이 학 석 사 학 위 논 문

애기장대 뿌리의 발달단계에 따른 RHD6와 RSL4의 뿌리털 발달에 대한 기능

The Functions of RHD6 and RSL4 in Root Hair Development along the Developmental zone of the *Arabidopsis* Root

서 울 대 학 교 대 학 원

생 명 과 학 부

조 현 민

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이 논문을 이학석사 학위논문으로 제출함

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조현민의 이학석사 학위논문을 인준함

２０１３ 년 ８ 월

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The Functions of RHD6 and RSL4 in Root Hair Development along the Developmental zone of the *Arabidopsis* Root

A dissertation submitted in partial fulfillment

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Hyun-Min Cho

Date Approved

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Abstract

The gene regulatory networks are important molecular basis of the cell differentiation in multicellular organisms. Root hair development system in *Arabidopsis* is frequently used to analyze the gene regulatory network in plant. Especially, hair cell-specific genes govern the root hair morphogenetic processes such as hair initiation and elongation at the later stages of the root hair development (Grierson and Schiefelbein, 2002). *RHD6* (*ROOT HAIR DEFECTIVE 6*, *AT1G66470*) and *RSL4* (*ROOT HAIR DEFECTIVE 6-LIKE 4*, *AT1G27740*) are the hair cell-specific genes that encode the basic-helix-loop-helix (bHLH) transcription factors (Menand and Dolan, 2007, Yi and Dolan, 2010). *RSL4* is direct target of RHD6. Both RHD6 and RSL4 are known to be expressed in the root meristem and elongation zone and regulate the root hair initiation and elongation. In this study, we investigated the importance of expression region of *RHD6* and *RSL4* and analyzed the inherent functions of RHD6 and RSL4 in the root hair formation. Our results showed the ectopic expression of *RHD6* and *RSL4* under various promoters, presents functions of RHD6 and RSL4 other than those known before. Auxin is the positive effector of root hair formation and modulates *RSL4* expression via unknown mechanisms (Schiefelbein, 2000, Yi and Dolan, 2010). Our results suggested that RHD6 and auxin regulate the *RSL4* expression in an independent manner and RHD6 has a target gene other than *RSL4*. We also found the possibility that RSL4 function has been conserved in angiosperms like RHD6 and EXPA7. These results extend our understanding of gene regulatory networks in the root hair development to angiosperm.

Key words: Root hair, RHS, Transcription factor, Auxin, Orthologous gene

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ABBREVIATION

ARE	Auxin response element
At	*Arabidopsis thaliana*
DNA	Deoxyribonucleic acid
E7, EXPA7	*EXPANSIN A7*
GL2	*GLABRA 2*
HYG	Hygromycin
IAA	Indole-3-acetic acid
MS	Murashige and Skoog
Os	*Oryza sativa*
OX	Over-expression
PCR	Polymerase chain reaction
Pt	*Populus trichocarpa*
RHE	Root hair element
RHS	Root hair specific genes
RNA	Ribonucleic acid
RT-PCR	Real time-polymerase chain reaction
Sm	*Selaginella moellendorffii*
WT	Wild type
YFP	Yellow fluorescence protein

1. Introduction

Recent molecular biology is focused on investigating the organization of complex gene regulatory networks. In multicellular organisms, transcriptional networks form the basis of cellular processes. Especially cellular differentiation is directed by transcriptional regulation of cell type specific genes. Root hair development system in *Arabidopsis* is ideal for deciphering gene regulatory networks during cell differentiation.

Root hair development is divided into three major stages (Grierson and Schiefelbein, 2002) : (1) fate determination, (2) hair initiation, (3) hair elongation by tip growth. Fate determination is the process that decides the hair or non-hair cell on root epidermis. This is established by position-dependent activities of a receptor-like kinase (*SCRAMBLED*, *SCM*), a complex made of a WD40 protein (*TRANSPARENT TESTA GLABRA*, *TTG*), /basic helix-loop-helix (*bHLH*) transcription factors (*GLABRA3/ENHANCER OF GLABRA3*, *GL3/EGL3*) and /a MYB transcription factor (*WEREWOLF*, *WER*), and a MYB-like protein (*CAPRICE*, *CPC*). The WD40/bHLH/MYB complex modulates the activity of a homeodomain-leucine-zipper (HD-ZIP) transcription factor (*GLABRA2*, *GL2*), which suppresses the hair cell-specific genes (*ROOT HAIR SPECIFIC*, *RHS*) activity on non-hair cell position. Fate determination occurs in the epidermis of the root meristem and elongation zone. Root hair morphogenetic processes occur in the elongation and differentiation zone of the root. These involve hair initiation, a stage when hair cell begins to show distinctive cytoplasmic characteristics and to bulge at the site of hair outgrowth, and elongation with the sustained tip growth to reach to the final size of a root hair. These are attributable to the activities of hair cell-specific genes (*RHS*), which are genetically downstream of GL2. Hormones also affect the root hair development (Schiefelbein, 2000). For example, auxin and ethylene are positive effectors of root hair formation.

RHD6 (*ROOT HAIR DEFECTIVE 6*, *AT1G66470*) is the hair cell-specific gene (*RHS*) that encodes the bHLH (basic-hexlic-loop-helix) transcription factor, which has mainly function related in the hair initiation. *RHD6* is expressed in meristem and elongation zones of the root (Menand and Dolan, 2007).

RSL4 (*ROOT HAIR DEFECTIVE 6-LIKE 4, AT1G27740*) is another hair cell-specific gene (*RHS*) encoding a bHLH transcription factor. It controls the hair growth and is expressed in elongation zone and early differentiation zone of the root (Yi and Dolan, 2010). *RSL4* expression is modulated by auxin through unknown mechanism. *EXPA7* (*EXPANSIN A7, AT1G12560*) is also the hair cell-specific gene (*RHS*) that encodes the cell loosening protein, which is important for tip growth and expressed in late elongation zone and differentiation zone of the root (Cho and Cosgrove, 2002). All of these three genes are essential for root hair morphogenetic processes and related to each other by transcriptional networks. So, it is crucial to determine the respective expression region for those genes and reveal their intrinsic functions for elucidation of root hair development.

In this study, we investigated the importance of expression region of *RHD6* and *RSL4*, and their inherent functions by generating several promoter driven gene expression constructs as well as treating auxin. Our results showed that the ectopic expression of these genes in different regions from their original ones, presented functions other than those known before. Moreover, we confirmed RHD6 and auxin regulate the *RSL4* expression independently. We also found that *RSL4* function might have been conserved in angiosperm like *RHD6* and *EXPA7*. These results extend the view of the existing genetic regulatory networks of the root hair development to angiosperm.

2. Materials and Methods

2.1 Plant materials and Growth Conditions

Arabidopsis thaliana, Columbia ecotype, was used as the wild-type plant in this study. The mutant seeds of *rhd6-3* were obtained from J. W. Schiefelbein (University of Michigan, Ann Arbor, MI) and *rls4-1* were obtained from Liam Dolan (University of Oxford, Oxford, United Kingdom). All seeds were grown on agarose plates containing 4.3g/L Murashige and Skoog (MS) nutrient mix (Sigma-Aldrich), 1% sucrose, 0.5g/L MES (PH 5.7), KOH, and 0.8% agarose. All seeds were cold-treated (4℃) for 3 days and germinated at 23℃ under 16-h-light/8-h-dark photoperiods. Transformed plants were selected on hygromycin-containing plates (50μg/mL). For all pharmacological experiments, 4-day-old seedlings of homozygous transformants were transferred to new plates containing the indicated chemicals and incubated for 12 hours before observation of root hairs.

2.2 Transgenic Constructs

For *ProE7:RHD6/RSL4/RSL4 orthologous genes*, *ProPIN2:RHD6/RSL4* and *ProGL2:RSL4/RSL4 orthologous genes* constructs, *RHD6* and *RSL4* genomic DNA were amplified using the polymerase chain reaction (PCR) and the primer sets listed in Table 1. Genomic DNA template was obtained from the 4-day-old *Arabidopsis* seedling. The *RHD6* and *RSL4* were inserted into the BamHI/SpeI sites of the *pCAMBIA1300-NOS* binary vectors, each had inserted the *EXPA7*, *PIN2* and *GL2* promoter (-470 from start codon, *ProE7*, -2166, *ProPIN2*, -2088, *ProGL2*). The *RSL4 orthologous genes* were inserted into the PacI/XbaI sites for *PtXP002302411* and *Os12g39850*, the PacI/XmaI sites for *SmXP002963143* and the XmaI/MluI sites for *Os07g39940* of the same vectors. For *RHD6-RNAi-1/2* construct, *RNAi* target regions in *RHD6* cDNA were amplified using the PCR and the primer sets listed in Table 2. cDNA template was obtained from the roots of *Arabidopsis* seedling. The two *RNAi*

target regions, *RNAi-1* and *RNAi-2*, are indicated in Fig 2. The *RNAi-1* and *RNAi-2* target regions were inserted into the XhoI/EcoRI and BamHI/XbaI sites of the *pHannibal* vector to generate sense and antisense fragments, respectively. Next, the XhoI/XbaI fragments from the cloned *pHannibal* vector were transferred into the SalI/XbaI sites of the binary vector *ProE7:pCAMBIA1300-NOS*. All transgenic constructs were confirmed by nucleotide sequencing and the genetic integrity of transgenic plants was confirmed by PCR amplification of genomic DNA.

2.3 Measurement of Root Hair Length and Cell-Type Pattern Analysis

The measurement of root hair length was carried out as described previously (Lee and Cho, 2006) with modification. For estimation of root hair length, digital photographs of roots were taken under a stereomicroscope (Leica MZ FLIII, Heerbrugg, Switzerland) at 40x magnifications. Hairs in the hair maturation region (~0.78mm from the tip) were counted. Eight to ten consecutive hairs protruding perpendicularly from each side of the root, representing a total of eighteen to twenty hairs from both sides of the root, were measured from 8-20 roots. Hair bulges shorter than 14μm were considered arrested at the early bulge stage, and were not counted. To characterize the distribution of hair cells and non-hair cells, 10 cells consecutively lied in the hair cell (H) position and 10 cells in the non-hair cell (N) position were assessed in each of 15-20, 4-day-old seedling roots for each line. Representative results from 2-5 independent experiments were presented.

2.4 Quantitative Reverse Transcriptase PCR (qRT-PCR) Analysis

Total RNA was isolated from the roots of 4-day-old seedlings (100 for each line) using an RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized by Primescript™ 1st Strand cDNA Synthesis Kit (Takara). qRT-PCR analyses were performed using an EvaGreen Master Mix (Applied Biological Materials Inc.). Gene-specific signals were normalized relative to Actin 7 which primer sets listed in Table 1. Each reaction was performed in triplicate and repeated three times using independent

preparations of RNA.

2.5 Sequence Alignments and Phylogenetic Trees

Nucleotide and protein sequences were aligned using CLUSTAL W (DNASTAR) with the default parameters (gap penalty, 10.0; gap length penalty, 0.2; delay divergent seqs, 30%; DNA transition weight, 0.5). Neighbor-joining phylogenetic trees were generated using MEGA version 4.0, adopting the Poisson correction distance for amino acid sequence. The number of bootstrap replicates was 1000.

2.6 Yeast One-Hybrid Assay

Yeast one-hybrid (Y1H) interactions were studied by using the BD Matchmaker™ One-Hybrid Library Construction & Screening Kit. Coding sequences of root hair specific-gene's cis-element (*Root Hair Element*, *RHE*) and *RSL4*, were amplified and fused to both pHIS2 Reporter and pGADT7-Rec2 AD cloning vectors. Y1H analyses were performed as following method. First, cultured the yeast on YPAD plates at 30℃ for 1-2 days, picked up the yeast and added the one-step buffer (comprised of 50% PEG, 1M LiAc, 4M DTT). Second, added the plasmid to the yeast-suspended solution and conducted heat shock at 42℃ for 45minute. Dropped the yeast-suspended solution on the SD (-Trp, -Leu and –Trp, -Leu, -His sets, for the removal of non-specific yeast, add the 3-AT solution) plates and cultured the yeast at 30℃ for several days. To quantify interactions strengths, three experimental and technical replicates were performed.

Table 1. List of Primers used for PCR

Type	Name	Sequence (5' to 3')
RHD6-PCR primers	gRHD6_BamHIF	TATTGGATCCTAATGGCACTCGTTAATGAC
	gRHD6_SpeIR	AAACACTAGTTTAATTGGTGATCAGATTCG
RSL4-PCR primers	RSL4_BamHIF	TCAGGGATCCCGAATGGACGTTTTTGTTGAT
	RSL4_SpeIR	TGTAACTAGTTCACATAAGCCGAGACAAAAGG
RHD6-RT-primers	R6_RT597F	AGTCTCTTTCGCCTAAATCCG
	R6_RT836R	AGGCTTTGTGGATCTTTAGGTG
RSL4-RT-primers	cRSL4_qF1	GTGCCAAACGGGACAAAAGT
	cRSL4_qR1	TTGTGATGGAACCCCATGTC
Actin primers	At_actin7F	GTGTGTCTTGTCTTATCTGGTTCG
	At_actin7R	AATAGCTGCATTGTCACCCGATACT
RSL4 orthologous gene-PCR primers	R4Pa_51PtF	AGCTTTAATTAACTAGTAGGCATCACCA
	R4Xb_204PtR	TGCTTCTAGAAACATGTGACGGGGAAAG
	R4Pa_153SmF	AAGATTAATTAAAGTCAACCCCCGTGGTCG
	R4Xm_178SmR	AAGGCCCGGGTAATCTCCTACAGGTAAA
	R4Pa_0OsF	ATATTTAATTAAATGGAGGGTGGAGGACTG
	R4Xb_0OsR	AATTTCTAGATCATGTATCAATGTTCAGATCG
	Os07g_EcXmF	ATATGAATTCCCCGGGATGGCGCAGTTTCTTGG
	Os07g_MlGXhR	TATACTCGAGTTAAACGCGTATGTATTTTTGCAGAGAAGAGATGTTC

3. Results

3.1 Ectopic expression of *RHD6* in differentiation zone has an effect on the root hair elongation

RHD6 is originally expressed in root hair cells in meristem and part of elongation zone for the root hair initiation. We asked whether *RHD6* essentially has the function related in root hair elongation. To address the question, we ectopically expressed *RHD6* under the *EXPA7* promoter from late elongation to differentiation zone of a root. Compared to the control (Cont, *ProE7:YFP*), wild-type plants harboring the *EXPA7* promoter:*RHD6* over-expressing (*ProE7:RHD6ox*) construct showed significantly longer root hair phenotype (On average, 37% increased) (Fig. 1A and 1B). The ProE7-driven expression of *RHD6* considerably increased the transcript level of *RHD6* in the root compared with the control, indicating that *RHD6* is overexpressed in *ProE7:RHD6ox* (Fig. 1C) construct. The result suggests that the ectopic expression of *RHD6* in differentiation zone exerts the root hair elongation. However, the transcript level of *RSL4*, which is a known direct target of *RHD6* (Yi and Dolan, 2010), in the *ProE7:RHD6ox* plants was less increased compared with the *RHD6* transcript level (Fig. 1C). The result implies many potential. For example, relatively small amount of *RSL4* comparing *RHD6* is sufficient to function, or *RHD6* needs another target gene which is not *RSL4* for the root hair elongation.

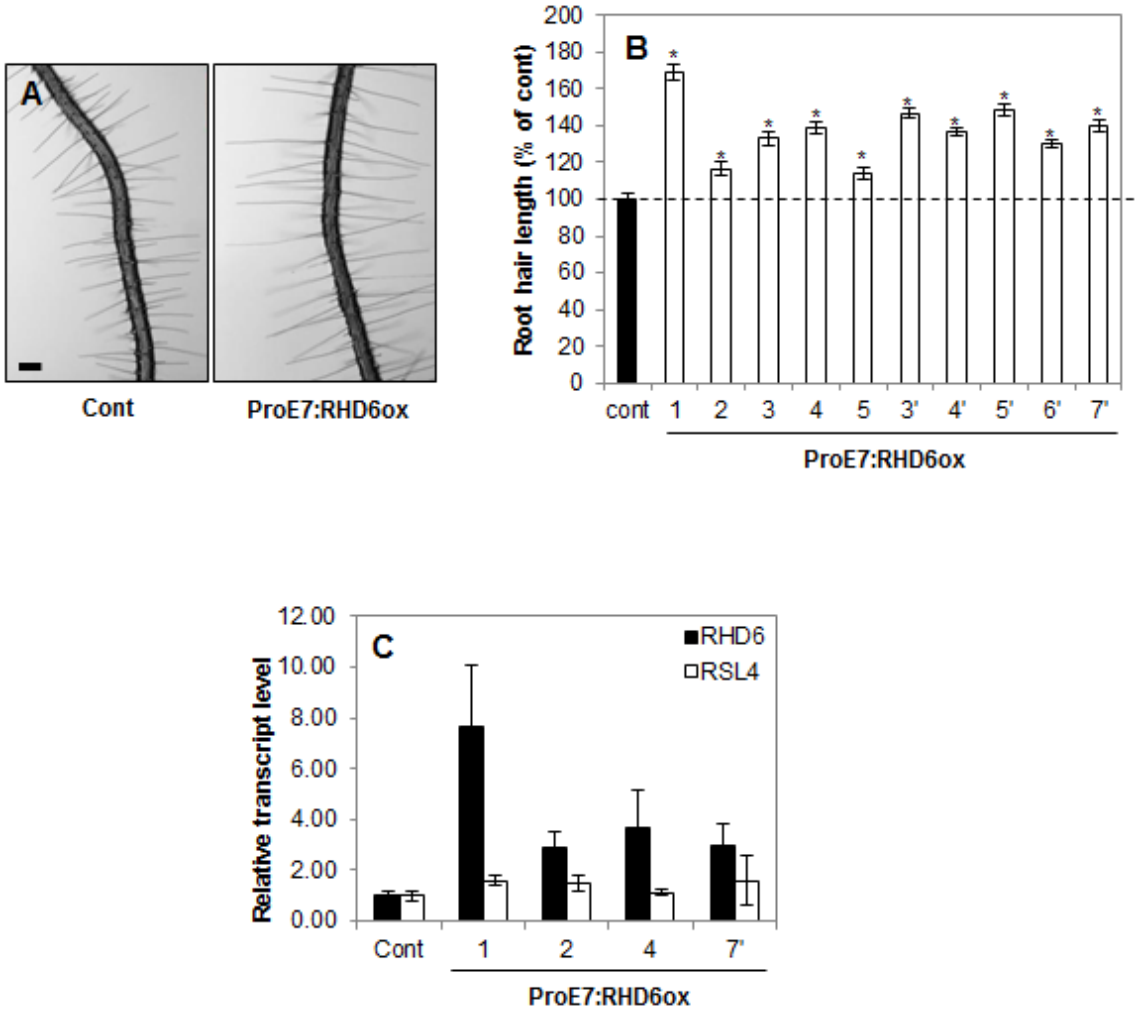

Fig 1. Effects of *RHD6*, which is root hair cell-specifically expressed, on root hair elongation in differentiation zone.

(A) Representative root images of control (Cont; *ProE7:YFP*) and *RHD6* over-expressing plants in differentiation zone (*ProE7:RHD6ox*). Bar=100μm.

(B) Root hair lengths of control (Cont; *ProE7:YFP*) and *ProE7:RHD6ox* plants. Numbers indicate independent over-expressing transgenic lines. Data represent means± S.E. (N = 660 to 980, average 790). *ProE7:RHD6ox* lines with root hair lengths significantly different from the control (p, 0.05) are indicated with an asterisk.

(C) Quantitative RT-PCR (qRT-PCR) analysis of control (Cont; *ProE7:YFP)* and *ProE7:RHD6ox* transcripts. Relative levels of *RHD6* and *RSL4* transcripts in the roots of control, *ProE7:RHD6ox* plants. Data represent means± S.E. from four independent experiments.

3.2 Knock-down transcript level of *RHD6* in separate zone of the root influences on the different root hair development

In order to compare the *RHD6* function in the originally expressed region (meristem and elongation zone) with in the ectopic expressed region (differentiation zone), we generated two sets of RNAi constructs to knock-down transcript level of *RHD6*. One set of RNAi constructs targeted in the epidermal cells in root meristem and elongation zone, another set of RNAi constructs targeted in the root hair cells in differentiation zone. The RNAi regions were 145 bp (RNAi-1, 271-415 bp from the start codon) and 190 bp (RNAi-2, 504-693 bp) in length (Fig. 2A). RNAi transcript expression was driven by the *PIN2* promoter (ProPIN2) and *EXPA7* promoter (ProE7) to obtain constructs fitting our objectives (Fig. 2B).

Eight *ProE7:RNAi-1* and Nine *ProE7:RNAi-2* construct lines were obtained, all of which showed shorter root hair lengths (*ProE7:RNAi-1*, decreased 39% on average, *ProE7:RNAi-2*, decreased 34%) than those of control (Cont, *ProE7:YFP*). Measurements were taken for 260-2500 root hairs per independent line, *ProE7:RNAi-1* lines exhibited slightly shorter root hairs than *ProE7:RNAi-2* (Fig. 3A and 3B). We analyzed whether the *ProE7:RNAi* constructs interfered with the *RHD6* transcript by performing quantitative RT-PCR (qRT-PCR). Target transcripts were reduced by 70 and 62% in *ProE7:RNAi-1* line 5 and 13, respectively, and by 73 and 60% in *ProE7:RNAi-2* line 7 and 20, respectively (Fig. 3C). Therefore, it is likely that reduction in the *RHD6* transcript level in the root hair cells in differentiation zone resulted in the inhibition of root hair elongation observed in *ProE7:RNAi* constructs.

We also obtained eight *ProPIN2:RNAi-1* and *ProPIN2:RNAi-2* construct lines. All the *ProPIN2:RNAi-1* lines showed a defective root hair initiation phenotype. The *ProPIN2:RNAi-2* lines showed shorter root hairs, which is similar to the phenotype of lines expressing *ProE7:RNAi* constructs (Fig. 4A, 4B and 4D). The magnified pictures of control and each of *RHD6-RNAi* transgenic construct plant showed the characteristic we mentioned above (Fig. 4E). In addition, the

ProPIN2:RNAi constructs were examined by quantitative RT-PCR (qRT-PCR). As expected, target transcripts were reduced by 24% and 45% in *ProPIN2:RNAi-1* line 1 and 2, respectively, and by 54% in *ProPIN2:RNAi-2* line 2 (Fig. 4C). Reduction in the *RHD6* transcript level in the epidermal cells in meristem and elongation zone resulted in the inhibition of root hair initiation, which is the original RHD6 function in the wild-type. These results suggest that expression of *RHD6* in a specific position of the root is crucial for making *RHD6* properly function.

Table 2. Primers used for PCR amplification of RNAi target regions in RHD6 cDNA

Type	Name	Sequence (5' to 3')
RNAi-1 primers	R6Xh_372ssF	TCATCTCGAGAGCTTTCCTCCTCCAGC
	R6Ec_538ssR	TGGGGAATTCCGGTGGATCTAGGGCTAATA
	R6Xb_371saF	TTTGATCTAGACGAGAGCTTTCCTCCTCC
	R6BH_530saR	TTCGGTGGATCCAGGGCTAATAATATCC
RNAi-2 primers	R6Xb_654caF	AACTCTAGAAGAAACTGAGTAGCGGTGTG
	R6BH_866caR	TTGAAGGATCCTGACATAACTAATAGCC
	R6Xh_647csF	AGCACTCGAGCGTCGAAGAAACTGAGTAGC
	R6Ec_830csR	GAAGGAATTCGACATAACTAATAGCCTTTTCAAG

Abbreviation of restriction enzyme sites in primers : Xh (Xho I), Ec (EcoR I), Xb (Xba I), BH (BamH I)

A

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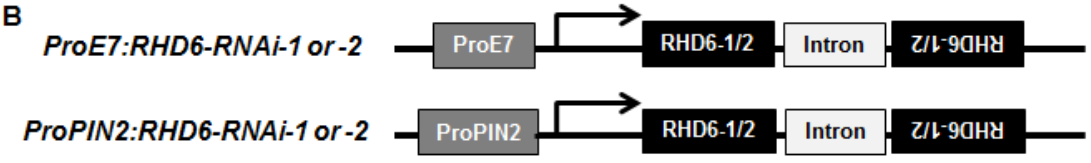


Fig 2. RNAi target regions in RHD6 cDNA.

(A) RHD6 cDNA sequence showing the coding and untranslated regions in upper and lower case, respectively. The start (ATG) and stop (TAA) codons are boxed. RNAi target regions are underlined by solid (*RNAi-1*) and broken (*RNAi-2*) lines.

(B) Expression of *RNAi-1* or *RNAi-2* constructs is driven by the *EXPA7* and *PIN2* promoter (ProE7 and ProPIN2). The arrow indicates the transcription initiation site.

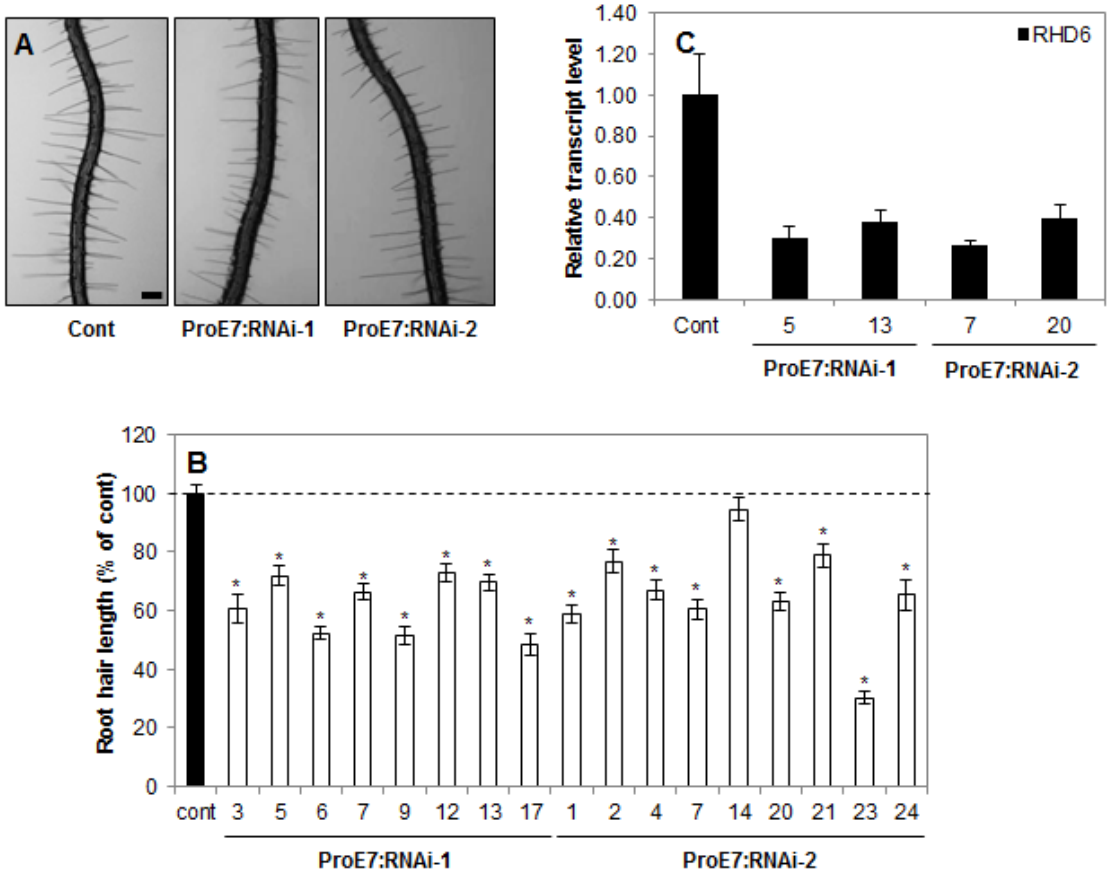
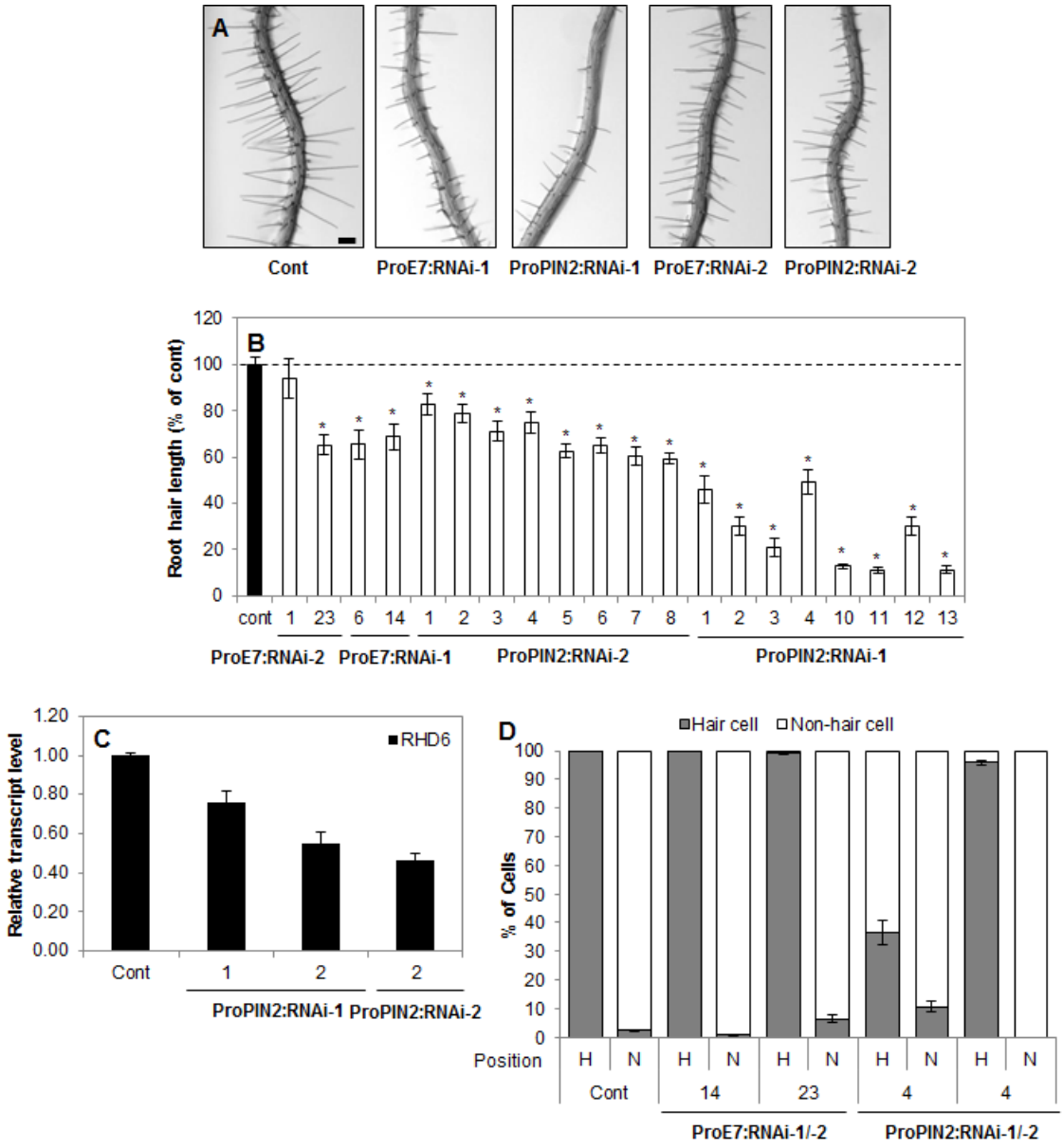


Fig 3. RNAi against RHD6 inhibited root hair elongation in differentiation zone.

(A) Representative root images of control (Cont; *ProE7:YFP*) and in differentiation zone root hair cell-specific RHD6-RNAi (*ProE7:RHD6-RNAi*) lines (*RNAi-1 and RNAi-2*). Bar=100μm.

(B) Root hair lengths of control (Cont; *ProE7:YFP*) and *ProE7:RNAi-1, ProE7:RNAi-2* plants. Numbers indicate independent RNAi transgenic lines. Data represent means± S.E. (N = 260 to 2500, average 864). *ProE7:RNAi-1, ProE7:RNAi-2* lines which root hair lengths differ significantly from the control (p, 0.05) are indicated with an asterisk.

(C) Quantitative RT-PCR (qRT-PCR) analysis of control (Cont; *ProE7:YFP)* and *ProE7:RNAi-1* and *ProE7:RNAi-2* transcripts. Relative levels of RHD6 transcripts in the roots of control, *ProE7:RNAi-1* and *ProE7:RNAi-2* plants. Data represent means± S.E. from three independent experiments.


A
Cont
ProE7:RNAi-1
ProPIN2:RNAi-1
ProE7:RNAi-2
ProPIN2:RNAi-2
B
Root hair length (% of cont)
cont 1 23 6 14 1 2 3 4 5 6 7 8 1 2 3 4 10 11 12 13
ProE7:RNAi-2
ProE7:RNAi-1
ProPIN2:RNAi-2
ProPIN2:RNAi-1
C
RHD6
Relative transcript level
Cont 1 2 2
ProPIN2:RNAi-1
ProPIN2:RNAi-2
D
Hair cell
Non-hair cell
% of Cells
Position
H N H N H N H N H N
Cont 14 23 4 4
ProE7:RNAi-1/-2
ProPIN2:RNAi-1/-2

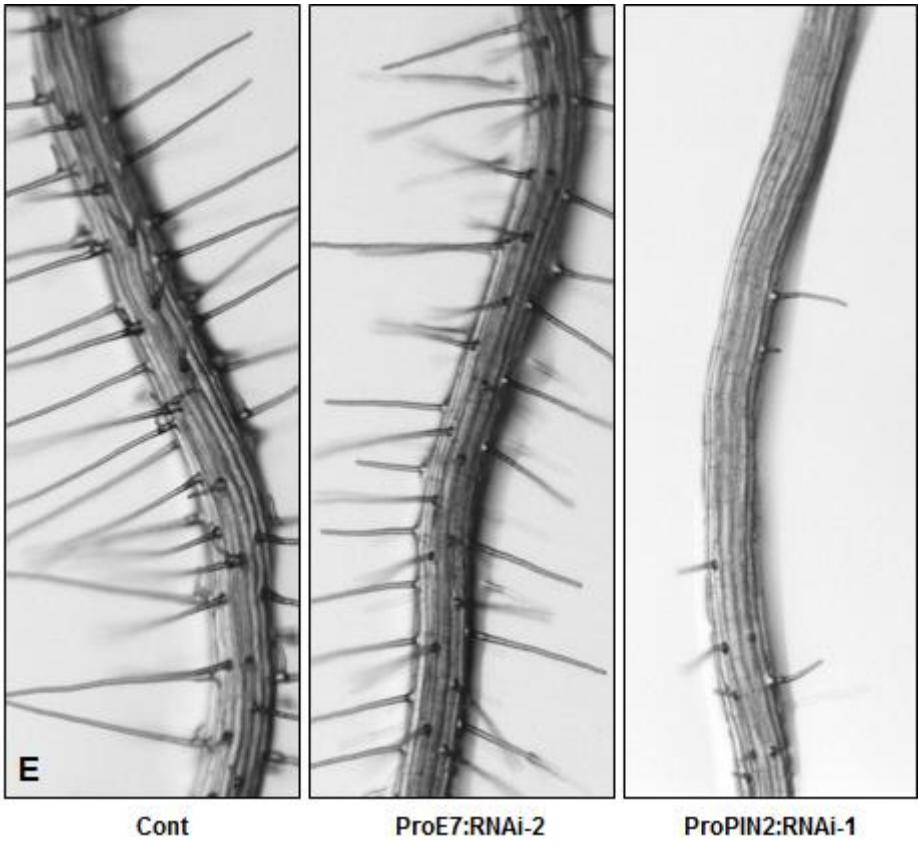


Fig 4. RNAi of RHD6 inhibited root hair initiation and elongation in the meristem region.

(A) Representative root images of control (Cont; *ProE7:YFP*), differentiation zone root hair cell specific RHD6-RNAi (*ProE7:RHD6-RNAi*) lines (*RNAi-1 and RNAi-2*) and meristem-focused RHD6-RNAi (*ProPIN2:RHD6-RNAi*) lines (*RNAi-1* and *RNAi-2*). Bar=100μm.

(B) Root hair lengths of control (Cont; *ProE7:YFP*), differentiation zone root hair cell-specific RHD6-RNAi (*ProE7:RHD6-RNAi*) lines and meristem-focused RHD6-RNAi (*ProPIN2:RHD6-RNAi*) lines. Numbers indicate independent RNAi transgenic lines. Data represent means± S.E. (N = 440 to 3180, average 855). *ProE7:RNAi-1*, *ProE7:RNAi-2*, *ProPIN2:RNAi-1*, *ProPIN2:RNAi-2* lines which root hair lengths differ significantly from the control (p, 0.05) are indicated with an asterisk.

(C) Quantitative RT-PCR (qRT-PCR) analysis of control (Cont; *ProE7:YFP)* and *ProPIN2:RNAi-1* and *ProPIN2:RNAi-2* transcripts. Relative levels of RHD6 transcripts in the roots of control, *ProPIN2:RNAi-1* and *ProPIN2:RNAi-2* plants. Data represent means± S.E. from three independent experiments.

(D) Cell-type pattern analysis, showing the fraction of root hair cells and non-hair cells that lie in the hair cell (H) and non-hair cell (N) positions, respectively, of the root epidermis (Cont, 100% (H), 2% (N), *ProE7:RNAi-1* line 14, 100% (H), 0.7% (N), *ProE7:RNAi-2* line 23, 99% (H), 6% (N),

ProPIN2:RNAi-1 line 4, 36% (H), 10% (N), *ProPIN2:RNAi-2* line 4, 95% (H), 0% (N), N = 350 to 530, average 396). The root hair initiation ratio was estimated by counting the root hairs that are longer than 14μm (root hair bulge).

(E) Representative root images of control (Cont; *ProE7:YFP*), *ProE7:RNAi-2* and *ProPIN2:RNAi-1* that magnified for the cell-type pattern analysis.

3.3 Suitable expression position can control the *RHD6* and *RSL4* primary function for the root hair development

Root hair development in *Arabidopsis* is regulated by developmental regulators, hormones, and environmental factors (Cho and Cosgrove, 2002). Developmental regulators like *RHD6* and *RSL4* are the components of transcriptional networks in the root hair cell, controlled exquisitely along the root longitudinal axis from the root tip to the basal regions. *RHD6* is known to be expressed in meristem and elongation zone of the root, *RSL4* is also known to the region of expression which is the elongation and part of differentiation zone of the root. We asked to what extent the expression of *RHD6* and *RSL4* in the specific position of the root is crucial for the root hair development. To this end, the expression of *RHD6* and *RSL4* was driven under the *PIN2* and *EXPA7* promoter in *rhd6-3* mutants. The *rhd6-3* mutant has defective root hairs, showing no root hair bulges (Masucci and Schiefelbein, 1994, 1996) (Fig 5A). *EXPA7* is one of downstream genes of *RHD6*, expression of *EXPA7* in *rhd6-3* mutant was blocked almost completely and *EXPA7* promoter could not be activated in *rhd6-3* mutant. So, plants expressing *ProE7:RHD6* or *ProE7:RSL4* constructs in the *rhd6-3* mutant background showed the same root hair phenotype as *rhd6-3* mutant (Fig 5B, 5C and 6C, 6D).

PIN2 is the independent component of transcriptional networks involved in root hair development but its expression region is similar to the one of *RHD6*. *ProPIN2:RHD6* expressed in *rhd6-3* mutant was able to restore the defective root hair phenotype to the wild-type's (Fig. 5D and 5F). Root hair initiations also occurred in non-hair cells in these transgenic plants, because *PIN2* promoter is not specific to the root hair cells in meristem and elongation zone of the root (Fig. 5E).

Ectopic expression of *RSL4* in differentiation zone driven by *EXPA7* promoter (background wild-type, *ProE7:RSL4ox*) showed the similar root hair phenotype of *RHD6* over-expressing one (*ProE7:RHD6ox*) (Fig. 6A, 6B and 6G). *RSL4*'s primary role is known to be to control the root hair growth, as opposed to *RHD6*. Thus, we suspected whether *RSL4* has the inherent function related to root hair initiation. When we expressed *ProPIN2:RSL4* in *rhd6-3* mutant background, the plants

showed the considerably restored root hairs in the hair cells compared with *rhd6-3* mutant (Fig. 6E, 6F and 6H). This result implies that *RSL4* has the inherent ability to regulate the root hair initiation.

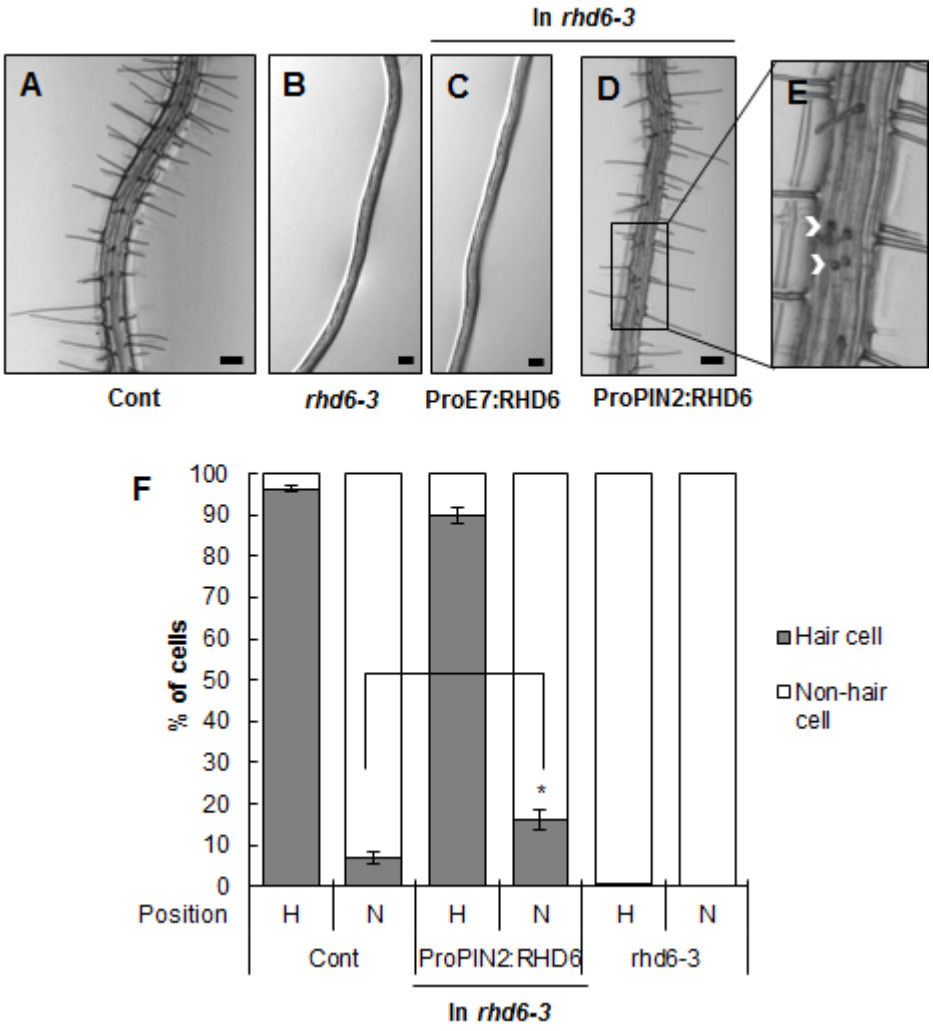


Fig 5. Defective root hair initiation phenotype of *rhd6-3* is not restored by *ProE7:RHD6* but restored by *ProPIN2:RHD6*.

(A) to (D) Representative root images of control (Cont; *ProE7:YFP*), *rhd6-3*, *ProE7:RHD6* and *ProPIN2:RHD6* in *rhd6-3* background plants. Bar=100μm.

(E) Representative root images of *ProPIN2:RHD6* in *rhd6-3* background that magnified for the showing root hairs in non-hair cell position.

(F) Cell-type pattern analysis, showing the fraction of root hair cells and non-hair cells that lie in the hair cell (H) and non-hair cell (N) positions, respectively, of the root epidermis (Cont, 96% (H), 7% (N), *ProPIN2:RHD6*, 90% (H), 16% (N), *rhd6-3*, 0.6% (H), 0% (N), N = 4 to 1158, average 594). *ProPIN2:RHD6* in *rhd6-3* background lines showed increasing hair cell percentages in non-hair cell positions, which increasing rate is different significantly from the control (p, 0.05) indicated with an asterisk.

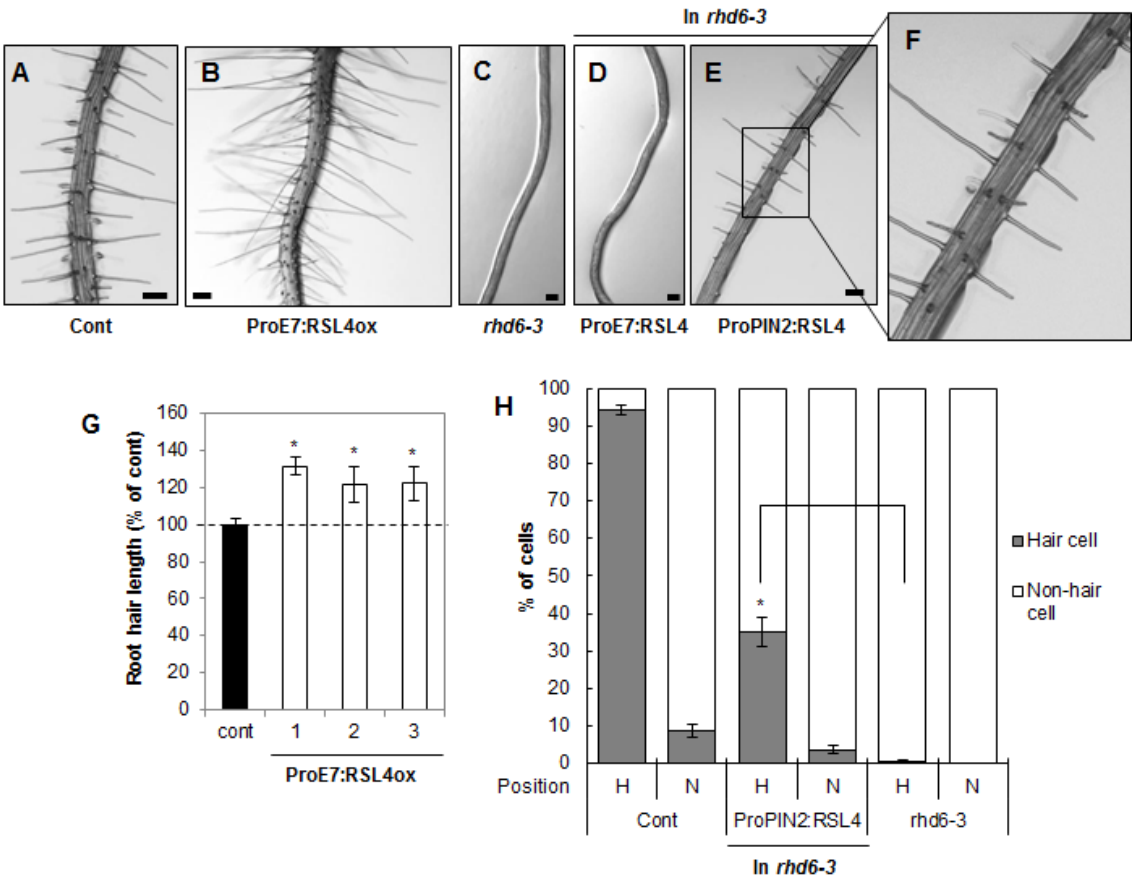


Fig 6. Effects of RSL4, which is root hair cell-specifically expressed, on root hair initiation or elongation in meristem and differentiation zone.

(A) to (E) Representative root images of control (Cont; *ProE7:YFP*), *ProE7:RSL4ox*, *rhd6-3*, *ProE7:RSL4* and *ProPIN2:RSL4* in *rhd6-3* background plants.

(F) Representative root images of *ProPIN2:RSL4* in *rhd6-3* background that magnified for the showing root hair initiation occurred in hair cell position.

(G) Root hair lengths of control (Cont; *ProE7:YFP*) and *ProE7:RSL4ox* plants. Numbers indicate independent over-expressing transgenic lines. Data represent means± S.E. (N = 320 to 520, average 400). *ProE7:RSL4ox* lines which root hair lengths differ significantly from the control (p, 0.05) are indicated with an asterisk.

(H) Cell-type pattern analysis, showing the fraction of root hair cells and non-hair cells that lie in the hair cell (H) and non-hair cell (N) positions, respectively, of the root epidermis (Cont, 94% (H), 9% (N), *ProPIN2:RSL4*, 35% (H), 4% (N), *rhd6-3*, 0.6% (H), 0% (N), N = 4 to 997, average 560). *ProPIN2:RSL4* in *rhd6-3* background lines showed increasing hair cell percentages in hair cell positions, which increasing rate is different significantly from the *rhd6-3* (p, 0.05) indicated with an asterisk.

3.4 Comparison of exogenous auxin (IAA) effects showed RHD6 might regulate the activation of genes other than *RSL4*

The differentiation of root hair cells is promoted by auxin, although the molecular mechanism is unclear (Bruex and Schiefelbein, 2012). In the *rhd6-3* mutant background, addition of exogenous auxin caused the *rhd6-3* mutant to overcome the blocked root hair formation, implying that auxin activates transcriptional networks at a certain point of the root hair development that are either downstream or independent of *RHD6*. On the other hand, auxin is known to modulate RSL4 expression (Yi and Dolan, 2010). To investigate the role of auxin in the root hair development, we compared auxin's effect on the *rhd6-3* mutant with the one on the *rhd6-3* expressing *ProE7:RHD6* and *ProE7:RSL4*. The results showed that *ProE7:RHD6* and *ProE7:RSL4* lines in *rhd6-3* mutant background are more sensitive to auxin than *rhd6-3* mutant control (Fig. 7A to 7F). From this results, we can infer that *RHD6* and auxin modulate *RSL4* expression independently of each other and that auxin reinforces the activity of downstream genes of *RSL4* involved in root hair formation (Fig 7D to 7F). *ProE7:RHD6* and *ProE7:RSL4* lines in *rhd6-3* also showed sensitivities different from each other, i.e. *ProE7:RHD6* lines were more sensitive to auxin than *ProE7:RSL4* lines (Fig. 7D to 7F). This result suggests that RHD6 might regulate the activation of genes other than *RSL4*, which regulate hair cell-specific gene's activations as upstream regulators (Fig. 7G).

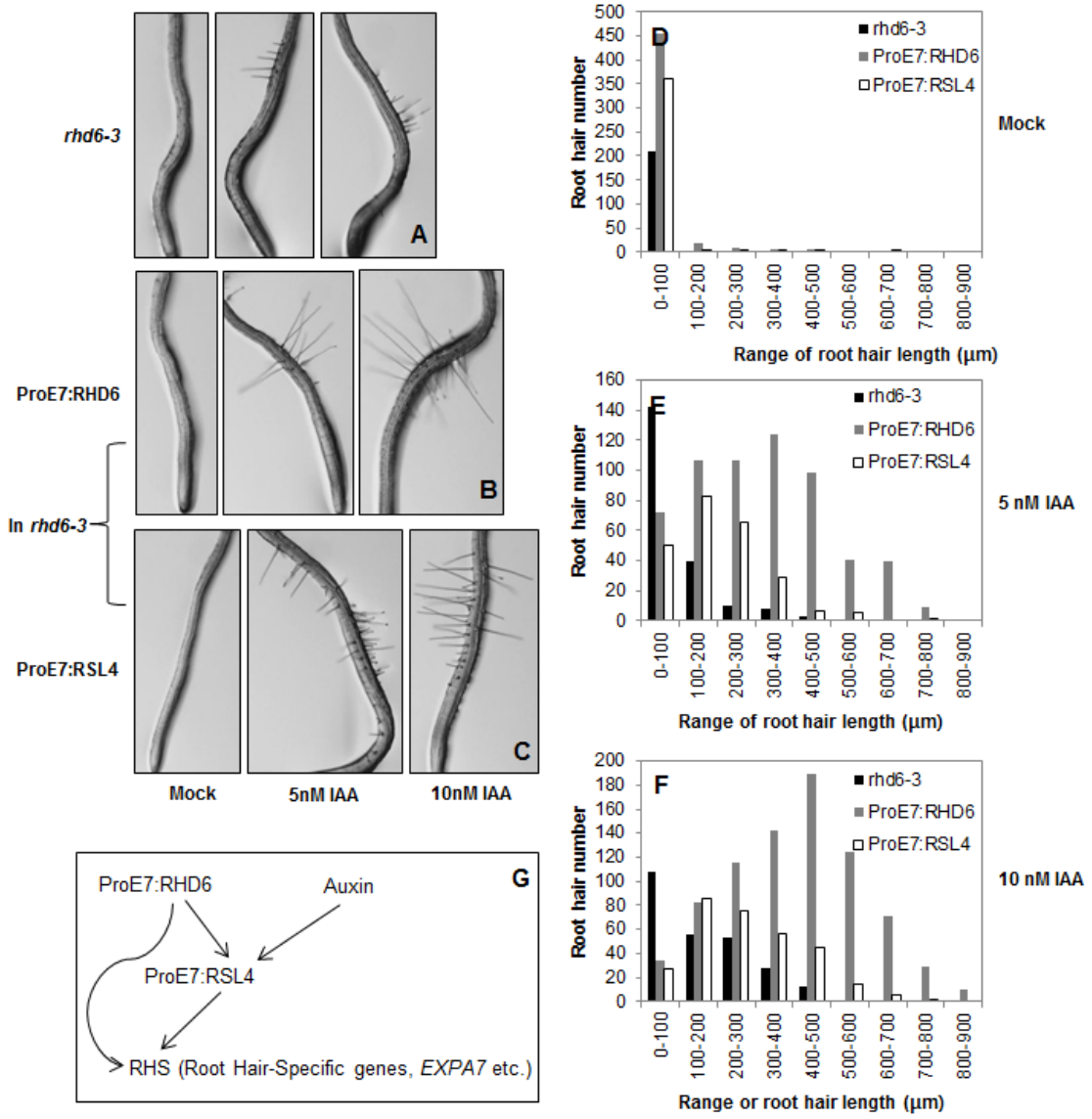


Fig 7. Exogenous auxin (IAA) induces more root hair initiation and elongation of *ProE7:RHD6* and *ProE7:RSL4* in *rhd6-3* than those of *rhd6-3*.

(A) to (C) Representative root images of control (Cont; *rhd6-3*) and *ProE7:RHD6* and *ProE7:RSL4* in *rhd6-3* background plants. Each plant grew for four days on antibiotic media (MS+HYG), transferred to either MS (Mock), MS+5nM IAA, or MS+10nM IAA and grew for 12 hours additionally.

(D) to (F) Quantitative analysis of root hair number scaled by length, ranged from 0 to 900 μm in control (Cont; *rhd6-3*) and *ProE7:RHD6* and *ProE7:RSL4* in *rhd6-3* background plants.

(G) The hypothetic model that explains the different phenotypes with transgenic (*ProE7:RHD6* and *ProE7:RSL4* in *rhd6-3*) constructs and *rhd6-3*.

3.5 Functions of *RSL4* orthologous genes from diverse angiosperm species might have been conserved

EXPA7 is known to have the orthologous genes in the angiosperm (Kim and Cho, 2006) and RHD6 has also RHD6-like proteins in the bryophytes (Menand and Dolan, 2007). *EXPA7* is supposed to be one of the direct target genes of *RSL4*, RSL4 protein exhibited to bind the root hair element (*Root Hair Element*, *RHE*), which is involved in hair cell-specific genes (*RHS*) (Fig. 8). Downstream and upstream genes of *RSL4* are conserved in the primitive plants, thus we questioned whether *RSL4* has also orthologous genes in the angiosperm. A NCBI BLAST search, using *Arabidopsis* RSL4 protein as query, identified closely related protein sequences from At (*Arabidopsis thaliana*), Al (*Arabidopsis lyrata*), Pt (*Populus trichocarpa*), Vv (*Vitis vinifera*), Rc (*Ricinus communis*), Lj (*Lotus japonicus*), Tr (*Trifolium repens*), Os (*Oryza sativa*), Sb (*Sorghum bicolor*), Ma (*Musa acuminata*), Pp (*Physcomitrella patens*) and Sm (*Selaginella moellendorffii*). Those RSL4 orthologous protein sequences were aligned by CLUSTAL W and a neighbor-joining phylogenetic tree was generated from this alignment (Fig. 9).

We selected three of RSL4 orthologous proteins from Pt, Os and Sm (Fig 9. black boxes). All of the selected RSL4 orthologous protein promoters contained multiple root hair elements (*RHEs*) in the proximal promoter region and these root hair elements (*RHEs*) were similar to the root hair elements (*RHEs*) in RSL4 promoter, including the hallmark CACG motif (Kim and Cho, 2006) (Fig. 10A and 10B).

To demonstrate three of RSL4 orthologous protein functions are similar to *Arabidopsis* RSL4 protein one, we aligned the proteins and generated the *ProE7:RSL4 orthologous genes* constructs (Fig. 11A and 11B). Compared with the control (Cont, *ProE7:YFP*), *ProE7:RSL4 orthologous genes* constructs showed various root hair phenotypes which are similar to *ProE7:RSL4ox* or not (Fig. 11B and 11C). The results were different from what we expected. So, for the check of those genes to complement RSL4 function in *rsl4-1* mutant, we made constructs which have *Arabidopsis RSL4* and *RSL4*

orthologous genes driven by *Arabidopsis RSL4* promoter in *rsl4-1* mutant (Fig. 12A). Though the constructs were T1 generations, the results showed the selected *RSL4 orthologous genes* may have the function similar to *Arabidopsis RSL4* one (Fig. 12C). Defective root hair growth in *rsl4-1* mutant (root hair length (% of control), 53%) was complemented by T1 generation transgenic constructs (*ProRSL4:At RSL4*, 95%, *ProRSL4:PtXP00232411*, 98%, *ProRSL4:Os12g39850*, 97%, *ProRSL4:SmXP002963143*, 92%) (Fig. 12C). We also found expression region is probably the important factor to what makes *Arabidopsis RSL4* and *RSL4 orthologous genes* function originally (Fig. 12B and 12D).

We generated another construct driven by *GL2* promoter which expressed the gene in non-hair cells in meristem (*ProGL2:RSL4 orthologous genes*) (Fig. 13A and 13B). *ProGL2:RSL4 orthologous genes* constructs showed the similar root hair phenotypes to *ProGL2:Arabidopsis RSL4*, which seems like a *gl2* mutant that forms root hair in non-hair cells (Fig. 13C, 13D, 13E and 13F). The result suggests the possibility, RSL4 orthologous proteins can function like *Arabidopsis* RSL4, but we need more studies to confirm the selected proteins are genuine RSL4 orthologs. Though the result that RSL4 also has been conserved in angiosperm was incomplete, it implies the potential that root hair development pathway, which is a downstream of fate determination, has been evolutionally conserved in angiosperm.

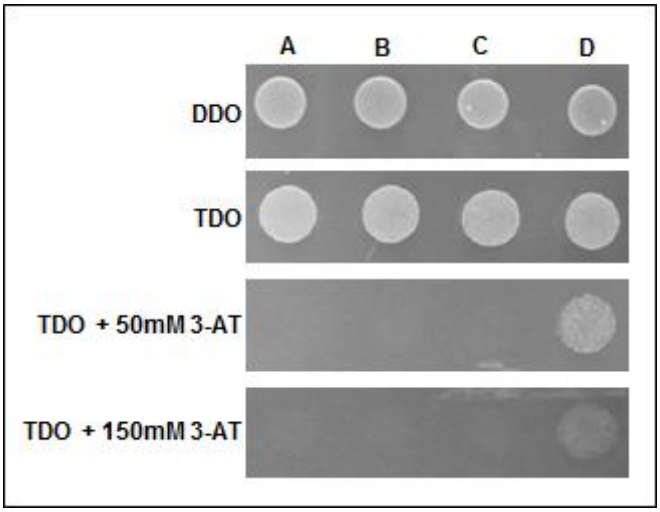


Fig 8. RSL4 showed specific binding to the root hair elements (*RHEs*).

20ul yeast culture, containing empty pGADT7 vector or pGADT7:RSL4 and empty pHIS vector pHIS:3x-E7-*RHE*, were grown on selection medium. SD medium lacked of leucine and tryptophane (DDO), indicated that both pGADT7 and pHIS vector were transformed into the yeast. SD medium lacked leucine, tryptophane and histidine (TDO), indicated the possible binding. Different concentrations 3-AT were used to inhibit spontaneous resistance to histidine.

A. empty pHIS vector + empty pGADT7 vector

B. empty pHIS vector + pGADT7:RSL4

C. pHIS:3x-E7-*RHE* + empty pGADT7 vector

D. pGADT7:RSL4+pHIS:3x-E7-*RHE*

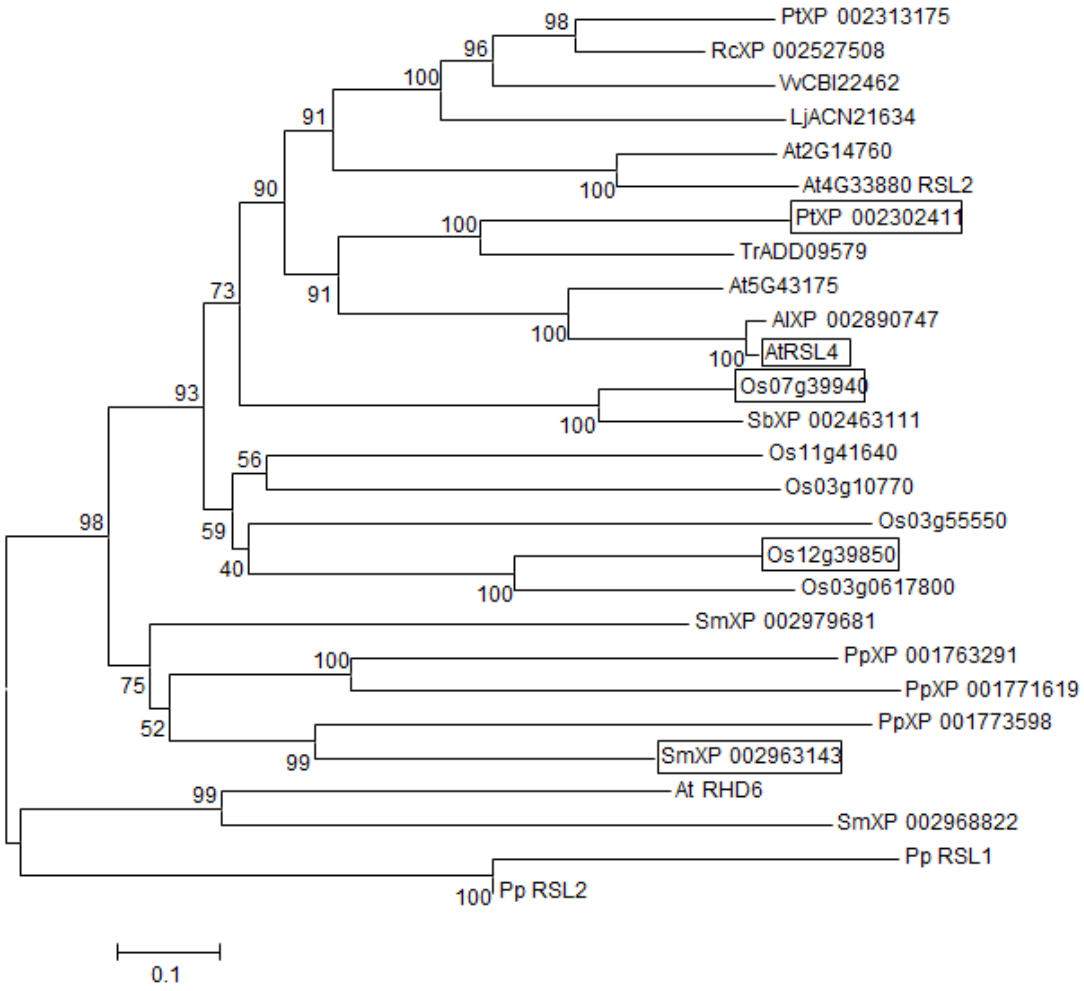


Fig 9. Neighbor-Joining phylogenetic tree of RSL4 protein sequences.

The phylogenetic tree, constructed by MEGA 4.0, demonstrates the evolutionary relationship of RSL4 orthologous proteins. The tree includes 27 proteins selected by NCBI(http://www.ncbi.nlm.nih.gov/) random blast and constructed after CLUSTALW alignment of the amino acid sequences. Bootstrap values are given at the nodes as a percentage of 1000 replicates. Black boxes are *Arabidopsis* RSL4 protein and selected RSL4 orthologous proteins. At (*Arabidopsis thaliana*), Al (*Arabidopsis lyrata*), Pt (*Populus trichocarpa*), Vv (*Vitis vinifera*), Rc (*Ricinus communis*), Lj (*Lotus japonicus*), Tr (*Trifolium repens*), Os (*Oryza sativa*), Sb (*Sorghum bicolor*), Ma (*Musa acuminata*), Pp (*Physcomitrella patens*), Sm (*Selaginella moellendorffii*).

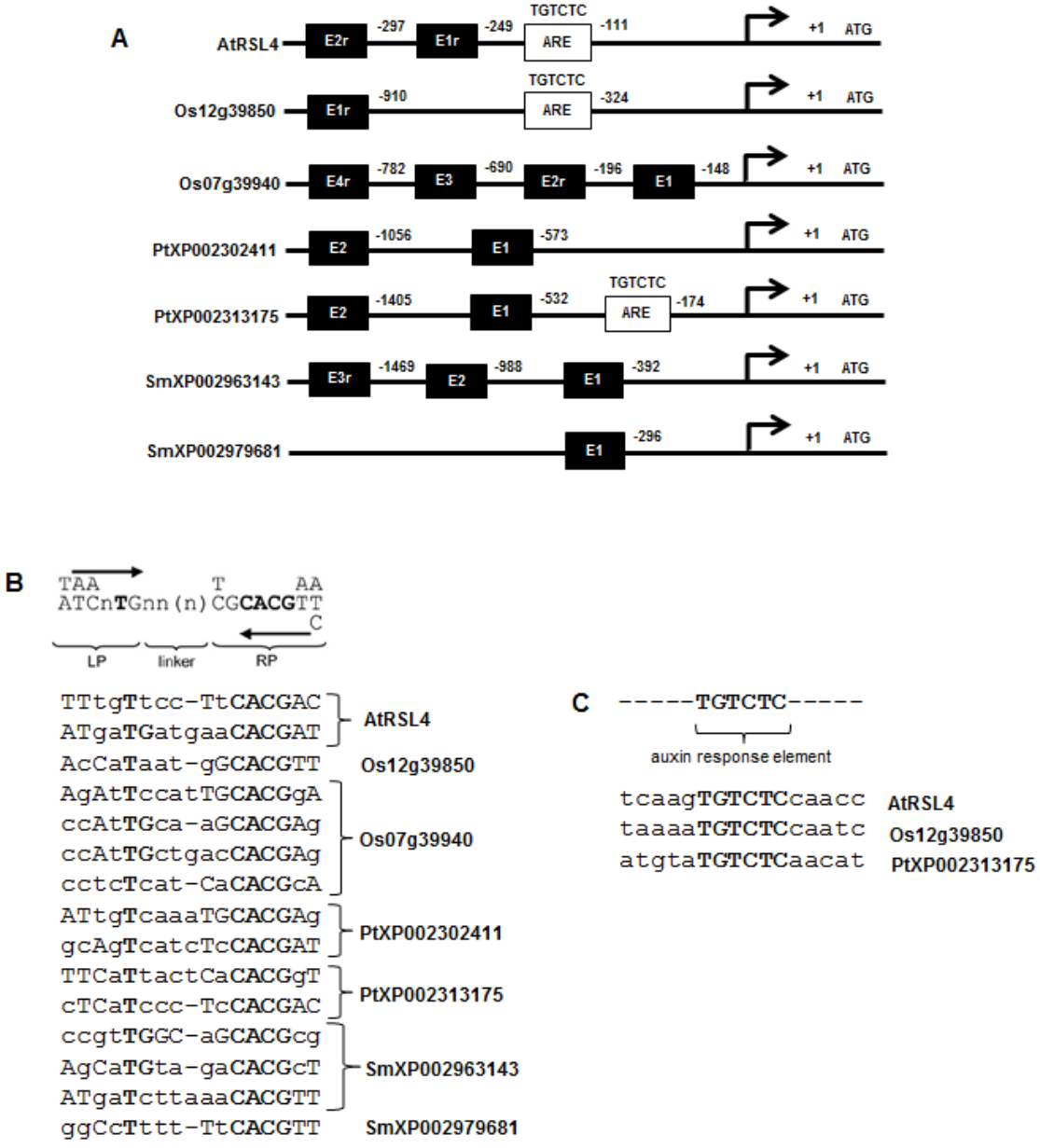


Fig 10. Root hair elements (*RHEs*) and auxin response element (*ARE*) from *Arabido*psis RSL4, RSL4 orthologous proteins and their paralogous proteins.

(A) Scheme of root hair elements (*RHEs)* and auxin response element (*ARE*) in the *Arabido*psis RSL4, RSL4 orthologous protein and their paralogous protein promoters. Numbers, by the sequence, are the last nucleotide positions of root hair elements (*RHE*, black boxes) and auxin response element (*ARE*). Nucleotide positions are numbered relative to the start codon (ATG). An ‘‘r’’ after the root hair elements (*RHE*) number indicates a reverse orientation of the root hair elements (*RHE*).

(B) The upper figure is the root hair element (*RHE*) consensus. The arrangements of nucleotide

sequences below the root hair element (*RHE*) consensus are alignments of the root hair element (*RHE*) sequences in the *Arabido*psis RSL4, RSL4 orthologous protein and their paralogous protein promoters. The alignments represent the conserved nucleotides by capitalized form.

(C) The upper figure is the auxin response element (*ARE*) consensus. The arrangements of nucleotide sequences below the auxin response element (*ARE*) consensus are alignments of the auxin response element (*ARE*) sequences in the *Arabido*psis RSL4, RSL4 orthologous protein and their paralogous protein promoters.

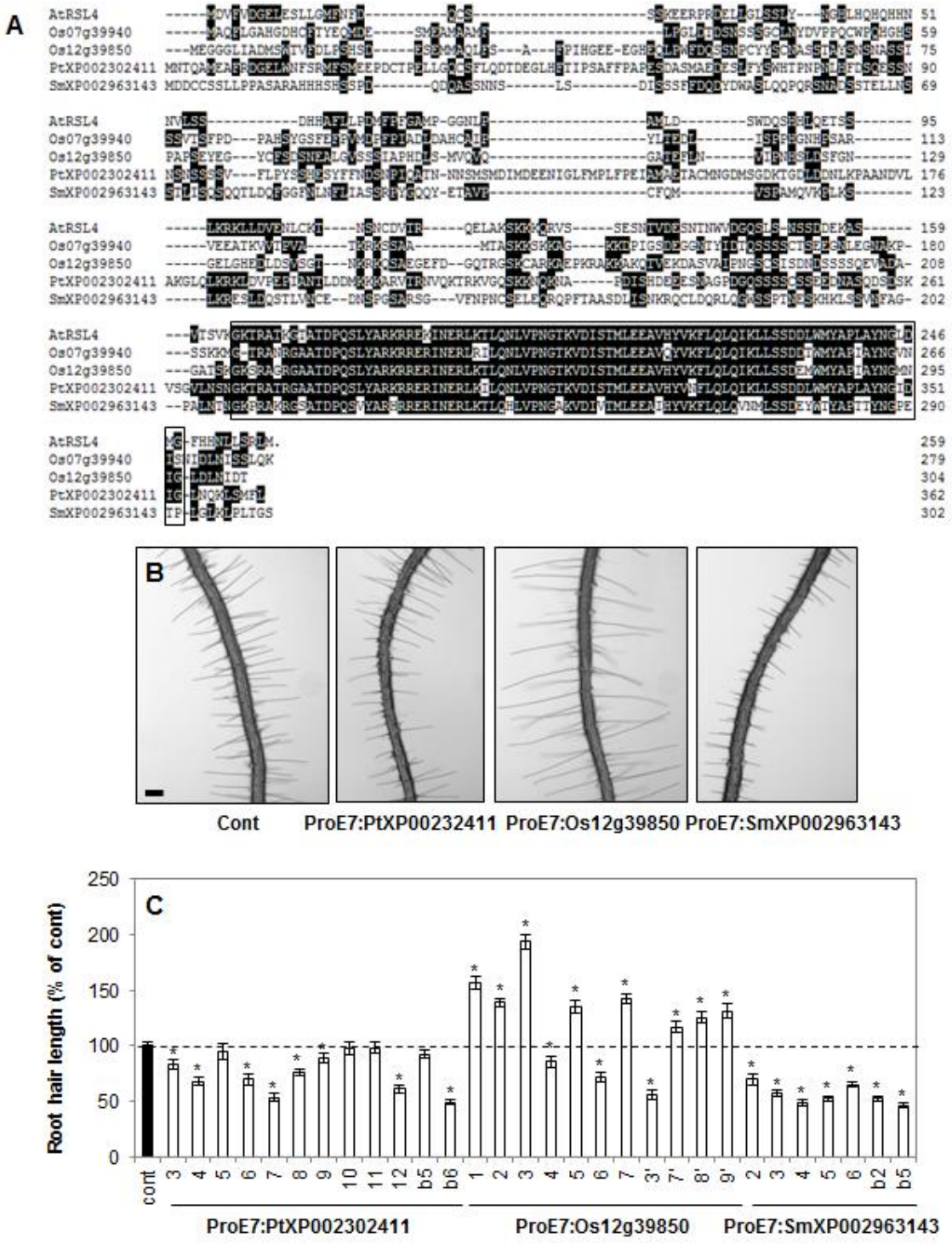


Fig 11. The effect of *ProE7:RSL4 orthologous genes* on the root hair growth.

(A) RSL4 orthologous protein sequences aligned with *Arabidopsis* RSL4 protein sequence. The black boxes indicate the *helix-loop-helix* (HLH) regions.

(B) Representative root images of control (Cont; *ProE7:YFP*) and *ProE7:RSL4 orthologous genes*

plants. Bar=100μm.

(C) Root hair lengths of control (Cont; *ProE7:YFP*) and *ProE7:RSL4 orthologous genes* plants. Numbers indicate independent *ProE7:RSL4 orthologous genes* transgenic lines. Data represent means± S.E. (N = 180 to 5520, average 921). *ProE7:RSL4 orthologous genes* lines which root hair lengths differ significantly from the control (p, 0.05) are indicated with an asterisk.

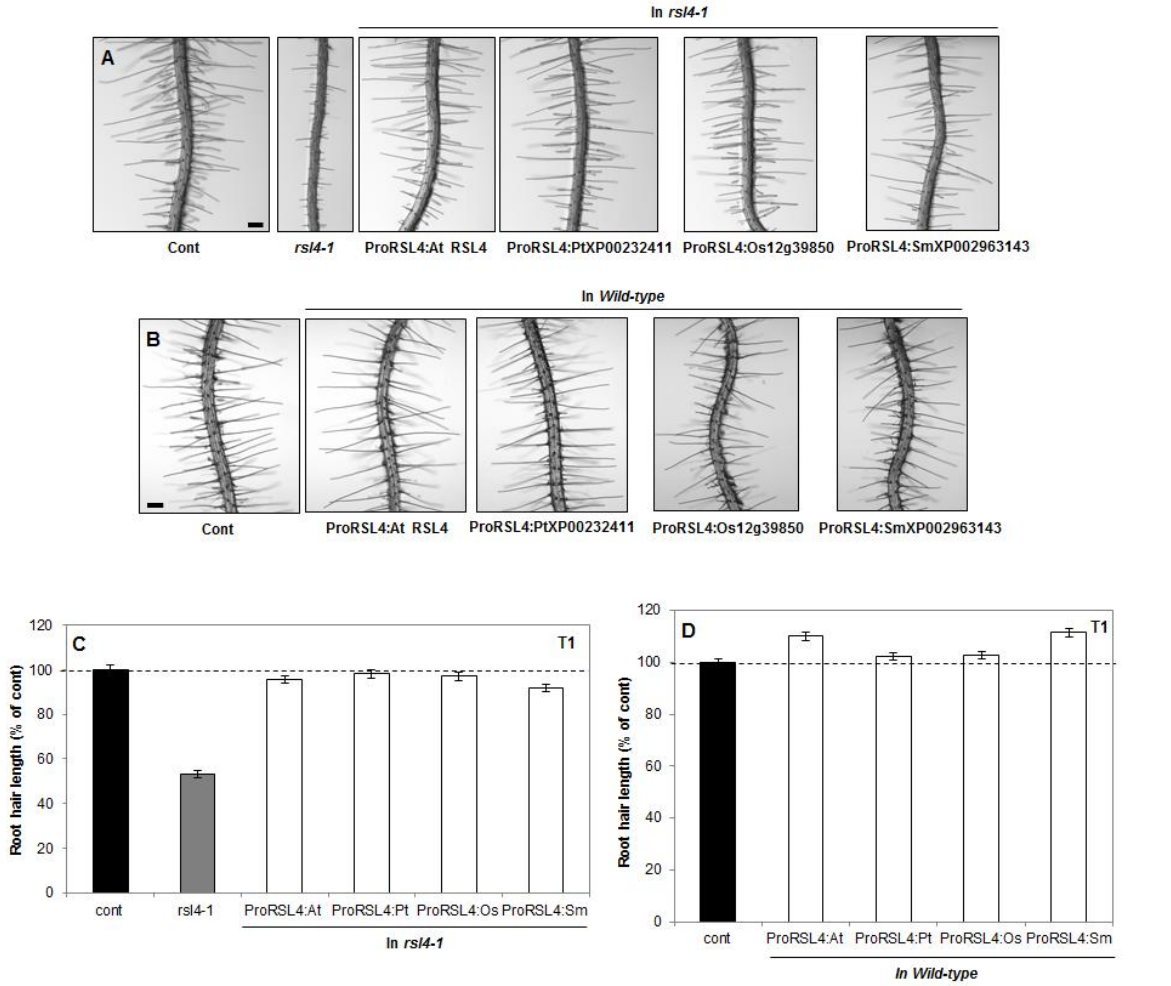


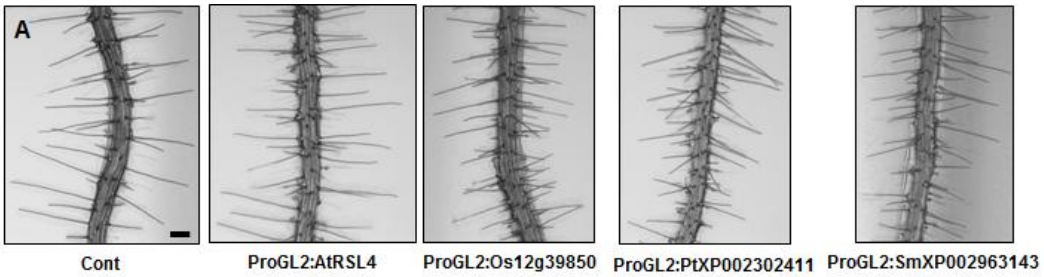
Fig 12. *RSL4 orthologous genes* may have the function similar to *Arabidopsis RSL4* and their function may be influenced by expression region.

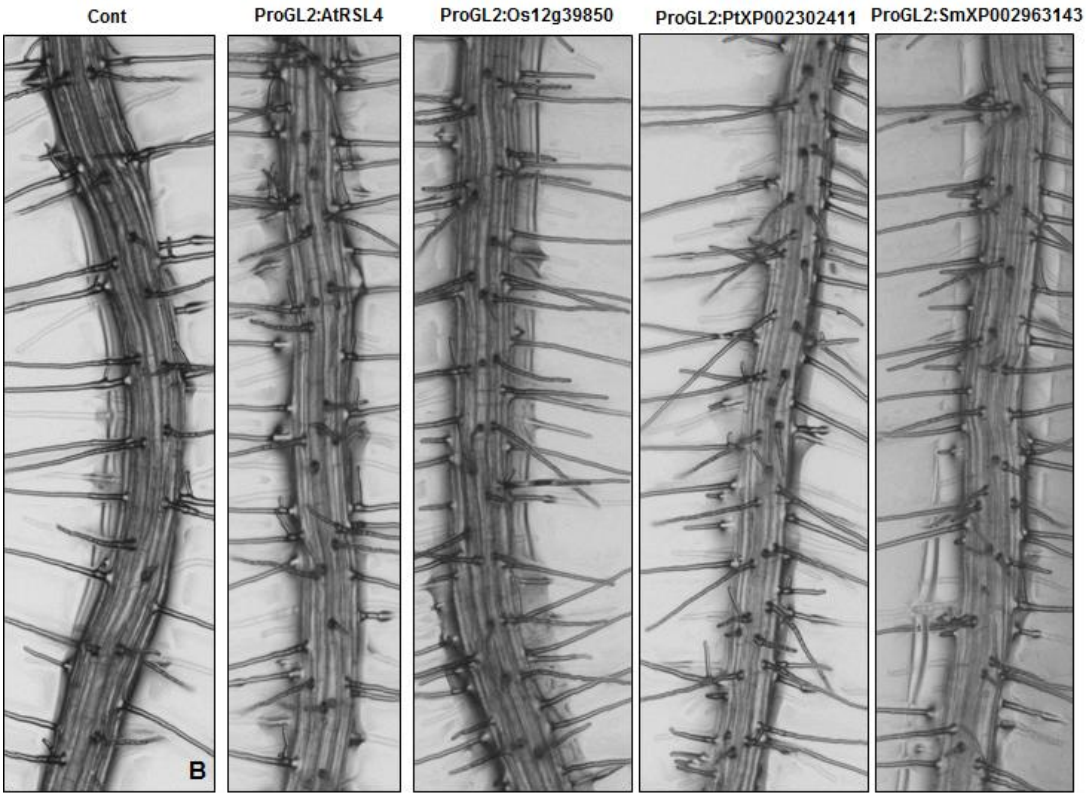
(A) Representative root images of control (Cont; *ProE7:YFP*), *rsl4-1* and *ProRSL4:RSL4 orthologous genes* in *rsl4-1* background T1 generation plants. Bar=100μm.

(B) Representative root images of control (Cont; *ProE7:YFP*), *ProRSL4:RSL4 orthologous genes* in *wild-type* background T1 generation plants. Bar=100μm.

(C) Root hair lengths of control (Cont; *ProE7:YFP*), *rsl4-1* and *ProRSL4:RSL4 orthologous genes* in *rsl4-1* background T1 generation plants. Data represent means± S.E. (N = 240 to 480).

(D) Root hair lengths of control (Cont; *ProE7:YFP*), *ProRSL4:RSL4 orthologous genes* in *wild-type* background T1 generation plants. Data represent means± S.E. (N = 208 to 320).


A
Cont
ProGL2:AtRSL4
ProGL2:Os12g39850
ProGL2:PtXP002302411
ProGL2:SmXP002963143


Cont
ProGL2:AtRSL4
ProGL2:Os12g39850
ProGL2:PtXP002302411
ProGL2:SmXP002963143
B

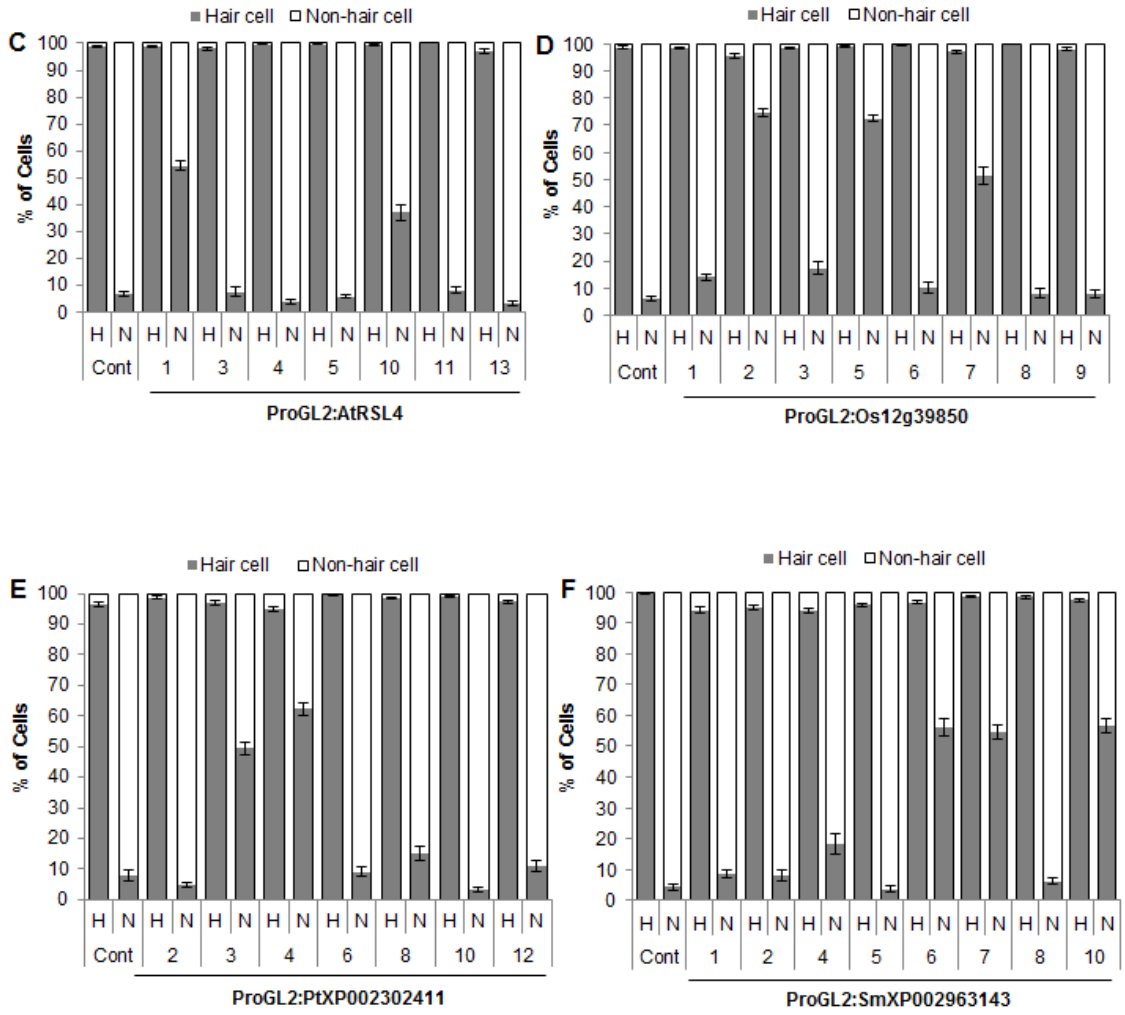


Fig 13. *ProGL2:Arabidopsis RSL4 and RSL4 orthologous genes* showed root hair initiation occurred in non-hair cell positions.

(A), (B) Representative root images of control (Cont; *ProE7:YFP*) and *ProGL2:AtRSL4 and RSL4 orthologous genes* plants. Yellow broken lines indicate the root hairs lied in hair cell position and red broken lines indicate the root hairs lied in non-hair cell position. Bar=100μm.

(C), (D), (E), (F) Cell-type pattern analysis, showing the fraction of root hair cells and non-hair cells that lie in the hair cell (H) and non-hair cell (N) positions, respectively, of the root epidermis. (N = 27 to 837, average 462).

4. Discussion

Recent studies have traced the gene regulatory networks involved in root hair development in *Arabidopsis*. RHD6 and RSL4 involved in the networks, have been known as the basic-helix-loop-helix (bHLH) transcription factors that regulate the hair initiation and elongation (Menand and Dolan, 2007, Yi and Dolan, 2010). Additionally, *RHD6* and *RSL4* have been known to be expressed root hair cells in meristem and elongation zone of the root. Reversely, our results showed the possibility that RHD6 has an inherent function of root hair elongation (Fig. 1 and 3) and RSL4 has a function of root hair initiation (Fig. 6). The results also showed that it is important to preserve their own expression region of RHD6 and RSL4 to function originally (Fig. 4 and 5). The morphogenetic process of root hair development is also controlled by auxin. Auxin is a positive effector of root hair formation, but the relationship of auxin and genetic regulator, like RHD6 and RSL4, remain less characterized. *RSL4* is direct target of RHD6 and its expression is modulated by auxin in the unknown way (Yi and Dolan, 2010). Our result showed the RHD6 and auxin regulate *RSL4* expression independently (Fig. 7). The possibility that RHD6 has another target gene, not RSL4, was also presented (Fig. 1 and 7). *EXPA7*, which encodes the hair cell-specific cell wall loosening protein and is expressed in differentiation zone, is the one of downstream target genes of RSL4 (Fig. 8). EXPA7 and RHD6 have been known to conserve the functions evolutionally. RSL4 has also the potential that RSL4 also conserves the function in angiosperm (Fig. 11 to 13).

We have to supplement the several things to propose detailed explanation about our assumptions. First, we have to check the importance of expression region of *RHD6* and *RSL4* by comparing the transcript expression region with protein expression region. Second, basic-helix-loop-helix (bHLH) transcription factors have motifs for DNA-binding, dimerization etc. Therefore we analyzed the RHD6 and RSL4 protein by alignment containing RHD6 orthologous protein sequences and RSL4 orthologous protein sequences to find the important motifs. Third, RSL4 protein promoters have an auxin response element (*ARE*, *TGTCTC*). We speculate auxin regulates the *RSL4* expression through

the element and will devise the experiment related to it. Fourth, each of the RSL4 related paralogous or orthologous protein promoters has RHEs (Root Hair Elements) or both of RHE and ARE (Auxin Response Element) (Fig. 10). Those results implied the possibility that RSL4 promoter has been differentiated for the root hair specific or auxin response protein function by evolutional change. The results of those studies will make up for the lacking point of our speculation. However, the results of our study are clearly the bases for the novel view of gene regulatory networks involved in root hair development.

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6. ABSTRACT IN KOREAN

애기장대 뿌리 내 발현 부위 차이에 의한 뿌리털 특이 단백질인 RHD6와 RSL4의 기능 비교

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다세포 생물의 세포 분화 과정에서 유전자 조절 네트워크는 중요한 분자생물학적 메커니즘이다. 식물에서는 이러한 분자생물학적 메커니즘을 연구하기 위해 애기장대의 뿌리털 발달 시스템을 자주 이용한다. 특히, 뿌리털 세포 특이적 유전자는 뿌리털 발달 과정의 후반 단계인 뿌리털의 형태형성 과정 (뿌리털 개시 및 신장)에 관여한다 (Grierson and Schiefelbein, 2002). *RHD6* (*ROOT HAIR DEFECTIVE 6, AT1G66470*)와 *RSL4* (*ROOT HAIR DEFECTIVE 6-LIKE 4, AT1G27740*)는 뿌리털 세포 특이적 유전자로써 basic-helix-loop-helix (bHLH) 전사인자를 암호화 하고 있다 (Menand and Dolan, 2007, Yi and Dolan, 2010). 이들은 뿌리의 분열조직과 신장 지역에서 발현이 된다고 알려져 있으며, *RSL4*는 RHD6의 직접적인 전사목표로 밝혀져 있다. RHD6와 RSL4는 뿌리털의 개시와 신장을 조절한다.

본 연구에서, 우리는 *RHD6*와 *RSL4*가 발현되는 부위의 중요성에 대해서 알아보았으며, 뿌리털 형성에 있어서 RHD6와 RSL4의 내재된 기능에 관한 분석을 해 보았다. 다

양한 프로모터를 사용하여 *RHD6*와 *RSL4*를 본래 발현되는 부위 외의 다른 부위에서 발현시키는 실험 결과를 통하여 RHD6와 RSL4가 기존에 알려진 것과 더불어 또 다른 기능을 가지고 있는 것을 알 수 있었다. 옥신은 뿌리털 형성에 있어서 양성적인 효과를 주는 호르몬이며, *RSL4*의 발현을 아직은 밝혀지지 않은 방식으로 조절하는 것으로 알려져 있다 (Schiefelbein, 2000, Yi and Dolan, 2010). 본 연구에서 실행한 실험의 결과는 RHD6와 옥신이 *RSL4*의 발현을 독립적인 방식으로 조절하는 것을 내포하며, RHD6가 *RSL4*가 아닌 또 다른 전사대상을 가지고 있을 가능성을 보여주었다. 또한 우리는 RSL4의 기능이 속씨식물 내에서 RHD6나 EXPA7와 마찬가지로 보존되어 있을 가능성을 발견하였다. 이러한 결과는 뿌리털 발달에 있어서 유전적인 조절 네트워크를 속씨식물 내에서 해석할 수 있게 해준다.

주요어: 뿌리털, RHS, 전사인자, 옥신, Orthologous 유전자

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