A novel Calmodulin-interacting Domain of Unknown Function 506 protein represses root hair elongation in Arabidopsis

Sheng Ying¹ and Wolf Scheible¹

¹Noble Research Institute LLC

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Abstract

DUF506 proteins are omnipresent in higher plants. Phosphorus (P) stress-inducible AtRXR1/REPRESSOR OF EXCESSIVE ROOT HAIR ELONGATION 1 gene, as the first functionally characterized DUF506 gene, is proved to inhibit root hair elongation through interaction of RXR2/RabD2c GTPase. However, the knowledge of other P-responsive DUF506 genes is still limited. Here, we identify four additional P-inducible DUF506 genes and select two of candidates for functional investigation. Expression analysis results reveal that both of candidates are induced by auxin. At3g07350, the duplicated pair of RXR1, expresses ubiquitously in seedlings under P-stress, whereas At1g62420 is mainly induced in roots. Overexpression and knockout mutants of At1g62420, which is called RXR3, exhibit shorter or longer root hair length, respectively. Cellular imaging results demonstrate RXR3 localizes in root epidermal cells. ChIP, synthetic peptide treatment and qRT-PCR assay results indicate RXR3 is transcriptionally activated by RSL4 or RALF1. BiFC and CaM-binding assay suggest that RXR3 interacts with various CaMs in presence of Ca²⁺. Moreover, the frequencies of [Ca²⁺]_{cyt} in rxr3 mutants are approximately 20% higher compared to that of wild type. Taken together, our results illustrate a divergent mechanism by which RSL4-directed RXR3 interacts with calmodulin and functions in parallel of RXR1, to prevent root hair excessive growth.

INTRODUCTION

Phosphorus (P) is essential for all life on earth as an indispensable structural component of nucleic acids, membrane phospholipids and energy metabolites. Plants assimilate P as inorganic phosphate (Pi) from the soil. However, the concentration of Pi in many unfertilized soils is low and limiting for plant growth (Hammond, Broadley, & White, 2004; Raghothama, 1999). In order to cope with a low P environment, plants have evolved various strategies to improve the acquisition, remobilization and efficient use of Pi, such as altering root morphology, secreting phosphatases and nucleases to mobilize or release Pi from soil insoluble organic sources (Lynch, 2011; Plaxton & Tran, 2011; Svistoonoff et al., 2007; Williamson, Ribrioux, Fitter, & Leyser, 2001). One morphological hallmark response of roots to low P is the increase of density and length of root hairs (RHs), which is thought to maximize the root surface area (~ 70%) and facilitate the absorbance of Pi present in the soil (could be responsible for up to 90%)(Bates & Lynch, 2001; Bucher, 2007; Crombez, Motte, & Beeckman, 2019; Lynch, 2019; Ma, Baskin, Brown, & Lynch, 2003). Additionally, RHs aid water uptake(Kwasniewski et al., 2016), improve root penetration of hard/drying soil (Choi & Cho, 2019; Haling et al., 2013), enhance plant tolerance to drought stress (Marin et al., 2020; Xiaomin Zhang et al., 2020), and enable root-microbe interactions (Brown, George, Dupuy, & White, 2013).

In Arabidopsis, RHs developed from specialized root epidermal cells, called trichoblasts, located outside the intercellular space between two underlying cortical cells (Grierson, Nielsen, Ketelaarc, & Schiefelbein, 2014). The genetics and molecular mechanisms of root hairs development, i.e. initiation, differentiation, and elongation, have been extensively studied (Bruex et al., 2012; Cho & Cosgrove, 2002; Hwang, Choi, Cho, & Cho, 2017; Lin et al., 2015; Parker, Cavell, Dolan, Roberts, & Grierson, 2000; Salazar-Henao, Vélez-Bermúdez, & Schmidt, 2016; Schiefelbein, 2000), and have identified a number of key genes. ROOT HAIR DEFECTIVE6

(RHD6), a basic helix-loop-helix (bHLH) transcription factor (TF), plays central roles in RH development (Masucci & Schiefelbein, 1994). As downstream bHLH TFs of RHD6, RHD6-LIKE2/4 (RSL2/4), dominantly regulate expression of various RH morphogenic genes (such as EXPANSIN A7/EXPA7, PEROXIDASE7/PER7, and CALCIUM-DEPENDENT PROTEIN KINASE11/CDPK11), through direct binding to root hair-specific *cis* -elements (RHEs) in their proximal promoter regions, and consequently control the elongation of RHs (Bhosale et al., 2018; Datta, Prescott, & Dolan, 2015; Hwang et al., 2017; Kim & Dolan, 2016; Kim, Han, & Dolan, 2017; Vijayakumar, Datta, & Dolan, 2016; Yi, Menand, Bell, & Dolan, 2010).

To date, many phytohormones (e.g. auxin/IAA, ethylene/ACC, Absciscic acid/ABA, Strigolactones/SLs and Jasmonate/JA), as well as chemical molecules (e.g. reactive oxygen species/ROS, Ca^{2+} , and short peptide/RAPID ALKALINIZATION FACTOR1/AtRALF1), have been demonstrated to regulate RH development (Abarca, Franck, & Zipfel, 2021; Y. Feng et al., 2017; Foreman et al., 2003; Han, Zhang, Yang, & Hu, 2020; Kapulnik et al., 2011; S. Mangano et al., 2017; Silvina Mangano, Denita-Juarez, Marzol, Borassi, & Estevez, 2018; Pitts, Cernac, & Estelle, 1998; Tian, Wang, Gao, Li, & Luan, 2020; Vissenberg, Claeijs, Balcerowicz, & Schoenaers, 2020; Wang et al., 2017; Zhu, Martínez Pacheco, Estevez, & Yu, 2020). Recently, two models have been proposed to illustrate the mechanism of RH elongation when exposed to low external P (Bhosale et al., 2018; Song et al., 2016). For instance, under P-deficiency condition, endogenous auxin level is significantly elevated in the root apex, which leads to activation of AUX1-mediated auxin transport and following the induction of ARF19, eventually stimulate the RSL2 and RSL4 to enhance RH elongation. In RH cell, NADPH oxidase (e.g. RHD2) trigger the accumulation of ROS, which subsequently stimulate the cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$) oscillation and gradient, through the activation of cyclic nucleotide-gated channels (CNGCs)(Foreman et al., 2003; Tian et al., 2020).

Domains of Unknown Function 506 (DUF506) gene family is widespread in many plant species. However, the basic information and knowledge of DUF506 genes are still lacking. The only functionally characterized DUF506 gene, *At3g25240/RXR1/Repressor of excessive root hair growth1*, which is highly induced by P-stress *via* PHR1/PHL1-dependent pathway, is demonstrated to repress RH elongation. Overexpression of its closest homolog (*Bdi2g58590*) in Brachypodium confers the identical short RH phenotype (Ying, Blancaflor, Liao, & Scheible, 2021). This intriguing trait promotes us to further investigate the P-response of other Arabidopsis DUF506 genes.

Here, additional P-inducible DUF506 genes are identified from public transcriptome database and their expression are validated by qRT-PCR. One of them, *At1g62420/RXR3*, is selected for comprehensively functional investigation. Our current results manifested that RXR3, differs from RXR1, is a novel root hair growth repressor through interaction with various cytosolic calmodulins.

MATERIALS AND METHODS

Plant material, growth and treatment

Arabidopsis thaliana (ecotype Col-0) seeds were sterilized and placed on half strength Murashige and Skoog (MS) solid medium according to (Ying et al., 2021). Petri dishes were stratified for 3 days at 4 °C in the dark before transfer to growth chamber (120 $\mu\text{mol}\cdot\text{s}^{-1}$ light intensity, 22°C/20 °C, 16-h-light and 8-h-dark cycle). For Phosphate-deprived (-P) treatment, the experiments were performed as described previously (MORCUENDE et al., 2007). In brief, Arabidopsis seedlings were germinated and grown in half strength MS liquid medium, and then transferred into -P liquid culture on day 7. Shoot and root tissues were respectively harvested on day 9 or as indicated, then quickly frozen in liquid N_2 , and stored in -80 °C freezer for further study. The *rxr3-1* (Salk_016908), *rxr3-2* (Salk_078603), *cam7* (CS873256), and *arf7 arf19* (CS24629) T-DNA insertion mutant lines were obtained from Arabidopsis Biological Resource Center (ABRC). The *rsl4* mutant was provided by Dr. Alison Blancaflor (Noble Research Institute). Homozygous mutant plants were identified or validated according to the of SiGnAL database protocol (<http://signal.salk.edu/>).

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using RNeasy[®] plant mini kit (QIAGEN) and genomic DNA was removed using

Turbo DNA-free kit (Invitrogen) following the manufacturer’s instructions. RNA (1 µg) was converted into cDNA using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacture’s instruction. Quantitative PCR was performed using QuantStudio 7 Flex Real-Time PCR System (Invitrogen) and KiCqStart®SYBR® Green qPCR ReadyMix(Millipore Sigma). The reaction procedures were executed as described previously (Ying et al., 2021). The Arabidopsis *GAPDH* (*At1g13440*) gene was used as an internal control (Czechowski, Stitt, Altmann, Udvardi, & Scheible, 2005). All the experiments were repeated at least three times using cDNAs prepared from two biological replicates. Sequences of primers used in the study are listed in Supplemental Table 1.

Measurement of root hair length.

Seedlings were germinated vertically for 3 days on half strength MS solid medium containing 0.4% Gelzan CM (Millipore Sigma), and then transferred to plates containing either 0 (-P) or 675 µM (+P) phosphate, and 1 µM synthesized AtRALF1 peptide (49 amino acids, Pepscan) respectively. Three days after transfer, root hair images were captured using a Nikon SMZ1500 stereo microscope. Root hair length was determined by measuring at least 700 root hairs located between 2 to 6 mm from the tip of the primary root in 10 individual plants from each line. Statistical analysis was performed as described previously (Ying et al., 2021).

Generation of transformants (Overexpressor and complementation lines)

For generation of *RXR3* gene overexpressing lines, full-length of coding region was amplified from Arabidopsis seedling cDNA using Phusion® High-Fidelity DNA polymerase (NEB) and cloned into GATEWAY® entry vector pENTR/SD/D-TOPO^(r) (Invitrogen). Sequencing-confirmed vector was used to recombine into destination vector pMDC32 (Curtis & Grossniklaus, 2003). For histochemical GUS analysis, a 1,300-bp upstream (from start codon) fragment of *RXR3* gene was amplified from Arabidopsis genomic DNA and subsequently cloned into the vector pBGWFS7 (Karimi, Inze, & Depicker, 2002). For complementation analysis in the *rxr2-1* mutant background, the promoter fragment of the *RXR3* gene and 852-bp coding region was respectively amplified, and subsequently constructed into destination vector pMU64 as described previously (Ying et al., 2021). For ChIP assay, the promoter fragment and full-length coding region of *RSL4* gene was amplified from Arabidopsis seedling genomic DNA or cDNA, and eventually recombined into pMU64 vector, resulting in the *proRSL4::RSL4-GFP* construct. For calcium oscillation analysis, the *proUBQ10::G-CAMP3-GFP* construct was transformed into individual *rxr3* mutant lines (Kwon, Sparks, Liao, & Blancaflor, 2018).

All constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 using a freeze-thaw procedure and were transformed into Arabidopsis by floral dipping (X. Zhang, Henriques, Lin, Niu, & Chua, 2006). Transformants were selected on half strength MS agar (0.8% w/v) medium containing 25 µg mL⁻¹ hygromycin (HYG, Omega Scientific).

Western blotting

For detection of GFP fusion protein in *RXR3* complementation plants, total protein was extracted as described previously (Ying et al., 2021). Equal amount (30 µg) of each sample was separated by SDS-PAGE, transferred into Immobilon-P PVDF membrane (0.45 µM, Millipore Sigma), and probed with 1/5000-diluted monoclonal mouse anti-GFP horseradish peroxidase (HRP)-conjugated antibody (MiltenyiBiotec). Chemiluminescence detection was performed with ECL Prime Western Blotting System (Millipore Sigma) and UVP ChemStudio imager (Analytik Jena, US).

Histochemical GUS staining and GFP imaging

The histochemical GUS activity analysis was performed as previously described (Jefferson, Kavanagh, & Bevan, 1987). Briefly, seedlings were incubated in a GUS staining solution containing 100 mM sodium phosphate (pH 7.0), 1 mM EDTA, 0.05% Triton X-100, 1 mM potassium ferricyanide/ferrocyanide, and 0.5 mg mL⁻¹ X-glucuronide (Goldbio) at 37°C for 1 to 3 hr. Then samples were gradually cleared in 30%, 50%, 70% and 100% (v/v) ethanol for 30 min. Images were acquired using Nikon SMZ1500 stereomicroscope. GFP

fluorescence of the *proRXR3::RXR3-GFP* or *proRSL4::RSL4-GFP* complementation plants was imaged with Leica TCS SP8 confocal laser-scanning microscope (Ex: 488 nm; Em 507 nm).

Chromatin immunoprecipitation (ChIP) assay

The root tissue of *RSL4-GFP* complementation seedlings, which were grown vertically on half strength MS agar (0.8% w/v) medium for 12 day, were harvested, cross-linked, and stored in -80 °C for further steps. ChIP assay was conducted as described previously (Haring et al., 2007; Nelson, Denisenko, & Bomsztyk, 2006; Saleh, Alvarez-Venegas, & Avramova, 2008).

Calmodulin binding assay

For heterologous expression of *RXR3*, its full-length (FL) and N-terminally ($\Delta 1-22$, residues 1 to 22, including CaM Binding Domain/CaMBD) truncated cDNA fragment was respectively subcloned into pMAL-c5X vector (NEB) carrying an N-terminal Maltose Binding Protein (MBP) tag. For recombinant protein production, constructs were separately introduced into the *Escherichia coli* (NEB Express Competent cells C2523H). The purification was performed using amylose resin (NEB) according to the manufacture's instruction. Protein concentration was quantified using Pierce rapid gold BCA protein assay kit (Thermo Fisher Scientific), and finally stored in -80°C freezer for further study.

CaM binding assay was carried out according to previous study with small modification (Kato, Aoyama, & Maeshima, 2013). Briefly, recombinant MBP-RXR3^{FL} or MBP-RXR3 ^{$\Delta 1-22$} protein (2 μ g) were incubated with 25 μ l CaM-agarose (Millipore Sigma) in the buffer containing 20 mM Tris (pH 7.0), 150 mM NaCl, and 0.5 mM CaCl₂ or 2 mM EGTA for 1 hr at 4°C. Then, the supernatant was discarded by centrifugation at 1470 g for 2 min. After three times washing with the same buffer, the bound protein was finally disassociated by incubation with 2 $\times$ SDS-PAGE protein sample buffer (Millipore Sigma). Samples were separated and transferred as described previously. Western blotting was performed using 1/5000-diluted monoclonal mouse anti-MBP-HRP antibody (MiltenyiBiotec).

Bimolecular fluorescence complementation (BiFC) assay

The BiFC analysis was performed as previously described (Kudla & Bock, 2016; Waadt, Schlücking, Schroeder, & Kudla, 2014). Briefly, *RXR3* was respectively fused to the N-terminal EYFP in the pSITE-nEYFP or pCambia1305-CFP (gift by Dr. Alison Blancaflor) vector, whereas various Arabidopsis *CaM* genes were fused with the C-terminal part of EYFP in pSITE-cEYFP. These different vector pairs were co-transformed into *N. benthamiana* through Agro-infiltration. Forty-eight hours after infiltration, YFP or CFP fluorescence was detected using a Leica TCS SP8 confocal laser-scanning microscope (CFP, Ex: 405 nm; Em: 485 nm; YFP, Ex: 514 nm; Em: 527 nm).

Measurement of phosphate content

The P-content measurement was conducted as described previously (Carter & Karl, 1982) with minor modification. In brief, 50 mg of Arabidopsis ground tissue was thoroughly resuspended in 600 μ L of MilliQ water. After centrifugation at 13000 g for 2 min, the clarified supernatant was used to quantify the phosphate content. Mixture of 20 μ L of supernatant, 90 μ L of 1 N HCl, and 90 μ L of malachite green dye solution (0.042%, w/v) was incubated at room temperature for 5 min, then the absorbance at 630 nm was recorded by spectrophotometer. The reading values were calibrated by the standard curve created by using various concentration of K₂HPO₄. All the measurements were repeated at least three times with representative results shown in the figures.

Statistical analysis

Statistical analyses and plotting were performed with GraphPad Prism 9.0 (GraphPad Software, Inc., San Diego, CA).

Accession Numbers

Sequence data from this article can be found in the Phytozome 12 online genomic resource (<https://phytozome.jgi.doe.gov/pz/portal.html>) under the following accession numbers: *AtRXR1* (*At3g25240*), *AtRabD2c/AtRXR2* (*At4g17530*), *AtRXR3* (*At1g62420*), *Arabidopsis DUF506* gene family members (*At1g12030*, *At1g77145*, *At1g77160*, *At2g20670*, *At2g38820*, *At2g39650*, *At3g07350*, *At3g22970*, *At3g54550*, *At4g14620* and *At4g32480*), *AtCAM1* (*At5g37780*), *AtCAM2* (*At2g41110*), *AtCAM6* (*At5g21274*), *AtCAM7* (*At3g43810*), *AtCNGC14* (*At2g24610*), *AtRALF1* (*At1g02900*), and

AtEXPA7 (*At1g12560*).

RESULTS

Expression profile of Arabidopsis DUF506 gene family under P-limitation

To functionally characterize *AtDUF506* genes under nutrient deprivation, we firstly examined their expression patterns from public RNA-seq database (i.e. Genevestigator), and found four additional *DUF506* genes (e.g. *At1g62420*, *At2g20670*, *At3g07350*, and *At4g32480*) were induced by P-limitation. Next, we investigated the transcript changes of *AtDUF506* genes in response to P-deprivation using qRT-PCR (Fig. 1). Similar to *RXR1*, *At3g07350* and *At4g32480* were significantly upregulated in both shoot and root tissue, whereas *At1g62420* and *At2g20670* respectively exhibited root or shoot-specific induction when exposed to P-stress. Owing to their high P-induction, we decided to detect the tissue-specific expression of *At3g07350* and *At1g62420* via GUS staining. As shown in Fig. S1, *At3g07350* gene expressed slightly in vascular bundles and lateral root tips under +P condition. Conversely, it was strongly induced throughout the entire seedling (e.g. cotyledon, primary/lateral root, root tips) after cultured in -P liquid medium for 24 hr. Meanwhile, *At1g62420* was mainly visualized in primary rather than lateral root, such as vascular bundle, root tip and root hairs. In contrast, no distinguishable signals were detected in P-stressed shoot and P-sufficient cultured seedlings (Fig. 2B). These results suggested that *Arabidopsis DUF506* genes broadly participated in P-stress response, and warranted to comprehensively demonstrate their roles by further study.

Transcriptional characterization of P-inducible At1g62420 gene

To investigate the specificity of P-inducible expression, time course experiment was employed to monitor the transcript changes of *At1g62420* gene during P-starvation. The transcript was unchanged for 6 hr, and reached the peak at 24 hr (Fig. 2A). After 3 hr of P re-supplement, expression level of *At1g62420* quickly resumed to regular level, indicating a direct response to the plant P-status.

PHOSPHATE STARVATION RESPONSE1 (PHR1) and its homolog PHR1-LIKE (PHL1) are the critical TFs of plant P-response (Bustos et al., 2010; NILSSON, MÜLLER, & NIELSEN, 2007; Rubio et al., 2001). *RXR1* was ascertained to be transcriptionally activated by PHR1 and PHL1 (Ying et al., 2021), we were interested to examine whether *At1g62420* is regulated by these two TFs. To test this, we firstly looked for PHR1 binding sequences (P1BS) in its promoter region, but none was found. Next, we investigated the transcript changes of *At1g62420* in *phr1 phl1* double mutant to P treatment. In *phr1 phl1* double mutant, *At1g62420* exhibited identical expression pattern when compared to wild type, especially in root tissue (Fig. 2C), indicating it responds to P stress independently of PHR1 and PHL1.

Under P-limitation, the concentration of endogenous auxin is significantly accumulated and re-distributed in various organs, that leading to altered root system architecture (RSA). Therefore, we looked into the expression changes of *At1g62420* to auxin treatment. Notably, the transcriptional inductions by auxin were comparable to those of P-stress (Fig. 2D), implying that auxin, not P status, directly modulated the transcription of *At1g62420*.

Overexpression of At1g62420 represses root hair growth

To elucidate the biological function of *At1g62420* in plant, we firstly generated the overexpression lines. Two independent homozygous transgenic lines (e.g. OX-5 and OX-12) exhibited significant short (~22%) RH length compared to that of wild type (e.g. +/-P, $0.189 \pm 0.003/0.242 \pm 0.005$ mm [OX-12] versus $0.237 \pm 0.003/0.308 \pm 0.004$ mm [WT]), when grown on P-sufficient or -deficient medium (Fig. 3B and C). Due to

high phenotypic similarity to *RXR1*, *At1g62420* was denoted as *RXR3* afterward. Secondly, we identified two independent *loss-of-function* mutants in ABRC, *rxr3-1* (SALK_016908) and *rxr3-2* (SALK_078603, Fig. 3A). The results of qRT-PCR analysis indicated that *RXR3* was undetectable in these mutants. RH length of mutants was longer than wild type (e.g. $\sim 41\%$, *rxr3-1*, -P) and overexpressors, in regardless of P-status (e.g. +/-P, $0.270 \pm 0.005/0.433 \pm 0.007$ mm [*rxr3-1*]; $0.299 \pm 0.005/0.437 \pm 0.007$ mm [*rxr3-2*], Fig. 3B and C).

To ascertain the RH traits were tightly related to *RXR3* dysfunction, we introduced endogenous promoter driven *RXR3-GFP* construct in *rxr3-1* mutant. GFP signal was detected from two independent complementation lines *via* Western blotting, implying that *RXR3* was correctly transcribed and translated *in vivo* (Fig. 3F). Moreover, the *RXR3-GFP* mainly presented in root epidermal cells and root hair tips (Fig. 3G), hinted the close relationship between *RXR3* and RH growth. RH lengths of complementation lines were similar to that of wild type, particularly under P-deficiency, indicating that *RXR3* appropriately rescued the abnormal phenotype of *rxr3-1* mutant (e.g. +/-P, $0.265 \pm 0.004/0.362 \pm 0.007$ mm [Comp#1] versus $0.233 \pm 0.004/0.327 \pm 0.006$ mm [WT], Fig. 3D and E).

Considering the similar inhibitory function of root hair elongation between *RXR1* and *RXR3*, we generated the *rxr1 rxr3-1* double mutant to validate their functional redundancy (Fig. 4). Under the P-replete condition, root hair length of double mutant (0.360 ± 0.005 mm) was similar to *rxr1* mutant (0.366 ± 0.006 mm), but significantly longer than *rxr3-1* mutant (0.309 ± 0.005 mm) and wild type (0.256 ± 0.004 mm). Under P-deficient condition, the double mutant (0.456 ± 0.007 mm) exhibited slightly additive effects ($P < 0.01$) in relative to each single mutant (*rxr1*, 0.421 ± 0.007 mm; *rxr3-1*, 0.430 ± 0.006 mm) or wild type (0.319 ± 0.005 mm), suggesting that *RXR3* inhibited RH growth possibly through separate pathway other than *RXR1*.

RHs are important for P-uptake, and as a result, the plant biomass increased accordingly (Crombez et al., 2019; Singh Gahoonia & Nielsen, 2004). Unsurprisingly, *rxr3-1* mutant exhibited distinguishable difference to wild type, regarding endogenous P-content or biomass (Fig. 3H, I and S3). In summary, these results suggested that *RXR3* repressed RH elongation without interfering plant growth.

RSL4 binds to the RXR3 promoter

RSL4 has been demonstrated as a critical TF that dominated RH initiation and elongation (see Introduction). Thus, we investigated whether *RXR3* participated in the *RSL4*-mediated regulatory pathway. According to previous reported consensus sequences (Hwang et al., 2017), four of RHE sites were identified in *RXR3* promoter region (Fig.5A). Additionally, the P-inducible expression of *RXR3* was reversed in *rsl4* mutant (Fig.5B). Therefore, we presumed that *RSL4* might directly bind to these RHEs to regulate *RXR3* expression. To test our hypothesis, we firstly generated the *RSL4* complementation line that expressed its endogenous promoter driven *RSL4-GFP* in *rsl4* mutant. Nucleus-localized GFP signal was observed in root, indicated that *RSL4-GFP* fused protein was succeeded in rescuing the *loss-of-function* phenotype of *rsl4* mutant (Fig.5D). Then, we conducted ChIP analysis to examine the interaction between *RSL4* and RHE motifs in *RXR3* promoter. *EXPA7* is well-known *RSL4* positive regulated gene (Yi et al., 2010), therefore, we employed it as positive control of ChIP assay (Fig. S4). As shown in Fig.5C, *RSL4* interacted with the probe 1 and 2, which contains the identified RHE motifs, whereas no interaction was observed between *RSL4* and probe 3. These results suggested that *RSL4* could directly bind to RHEs within the *RXR3* promoter.

AtRALF1 activates RXR3 expression

Recently, Zhu and colleagues (Zhu et al., 2020) reviewed that *RALF1* and receptor *FERONIA* (*FER*) act central roles in RH growth, through translational regulation *RSL4* protein by phosphorylated eIF4E1. Thus, we thought that *RALF1* could possibly activate the expression of *RXR3* *via* *RSL4*. Unsurprisingly, RH growths were highly promoted in wild type and *rxr3-1* mutant after *RALF1* treatment (Fig.6A). Also, transcript level of *RXR3* in wild type, but not in *rsl4*, increased in response to *RALF1* (Fig.6B and C). Lastly, we examined whether *RALF1* affected the subcellular localization of *RXR3*. Five-day-old of *RXR3* complementation seedlings were incubated with half strength MS liquid medium supplemented 1 μ M

RALF1 for 6 hr. In Fig.6D, RXR3 exhibited the same pattern as it responding to P-stress, which was mainly presented in root epidermis. The results implied that AtRALF1 induced *RXR3* expression *via* RSL4.

RXR3 interacts with Calmodulins

Previously, no featured motifs other than DUF506 domain was found in RXR1, whereas in the current study, one N-terminal CaMBD was identified in between amino acids 11 and 22 (motif 1-12, [FILVW]xxxxxxxx[FILVW]) of RXR3. To determine whether RXR3 could interact with CaMs, we heterogeneously expressed and purified the MBP-tagged recombinant RXR3 protein (MBP-RXR3^{FL}), as well as the N-terminal truncated mutant (MBP-RXR3^{Δ1-22}, Fig.7A and Fig. S5). Both of recombinant proteins were individually incubated with CaM-agrose in the presence or absence of Ca²⁺. The bound protein was dissociated, subjected to SDS-PAGE, and detected by immunoblotting with antibodies against MBP tag. As shown in Fig.7B, RXR3^{FL} bound to CaM-agrose in the presence of Ca²⁺, but not in the presence of EGTA (Ca²⁺ chelator). Meanwhile, RXR3^{Δ1-22} did not bind to CaM-agrose, in regardless of Ca²⁺ status. These results indicated that the N-terminal CaMBD of RXR3 was critical and sufficient for binding to Ca²⁺-CaM complex.

To validate the interaction between RXR3 and CaMs *in vivo*, we further conducted the BiFC assay by co-infiltration to *N. benthamiana* leave. In Arabidopsis, four isoforms (e.g. CaM1/4, CaM2/3/5, CaM6 and CaM7) which derived from seven of CaM encoding genes have been identified and studied (Bender & Snedden, 2013; Zielinski, 1998). We selected four different *CaM* genes (i.e. *CaM1*, *CaM2*, *CaM6* and *CaM7*) that represented all isoforms to carry on the BiFC assay. In Fig.7C, strong cytosolic yellow fluorescence signal was observed in each combination, illustrating that RXR3 interacted with various CaMs *in vivo*. Conversely, no signal was detected between CaM7 and RXR1, which served as negative control. Overall, these results demonstrated that RXR3 physically interacted with CaMs.

RXR3 influences on root hair [Ca²⁺]_{cyt} oscillation

Calmodulins function as versatile Ca²⁺-binding proteins that regulate the activity of numerous effectors (e.g. CNGCs) in Ca²⁺ signaling. It is reported that CaM7 bound with CNGC14 to regulate RH polar growth by controlling intrinsic Ca²⁺ status (Zeb et al., 2020). Thus, we investigated the effect of RXR3 protein on the interaction of CaM7 with CNGC14. When RXR3-CFP was co-expressed with CaM7-nEYFP and CNGC14-cEYFP in tobacco, the yellow fluorescence was not noticeably affected (Fig.8A and B). RXR3 was not only co-localized with CNGC14-CaM7 complex in cytosol, but also presenting in nucleus (Arrow, Fig.8B). These results indicated that RXR3 did not interfere in the interaction of CaM7 with CNGC14, and might conduct other functions in nucleus. Furthermore, we expressed GCaMP-GFP construct in wild type, *rxr3-1* and *rxr3-2* mutant respectively, to quantitatively examined whether RXR3 influences the apical [Ca²⁺]_{cyt} oscillation in root hair. Both *rxr3* mutants (*rxr3-1*, 0.0517 ± 0.0027 Hz; *rxr3-2*, 0.0542 ± 0.0020 Hz) exhibited ~20% higher frequency of [Ca²⁺]_{cyt} oscillations compared to that in wild type (0.0399 ± 0.0009 Hz), indicating that RXR3 repressed RH growth possibly by regulating [Ca²⁺]_{cyt} oscillations (Fig.8C and D).

Overexpression of *CaM7* dramatically represses RH growth by inhibition of CNGC14 activity (Zeb et al., 2020). Here, we found that *cam7* mutant exhibited longer RH length in relative to wild type. Additionally, when constitutively overexpressing *RXR3^{FL}* in *cam7* mutant, the long RH phenotype was not restored (Fig. S7). These results suggested that RXR3 affected RH growth and [Ca²⁺]_{cyt} oscillation through interaction of CaM7.

DISCUSSION

Domain of unknown function (DUF) is a conserved protein domain that has not been functionally characterized or annotated. Recently, we demonstrated that P-inducible *RXR1*, which belongs to DUF506 family, negatively regulates RH growth through interacting with small GTPase RabD2c/RXR2 (Ying et al., 2021). Here, we have expanded our study with identification and characterization of four other P-inducible DUF506 genes in Arabidopsis.

Based on the relatively high P-induction expression, we selected two of the *AtDUF506* genes for in-depth characterization. *At3g07350*, which is the duplicated pair of *RXR1*, was previously recognized as uncharacterized and putative root hair specific gene with two RHE in its promoter region (Won et al., 2009). At that time, a set of 37 Arabidopsis RHS genes were identified through genome-wide RHE motif screening in gene promoter region together with transcriptome analysis. Meanwhile, 65% (24/37) of RHS genes were experimentally validated their relationship of RH morphology (Won et al., 2009). For instance, one cell-wall associated receptor kinase encoding gene, *RHS10* (*At1g70460*), was demonstrated to be activated by RSL4 and inhibit RH growth through suppression of ROS accumulation and RNA metabolism (Hwang et al., 2017; Hwang, Lee, Lee, & Cho, 2016). Here, GUS staining results discovered that under P-stress *At3g07350* was highly induced in roots, especially root tips and root hairs (Fig. S1). The root tip focused expression pattern differs from *RXR1*, which mostly presents in vascular bundle but is undetectable in primary root tip. Moreover, expression analysis revealed that *At3g07350* in root was induced by auxin (Fig. S2A), suggesting its involvement in auxin-dependent regulatory pathway. It is reported that *TAA1*, which expresses at root apex and manages auxin synthesis, is up-regulated under low-P condition that led to activation of AUX1-dependent shootward auxin transport pathway accompanied with *RSL4* transcript up-regulation, and eventually root hairs elongation (Bhosale et al., 2018; Datta et al., 2015). It is noteworthy that *At3g07350* is the only RHS gene showing up-regulation (i.e. 2-fold) in *rhd6* mutant background (Won et al., 2009), suggesting that it might be directly or indirectly suppressed by RHD6 in root epidermis. *At3g07350* was not included in the set of 208 core root epidermal genes in Arabidopsis (Bruex et al., 2012), possibly owing to the paucity of transcript under normal condition, or irrelevance of RH initiation. Collectively, we speculated that *At3g07350* acted a role in aforementioned auxin-mediated P-signal pathway, different from *RXR1*. In future, additional experiments (e.g. characterization of its overexpressor and *loss-of-function* mutants) are necessary to discover the functions of *At3g07350* in respect to P-signaling and RH development.

In order to investigate the biological function of the third P-inducible DUF506 gene, we performed additional genetic, molecular, biochemical and cytological experiments (Fig. 2 to 8). Similar expression pattern (e.g. tissue-specific and P-response) as well as GUS staining results (i.e. root hair) between *RXR1* and *RXR3*, implicate the possibility of functional redundancy. Overexpression of *RXR3* significantly limits the RH length, whereas the knock-out mutants exhibit opposite phenotype (Fig. 3), that remind us the inhibitory role of RXR1 to RH elongation. Despite both of RXR1 and RXR3 shares highly similar molecular and functional features, our studies reveal that the regulatory mechanism is distinct from each other. For instance, *RXR1* magnificently responds to P-stress (i.e. >1000-fold up-regulation) through PHR1/PHL1-dependent pathway, and independently of auxin (Ying et al., 2021). In contrast, *RXR3* in root functions in PHR1/PHL1-independent pathway and is induced by auxin (Fig. 2). Furthermore, the *rxr1 rxr3-1* double mutant exhibits extra longer root hair when compared to each single mutant (Fig. 4), implicated the functional divergency between RXR1 and RXR3.

RSL4 is moderately up-regulated by P-limitation, and promotes RH growth accordingly (Bhosale et al., 2018; Datta et al., 2015; Yi et al., 2010). Our results demonstrate that RSL4 TF binds to RHE motifs in *RXR3* promoter, resulting in its transcript accumulation (Fig.5). Zhu et al. (2020) summarized an autocrine signaling pathway that involved RALF1-FERONIA-RSL4 to synergistically regulation RH size. RALF1 was proved to promote the expression of *RSL4*, which consequently caused the accumulation of *RXR3* transcript (Fig.6). Thus, we could conclude that RXR3 participates in auxin and RSL4-dependent signaling pathway, in order to manage P-induced root hair elongation (see Fig.9).

Beside of the RHE motifs, three copies of auxin responsive element (i.e. AACGAC) were found in *RXR3* promoter (i.e. respectively localized in -116, -184 and -454 bp upstream of start codon) as well. Previously, ChIP assay and genetic evidences demonstrated that both *RSL2* and *RSL4* are transcriptionally activated by auxin response factors (ARFs) (Bhosale et al., 2018; S. Mangano et al., 2017). Under P-limitation, high auxin level induced the expression of several ARFs (e.g. *ARF5*, 7, 8 and 19), leading to the activation of *RSL4* and possible *RSL2*. On the other hand, when Pi is sufficient in soil, endogenous auxin activates *RSL2* expression, but not *RSL4*, to regulate ROS production, and RH growth in subsequent (Bhosale et al., 2018; Silvina Mangano et al., 2018). qRT-PCR analysis results indicated that transcript of *RXR3* was

moderately reduced in *arf7 arf19* double mutant, in regardless of P-status (Fig. S2B). Therefore, we propose that *RXR3* might not only be managed directly by RSL4, but also ARFs, especially under P-deficient condition, to coordinatively control RH growth (Fig.9).

Ca^{2+} , as indispensable macronutrient and second messenger molecule, could associate with various Ca^{2+} -binding protein (e.g. CaM and CDPK) by EF-hand motifs, to vastly modulate many biological processes in plants (Hepler, 2005; Himschoot, Beeckman, Friml, & Vanneste, 2015; Thor, 2019; Yuan, Jauregui, Du, Tanaka, & Poovaiah, 2017). Accumulating evidences suggested that rapid and gradient oscillation of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) was critical for sustained root hair polar tip growth (Bibikova, Zhigilei, & Gilroy, 1997; W. Feng et al., 2018; Konrad, Wudick, & Feijó, 2011; Monshausen, Messerli, & Gilroy, 2008). Recently, CNGC14 is reported to be in charge of stabilizing the integrity of growing RH and supporting cell expansion as well, and prone to interact with CaM7, to inhibit RH tip growth (Brost et al., 2019; Zeb et al., 2020; S. Zhang et al., 2017). Except of RXR1, CaMBD has been identified in other members of Arabidopsis DUF506 family. Protein microarray analysis have demonstrated that At1g77145 interacted with several CaM or CaM-like (CML) proteins (Popescu et al., 2007). Here, we found that RXR3 could interact with various Ca^{2+} -bound CaMs *in vivo* (Fig.7). When overexpressing *PEP3 Δ 1-22* in *rxr3-2* mutant, it could not restore the long root hair phenotype (Fig. S6), suggested the CaMBD was critical for inhibitory function. Moreover, RXR3 was unable to interrupt the association between CaM7 and CNGC14 (Fig.8B). Similar to *RXR3*, overexpression of *CaM7* gene conferred to shorter RH length (Zeb et al., 2020). The long RH phenotype of *cam7* mutant could not be restored by constitutively expressing *RXR3* gene (Fig. S7), demonstrated that *CaM7* was required for *RXR3* conducting its inhibitory roles. Considering the root hair tips focused localization (Fig. 3G and 6D), we hypothesized that, under P-limitation, RSL4-activated RXR3 might play as an escort or stabilizer, associates with and delivers Ca^{2+} -bound CaM7 to plasma membrane, to simultaneously inhibit CNGC14 activation and RH growth (Fig.9).

Besides CNGC14, other CNGCs, have been demonstrated to be important regulators of root hair or pollen tube tip growth (Brost et al., 2019; Chang, Yan, Zhao, Wu, & Yang, 2007; Gao et al., 2016; Pan et al., 2019; Tan et al., 2020; Tunc-Ozdemir et al., 2013; S. Zhang et al., 2017). Moreover, some of CNGCs could spontaneously form heteromeric complexes, such as CNGC9/14 and CNGC2/4, to conduct their regulatory roles (Brost et al., 2019; Chin, DeFalco, Moeder, & Yoshioka, 2013; Yu et al., 2019). In contrast to CaM7-arrested CNGC14, the activity of other CNGCs could be positively regulated by CaM binding (e.g. CaM1 to CNGC12)(Dietrich, Moeder, & Yoshioka, 2020; Jarratt-Barnham, Wang, Ning, & Davies, 2021). These aforementioned studies have shed light on the diverse regulatory mechanism of CNGC. We have characterized the capacity of RXR3 interacting with different kinds of CaM isoforms, hint that RXR3, and perhaps other DUF506 proteins (e.g. At1g77145), are novel components in regulation of CaM-CNGC-mediated Ca^{2+} dynamics and oscillation. Further research is required to explore DUF506-CaM interacting specificity and to understand the biological function of individual DUF506 proteins.

In an effort to assess the influences of *RXR3* on $[\text{Ca}^{2+}]_{\text{cyt}}$ changes, we introduced GCaMP3-GFP construct to two individual *rxr3* mutant lines, and recorded the $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillation in root hair apex zone (Fig.8C and D). Frequency of $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillation elevated about 20% in both *rxr3* mutants compared to that of wild type, manifested that RXR3 negatively affected tip-focused $[\text{Ca}^{2+}]_{\text{cyt}}$ gradients. Interestingly, the frequency of $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillation in *rxr1* mutant showed the similar level (~25%) of elevation (Ying et al., 2021). Therefore, we could not rule out the possibility of the interaction between RXR2 and RXR3, because of the widespread of conserved domain 3 in DUF506 family. On the other hand, these results confirmed the importance of elements outside of the conserved domain of DUF506 protein (Fig. S6).

While additional insights would be required to systematically illustrate the mechanism, evidence here supports a model in which RXR3 has inhibitory function that are essential for the maintenance of environmental stress induced RH growth (Fig.9). It remains to be determined as to whether and/or how RXR3 could connect with the other tip growth focused CNGCs. Our present evidence, together with previous studies, suggest that the RXR1 and RXR3 function as novel complements of P-inducible RH growth regulatory network, and could be considered as crop engineering targets to improve crop/forage resilience against rhizosphere

nutrient deficiency.

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SUPPORTING INFORMATION

Fig. S1 . GUS staining assay of the *proAt3g07350::GUS* transgenic line.

Fig. S2 . Expression analysis by qRT-PCR.

Fig. S3 . Phenotype of 10-day-old Arabidopsis seedlings (WT and *rrx3-1*) grown on +P or -P half strength MS solid medium.

Fig. S4 . RSL4 binds to the *EXPA7* promoter.

Fig. S5 . Recombinant expression of MBP-RXR3^{FL} (75.2 kDa) and MBP-RXR3^{Δ1-22} (72.7 kDa) protein.

Fig. S6 . Constitutive overexpression of CaMBD-truncated *RXR3* (*proΔM³⁵Σ::PΞP3^{Δ1-22}*) in *rrx3-2* mutant.

Fig. S7 . Constitutive overexpression of *RXR3^{FL}* (*proCaMV35S::RXR3^{FL}*) in *cam7* mutant.

Table S1 . Primer sequences used in this study.

AUTHOR CONTRIBUTION

S.Y. and W.-R. S. conceived and designed the research. S.Y. performed experimental work, analyzed data and wrote manuscript. W.-R.S. coordinated the project, and provided overall supervision.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Fig. 1 . qRT-PCR analysis of *Arabidopsis* DUF506 genes responds to phosphate starvation. Five-day-old wild-type (WT) seedlings were transferred to fresh regular (+P) or no phosphate (-P) half strength MS liquid medium, then the shoots and roots were harvested separately after 24 hr. *At3g54550* gene was unable to detect because of its silique-specific expression pattern. Changes of gene expression level between tissues are interpreted as described previously (Bari, Datt Pant, Stitt, & Scheible, 2006). Expression levels are given on a log scale expressed as $40 - \Delta CT$, where ΔCT is the difference in qRT-PCR threshold cycle number between the respective gene and the reference gene (*GAPDH*); 40 therefore equals the expression level of *GAPDH*; the number 40 was chosen because the PCR run stops after 40 cycles. The fold difference in expression is $2^{\Delta\Delta CT}$ when PCR efficiency is 2 (e.g. an ordinate value of 34 represents 16-fold lower expression than a value of 38).

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Fig. 3 . Overexpression of *RXR3* represses *Arabidopsis* root hair elongation. A, Diagram of two T-DNA insertions mutants used in the study. Exons, black boxes; UTRs, white boxes; Intron, black line. B and D, Representative images showing the effect of *RXR3* on root hair elongation. Three-day-old seedlings were transferred to +P or -P half strength MS solid medium, then grew vertically for 2 day. While the loss-of-function mutants (*rxr3-1* and *rxr3-2*) grow longer root hairs, constitutively overexpressing *RXR3* (*proCaMV35S::RXR3*, OX-5 and 12) repress root hair growth (B). The root hair growth are resumed by complementing *RXR3* in *rxr3-1* mutant (*proRXR3::RXR3-GFP*, Com#1 and #2, D). Scale, 1 mm. C and E, Violin plots of root hair lengths in WT, overexpressors, *rxr3* mutants, and complemented lines, under P sufficient or deficient condition. The plot illustrates kernel probability density in which the width represents distribution of data points. Dash lines indicate 25th and 75th percentiles and horizontal black line is the median value. Between 885 and 1026 root hairs from 10 seedlings were measured for each genotype and numbers at bottom indicate median values (in mm) for each genotype. F, Immunoblot analysis of RXR3-GFP fusion protein in complementation lines (#1 and #2) under different P-status. Coomassie-stained RuBisCO protein is shown as loading control. G, Root hair apical (a) and root epidermal (b, transection view) localization of RXR3-GFP fusion protein in *Arabidopsis* complemented line (Comp#1) under P-stress. Scale, 100 μm . H and I, Box plots of shoot phosphate content (H) or fresh biomass (I) of 10-day-old wild type (blue) and *rxr3-1* mutant (orange) seedlings that grown on +P or -P half strength MS solid medium ($n = 3$). Horizontal line is the

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Fig.5 . RSL4 binds to the *RXR3* promoter. A, Diagram of the *RXR3* promoter region showing the relative positions of the RHEs. The RHEs are marked by gray rectangles, and relative positions and sizes of the different PCR-amplified fragments are indicated by black lines under the RHE(s). B, qRT PCR analysis of *RXR3* expression in *rsl4* mutant. Five-day-old Arabidopsis seedlings were transferred to fresh +P or -P half strength MS liquid medium, then the roots were harvested after 24 hr. The data represent the mean values of three replicates $\pm$ SD. C, ChIP analysis to detect the association between RSL4 and the RHEs within the *RXR3* promoter in the RSL4-GFP transgenic line. The ChIP signals with (anti-GFP) and without (No Ab) addition of anti-GFP are indicated. D, Nucleus-localized GFP signal (Z-stack) in roots of *rsl4* complementation line (Comp#1) harboring *proRSL4::RSL4-GFP*. Scale, 30 μ m.

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Fig.8 . RXR3 affects root hair apical [Ca²⁺]_{cyt} oscillation. A, BiFC analysis to confirm the interaction between CNGC14 and CaM7 in *N. benthamiana*. B, BiFC analysis to investigate the interaction between CNGC14 and CaM7 is independent of RXR3. Arrow, nucleus. Scale, 20 μ m (A) or 40 μ m (B). C, Representative normalized oscillograms of wild type and *rrx3-1* mutant. The present data has been processed by baseline simulation analysis. D, Box plot of [Ca²⁺] oscillations frequencies in root hairs of *rrx3* mutants and wild type over 600 sec. Box limits indicate 25th and 75th percentiles, horizontal line is the median, and whiskers display minimum and maximum values. Each semitransparent dot represents individual measurements from 7 to 11 root hairs per group from 4 to 6 plants. ** $P < 0.01$ indicates statistical significance as determined by Student's *t* test. Ns, no significance.

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