOC_Os01g61880)* are related to early oxidative burst and late oxidative burst pathways in rice (Yoshie et al. 2005). In addition, it has been shown that *RBOH* mediates plant growth while activating calcium channels by producing superoxide radicals (Very and Davies 2000; Foreman et al. 2003). Since the first *RBOH* found in rice, *OsRBOHA* (Groom et al. 1996), a total of nine *RBOH* members have been found in rice (Yang et al. 2014). Thereafter, ten *RBOH* genes in Arabidopsis (Torres et al. 1998; Sagi and Fluhr 2006) and four *RBOH* genes in *Zea mays* (maize) were found (Lin et al. 2009).

In *A. thaliana*, the mutation that interferes with calcium absorption causes root hairs to be shorter (Wymer et al. 1997). It was later found that the *root hair defect 2* (*RHD2*) gene is *RBOH*, based on the result that active oxygen is decreased in the root hairs of the *rhd2* mutant and treatment of the wild type with diphenylene iodonium (DPI), an inhibitor of RBOH, results in the same phenotype as that of the *rhd2* mutant (Foreman et al. 2003). Afterwards, the auxin-mediated root hair growth model was proposed in Arabidopsis. Auxin activates auxin response factors (ARFs) to regulate the expression of *ROOT HAIR DEFECTIVE SIX-LIKE 4* (*RSL4*) encoding basic helix loop helix (bHLH) transcription factor (TF), which increases the expression of *AtRBOHC*, *AtRBOHH*, and *AtRBOHJ* (Mangano et al. 2017). Similarly to the studies of Arabidopsis model, a recent research reported the positive regulation of OsRBOH by OsRSL to in rice root hair development (Moon et al. 2019). The knock-out of auxin biosynthesis genes in Arabidopsis, such as *auxin response factor 1* (*ARF1*) and *auxin resistant 1* (*AUX1*), and ethylene biosynthesis genes, such as *ethylene response factor 1* (*ETR1*) and *ethylene insensitive 2* (*EIN2*), causes the reduction of root hair length and the exogenous application of these hormones recovers the defects (Masucci and Schiefelbein 1996). These results indicate that both auxin and ethylene are necessary for normal root hair growth.

RBOH is conserved in monocot plants. The *roothairless5* (*RHL5*) gene from maize encodes monocot-specific *RBOH*. The *rth5* mutant inhibits the initiation and growth of root hairs and also reduces the length and density of root hairs (Nestler et al. 2014). Currently, there are many studies on root hair development in Arabidopsis. However, research on *RBOH* in monocot species including rice has been very limited. *OsRBOH3* is an ortholog with maize *RHD2* and Arabidopsis *RHD2*. Mutation of the *OsRBOH3* gene causes the reduction of ROS in the root hair tip (Wang et al. 2018). Functions for the other members in rice and other monocot species remain to be elucidated.

In this study, we investigated the functions of *OsRBOH* genes at the genome level and researched the mechanism of root hair development associated with these genes. We analyzed the expression patterns of these genes in response to the treatment of auxin, a hormone known to be involved in root hair and root growth in rice. Based on this, we identified the functional relationship between seven *OsRBOH* genes and auxin. Among these, three genes showed root hair preferred expression patterns and we analyzed their promoters for cis-acting regulatory elements (CREs). We also examined the relationship between ROS changes and root hair development after treatment with an *RBOH* inhibitor. Based on our findings, we propose diverse roles for *OsRBOH* genes in root hair development.

Results

Expression Profiling Analysis of Rice *RBOH* Family Revealed the Association of Three *RBOH* Genes in Root Hair Development

First, we made a phylogenic tree with nine *OsRBOH* family genes and divided them into three color-coded groups by using the information from a recent study (Kaur et al. 2018). Next, we analyzed meta-expression data for the nine *OsRBOH* genes in special and temporal samples based on Agilent 44K array data by incorporating the heatmap image into the context of the phylogenetic tree (Fig. 1A). As a result, we saw that *OsRBOH2* (*LOC_Os01g53294*), *OsRBOH3* (*LOC_Os01g61880*), and *OsRBOH5* (*LOC_Os05g45210*) were preferentially expressed in root hairs. In addition, *OsRBOH2* showed weak expression in stem and endosperm, *OsRBOH5* in inflorescence, palea, and lemma, and *OsRBOH3* in the leaf blade. Of these genes, *OsRBOH2* and *OsRBOH5* were clustered together in the phylogenetic tree, while *OsRBOH3* was clustered together with *OsRBOH4* showing relatively higher expression in inflorescence, embryo, and endosperm than other tissues/organs.

Next, to verify the meta-expression data, we performed quantitative real-time PCR (qRT-PCR) using six samples: shoots, roots, leaf sheaths, seeds, panicles, and root hairs. We first checked the quality of root hair samples by using *rice cellulose synthase-like D1* gene (*OsCSLD1*) as a positive marker for root hair preferred expression patterns and saw that *OsCSLD1* exhibited root hair preferred expression patterns (Fig. 1). We then tested the expression pattern of nine *OsRBOH* genes (Fig. 1). As a result, we found that *OsRBOH2*, *OsRBOH3*, and *OsRBOH5* were highly expressed in root hairs as expected, and *OsRBOH8* and *OsRBOH9* showed a relatively higher expression level in root hairs than in other tissues/organs. In addition, *OsRBOH1* and *OsRBOH6*

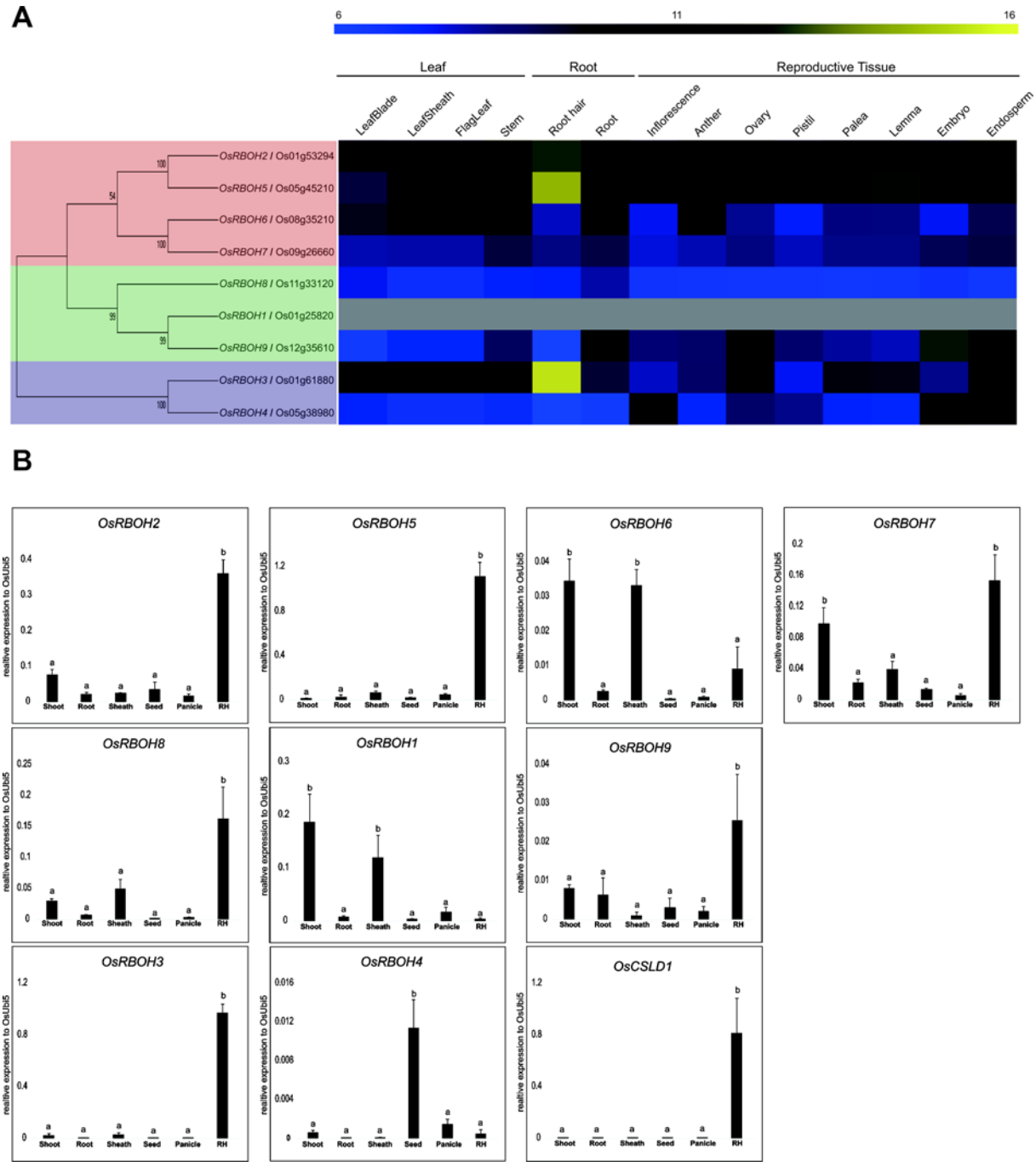

Fig. 1. Comparative meta-expression analysis of the nine *OsRBOH* genes and validation of expression patterns using qRT-PCR. (A) Microarray data displayed by using a Heatmap of *OsRBOH* genes in 14 tissue/organs. Yellow, high level of expression; blue, low level. Numeric values indicate an average of the normalized log2 intensity of microarray data. Heatmap data were combined with a phylogenic tree of rice *RBOHs*, which were divided into three groups by using the information from a recent study. (B) Validation of meta-expression patterns in six tissues/organs for nine *RBOH* genes in rice based on the qRT-PCR analysis. RH, root hair. Rice ubiquitin 5 (*OsUbi5, LOC_Os01g22490*) was used as internal control. Y-axis, expression level relative to *OsUbi5*; X-axis, samples used for analyses. Error bars represent the standard errors (SE) of three biological replicates. Significant differences are indicated by different letters. b, p-value < 0.01. Data were analyzed by employing a one-way ANOVA with repeated measures using a Tukey's pairwise comparison.

showed preferred expression patterns in shoots and leaf sheaths, and *OsRBOH4* in seeds, but *OsRBOH7* did not show preferred expression patterns in any tissues/organs (Fig. 1B). These results informed us that more than half of *OsRBOH* genes are related to root hair development.

Root Hair-Preferred RBOH Genes Contain Various CREs including RHE in the Promoter

We performed a promoter analysis to find the CREs that were conserved in the promoters of three *OsRBOHs*, *OsRBOH2*, *OsRBOH3*, and *OsRBOH5,* and associated with root hair preferred expression. The 2 kb upstream sequences of *OsRBOHs* and three Arabidopsis *RBOHs*, *AtRBOHC*, *AtRBOHH*, and *AtRBOHJ*, which are related to root hair elongation, were analyzed. This was accomplished by using the SOGO/PLACE database (Higo et al. 1999) and the Multiple Em for Motif Elicitation (MEME)-suite (Bailey et al. 2009) (Table S1). We first checked two well-known root hair *cis*-elements (RHEs) from three *OsRBOH* and three *AtRBOH* promoters showing root hair preferred expression and indicated them as yellow or red boxes in the promoter region (Fig. 2); the yellow box is RHE/WHHDTGNNNNKCACGWH and the red box is RHERPATEXPA7/KCACGW (Kim et al. 2006). The RHERPATEXPA7 CRE is a conserved sequence of root hair-specific cis-elements, from the Arabidopsis expansin 7 and its ortholog genes (PlantPAN; Plant Promoter Analysis Navigator). In the case of RHE, we found four RHEs in the *AtRBOHH* promoter, two in *AtRBOHC*, and one of each in *AtRBOHJ, OsRBOH3*, and *OsRBOH5* (Yellow box in Fig. 2). In the case of RHERPATEXPA7, we identified it five times in the *OsRBOH5* promoter, four times in the *AtRBOHH* promoter, and two times in each the *OsRBOH3* and *AtRBOHC* promoters, indicating that the expression of these *RBOH* genes might be regulated by known upstream TFs such as RSL class I bHLH proteins. Especially, multiple RHERPATEXPA7 elements in the promoter might play roles in the root hair preferred expression patterns of *OsRBOH3, OsRBOH5, AtRBOHC,* and *AtRBOHH*.

We also identified three conserved CREs among the promoters of *OsRBOH2, OsRBOH3,* and *OsRBOH5*: SGCCCGCGCC (Green in Fig. 2), GGTCASGCYC (Purple in Fig. 2), and GCCGKSGATC (Pink in Fig. 2). The first CRE was SGCCCGCGCC and there were seven in the *OsRBOH2* promoter, two in the *OsRBOH5* promoter, and one in the *OsRBOH3* promoter. An analysis using the Tomtom tool of the MEME-suite indicated that this CRE is mainly present in AP2/EREBP TFs in *A. thaliana*. AP2/EREBP TFs regulate the gene expression in plants under various stress conditions (Chen et al. 2016; Yamaguchi-Shinozaki and Shinozaki 2006). We speculated that the expression of three *OsRBOH* genes through SGCCCGCGCC in root hairs would be involved in root hair elongation in a variety of stress situations encountered during rice growth. The second CRE is GGTCASGCYC. We found this CRE twice in the *OsRBOH2* promoter and once in the *OsRBOH3, OsRBOH5*, and *AtRBOHJ* promoters, suggesting that new cis-acting elements for root hair preferred expression were conserved between rice and Arabidopsis. As a result of a Tomtom search, we found that this CRE exists in the Arabidopsis TEOSINTE BRANCHED 1, CYCLOIDEA, PCF1 (TCP) TF gene promoter. The TCP gene with this CRE is involved in plant growth and development (Cubas et al. 1999; Koyama et al. 2010). The third CRE is GCCGKSGATC which occurs three times in *OsRBOH2*, twice in *OsRBOH5*, and once in *OsRBOH3*. In addition, this CRE exists in Arabidopsis TCP and AP2/EREBP TF gene promoters. Functions of these potential CREs should be clarified through further studies.

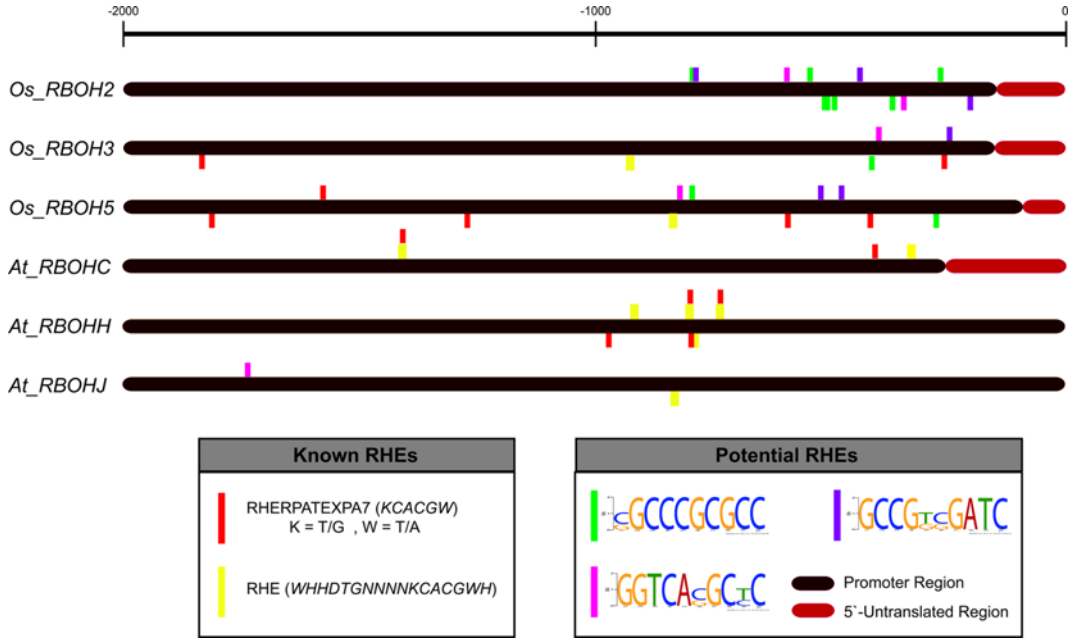

Fig. 2. Identification of *cis*-acting regulatory elements (CREs) responsible for preferred expression patterns of rice and Arabidopsis *RBOH* genes. Mapping the location, names, and logos of two well-known root hair *cis*-elements (RHE) CREs and three CREs, which we found in promoters of *OsRBOH2, OsRBOH3, OsRBOH5*, *AtRBOHC, AtRBOHH,* and *AtRBOHJ.* The number of scale bars above the figure shows the upstream position of the promoter basepair when taken at +1 of ATG. We analyzed up to the upstream 2,000 base pair.

Phenotype Differences and ROS Changes in the Seminal Roots and Root Hair_Tips After Treatment with an NADPH Oxidase Inhibitor and Auxin

One of the most distinctive features of root hair formation is the rapid elongation of the hair via tip growth. ROS homeostasis has been demonstrated to play a critical role during root hair development in Arabidopsis. To determine whether *OsRBOH*-mediated ROS balance and distribution are also important in rice root hair formation, we treated 5-day-old seedlings with the NADPH oxidase inhibitor DPI, which reduces the level of O^{2-}. Since root hairs in rice roots actively grow out in both elongation and maturation zones of the root, we closely observed these zones using an optical microscope and laser scanning confocal fluorescence microscope. When observed under an optical microscope, the root hair of the seminal root of rice grown for 5 days in DPI condition, tended to decrease in length and density, compared to the control (Fig. 3A, C). In addition, ROS staining with 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA) solution of non-treated samples clearly showed a higher level of ROS in the root epidermis and root hair tips (Fig. 3D). In contrast, DPI treated roots and root hairs were almost devoid of detectable levels of ROS (Fig. 3F; Fig. S2). We also observed root hair of rice grown in auxin treated media for 5-days to demonstrate the association of auxin and root hair in rice. As we expected, the root hairs of the seminal root treated with auxin showed the significant increase in length and density, compared to control (Fig. 3A, B). ROS level stained by H_2DCF-DA increased than control, showing a tendency to increase not only in the root hair tip but also root hair as a whole (Fig. 3E, F). Based on previous reports, the radical cell pattern seen in the root epidermis is predicted to be trichoblast, the root hair initiating cell (Kim et al. 2007; Moon et al. 2019; Salazar-Henao et al. 2016). Therefore, our observation that ROS generation in the trichoblast cells and the tip of the root hair is activated by auxin and inhibited by RBOH inhibitor, DPI, confirms the association of RBOH with ROS generation in rice root hair development. These data indicated that ROS homeostasis and distribution are critical for root hair differentiation and elongation similar to Arabidopsis root hairs.

Most of the *OsRBOHs* Showed Transcriptional Alteration in Root Hair in Response to Auxin

It is well known that root hair-specific TFs and its downstream targets are positively or negatively regulated by auxin during root hair development. To determine if *OsRBOHs* are differentially regulated by auxin, the effect of auxin treatment on steady-state levels of *OsRBOHs* mRNA was investigated. cDNA was isolated from auxin-treated 3-day old root hairs and compared to those without treatment. *OsIAA9*, an auxin response gene, was used as a positive marker gene for this analysis (Fig. 4). The transcriptional level of *OsIAA9* was highly induced by exogenously applied auxin, demonstrating that the concentration of auxin used in this study could activate the auxin signaling pathway. The expression of *OsRBOH5* and *OsRBOH6* did not show any detectable changes

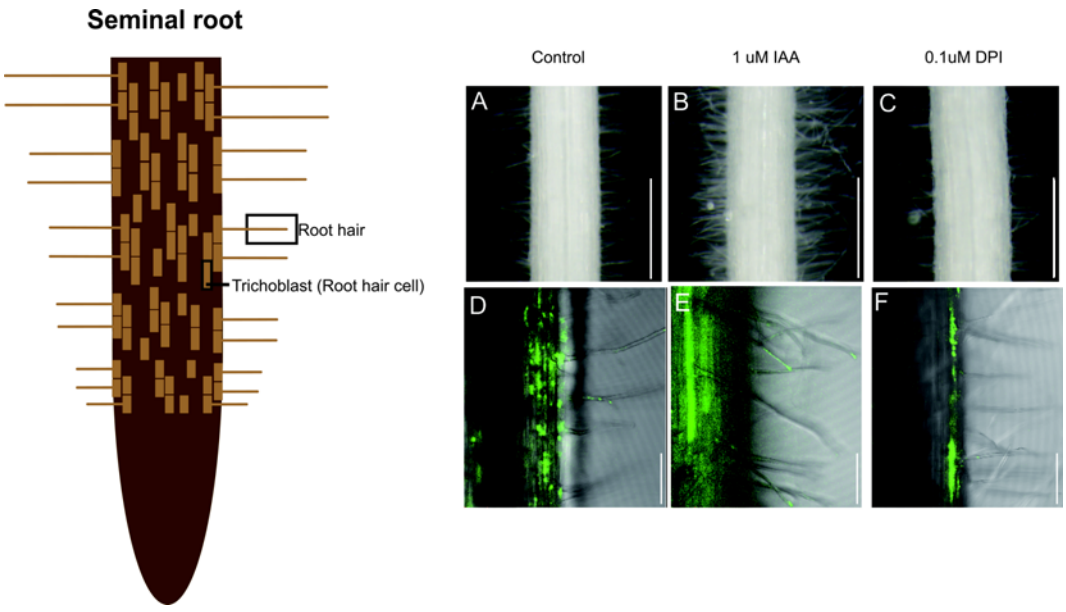

Fig. 3. Analysis of variation in the phenotype and ROS content of root hairs with or without diphenylene iodonium (DPI) and auxin treatment by using optical microscope and H_2DCF-DA staining followed by laser scanning confocal fluorescence microscope. (A-C) Changes in length and density of root hair. (D-F) Changes of ROS content observed in root hair of seminal roots. The figure on the left side is a schematic view showing the trichoblast (root hair cell) and root hairs on the roots. A-C, Bar = 0.5 mm, D-F, Bar = 100 μm.

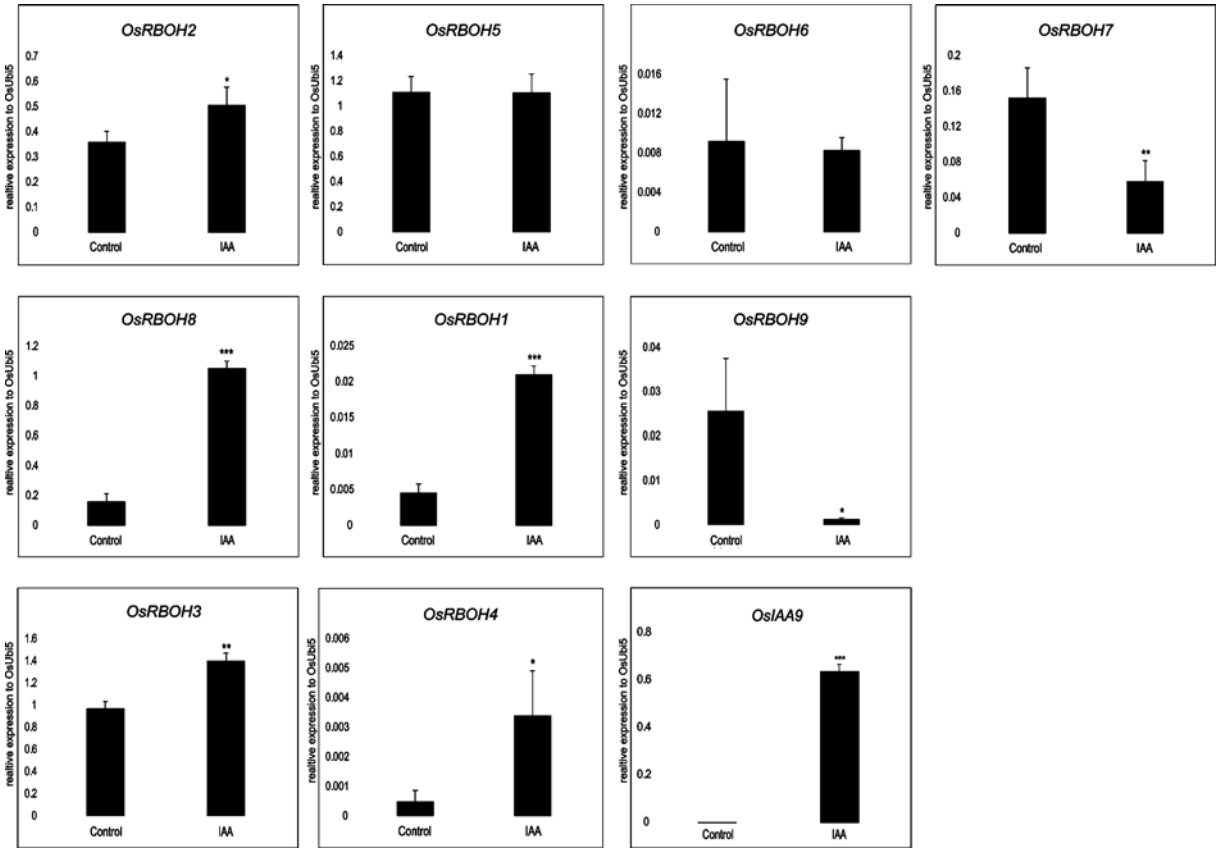

Fig. 4. Expression analysis of nine *RBOH* genes in root hairs using qRT-PCR compared with the control group after treatment with exogenous auxin. The *RBOH* genes were arranged in groups shown in the phylogenic tree of Fig. 1A. Rice ubiquitin 5 (*OsUbi5, LOC_Os01g22490*) was used as an internal control. Y-axis, expression level relative to *OsUbi5*; X-axis, samples used for analyses. Error bars represent the standard errors (SE) of three biological replicates. ***, p-value <0.001, **, p-value <0.01, *, p-value <0.05, based on a t-test.

under auxin treatment (Fig. 4). In contrast, the transcriptional levels of *OsRBOH1*, *OsRBOH4*, and *OsRBOH8* were highly induced (over 6-fold) by auxin treatment. Among three root hair-specific genes, the steady-state levels of *OsRBOH2* and *OsRBOH3* were slightly increased (1.4 fold) by auxin treatment. Interestingly, the expression of *OsRBOH7* and *OsRBOH9* were noticeably downregulated by 2.59 and 20.6-fold, respectively. Most members of the rice *RBOH* family are positively (five) or negatively (two) regulated by auxin treatment.

Discussion

With an increase in the human population and climate change, the global production of major crops needs to be improved. Previous studies clearly demonstrated that rice roots can play an important role in response to biotic and abiotic stresses (Suzuki and Mittler 2006; Wang et al. 2013). Plants have to absorb nutrients and water effectively through roots to sustain a healthy life and increase productivity. Unfortunately, compared to Arabidopsis, the molecular mechanism of root hair development in rice is poorly understood.

Using the transcriptome data of root hairs from rice seedlings, we have identified three root hair preferred *RBOH* genes (*OsRBOH2, OsRBOH3,* and *OsRBOH5*) and confirmed their expression patterns through qRT-PCR analysis. These *OsRBOHs* have orthologs in *A. thaliana*, *AtRBOHF, AtRBOHH, AtRBOHI*, and *AtRBOHJ* (Mangano et al. 2017) (Fig. S1). Of these, *AtRBOHH* and *AtRBOHJ* are involved in ROS-mediated root hair growth in Arabidopsis (Mangano et al. 2017). In addition, promoters of these genes retain a putative RSL4 response element (RSL4-RE) which is very similar to the RHE. In the case of rice orthologs, *OsRBOH5* has an RHE in the promoter, which suggests a conserved mechanism for the development of root hairs between rice and Arabidopsis (Fig. 2). Moreover, the *OsRBOH5* promoter has five RHERPATEXPA7 which is another RHE for root hair-specific expression (Kim et al. 2006). The *OsRBOH3* promoter retains two RHERPATEXPA7 for root hair-specific expression but *OsRBOH2* did not have known *RHEs* in the promoter. Instead, we identified three potential CREs conserved in their promoters which exist in Arabidopsis TCP or AP2/EREBP TF gene promoters (Fig. 2). Thus, we anticipated that these *OsRBOH* genes would be involved in transcriptionally regulating the

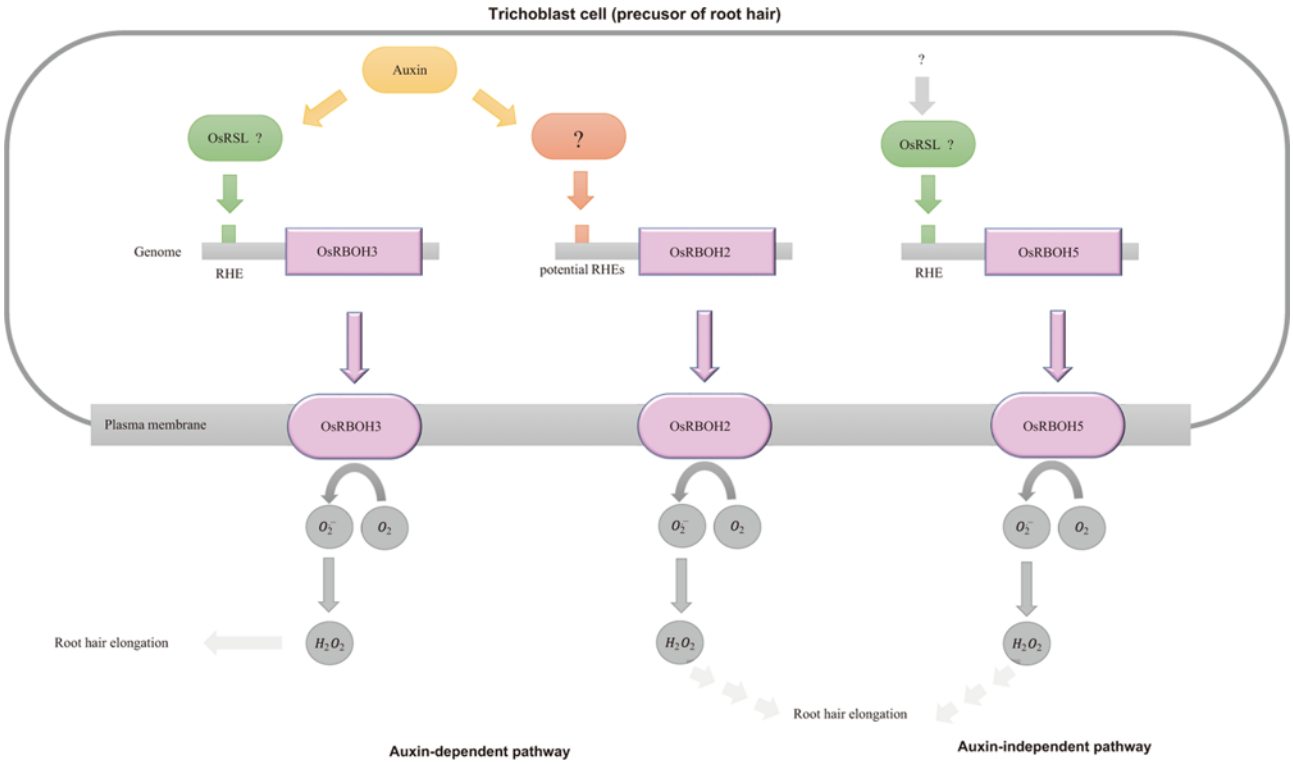

Fig. 5. A model summarizing auxin-RHE mediated expression mechanism for three rice *RBOH* genes with high expression in root hair cells. This model is based on the auxin-mediated root hair growth model of Arabidopsis.

initiation and elongation of root hairs via the RHE or other CREs.

To determine whether *RBOHs* function in the entire roots or not, we observed changes in ROS when DPI, an inhibitor of RBOH, was applied to rice roots and root hairs. Strong ROS signals were observed in trichoblast cells in the maturation zone of the root and the tip part of the root hair. In addition, rice seedlings containing auxin showed increased length and density of root hair as well as accumulated ROS, indicating that RBOH, which produces ROS, is closely related to the growth of root hairs. Previous studies have shown that *AtRBOHC*, *AtRBOHJ*, and *AtRBOHH* affect root hair elongation by producing ROS in Arabidopsis, and the expression of *AtRBOH* is affected by TFs, RSLs, and auxin (Mangano et al. 2017). Moreover, it was recently reported that *OsRBOH3* affected root hair initiation and elongation (Wang et al. 2018). We hypothesized that highly expressed *RBOH* in rice root hairs would act by a mechanism similar to the model for *RBOH*-mediated root hair development in Arabidopsis. Based on comparative analysis with Arabidopsis and rice *RBOH* genes, we found that *OsRBOH2* and *OsRBOH5* are orthologs of *AtRBOHE* and *AtRBOHF,* and *OsRBOH3* is an ortholog of *AtRBOHH* and *AtRBOHJ* (Fig. S1). In response to auxin treatment, expression of *OsRBOH2* and *OsRBOH3* was slightly increased (Fig. 4), indicating that these are in the auxin-responsive regulatory pathway. However, *OsRBOH5* did not change in response to auxin treatment, indicating that this gene might function in an auxin-independent pathway. Out of the rice *RBOH* genes (*OsRBOH7, OsRBOH8,* and *OsRBOH9*) with a relatively high level of expression in root hairs, *OsRBOH8* showed a significant upregulation in response to auxin treatment, indicating that this RBOH might be a main regulator of auxin-dependent root hair development. In contrast, *OsRBOH7* and *OsRBOH9* were downregulated in response to auxin treatment, suggesting their roles as fine-tuning members of aux in-dependent root hair development by other *RBOH* genes in rice.

Together with CREs analysis, we have created a root hair model associated with *RBOH* family genes in rice (Fig. 5). Although this model is focused on the auxin response, ROS, and *RBOH*, it can be a useful basis for future research on rice root hair development. In addition, Arabidopsis *RBOH* genes also play an important role in the pollen tube, which can also be used as a model for rice pollen tubes and other expanding tissues.

Materials and Methods

Multiple Sequence Alignment and Phylogenetic Tree Analysis

To perform a phylogenomic analysis of *RBOHs* in rice, we collected the amino acid sequences of nine family members from the Rice Genome Annotation Project (RGAP) using locus IDs (http://rice.plantbiology.msu.edu/). The multiple-alignment of amino acid sequences

was conducted with the ClustalW program (Higgins et al. 1996) under the following parameters: gap opening penalty of 10 and gap extension penalty of 0.2. The phylogenic analysis was performed by using MEGA 7.0 under the following parameters: Neighbor-Joining tree method, complete deletion, and bootstrap with 1000 replicates (Kaur and Pati 2016). We divided the *OsRBOH* proteins into three subfamilies based on previous studies on *RBOH* classification (Kaur et al. 2018).

Meta-Analysis of Tissue-Specific Expression Profiles

We used a meta-analysis of tissue-specific expression profiles based on Agilent 44K array data (GSE21396, GSE109811, and GSE111350). This Agilent 44K array data can be found in NCBI's 'GEO Accession viewer' (https://www.ncbi.nlm.nih.gov/geo/). We then uploaded the $\log_2$ normalized intensity data in a tab-delimited text format into Multi Experiment Viewer (MeV, http://www.tm4.org/mev/). The generated heatmap and the image were edited by Adobe illustrator (Fig. 1A). The $\log_2$ expression value was set between 6 to 16 and the mean value was 11.

Plant Materials

To sample rice root hairs, we sterilized rice seeds (*Dongjin* variety) with 50% sodium hypochlorite for 30 min with gentle shaking. Rice seeds were then germinated on Murashige and Skoog (MS) media under controlled conditions (28/25°C day/night, 8-h photoperiod, and 78% relative humidity) for 3 days. The seminal root samples were cut and directly frozen in liquid nitrogen and the root hairs were swept with a brush for sampling. Samples were also prepared from six other tissues (7-day-old shoots and roots, 10-day-old leaf sheath, young panicle, and seeds).

RNA Extraction, RT-PCR, and qRT-PCR Analyses

For RNA extraction, RT-PCR, and qRT-PCR, all tissue and organ samples except root hairs were frozen in liquid nitrogen and ground with a Tissue Lyser II (Qiagen, Hilden, Germany). All samples were then extracted with an RNAiso Plus Kit according to the manufacturer's protocol (Takara Bio, Kyoto, Japan). To make cDNA by RT-PCR, we used 2×SuPrime Script RT-PCR Premix (GeNet Bio, Nonsan-Si, Korea). The PCR conditions included 22-38 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min 30 s. qRT-PCR was performed using a Rotor-Gene Q instrument system (Qiagen). The *rice ubiquitin 5* gene (*OsUbi5, LOC_Os01g22490*) was used as an internal control. We used 2× Prime Q-Mastermix with SYBR Green (GeNet Bio) as the qRT-PCR buffer and cycling conditions were set at 95°C for 15 s, 56°C for 30 s, and 72°C for 60 s for 23 cycles. Relative transcript levels and fold changes were calculated by the $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ methods (Schmittgen and Livak 2008). *OsCSLD1* was used as a positive marker gene to identify the quality of root hair samples (Kim et al. 2007). All primers used for analysis are listed in Table S2. Primer sets for *OsRBOH1* to *OsRBOH8* were those used in previous studies (Yang et al. 2014). We constructed three repeat samples for each tissue/organ and calculated the average of the qRT-PCR results.

Auxin Treatment

For auxin treatment in rice, we prepared MS media with 1 μM indol-3-acetic acid (IAA; Sigma-Aldrich, St. Louis, USA) and the germinated seeds were grown for 5 days under controlled conditions (28/25°C day/night, 8-h photoperiod, and 78% relative humidity). To determine the best IAA concentration which maximizes the length and density of root hairs, we observed the growth of root hairs after growing the rice in a medium with control, 0.1, 1, 10, and 30 μM IAA. As a result, the density and length of root hairs were maximized in MS media with 1 μM IAA. We then conducted further analyses with IAA treatment at this concentration. Three biological replicate samples were prepared for the experimental control and the IAA treated plants. Based on previous research, *OsIAA9* (*LOC_Os02g56120*) was used as an auxin response marker gene to identify the quality of auxin treatment (Jain et al. 2006). All primers used for analysis are listed in Table S2.

DPI Treatment, Root Hair Observation and ROS Detection

The RBOH inhibitor, DPI was purchased from Sigma-Aldrich. We made a medium with a DPI concentration of 0.1 μM as used previously (Achard, et al. 2008). DPI was dissolved in dimethyl sulfoxide (DMSO), diluted with diethyl pyrocarbonate (DEPC) and Murashige and Skoog (MS) media used as the experimental control. Plants were grown in MS media for 3 days and be transferred with DPI media for 2 days in controlled conditions (28/25°C day/night, 8-h photoperiod, and 78% relative humidity). The assayed roots were photographed with a SZX61 microscope (Olympus, Tokyo, Japan). ROS was detected after treatment. For ROS detection, we measured the production of hydrogen peroxide using 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Sigma-Aldrich). The H_2DCF-DA staining was conducted according to the manufacturer's instructions. Fluorescence in living tissue was detected using a confocal laser scanning microscopy (LSM 510 META; Carl Zeiss, Jena, Germany). Green fluorescent protein (GFP) was detected using 488-/505- to 530-nm excitation/emission filter sets. Fluorescence images were digitized with the Zeiss LSM image browser. More than five plants were examined and the experiment was biological repeated three times.

Cis-Acting Regulatory Element Analysis

The 2 kb upstream promoter region sequences for three rice *RBOH* and three *RBOH* genes of *A. thaliana* were extracted from Phytozome (Goodstein, et al. 2011). SOGO (previously PLACE: A database of plant cis-acting regulatory DNA elements) was used to find already known RHEs (Higo et al. 1999). The MEME-suite was used to identify CREs conserved in the promoters and the resulting CREs were further analyzed using FIMO to find individual motif occurrences and Tomtom tools were used to compare a queried CRE against a database of known CREs. With regards to the 5′ to 3′ direction, the elements shown on the upper strand of the promoter indicate that they are located in the forward direction and the elements shown on the lower strand are in the reverse direction.

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Authors' Contributions

EJK, YJK, WJH, JSJ, and KHJ designed experiments; EJK, YJK, and WJH carried out experiments and analyzed data in figures and tables; EJK, YJK, WJH, CL, JSJ, and KHJ wrote the manuscript. All the authors agreed on the contents of the paper and post no conflicting interest.

Supporting Information

Fig. S1. Phylogenetic tree and ortholog analysis of nine *RBOH* genes of *Oryza sativa* and ten *RBOH* genes of Arabidopsis.
Fig. S2. A fluorescence microscope photograph showing the extent of

ROS change in the root hair when plants were treated with DPI, which focuses on the root hair tip.

Table S1. Chromosomal location information of *cis*-acting regulatory element (CRE) analysis of three highly expressed *OsRBOH* genes and three *AtRBOH* genes in root hairs.

Table S2. Information of gene locus identifiers (IDs) used in this study and primer sequences used for qRT-PCR analysis.

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