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Comparative analyses of ROS-producing enzymatic activity of Arabidopsis NADPH oxidases

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Running head: Comprehensive comparative analysis of AtRbohs

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Summary

Reactive oxygen species (ROS) produced by NADPH oxidases, respiratory burst oxidase homologs (Rbohs), play crucial roles in development as well as biotic and abiotic stress responses in plants. *Arabidopsis* has ten *Rboh* genes, *AtRbohA* to *AtRbohJ*. Five *AtRbohs* (*AtRbohC*, *D*, *F*, *H* and *J*) are synergistically activated by Ca^{2+} and protein phosphorylation to produce ROS that play various roles *in planta*, although the activities of the other Rbohs remain unknown. With a heterologous expression system, we found a range in ROS–producing activity among the AtRbohs with differences up to 100 times, indicating that the required ROS amount is different in each situation where the AtRbohs act. To specify the functions of AtRbohs involved in cell growth, we focused on AtRbohC, H and J, which are involved in tip growth of root hairs or pollen tubes. Ectopic expression of the root hair factor *AtRbohC*/*ROOT HAIR DEFECTIVE 2* (*RHD2*) in pollen tubes restored the *atrbohH atrbohJ* defects in tip growth of pollen tubes. However, expression of *AtRbohH* or *J* in root hairs did not complement the tip growth defect in the *atrbohC/rhd2* mutant. Our data indicate that Rbohs possess different ranges of enzymatic activity, and that some Rbohs have evolved to carry specific functions in cell growth.

Introduction

Aerobic organisms need to deal with unavoidable generation of reactive oxygen species (ROS) by aerobic respiration, including superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$), because they can damage DNA, protein, and lipids, and thus are highly toxic in cells (Kehrer, 2000). This situation is particularly severe in plants due to ROS generation through photosynthesis. Recent findings have revealed that most organisms produce ROS by several kinds of enzymes and use them as signaling molecules for environmental responses and development (Kärkönen and Kuchitsu, 2015). In plants, ROS are involved in numerous processes such as responses to disease resistance (Levine *et al.*, 1994), salinity stress responses (Kurusu et al. 2015; Hossain and Dietz, 2016), cell growth by scission or cross-linking of cell wall (Everdeen *et al.*, 1988; Fry, 1998; Schweikert *et al.*, 2000; Schopfer, 2001; Rodríguez *et al.*, 2002; Schopfer *et al.*, 2002), root gravitropism (Joo *et al.*, 2001), lateral root emergence (Orman-Ligeza *et al.*, 2016), cell-fate transition in roots (Tsukagoshi *et al.*, 2010), development of male and female gametophytes (Jiménez-Quesada *et al.*, 2016), mutualistic symbiosis with fungus (Tanaka *et al.*, 2006), long distance signal transduction (Miller *et al.*, 2009), development of Casparian strips (Lee *et al.*, 2013) and organ abscission (Lee *et al.*, 2018).

NADPH oxidases (Nox) are ROS-producing enzymes conserved in many eukaryotes and play roles in signal transduction in defense and developmental processes. Nox proteins carry six transmembrane domains and a conserved C-terminal region containing the NADPH binding and FAD binding motifs, which are crucial for transferring

electrons to generate superoxide anions (Sumimoto, 2008). The mammalian gp91^{phox}/Nox2, acting together with p22^{phox}, requires other protein components such as p47^{phox}, p67^{phox}, and Rac small GTPase for its activation, but plants do not appear to carry the clear homologues of p47^{phox} or p67^{phox} (Sumimoto, 2008). Generally, plant Nox proteins, called respiratory burst oxidase homologues (Rbohs), carry two EF-hand motifs and phosphorylation sites at the cytological N-terminal region, in addition to the transmembrane domains and C-terminal region (Fig. 1A; Sumimoto, 2008; Oda *et al.*, 2010; Kärkönen and Kuchitsu, 2015). This indicates that organisms have established their own regulatory system to activate the Nox enzymes during evolution.

Rboh genes have been first isolated from rice and then from *Arabidopsis thaliana* as homologues of mammalian *gp91^{phox}/Nox2* (Groom *et al.*, 1996; Torres *et al.*, 1998). So far nine and ten *Rbohs* have been identified in rice and *Arabidopsis*, respectively (Kaur *et al.*, 2014; Kaur and Pati, 2016). They have also been identified in other plants including tobacco, potato, tomato, *Medicago*, and maize, where they regulate pathogen defense, abiotic stress responses, symbiosis or cell growth (Yoshioka *et al.*, 2001; Simon-Plas *et al.*, 2002; Yoshioka *et al.*, 2003; Liszkay *et al.*, 2004; Sagi *et al.*, 2004; Marino *et al.*, 2011; Nestler *et al.*, 2014). The ten *Rboh* genes in *Arabidopsis* are referred to as *AtRbohA* to *AtRbohJ*, and genetic studies have suggested their distinct roles (Table 1).

While the spatio-temporal control of *AtRboh* expression is important for their functions (Morales *et al.*, 2016; Orman-Ligeza *et al.*, 2016), control of enzymatic activity of AtRboh proteins is also crucial at a cellular level, as high levels of ROS can be toxic for cells (Yun *et al*., 2011; Kadota *et al*., 2014). Their activities can be successfully analyzed by heterologous expression in the human embryonic kidney (HEK) 293T cell line (Ogasawara *et al.*, 2008), which lacks expression of endogenous NADPH oxidases, *Nox1* and *Nox2,* and does not produce significant amounts of extracellular ROS (Shiose *et al.*, 2001). Transient expression in HEK293T cells has shown that Ca^{2+} binding to the EF-hand and protein phosphorylation synergistically activate the ROS-producing activity of AtRbohC/ ROOT HAIR DEFECTIVE 2 (RHD2), AtRbohD, AtRbohF, AtRbohH and AtRbohJ (Ogasawara *et al.*, 2008; Takeda *et al.*, 2008; Kimura *et al*., 2012; Kaya et al., 2014). Co-expression analyses of AtRbohs and their putative regulators such as AtCIPK26 or AtSRC2 have also suggested regulatory mechanisms of the activity of AtRboh proteins (Kawarazaki *et al.*, 2013; Kimura *et al.*, 2013). However, the ROS-producing activity of other AtRbohs remains unclear.

Here we examined the enzymatic activity of all 10 AtRbohs in the HEK293T cell line to compare their ROS-producing ability. Our data revealed striking differences in the ROS-producing activity of these enzymes. Moreover, we performed complementary analysis of AtRbohs involved in tip growth of pollen tubes or root hairs, indicating the functional diversification of AtRbohs during evolution.

Results and Discussion

Comparison of the ROS-producing enzymatic activity among AtRbohs

To compare the ROS-producing enzymatic activity of AtRbohs, we used the heterologous expression system with HEK293T cells. Coding sequences of *AtRboh* were fused to a FLAG epitope tag (Fig. 1A) to detect expressed proteins by Western blot analysis, confirming comparable expression in HEK293T cells (Fig. 1B). After baseline measurements of ROS production for 5 min, 1 μM of ionomycin, a Ca^{2+} ionophore that induces Ca^{2+} influx into the cells, was added to medium. Ionomycin triggered rapid and transient ROS production in all *AtRboh*-transfected cells, but not in empty vector-transfected controls (Fig. 1C-E), suggesting that Ca^{2+} influx induces the ROS-producing enzymatic activity of AtRbohs. The AtRbohH carried the highest activity, followed by AtRbohJ, C and A (Fig. 1C). AtRbohA, D, E and G produced around 10 times less ROS, and AtRbohG, F, I and B did further less (Fig. 1D and E). The specific ROS-producing activity varies 100-fold between different AtRbohs in HEK293T cells (Fig. 1F).

Ca^{2+}-regulation in ROS production is conserved among AtRbohs

The cytosolic N-terminal region of AtRbohs contains two EF-hand motifs, where the conformation changes upon Ca^{2+} binding, leading to the activation of ROS-production (Ogasawara *et al.,* 2008; Oda *et al.*, 2010). Point mutation in the conserved glutamic acid (E at the position –Z) of the 1st EF-hand Ca^{2+}-binding loop reduces Ca^{2+} affinity and significantly abolish the ROS-producing activity of AtRbohC/RHD2, AtRbohD, F, H, and J (Ogasawara *et*

al., 2008; Takeda *et al.*, 2008; Kimura *et al*., 2012; Kaya *et al.*, 2014). To examine whether the importance of this amino acid residue is conserved among AtRbohs, we analyzed the ROS-producing activity of mutated AtRbohs where the E at the position –Z was converted to glutamine. Mutated proteins of AtRbohA, B, E, and G showed reduced ionomycin-triggered ROS-producing activity, without affecting the level of expression (Fig. 2A–D). The E residue at the –Z position of the 1st EF-hand is predicted to bind Ca^{2+}, inducing a conformational change of the EF-hand region (Ogasawara *et al.*, 2008; Oda *et al.*, 2010). Together with the previous data, our results suggest that Ca^{2+} binding to the EF-hand motifs is a common regulatory mechanism for ROS production of AtRbohs.

Alignment of the two EF-hand motifs of AtRbohs revealed that AtRbohI carries unique amino acid residues and lacks the conserved aspartic acid (D) in the first EF-hand motif (Fig. 3A), which is involved in Ca^{2+} binding (Oda *et al.*, 2010). This may cause the low ionomycin-induced ROS production activity of AtRbohI in HEK293T cells (Fig. 1E). Point mutation of this conserved aspartic acid (D) residue in the other AtRbohs resulted in the decrease in Ca^{2+} affinity and the ROS-producing activity, indicating the importance of this residue in Ca^{2+} binding and Ca^{2+}-triggered activation (Ogasawara *et al*., 2008; Kimura *et al*., 2012; Kaya *et al.*, 2014). We generated AtRbohI_ins268D mutant, which has an additional D to mimic the sequence of the conserved EF-hand motif. The insertional mutation did not affect the level of expression (Fig. 3B), and contrary to our expectation, the mutated protein showed lower enzymatic activity, rather than improving the ionomycin-triggered ROS-production (Fig. 3C).

In the second EF-hand motif of AtRbohI, the conserved glycine (G318) is converted to glutamine, and two additional amino acids (alanine and proline) are inserted (Fig. 3A). To examine whether these variations in EF-hand motifs of AtRbohI contribute to the ionomycin-triggered ROS-producing activity, we generated AtRbohI_Q318G and AtRbohI_ins268D_Q318G mutated proteins (Fig. 3A). These mutations did not affect the level of protein expression (Fig. 3B). AtRbohI_Q318G mutation increased the overall activity and did not affect the response to ionomycin (Fig. 3C), suggesting that the conserved G318 is not critical in the Ca^{2+} response. The AtRbohI_ins268D_Q318G mutants showed reduced ROS-producing activity similarly to AtRbohI_ins268D (Fig. 3C), indicating that the insertion of the conserved D to mimic the conserved EF-hand motif is rather inhibitory to the ROS-producing activity of AtRbohI. Taken together, AtRbohI has unique EF-hand structure and carries lower ionomycin-triggered ROS-producing activity.

Protein phosphorylation-induced activation is conserved among all AtRbohs

Previous studies have shown that protein phosphorylation of several AtRbohs stimulate the ROS–producing activity. Calyculin A (CA), a protein phosphatase inhibitor, induces ROS production in the HEK293T cells transfected with *AtRbohC/RHD2, AtRbohD, F, H* or *J* (Ogasawara *et al.*, 2008; Takeda *et al.*, 2008; Kimura *et al*., 2012; Kaya *et al.*, 2014), suggesting that AtRbohC, D, F, H and J are activated by protein phosphorylation. Several putative phosphorylation sites including serine residues in the N-terminal cytosolic region have been proposed to be critical for activation (Takeda *et al.*, 2008; Dubiella et al., 2013;

Kadota *et al.*, 2014; Li *et al.*, 2014; Chen et al., 2017). To investigate whether phosphorylation-dependent activation is a common regulatory mechanism in all AtRbohs, we comparatively examined the CA-induced ROS production in HEK293T cells transfected with each AtRboh. As shown in Fig. 4, all AtRbohs showed CA-induced ROS producing activity with strikingly difference, although the protein expression level did not differ much in the HEK293T cells (Fig. 1B). AtRbohA showed the highest activity and AtRbohB did the lowest. CA induced rapid and transient ROS production that decreased within a couple of minutes in AtRbohA, whereas the other AtRbohs showed sustained ROS production for at least 30 minutes after CA application (Fig. 4). Together with the results in response to ionomycin, our data suggest that each AtRboh possess different activity indicating different responses to Ca^{2+} and protein phosphorylation.

Functional conservation of AtRbohs involved in tip growth

To investigate the functional conservation of AtRbohs in plant development, we comparatively analyzed AtRbohC, H and J that are involved in tip growth of plant cells. AtRbohC/RHD2 locates to the root hair tip, where it maintains the tip growth (Foreman *et al.*, 2003; Takeda *et al.*, 2008), and AtRbohH and J, localized to the tip of pollen tubes, regulates their tip growth in a similar manner (Kaya *et al.*, 2014). Root hairs and pollen tubes develop into long tubular structures, which allow them to play critical roles for plants: root hairs absorb water and nutrients from soil and are also involved in plant-microbe interactions, while pollen tubes deliver sperm nuclei to the female gametophyte. Tip growth in these two types of cells

involves numerous common factors including Rboh proteins and ROS, Ca^{2+}, and ROP GTPase (Mangano *et al.*, 2016).

We expressed *AtRbohC, H* and *J* genes under the control of *cis*-regulatory sequences (promoter and terminator) of *AtRbohH* or *AtRbohJ* in the *atrbohH-3 atrbohJ-2* double mutant, where the tip growth of pollen tubes is impaired resulting in reduced seed production (Fig. 5) (Kaya *et al.*, 2014). Expression of either *AtRbohH* or *AtRbohJ* under the control of the *AtRbohH or AtRbohJ cis*-regulatory sequences complemented the defects of the double mutant in pollen tube elongation and seed number (Fig. 5). Intriguingly the expression of *AtRbohC/RHD2* under the control of the *AtRbohH or AtRbohJ cis*-regulatory sequences restored pollen tube growth and seed number in the *atrbohH-3 atrbohJ-2* double mutant (Fig. 5). This indicates that AtRbohC/RHD2 can compensate for the AtRbohH/J function.

Next we examined the functional replacement in root hairs. In the *atrbohC/rhd2-5* mutant, root hairs initiate but elongation by tip growth is impaired (Fig. 6, Supplementary Fig. S1). Expression of *AtRbohC/RHD2* under the control of the *AtRbohC/RHD2 cis*-regulatory sequences restored tip growth (Fig. 6, Figure S1). However, the root hair defects were not fully complemented with the expression of *AtRbohH* or *J* under the control of the same *AtRbohC/RHD2 cis*-regulatory sequences (Fig. 6, Supplementary Fig. S1). We examined ROS accumulation at the root hair tip. NBT staining was observed in the wild type and *AtRbohC*-expressing line but not in the *AtRbohH* and *J*-expressing lines (Figure S2 & S3).

Alignment of AtRbohC, AtRbohH and AtRbohJ shows some specific insertions/deletions (Figure S4) that may be involved in the different activation mechanism in root hairs and pollen tubes.

Pollen tubes deliver male nuclei to the female gametophyte, so tip growth of pollen tubes is critical for plant reproduction, while root hairs are more primitive somatic cells that absorb water and nutrient from soil. Phylogenetic analysis shows that AtRbohC and AtRbohH/J belong to different subfamilies (Figure S5). AtRbohC/RHD2 may be a primitive type so that it can function in the other cell types, while AtRbohH and AtRbohJ cannot function in root hairs because they have acquired specific function in later-arisen reproductive cells. This can be explained by different regulators of AtRboh protein, such as ROP (Rho in plants) GTPases, Ca^{2+}-dependent protein kinases, or other unknown factors, but this needs to be analyzed further in future.

AtRbohC, H, and J possessed higher ROS-producing activity, while AtRbohD and AtRbohF showed rather lower enzymatic activity. AtRbohC is root specific-, and AtRbohH and J are pollen specific-AtRbohs. AtRbohC, H and J are required continuously at the tip of growing root hair or pollen tube, where they may be involved in Ca^{2+}-dependent positive feedback regulation and/or cell-wall regulation for tubular morphogenesis. While AtRbohD and AtRbohF are expressed in many tissues throughout the plant, and they are important for abiotic and biotic stresses (e.g. AtRbohD is the main component of PAMPs-induced transient ROS production) (Torres *et al*., 2005; Kadota *et al*., 2015). We suppose that the amount of

ROS required for endogenous development and stress responses may be different, so that the activities of AtRbohs are different depending on their biological function.

Conclusions and perspectives

Rbohs are involved in ROS production in numerous aspects of plant life. Our results show that ROS-producing activity among ten AtRbohs is dramatically different in their regulation of Ca^{2+} in HEK293T cells, and suggest that all AtRbohs are activated by protein phosphorylation. We propose that each AtRboh is involved in tuning of ROS production at different sites. The point mutation in the regulatory motifs may at least in part account for the functional differentiation of AtRbohs during evolution. Complementation analysis revealed the functional conservation and distinction of the AtRbohs, suggesting that they have been modified to have specific roles in different cell types. Our data propose the functional diversification of Rboh proteins, and thus it would be interesting to comparatively examine activity and functions in other plants. Heterologous expression in HEK293T cells would be a powerful tool for these comparative studies.

Experimental procedures

Plasmid construction and transgenic lines

To express AtRboh in HEK293T cells with a 3xFLAG epitope tag, a synthetic DNA fragment of Kozak (GCCGCCACC)-3xFLAG epitope tag (Sigma-Aldrich)-*Bam*HI-*Eco*RV-stop(TAATAG) was inserted into *Nhe*I and *Kpn*I sites in

pcDNA3.1(-) vector (Invitrogen), resulting in pcDNA3.1-Kozak-3xFLAG vector. The CDS of *AtRboh* genes (except for *AtRbohG*) was PCR-amplified from a cDNA library and cloned into the *Bam*HI or *Eco*RV site of pcDNA3.1-kozak-3xFLAG vector. For *AtRbohG*, a synthetic gene for *Homo sapiens* was used. EF-hand mutants were generated by point-mutation primers and the mega-primer PCR method. Oligonucleotide primer sequences for all constructions are shown in Table S1. *AtRbohH-pro::AtRbohC::AtRbohH-ter* and *AtRbohJ-pro::AtRbohC::AtRbohJ-ter* constructs were generated by replacing the CDS of *AtRbohH-pro::AtRbohH::AtRbohH-ter or AtRbohJ-pro::AtRbohJ::AtRbohJ-ter* (Kaya *et al*., 2014) with the CDS of *AtRbohC*, and were expressed in *atrbohH3 atrbohJ-2* double mutant. *AtRbohC-pro::AtRbohC/AtRbohH/AtRbohJ::AtRbohC-ter* constructs were generated by cloning the promoter, terminator of *AtbohC* and CDS (*AtRbohC*, *AtRbohH* or *AtRbohH*) into pZP2H-lac binary vector for expression in *atrbohC/rhd2-5* mutant. These plasmids were introduced into plants by *Agrobacterium*-mediated transformation.

Cell culture and Transfection

HEK293T cells were maintained at 37°C in 5% CO_2 in Dulbecco's Modified Eagle's Medium nutrient mixture Ham's F-12 (WAKO, Japan) supplemented with 10% fetal bovine serum (HyClone). HEK293T cells were transiently transfected with *pcDNA3.1-3FLAG-AtRbohs* or empty vector by GeneJuice transfection regent (Novagen) according to the manufacturer's instructions.

Measurement of ROS production in HEK293T cells

The ROS producing activity of AtRbohs was assayed as described previously (Ogasawara *et al.*, 2008). ROS production was detected by a peroxidase-dependent luminol-amplified chemiluminescence technique. Chemiluminescence was measured for 1 second every 60 seconds at 37 °C using a microplate luminometer Centro LB960 (Berthold Technologies). ROS production was expressed in relative luminescence units (RLU) per second. Data are presented as the average of three samples in a representative experiment. We independently replicated this experiment more than five times with similar results.

Aniline Blue Staining

The aniline blue staining was performed as described previously (Kaya *et al.*, 2014). Briefly, pistils were collected 12 h after hand pollination and fixed in acetic acid: ethanol solution (25:75). The fixed pistils were softened in 1 N NaOH for 30 min at 60 °C and then stained with 0.01% aniline blue in 2% potassium phosphate buffer (K_3PO_4).

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Conflict of Interest:

The authors declare no conflict of interest.

Author contribution

H.K. designed the experiments. K.K. supervised this study. A.I., A.I., H.H., T.K., performed the measurement of ROS production. T.K., K.M., Y.Y., Y.M., A.N., contributed to the functional analysis of *AtRbohs* in plants. M.K. performed the phylogenetic tree analysis. H.K., S.T., M.K., S.K., M.A., K.K. wrote the manuscript.

Short supplementary material legends

Table S1 Oligonucleotide primer sequences used in this work.

Figure S1 Complementation analysis of *atrbohC/rhd2* mutant with *AtRbohH* and *J.*

Figure S2 Detection of ROS in root hair.

Figure S3 Expression analysis by RT-PCR in roots.

Figure S4 Alignment of AtRbohC, AtRbohH and AtRbohJ.

Figure S5 Phylogenetic tree of Rboh in plants.

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Figure Legends

Figure 1 Comparison of ionomycin-induced ROS producing activities among AtRbohs. (**A**) Schematic representation of the construct for AtRboh expression. AtRbohs carry N-terminal EF-hand motifs and C-terminal FAD– and NADPH–binding domains, connected by six transmembrane (TM) helices. (**B**) Western blotting analysis of the expression levels of N-terminal FLAG tagged AtRbohA−J proteins in transfected HEK293T cells. β-actin was used

as a loading control. (**C−E**) Ionomycin-induced ROS production in *AtRbohA−J* transfected HEK293T cells. Empty vector-transfected cells were used to measure an internal ROS producing activity in HEK293T. After baseline measurements of ROS production for 5 min, 1 μM ionomycin was added to culture medium. ROS production was measured by chemiluminescence and shown as relative luminescence units (RLU) per second. Results given are means ± standard error (SE; n=3). (**F**) Comparison of maximum ROS producing activities between *AtRbohA−J* transfected HEK293T cells. The *p*-values were determined using the Student's t-test (**p*<0.05, ***p*>0.05).

Figure 2 Effect of mutation in the canonical EF-hand motif of AtRbohA, AtRbohB, AtRbohE and AtRbohG on ionomycin-induced ROS production.

(**A−D**) Ionomycin-induced ROS production in *AtRbohA*-, *AtRbohB*-, *AtRbohE*-, *AtRbohG*- and their EF-hand mutant transfected HEK293T cells, respectively. The underlines on the amino acid sequences indicate the Ca^{2+}-binding loop in the 1st EF-hand motif in each AtRboh. The bold letters indicates the substitution of the bidentate (E) to the non-bidentate Asn (Q) in each EF-hand mutant. Open circle, wild-type Rboh; closed triangle, EF-hand mutant; open square, empty vector-transfected cells. The maximum value of the luminescence unit in wild type transfected cells was set at 1.0. Results given are means ± standard error (SE; n=3). The protein expression level of wild type and EF-hand mutant was checked with anti-FLAG antibody. β-actin was used as a loading control.

Figure 3 Effect of mutation in the non-canonical EF-hand motif of AtRbohI on ionomycin-induced ROS production. (**A**) The alignment of amino acid sequences of the Ca^{2+}-binding loop in the 1st and 2nd EF-hand motif of 10 AtRbohs. The Ca^{2+}-binding loops in the EF-hand motifs are underlined. The consensus Ca^{2+}-binding site is shown: first (X), third (Y), fifth (Z), seventh (#), ninth (**-**X)**=**Ca^{2+} ligand; twelfth (-Z)**=**Ca^{2+} ligand, a bidentate Glu (E); fifth (G)**=**Gly; eighth (I)**=**Ile or other aliphatic residues are found at this position; *, any residue. (**B**) Western blotting analysis of the expression levels of N-terminal FLAG tagged wild-type *At*RbohI and the EF-hand mutant proteins in transfected HEK293T cells. β-actin was used as a loading control. (**C**) Ionomycin-induced ROS production in wild-type *AtRbohI* or the EF-hand mutant transfected HEK293T cells. Empty vector-transfected cells were used to measure an internal ROS producing activity in HEK293T. Results given are means ± standard error (SE; n=3).

Figure 4 Comparison of calyculin A-induced ROS producing activities among AtRbohs. Calyculin A-induced ROS production in *AtRbohA−J* transfected HEK293T cells. Empty vector-transfected cells were used to measure an internal ROS producing activity in HEK293T. After baseline measurements of ROS production for 5 min, 0.1 μM calyculin A was added to culture medium without Ca^{2+}. Results given are means ± standard error (SE; n=3).

Figure 5 Complementation analysis of *atrbohH atrbohJ* double mutant phenotype with *AtRbohC*.

In *atrbohH-3 atrbohJ-2* double mutant, *AtRbohC* was ectopically expressed under the control of the *AtRbohH* and *AtRbohJ cis*–regulatory sequences, respectively. Each *AtRbohH* and *AtRbohJ* was expressed by its own *cis*–regulatory sequences as control experiments. Pollen grains were from wild type (WT: Col), *atrbohH-3 atrbohJ-2* double mutant and *AtRbohC*-, *AtRbohH*- and *AtRbohJ*-expressing *atrbohH-3 atrbohJ-2* double mutant. Pistils were WT. (**A**) Pistils were harvested 12 h after hand pollination and stained with aniline blue. Scale bar = 250 µm. (**B**) Each bar represents the mean number of seeds in a silique and ± standard error (SE; n=7−10). Siliques were harvested about 10 days after hand pollination. There is no statistically significant difference with the same letter (Tukey's test, $p > 0.05$).

Figure 6 Complementation analysis of *atrbohC/rhd2* mutant with *AtRbohH* and *J*.

In *atrbohC/rhd2-5* mutant, each *AtRbohC*, *AtRbohH* or *AtRbohJ* was ectopically expressed under the control of the *AtRbohC cis*–regulatory sequences. *AtRbohC* was expressed by the same promoter region as control experiments. (**A**) Rescue of root hair defects of *atrbohC/rhd2* by expression of *AtRbohH* and *J*. (**B**) Root hairs located within in 5 mm from the root tip were measured in different genetic backgrounds and represented by percentage of hair length. Examined root hair number is shown above the graph.

Table 1. Rboh genes in *Arabidopsis thaliana*

	clade	AGI	Molecular weight[a]	Functions	References
Subfamily I & II					
AtRbohA	ACG	At5g07390	102934.6		
AtRbohB	B	At1g09090	96389.3	seed after-ripening[1]	[1]Müller et al 2009
AtRbohC	ACG	At5g51060	102517.4	root hair tip growth[2,3], root hydrotropism[4], mechanosensing[5]	[2]Foreman et al 2002, [3]Takeda et al 2008, [4]Krieger et al 2016, [5]Monshausen et al 2009
AtRbohD	D	At5g47910	103907.7	pathogen defence[6,7], stomata closure[8], osmosensitive metabolic changes induced by cellulose biosynthesis inhibitor[9], rapid systemic signaling in response to exogenous stimuli[10], lignin deposition caused by cell wall biosynthesis inhibitor[11,12], salinity-stress response[13], systemic acquired acclimation by abiotic stress[14], JA-induced ROS production[15], abscission of floral organs[16]	[6]Torres et al 2002, [7]Torres et al 2005, [8]Kwak et al 2003, [9]Wormit et al 2012, [10]Miller et al 2009, [11]Hamann et al 2009, [12]Denness et al 2011, [13]Ma et al 2012, [14]Suzuki et al 2013, [15]Maruta et al 2011, [16]Lee et al 2018
AtRbohG	ACG	At4g25090	96861.7		
Subfamily III & IV					
AtRbohE	E	At1g19230	107701.6	programmed cell death in tapetum[16]	[16]Xie et al 2014
AtRbohF	F	At1g64060	108417.3	pathogen defence[6,7], stomata closure[8],osmosensitive metabolic changes induced by cellulose biosynthesis inhibitor[9], lignin deposition caused by cell wall biosynthesis inhibitor[11,12], salinity-stress response[13], JA-induced ROS production[15], Casparian strip formation[16], abscission of floral organs[17]	[6]Torres et al 2002, [7]Torres et al 2005, [8]Kwak et al 2003, [9]Wormit et al 2012, [11]Hamann et al 2009, [12]Denness et al 2011, [13]Ma et al 2012, et al J Exp Bot 2011, [15]Maruta et al 2011, [16]Lee et al 2013, [17]Lee et al 2018
AtRbohI	I	At4g11230	106951	drought stress response in seeds and roots[17]	[17]He et al 2017

Subfamily V					
AtRbohH	HJ	At5g60010	100626.7	Pollen tube tip growth[18,19,20], Polar root hair growth[21]	[18]Kaya et al 2014, [19]Boisson-Dernier et al 2013, [20]Lassig et al 2014, [21]Mangano et al 2017
AtRbohJ	HJ	At3g45810	102936.2	Pollen tube tip growth[18], Polar root hair growth[21]	[18]Kaya et al 2014, [21]Mangano et al 2017

[a] Molecular weight information from the TAIR website

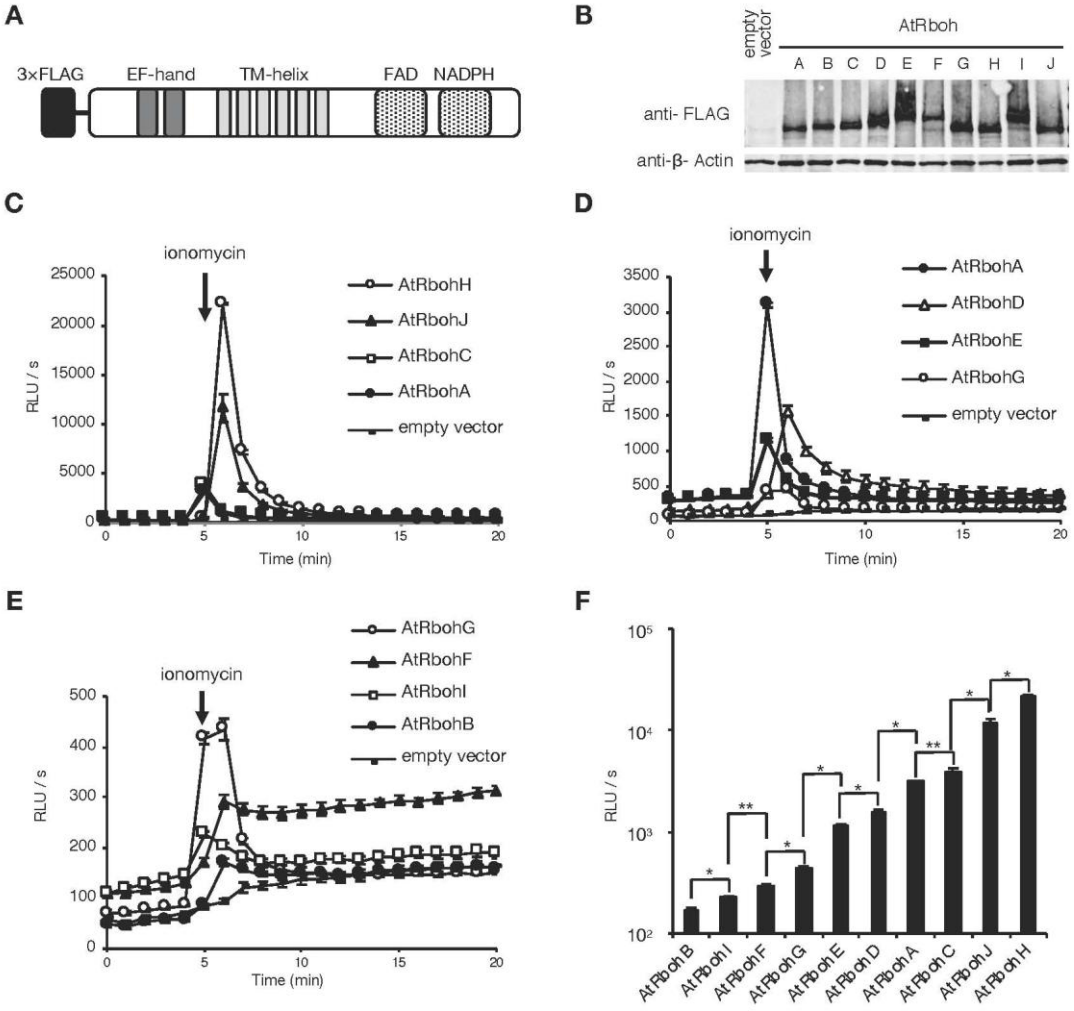

Figure 1

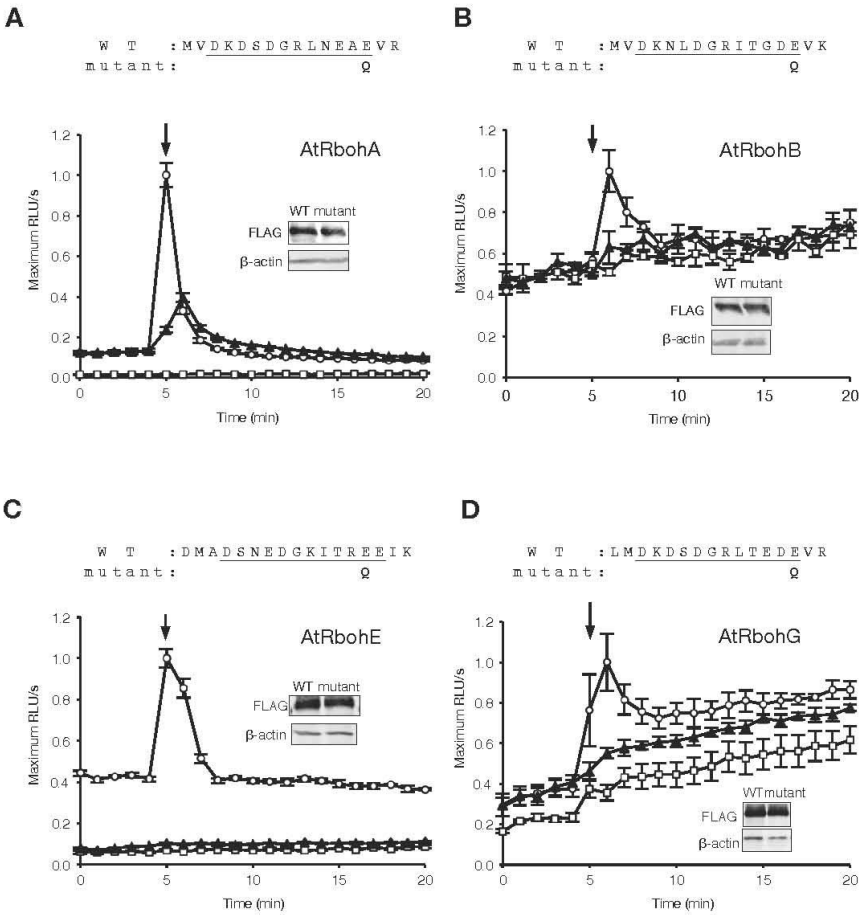

Figure 2

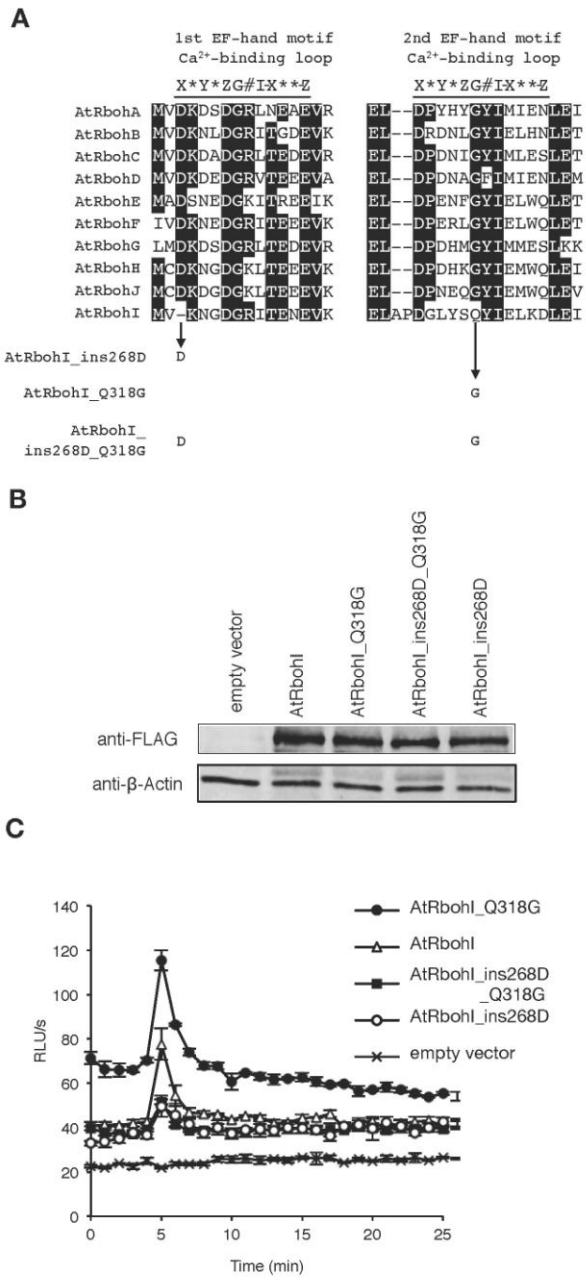

Figure 3

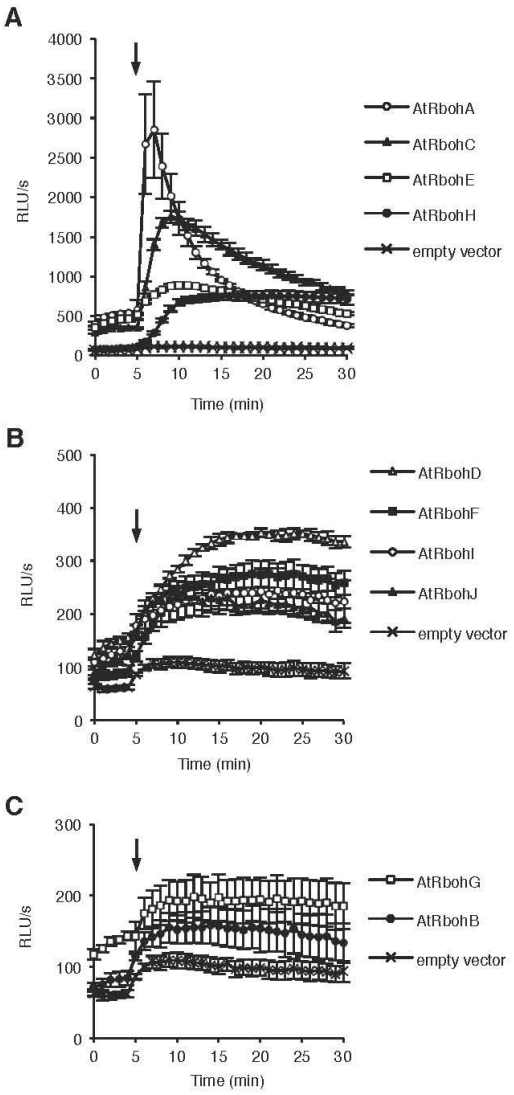

Figure 4

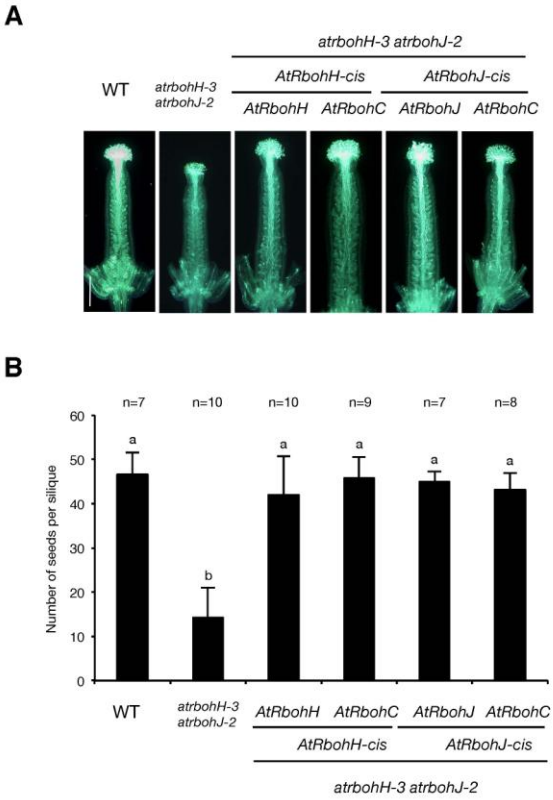

Figure 5

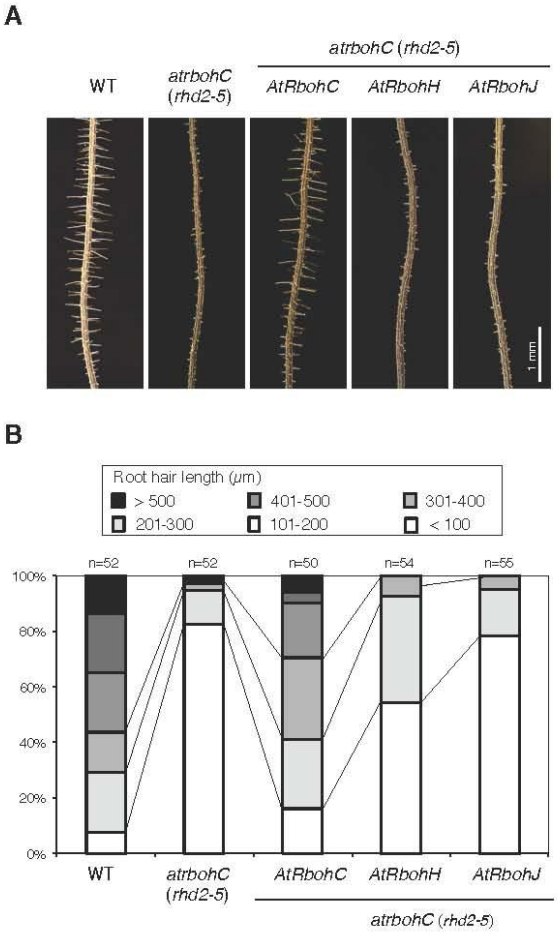

Figure 6