ABA Suppresses Root Hair Growth via the OBP4 Transcriptional Regulator[1][OPEN]

Bart Rymen, Ayako Kawamura, Sabine Schäfer, Christian Breuer[2], Akira Iwase, Michitaro Shibata, Miho Ikeda, Nobutaka Mitsuda, Csaba Koncz, Masaru Ohme-Takagi, Minami Matsui, and Keiko Sugimoto*

RIKEN CSRS, Yokohama 230-0045, Japan (B.R., A.K., C.B., A.I., M.S., M.M., K.S.); Max-Planck Institute for Plant Breeding Research, D-50829 Cologne, Germany (S.S., C.K.); Graduate School of Science and Engineering (M.I.) and Institute for Environmental Science and Technology (M.O.-T.), Saitama University, Saitama 338-8570, Japan; Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba 305-8566, Japan (M.I., N.M., M.O.-T.); and Institute of Plant Biology, Biological Research Center of the Hungarian Academy of Sciences, H-6724 Szeged, Hungary (C.K.)

ORCID IDs: 0000-0003-3651-9579 (B.R.); 0000-0003-3294-7939 (A.I.); 0000-0002-2713-5293 (M.I.); 0000-0003-4422-9993 (C.K.); 0000-0002-9209-8230 (K.S.).

Plants modify organ growth and tune morphogenesis in response to various endogenous and environmental cues. At the cellular level, organ growth is often adjusted by alterations in cell growth, but the molecular mechanisms underlying this control remain poorly understood. In this study, we identify the DNA BINDING WITH ONE FINGER (DOF)-type transcription regulator OBF BINDING PROTEIN4 (OBP4) as a repressor of cell growth. Ectopic expression of *OBP4* in Arabidopsis (*Arabidopsis thaliana*) inhibits cell growth, resulting in severe dwarfism and the repression of genes involved in the regulation of water transport, root hair development, and stress responses. Among the basic helix-loop-helix transcription factors known to control root hair growth, OBP4 binds the *ROOT HAIR DEFECTIVE6-LIKE2* (*RSL2*) promoter to repress its expression. The accumulation of OBP4 proteins is detected in expanding root epidermal cells, and its expression level is increased by the application of abscisic acid (ABA) at concentrations sufficient to inhibit root hair growth. ABA-dependent induction of *OBP4* is associated with the reduced expression of *RSL2*. Furthermore, ectopic expression of *OBP4* or loss of *RSL2* function results in ABA-insensitive root hair growth. Taken together, our results suggest that OBP4-mediated transcriptional repression of *RSL2* contributes to the ABA-dependent inhibition of root hair growth in Arabidopsis.

Plant growth is constantly adjusted during the plant's life cycle to ensure an optimal balance between endogenous and environmental demands. At the cellular level, growth control is the result of the progression of cells through a cell proliferation phase dictating the number of cells in an organ and sequentially a cell growth phase determining the size of these cells (Beemster and Baskin, 1998; Rymen et al., 2007; Gonzalez et al., 2012). Accumulating evidence suggests that plants adjust organ growth by modulating either one or both of these two cellular processes (Rymen and Sugimoto, 2012). Therefore, it is likely that transcription regulatory networks integrate developmental and environmental factors to optimize cell proliferation and cell growth.

In this study, we focus on the transcriptional regulation of cell growth and investigate how endogenous and environmental signals integrate with this regulation. Ideal models to study cell growth are cells in which cell division and cell growth are uncoupled (Franciosini et al., 2016). A great example of such a model are root hairs, since, concurrent with the establishment of root hair identity, these cells lose their ability to divide (Ikeuchi et al., 2013). The outgrowth of root hairs is restricted to specialized epidermal cells, referred to as trichoblasts, in contrast to atrichoblasts, which represent the non-hair-forming type of epidermal cells (Ishida et al., 2008). A subfamily of basic helix-loop-helix (bHLH) transcription factors, ROOT HAIR DEFECTIVE6 (RHD6) and its homologs RHD6-LIKE (RSL) genes, and a family of LOTUS JAPONICA ROOTHAIRLESS-LIKE1 (LRL1) to LRL5 transcription factors, have been identified as important regulators for root hair growth (Menand et al., 2007; Yi et al., 2010; Jang

[1] This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (grant no. 26291064 to K.S.) and Scientific Research B (grant no. 15H05961 to K.S.) and by the Japan Society for the Promotion of Science (postdoctoral fellowship to B.R.).

[2] Present address: PtJ, Division Bioeconomy BIO7, Forschungszentrum Jülich, 52425 Juelich, Germany.

* Address correspondence to keiko.sugimoto@riken.jp.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Keiko Sugimoto (keiko.sugimoto@riken.jp).

B.R. and K.S conceived the research and planned the experiments; B.R. performed most of the experiments with help from A.K. and M.S.; A.I. provided constructs; S.S., C.B., C.K., M.I., N.M., M.O.-T., and M.M. provided transgenic plants; B.R. and K.S. wrote the article with contributions of all the authors.

[OPEN] Articles can be viewed without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.16.01945

et al., 2011; Breuninger et al., 2016). Within the bHLH transcription factors, RHD6 and RSL1 are expressed early in development and orchestrate root hair outgrowth by driving the expression of *RSL2*, *RSL4*, and *LRL3* (Yi et al., 2010). RSL4 subsequently activates a set of genes expressed specifically in root hairs and/or required for root hair growth, such as *EXPANSIN A7* (*EXPA7*), *EXPA18*, *MORPHOGENESIS OF ROOT HAIR6* (*MRH6*), *PROLINE-RICH PROTEIN3*, and *ROOT HAIR SPECIFIC* (*RHS*; Cho and Cosgrove, 2002; Jones et al., 2006; Won et al., 2009; Yi et al., 2010; Datta et al., 2015). In addition, several other genes, including other *MRHs*, *COBRA-LIKE9*, *CELLULOSE SYNTHASE LIKE D2* (*CSLD2*), and *CSLD3*, display root hair growth defects when mutated (Favery et al., 2001; Roudier et al., 2002; Jones et al., 2006; Bernal et al., 2008; Karas et al., 2009); however, so far, less is known about their upstream transcriptional regulation.

Root hair growth is highly plastic and depends on a wide range of endogenous and environmental inputs. The best-studied example is the response to phosphate starvation that results in an extensive outgrowth of root hairs. This response is associated with increased activity of two MYB transcription factors, PHOSPHATE STARVATION RESPONSE (PHR1) and PHR1-LIKE1 (PHL1), the homeodomain transcription factor ALFIN-LIKE6/ PI DEFICIENCY ROOT HAIR DEFECTIVE2 (PER2), and RSL4 (Bustos et al., 2010; Yi et al., 2010; Chandrika et al., 2013). Accordingly, the *phr1 phl1* double mutant, the *per2* mutant, and the *rsl4-1* mutant show strong decreases in root hair length and compromised root hair growth responses to phosphate availability (Bustos et al., 2010; Yi et al., 2010; Chandrika et al., 2013). In addition, root hair growth is tuned by other environmental factors and associated hormone signaling, such as the availability of iron, magnesium, potassium, and manganese and changes in abscisic acid (ABA), auxin, or ethylene levels (Schnall and Quatrano, 1992; Schmidt and Schikora, 2001; Giehl and von Wirén, 2014). It was suggested that these different signals utilize different signaling pathways (Schmidt and Schikora, 2001), since, for instance, the *per2* mutant, which displays defects in root hair growth under the low-phosphate condition, still responds normally to iron and manganese deficiencies (Chandrika et al., 2013).

In this study, we searched for transcription factors that display differential expression along the developmental gradient and identified a DNA BINDING WITH ONE FINGER5.4 (DOF5.4)/OBF BINDING PROTEIN4 (OBP4) transcription factor as a repressor of cell growth. We show that OBP4 represses the transcription of the *RSL2* gene underlying the cessation of root hair growth in response to ABA.

RESULTS

OBP4 Is Expressed Preferentially in Differentiating Cells

In order to identify novel transcriptional regulators that control cell growth in Arabidopsis (*Arabidopsis thaliana*), we have ranked about 2,000 entries present in the plant transcription factor database (PlnTFDB; Pérez-Rodríguez et al., 2010) for their likelihood to be involved in cell growth. Since cells experience massive transcriptional reprogramming as they transit from proliferative to growth phase, we reasoned that transcription factors whose expression changes along this developmental gradient are likely to play a role in cell growth. Therefore, we extracted transcription factors showing differential expression along the developmental gradient in Arabidopsis leaves and roots using publicly available transcriptome studies (Birnbaum et al., 2003; Beemster et al., 2005; Andriankaja et al., 2012). We identified 204 candidates that display differential expression in both leaves and roots based on the significance cutoff used in the respective studies. We subsequently clustered the transcript profiles of the selected genes over the leaf and root developmental gradients detected by Birnbaum et al. (2003) and Andriankaja et al. (2012) using the k-means algorithm (Gasch and Eisen, 2002). This clustering yielded six different groups with distinct expression patterns along the developmental gradient (Fig. 1A; Supplemental Data Set S1). Transcription factors in groups I, II, and III generally display high expression in proliferating cells. The 50 genes in group I show this pattern of expression in both leaves and roots, whereas this trend is relatively specific to roots for 33 genes in group II and to leaves for 14 genes in group III (Fig. 1A). By contrast, the expression of genes in groups IV and V increases as cells start to differentiate (Fig. 1A). This tendency is common for both leaves and roots for 36 genes in group IV and more pronounced in roots for 51 genes in group V. The last cluster of 20 genes in group VI display an opposite trend in shoots and roots, with relatively high expression in proliferating cells of leaves and in expanding cells of roots (Fig. 1A).

Since we wanted to find general regulators of cell growth, we decided to focus on the 36 genes in group IV that show consistently high expression during cell growth in both leaves and roots. We further ranked these transcription factors based on the correlation of their expression profile with known regulators of cell proliferation and cell growth using the ATTED-II algorithm (Obayashi et al., 2009). We negatively valued the correlation with genes that show peaked expression in proliferating cells, such as *PROLIFERATING CELL NUCLEAR ANTIGEN2* (Menges et al., 2005) and *CYCLIN B1;1* (Shaul et al., 1996), and positively valued the correlation with genes preferentially expressed during cell growth, such as *EXP10* (Cho and Cosgrove, 2000) and *CELL CYCLE SWITCH52 A2* (Lammens et al., 2008). Based on this analysis, a transcription factor that has a characteristic DOF motif, ATDOF3/DOF5.4/OBP4, was ranked first (Supplemental Table S1) and, therefore, chosen for further characterization.

To validate *OBP4* expression in vivo, we generated an OBP4-GFP marker line by fusing a genomic region of *OBP4*, including a 3-kb upstream sequence and a 921-bp coding sequence, to GFP. As shown in Figure 1B, confocal microscopy revealed an abrupt increase in

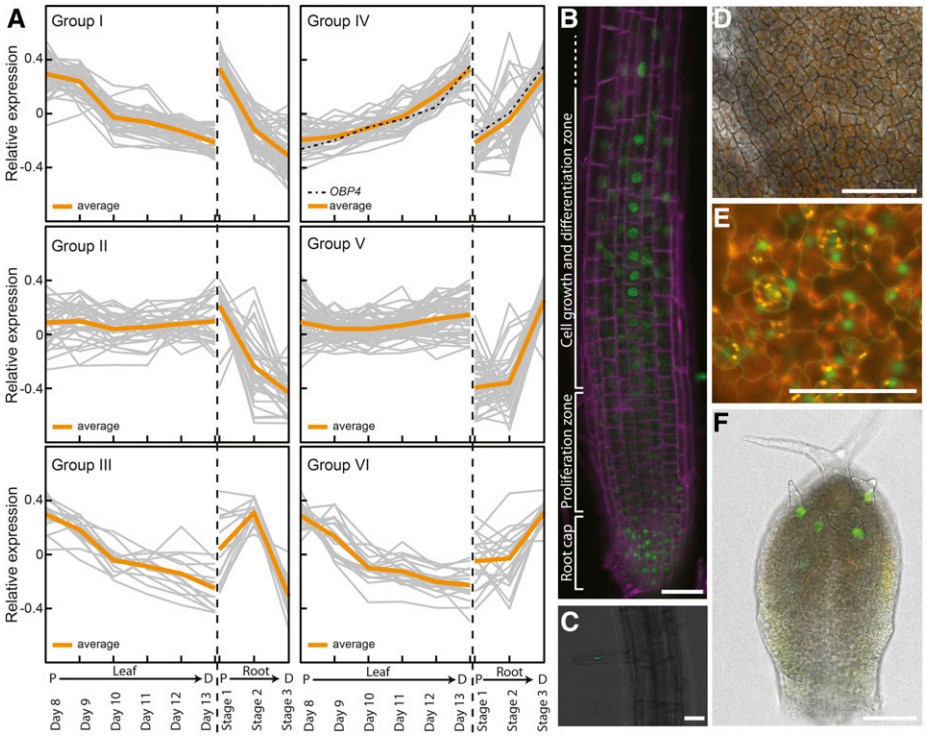

Figure 1. OBP4 expression is associated with cell growth. A, Clustering of differentially expressed genes along the developmental gradient from proliferating cells (P) to differentiating cells (D) in Arabidopsis leaves and roots. The transcriptional data are taken from Andriankaja et al. (2012) and Birnbaum et al. (2003). For leaves, five different time points after germination were taken corresponding to the proliferative phase (days 8 and 9), the transition phase (days 10–12), and the expansion phase (day 13), while for roots, three zones were harvested: the proliferation zone (stage 1), the expansion zone (stage 2), and the fully mature zone (stage 3). B, Confocal optical section of a root epidermis expressing OBP4-GFP under the control of its own promoter. Cell membranes are visualized with propidium iodide and shown in magenta. C, Expression of OBP4-GFP in a mature root hair. D, Expression of OBP4-GFP in epidermal cells of young leaves. E, Expression of OBP4-GFP in epidermal cells of older leaves. F, Expression of OBP4-GFP in maturing trichomes. Bars = 50 μm.

OBP4-GFP expression when epidermal cells enter the cell growth zone at the root meristem. The OBP4 expression persists in mature epidermal cells, including root hair cells and root cap cells (Fig. 1, B and C). The pattern of OBP4-GFP expression is similar in shoots, since we detect its expression in maturing leaf epidermal cells but not in proliferating cells (Fig. 1, D–F). Together, these data confirm that OBP4 is expressed preferentially in differentiating cells and, thus, may regulate gene expression associated with cell growth.

Ectopic Expression of OBP4 Leads to Growth Retardation Due to Defects in Cell Growth

To explore the function of OBP4 in planta, we first generated transgenic plants expressing the OBP4-glucocorticoid receptor (GR) fusion protein under the control of the cauliflower mosaic virus (CaMV) p35S promoter. This system allows ectopic activation of OBP4 function by the application of dexamethasone (DEX), since GR fusion proteins enter the nucleus only in the presence of DEX (Zuo and Chua, 2000). As shown in Figure 2A, heterozygous populations of OBP4-GR plants, grown on DEX-containing plates from germination, segregate for wild-type-like plants and those that show a drastic growth retardation. PCR genotyping confirmed that large plants were indeed wild type and dwarf offspring carried the *p35S:OBP4-GR* construct, causing a 24-fold higher *OBP4* transcript level (Supplemental Fig. S1A). Further phenotypic examination of OBP4-GR plants confirmed that the strong reduction in organ growth occurs in both leaves and roots (Fig. 2, A–D). Within 12 h after transfer to DEX-containing plates, the root growth of OBP4-GR plants is already reduced by 42% compared with the wild type, while they grow at a similar rate to the wild type in the absence of DEX (Fig. 2E). To determine the cellular effects of ectopic OBP4 expression, we analyzed the developmental profile of cortical cell length for wild-type and OBP4-GR roots after transfer to DEX-containing plates. As shown in Figure 2F and Table I, the length of mature cortical cells in OBP4-GR roots is reduced by 45% compared with the wild type, causing a strong reduction in the size of the root cell growth zone at 48 h after DEX treatment. Based on these cell length profiles,

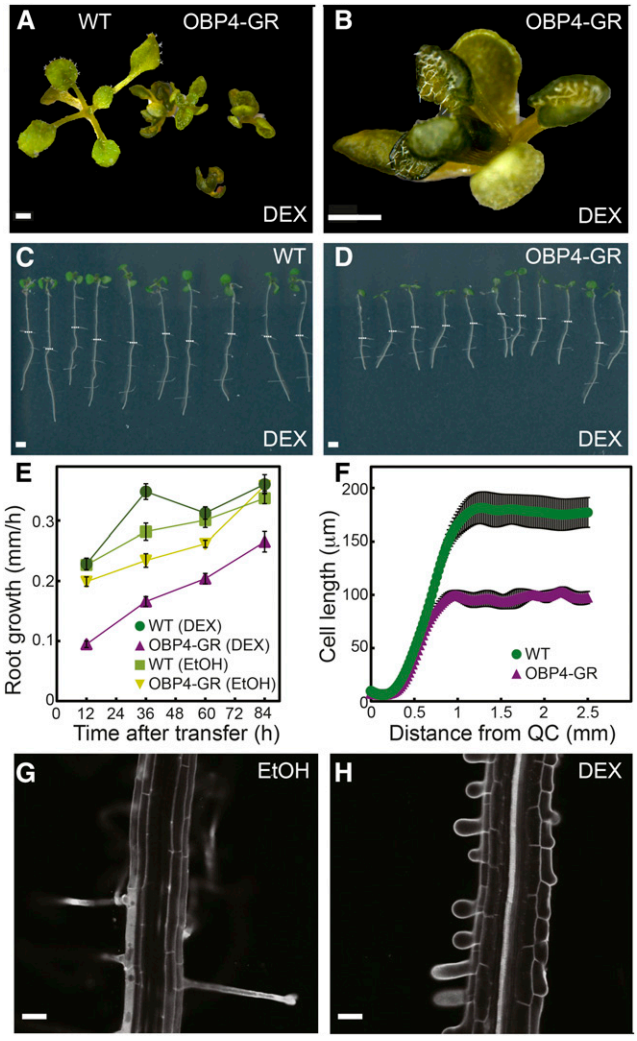

Figure 2. Ectopic expression of OBP4 impairs cell growth. A and B, Representative photographs of 10-d-old wild-type (WT; left in A) and OBP4-GR (right in A and enlarged image in B) plants grown on 10 μM DEX-containing medium. C and D, Representative photographs of wild-type (C) and OBP4-GR (D) plants after transfer to DEX-containing medium. Horizontal white lines indicate root length at the time of transfer. E, Rate of root growth after transfer to DEX- or ethanol (EtOH)-containing medium. Data are means ± SE ($n = 10$). F, Cell length profile determined 2 d after transfer to DEX-containing medium. Data are means ± SE ($n = 5$). G and H, Optical longitudinal sections of propidium iodide-stained roots 2 d after transfer to DEX-containing medium. Bars = 10 mm (A–D) and 50 μm (G and H).

we also estimated the rate of cell proliferation and cell growth and found that both of these parameters are not significantly different between wild-type and OBP4-GR roots after transfer to DEX-containing plates (Table I). Together, these results indicate that ectopic OBP4 expression causes a premature termination of cell growth, leading to the reduction in final cell size. In agreement with this, both root hair cells and epidermal cells in mature leaves also are severely reduced in final cell size (Fig. 2, G and H; Supplemental Fig. S1, B and C), demonstrating that excess levels of OBP4 generally inhibit cell growth.

To test the functional requirement of OBP4 genetically, we isolated four loss-of-function mutants for *OBP4*, *obp4-1* to *obp4-4*, that display 50% to 80% reduction of *OBP4* transcripts (Supplemental Fig. S2). However, we did not detect any obvious growth defects in these mutants (Supplemental Fig. S2).

OBP4 Represses Genes Involved in Root Hair Development

To gain molecular insights into how OBP4 represses cell growth, we examined transcriptional changes after *OBP4* induction in OBP4-GR plants. We treated 7-d-old OBP4-GR plants with either ethanol or 10 μM DEX and isolated RNA from their root tips, which included proliferating, expanding, and maturing cells. Allowing a false discovery rate of 1%, we identified 640 and 1,132 genes differentially expressed at 12 and 24 h, respectively, after *OBP4* induction (Fig. 3A; Supplemental Data Set S2). After 12 h of *OBP4* induction, more than 80% of differentially expressed genes were repressed, while at 24 h, similar numbers of induced and repressed genes were found. For a small set of genes, we compared the relative expression quantified by RNA sequencing and quantitative reverse transcription (qRT)-PCR and found a strong correlation between the two techniques, confirming the accuracy and reproducibility of RNA sequencing data (Supplemental Fig. S3A). Gene Ontology (GO) enrichment analysis revealed that genes repressed after 12 h include those implicated in water transport, root hair differentiation, and response to external stimuli (Fig. 3B; Supplemental Data Set S3). Similar GO enrichment also is found for genes repressed after 24 h; in addition, GO categories such as cell wall modification/organization and processes related to secondary metabolism are found (Supplemental Data Set S3). On the other hand, genes involved in several secondary metabolic pathways and stress responses are enriched among the genes induced after ectopic activation of OBP4 (Supplemental Data Set S3).

The enrichment of genes involved in root hair differentiation among the OBP4-repressed genes in combination with the strong reduction in root hair length after OBP4 induction (Fig. 2, G and H) made us further investigate the potential role of OBP4 in root hair growth regulation. Therefore, we compared the OBP4-repressed genes with known root hair regulatory genes listed on iroothair.org in more detail (Kwasniewski et al., 2013). Among the 138 genes listed on iroothair.org, the expression of 29 root hair genes is altered significantly after OBP4 induction, and 90% of these genes are repressed by OBP4 (Fig. 3C; Supplemental Table S2). Interestingly, the majority of root hair genes repressed by OBP4 are implicated in the control of tip growth rather than earlier processes such as root hair patterning and growth initiation (Supplemental Table S2). In agreement with this, a comparison with a transcriptional network

Table I. *Comparison of cellular growth parameters between wild-type and p35S:OBP4 roots growing on DEX-containing plates*
All parameters were determined at 48 h after transfer to DEX. Values are means ± SE.

Growth Parameter	Wild Type	OBP4-GR	P [a]
Rate of root growth[b] (mm h^{-1})	0.20 ± 0.05	0.12 ± 0.04	0.0000
Size of the proliferation zone[c] (μm)	331.87 ± 28.55	401.06 ± 35.88	0.0041
Size of the cell growth zone[c] (μm)	651.47 ± 115.36	348.94 ± 41.97	0.0017
Mature cell size[c] (μm)	177.57 ± 31.44	97.06 ± 8.61	0.0001
Rate of cell production[c] (cells h^{-1})	1.41 ± 0.27	1.21 ± 0.36	0.7092
Maximum strain rate[c] ($\mu m\ \mu m^{-1}\ h^{-1}$)	377.09 ± 65.72	313.82 ± 55.27	0.1293

[a]Statistical significance is calculated based on Student's t test. [b]$n = 35$. [c]$n = 6$.

reported previously for root hairs (Bruex et al., 2012) revealed that the OBP4-repressed genes are present mainly in clusters associated with late phases of root hair development (Supplemental Fig. S4).

OBP4 Represses the Expression of *RSL2* and *RSL3* Genes

A small subfamily of bHLH transcription factors, including RHD6 and its close homologs RSL1 to RSL5, play central roles in the regulation of root hair growth (Fig. 4A; Menand et al., 2007; Jang et al., 2011). Among them, RNA sequencing data showed that OBP4 strongly represses the transcription of *RSL2* at 12 and 24 h after DEX application (Fig. 3C). Our qRT-PCR studies confirmed the repression of *RSL2* expression after OBP4 induction and additionally revealed a significant decrease in the expression of *RSL3*, the closest homolog of *RSL2* expressed below the detection limit in our RNA sequencing experiment (Fig. 4B). This transcriptional repression is already very strong by 3 h after DEX application and clearly caused by OBP4 induction, since DEX treatment of wild-type plants or plants expressing the GR receptor fused with LEAFY COTYLEDON2 (LEC2), a protein unrelated to OBP4, does not induce the same transcriptional response (Supplemental Fig. S3, B–D). We also confirmed that OBP4 does not alter the expression of *RHD6*, *RSL1*, and *RSL4* genes, indicating that OBP4 down-regulates only *RSL2* and *RSL3* of the *RHD6* family genes (Fig. 4B). To test the possibility that OBP4 directly represses *RSL2* and *RSL3* expression, we evaluated their expression in OBP4-GR plants treated simultaneously with DEX and CHX, an inhibitor for protein translation. The addition of CHX does not block the OBP4-mediated repression of *RSL2* and *RSL3* expression, indicating that OBP4 represses *RSL2* and *RSL3* promoter activity without new protein synthesis (Fig. 4, C and D).

To corroborate these transcriptional relationships further in vivo, we cobombarded the *p35S:OBP4* construct together with the promoters of *RSL2* and *RSL3* fused to

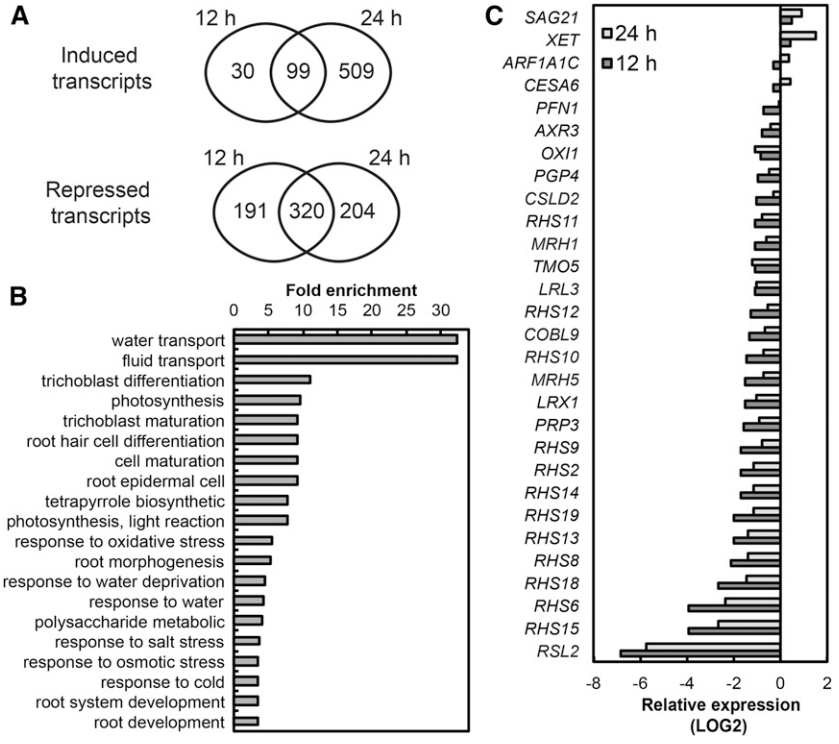

Figure 3. OBP4 activation represses genes associated with root hair development. A, Venn diagram analysis of genes induced or repressed at 12 and 24 h in OBP4-GR plants after DEX application based on a 1% false discovery rate cutoff. B, GO terms overrepresented among genes repressed after 12 h compared with all transcripts in the Arabidopsis genome. Genes with less than a 1% false discovery rate were subjected to BiNGO analysis. C, Transcript changes of genes implicated in root hair development based on the root hair information database (www.iroothair.org). Genes with less than a 10% false discovery rate are listed. Relative transcript levels are shown as fold enrichment between ethanol-treated and DEX-treated OBP4-GR plants.

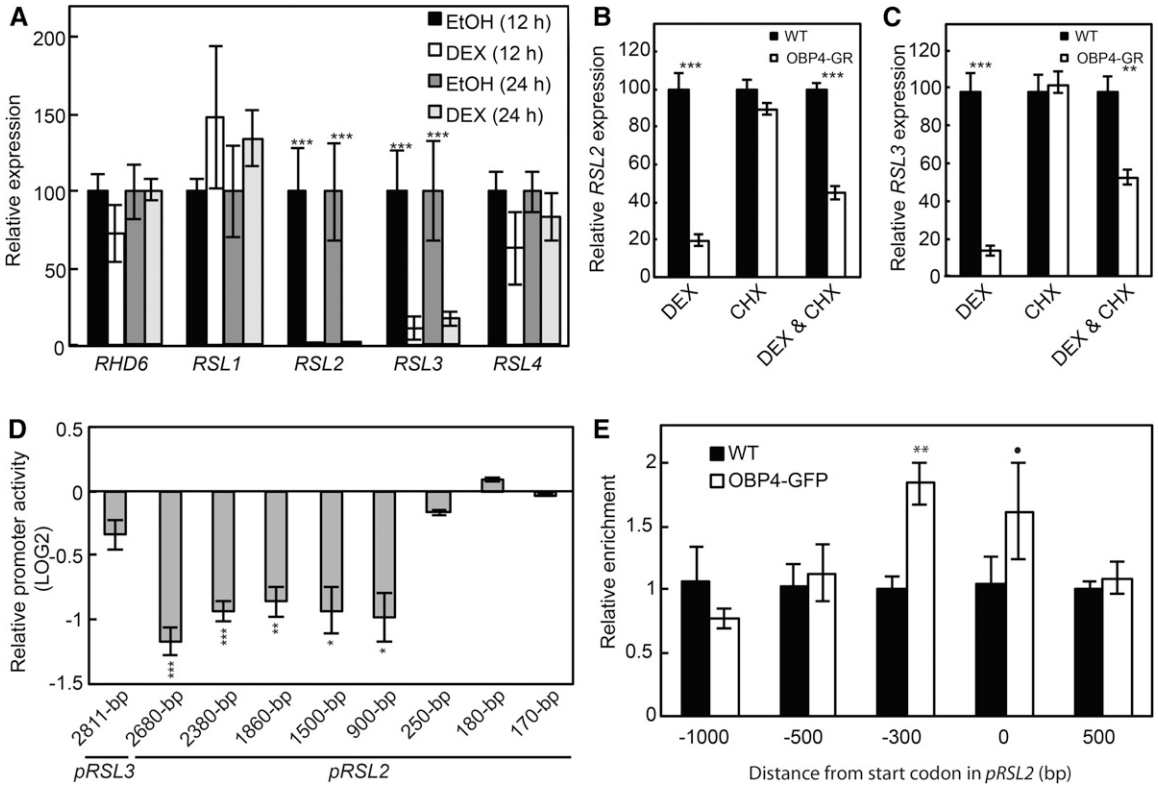

Figure 4. OBP4 represses the expression of *RSL2* and *RSL3*. A, qRT-PCR analysis of *RHD6* and *RSL* expression after OBP4 activation. B and C, qRT-PCR analysis of *RHD6* and *RSL* expression after OBP4 activation with and without cycloheximide (CHX). D, Activity of the *RSL2* and *RSL3* promoters fused to firefly luciferase when coexpressed with p35S:OBP4 in cultured Arabidopsis cells. E, Chromatin immunoprecipitation of OBP4-GFP fusion proteins followed by quantitative PCR analysis, using primers designed within the promoter and coding sequence of *RSL2*. Data are normalized against input DNA and shown as relative enrichment of DNA immunoprecipitated from the wild type (WT). Data and error bars represent means ± SE ($n = 3$). Symbols indicate significance determined by Student's *t* test: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; and ●, $P < 0.01$. EtOH, Ethanol.

luciferase and tested whether OBP4 can repress *RSL2* and *RSL3* promoter activity in cultured Arabidopsis cells. As shown in Figure 4E, the *RSL2* promoter proved most sensitive to OBP4 expression, and its activity decreased in the presence of OBP4. In contrast, the activity of *RSL3* is repressed only marginally by OBP4, indicating that OBP4 mainly regulates the expression of *RSL2*. By generating a deletion series of the *RSL2* promoter, we found that the DNA sequence between 900 and 250 bp upstream of the start codon is responsible for the full responsiveness to OBP4 activity (Fig. 4D). Furthermore, we immunoprecipitated the chromatin bound by OBP4-GFP roots using GFP antibodies. Our quantitative PCR analysis revealed an enrichment of OBP4-GFP binding within the chromatin around 300 bp upstream of the *RSL2* start codon (Fig. 4E), indicating that OBP4 directly binds the *RSL2* promoter in vivo to regulate its expression.

ABA-Induced Repression of Root Hair Growth Is Accompanied by Increased OBP4 Expression

ABA plays major roles in tuning plant growth in response to various environmental changes. In particular, we confirmed a previously reported observation that ABA inhibits root hair growth in Arabidopsis (Schnall and Quatrano, 1992). As shown in Figure 5, A and B, root hair growth is compromised significantly at all ABA concentrations we tested; for instance, the final length of root hairs in 1 μM ABA-treated roots is reduced by ~50% compared with untreated plants (Fig. 5B). To test whether these physiological responses are associated with changes in OBP4 expression, we examined the accumulation of OBP4-GFP protein in the presence of 1 μM ABA. Confocal microscopy examination revealed increased accumulation of OBP4-GFP within 3 h after ABA application (Fig. 5C). We observed statistically significant increases in OBP4-GFP abundance in all root epidermal cells along the developmental gradient, causing OBP4-GFP accumulation even in meristematic cells, where OBP4 expression is barely detectable in the absence of ABA (Fig. 5, D and E). Increased OBP4-GFP expression appeared to be relatively stable and persisted up to 48 h after ABA application.

To assess the extent to which this increase in OBP4 contributes to the reduction of root hairs upon ABA

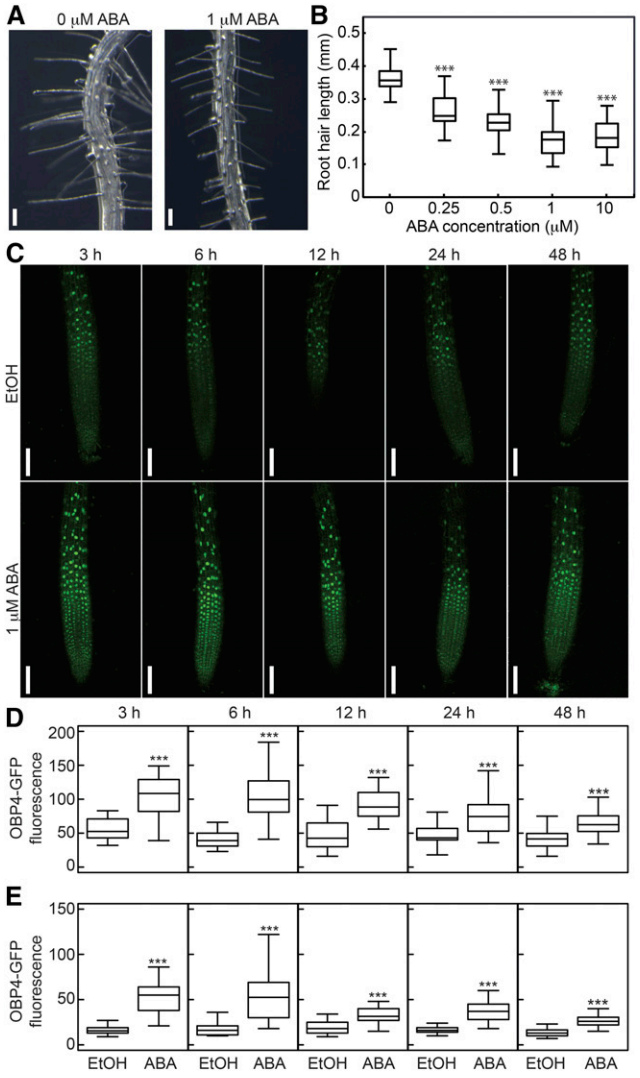

Figure 5. ABA up-regulates OBP4 expression and represses root hair growth. A, Representative photographs of mature root hairs 2 d after transfer to ABA-containing medium. B, Box-plot representation of root hair length 2 d after transfer to ABA-containing medium (n = 10). C, Accumulation of OBP4-GFP proteins in pOBP4:OBP4-GFP plants after transfer to ethanol (EtOH)- or ABA-containing medium. The OBP4-GFP proteins in root epidermal cells are visualized by confocal microscopy. D and E, Box-plot representations of the quantitative analysis of OBP4-GFP accumulation in the expansion zone (D) and the proliferation zone (E) in pOBP4:OBP4-GFP plants after transfer to ethanol- or ABA-containing medium ($n > 10$). Asterisks indicate significance determined by Student's t test: ***, $P < 0.001$. Bars = 200 μm.

treatment, we tested whether OBP4-GR plants with increased levels of OBP4 show altered responses to ABA. As shown in Supplemental Figure S2C, OBP4-GR plants overexpressing OBP4 on 0.01 or 0.05 μM DEX-containing plates are insensitive to 1 μM ABA, suggesting that increased OBP4 expression is indeed a major contributor to the root hair growth response to ABA. To further elaborate on the role of OBP4 in the ABA response, we also tested whether the *obp4-1* to *obp4-4* mutants show altered responses to ABA. However, we did not detect any significantly altered response to ABA in all *obp4* mutants (Supplemental Fig. S2D).

The Down-Regulation of *RSL2* Expression Contributes to ABA-Mediated Repression of Root Hair Growth

Having uncovered a strong increase in OBP4 abundance by ABA, we next examined whether the expression of *RSL2* and *RSL3* genes is reduced under these conditions. Our qRT-PCR analysis indeed revealed that the expression of both genes is reduced strongly at both 24 and 48 h after 1 μM ABA application, while the expression of other RHD6 family genes is either unchanged or increased (Fig. 6A). Confocal microscopy of *pRSL2-GFP-RSL2* plants (Yi et al., 2010) further showed that the decreased *RSL2* transcription leads to a reduced accumulation of GFP-RSL2 proteins, as assessed by the abundance of GFP signal intensities (Fig. 6B).

To test the possibility that the reduction in the *RLS2* and *RSL3* expression contributes to the repression of root hair growth by ABA, we asked whether mutations in these genes reduce the responsiveness of root hair growth to ABA. As reported previously by Yi et al. (2010), the *rsl2-1* mutants display shorter root hairs compared with the wild type under control conditions (Fig. 6C). Interestingly, these mutants are almost completely insensitive to ABA (Fig. 6, C and D), suggesting that the reduction in *RSL2* expression contributes to the root hair response to ABA. By contrast, the *rsl3-1* mutant that we isolated (Supplemental Fig. S5) shows a similar response to ABA as the wild type (Fig. 6, E and F). We also examined whether *rsl4-1* mutants that have short root hairs under control conditions also are insensitive to ABA, but the root hair growth in these mutants is as strongly inhibited by ABA as those in wild-type roots (Supplemental Fig. S6). Together, these results strongly suggest that ABA-dependent root hair repression is, to a large extent, mediated by the repression of *RSL2* expression. In agreement with this idea, *RSL2* expression is comparable between wild-type and *obp4-1* to *obp4-4* mutant plants that do not display an altered response to ABA (Supplemental Fig. S2E).

DISCUSSION

In this study, we searched for novel transcriptional regulators that control plant cell growth and identified a previously uncharacterized transcription factor, DOF5.4/OBP4, as a repressor of root hair growth. The DOF proteins belong to a plant-specific zinc-finger transcription factor family that consists of 37 members in Arabidopsis. Based on a phylogenetic study, OBP4 belongs to DOF subfamily B (Moreno-Risueno et al., 2007). The DOF transcription factors have been implicated in diverse developmental pathways, including flower induction (Fornara et al., 2009), stomata maturation (Negi et al., 2013), vasculature development (Le

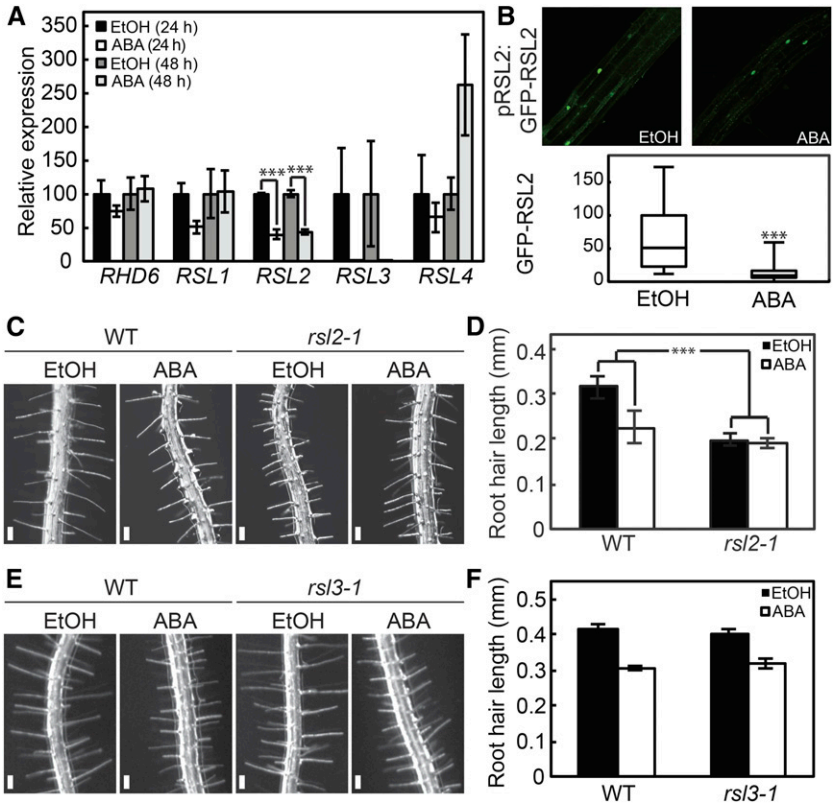

Figure 6. ABA down-regulates *RSL2* expression to repress root hair growth. A, qRT-PCR analysis of *RHD6* and *RSL* expression after transfer to ABA-containing medium. Data represent means ± SE ($n = 3$). EtOH, Ethanol. B, Confocal microscopy of GFP-RSL2 expression and its quantitative analysis in pRSL2:GFP-RSL2 represented in a box plot, with asterisks indicating significance determined by Student's *t* test: ***, $P < 0.001$ ($n > 10$). C and D, Representative photographs (C) and quantitative analysis (D) of wild-type and *rsl2-1* root hairs 2 d after transfer to ABA-containing medium. Data represent means ± SE ($n = 10$). E and F, Representative photographs (E) and quantitative analysis (F) of wild-type and *rsl3-1* root hairs 2 d after transfer to ABA-containing medium. Data represent means ± SE ($n = 10$). Asterisks indicate significance determined by two-way ANOVA: ***, $P < 0.001$. Bars = 200 μm.

Hir and Bellini, 2013), shoot branching (Zou et al., 2013), seed coat maturation (Zou et al., 2013), and cell cycle progression (Skirycz et al., 2008). Our findings strongly support that a DOF transcription factor also functions in the regulation of cell growth.

Identification of a Novel Set of Transcription Factors Involved in the Control of Cell Growth

To identify new regulators of plant cell growth, we first grouped the differentially expressed transcription factors into two groups: cell proliferation-associated genes (groups I, II, and III in Fig. 1A) and cell growth-associated genes (groups IV and V in Fig. 1A). A similar approach previously proved that cell cycle regulators are enriched among genes with high expression in the proliferative phase (Beemster et al., 2005). Similarly, we found transcription factors that were described previously as regulators of cell proliferation in the groups showing high expression in the proliferative phase (Supplemental Data Set S1). These included AINTEGUMENTA-LIKE6 (AIL6; Mizukami and Fischer, 2000), CELL DIVISION CYCLE5 (CDC5; Lin et al., 2007), GROWTH-REGULATING FACTOR2 (GRF2; Gonzalez et al., 2010), ULTRAPETALA1 (ULT1; Carles et al., 2005), and MONOPTEROS (MP) and several of its targets, TARGET OF MONOPTEROS (TMOs; Schlereth et al., 2010). Roles of AIL6, CDC5, GRF2, ULT1, MP, and TMOs in the regulation of cell proliferation (Mizukami and Fischer, 2000; Carles et al., 2005; Lin et al., 2007; Gonzalez et al., 2010; Schlereth et al., 2010) have been demonstrated based on mutant analysis (Mizukami and Fischer, 2000; Lin et al., 2007; Rodriguez et al., 2010).

In groups IV and V, showing an increase in transcription associated with cell growth, we identified factors with an expected potential to play roles in cell growth regulation, such as HOMEOBOX-1 (HB1; Capella et al., 2015), ETHYLENE RESPONSE FACTOR71 (ERF71; Lee et al., 2015), KNOTTED1-LIKE HOMEOBOX GENE 5 (KNAT5; Truernit and Haseloff, 2007), MINI ZINC FINGER1 (MIF1; Hu and Ma, 2006), SPEEDY HYPONASTIC GROWTH (SHYG; Rauf et al., 2013), OVATE FAMILY PROTEIN13 (OFP13; Wang et al., 2016), GT-2-LIKE1 (GTL1; Breuer et al., 2009), and HOMEODOMAIN-16 (HB16; Wang et al., 2003). For *KNAT5* and *GTL1*, previous studies showed high expression during the cell growth phase in the root and trichomes, respectively (Wang et al., 2003; Breuer et al., 2009). For the other genes, expression profiling at a high cellular resolution is not reported so far, but the expression of *HB1* and *SHYG* was shown to be increased during conditions inducing cell growth (Rauf et al., 2013; Capella et al., 2015). Furthermore, overexpression of *HB1*, *SHYG*, or *ERF71* leads to increased cell size, while mutants for *HB1* or *SHYG* are associated with reduced cell size (Rauf et al., 2013; Capella et al., 2015; Lee et al., 2015), indicating their roles in promoting cell growth. Interestingly, transcription factors with a repressive role in cell growth also were represented in groups IV and V. Reduced expression of *GTL1* or *HB16* is shown to lead to larger cells in trichomes and leaf epidermis, respectively,

while overexpression of *GTL1*, *HB16*, *MIF1*, or *OFP13* leads to smaller organs correlated with reduced cell size (Wang et al., 2003, 2011; Hu and Ma, 2006; Breuer et al., 2012).

These examples thus highlight the power of our grouping to identify genes regulating cell proliferation and/or cell growth. Given that many of the transcription factors we selected have not been studied before, further functional characterization of these genes should help uncover novel transcriptional control of cell growth in development and environmental response.

Role of OBP4 as a Transcriptional Repressor of Cell Growth

We mainly characterized the effects of OBP4 on root hair growth because of the dramatic decrease in root hair growth and the associated repression of a large set of root hair growth regulators in plants ectopically expressing *OBP4* (Figs. 2 and 3). Our data show that the hair growth defects in OBP4-overexpressing plants are associated with the down-regulation of *RSL2* and *RSL3* genes (Fig. 4). We predict that this transcriptional regulation may involve direct transcriptional repression by OBP4, since our qRT-PCR analysis using CHX suggests that the *RSL2* repression by OBP4 does not involve new protein synthesis (i.e. the production of additional transcriptional regulators; Fig. 4, C and D). Our cobombardment assay and chromatin immunoprecipitation assay together demonstrate that OBP4 directly binds the *RSL2* promoter in a region between 900 and 250 bp upstream of the *RSL2* translational start site (Fig. 4, E and F). Within this region, a previously predicted DOF-binding element (Cominelli et al., 2011) is present at 553 bp upstream of the *RSL2* translational start codon. Whether OBP4 binds this putative sequence, and, if so, whether this binding is sufficient to repress *RSL2* expression, should be investigated in future studies. Our data, in contrast, suggest that the *RSL3* promoter is not responsive to OBP4 nor does its loss-of-function mutation cause any obvious root hair phenotypes (Figs. 4E and 6E), suggesting that RSL3 alone does not play major roles in root hair growth. We should note, however, that the *rsl3-1* allele we isolated may not represent a complete loss-of-function mutant, although it bears a T-DNA insertion in its first exon (Supplemental Fig. S5C). The generation of additional loss-of-function alleles by CRISPR-Cas9 should further clarify the involvement of RSL3 in normal root hair development and its ABA-induced repression.

Given that OBP4-expressing plants display much stronger hair growth defects compared with the *rsl2-1* mutants (Figs. 2 and 6), it is also possible that additional factors act downstream of OBP4 to repress root hair growth. Among other root hair genes down-regulated by OBP4 induction, several of them display shorter root hairs when mutated (Supplemental Table S2). These genes, including several *RHS* genes and the *MRH1* gene, are strong candidates that act downstream of OBP4 to repress root hair growth. These genes may be targeted directly by OBP4 and act in parallel with RSL2, or they might act downstream of RSL2 and be only indirectly regulated by OBP4. So far, no direct targets of RSL2 are reported, but RSL4 is known to induce the expression of several *RHS* and *MRH* genes (Yi et al., 2010). To fully understand the OBP4-mediated regulatory network of root hair growth, further studies should uncover which genes are directly (or indirectly) targeted by OBP4 and how much each gene product contributes to root hair growth.

In addition to root hairs, OBP4-GFP fusion proteins were detected in a broad spectrum of organs, and their expression was associated with the onset of cell growth (Fig. 1). Although we could not prove the functionality of the OBP4-GFP fusion protein experimentally, due to the lack of phenotypic defects in the *obp4* mutants (Supplemental Fig. S2), our data are consistent with publicly available data (Birnbaum et al., 2003; Andriankaja et al., 2012), strongly suggesting that OBP4 plays additional roles in plant development. The overall size reduction of plants ectopically expressing *OBP4* further indicates that OBP4 is able to repress cellular growth in other organs (Fig. 2). How OBP4 represses the cell growth of nonroot hairs is not clear at present. In our RNA sequencing experiment, OBP4 represses several cell wall enzymes, such as expansins and xyloglucan transferases repressed by OBP4 (Supplemental Data Set S2), implicated in the modulation of cell growth (Braidwood et al., 2014). Thus, it is possible that they participate in the downstream pathway, leading to OBP4-induced growth retardation. Given that we detect strong OBP4 expression in the vasculature (data not shown), OBP4 also may contribute to vascular

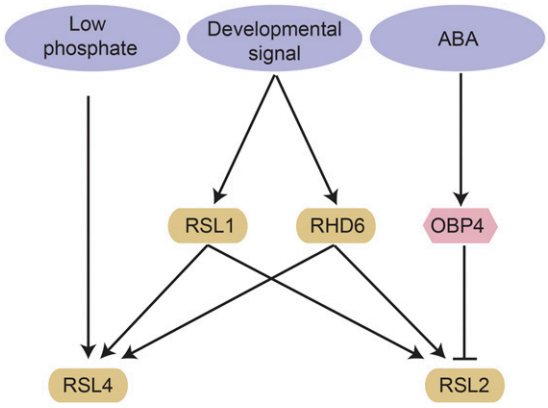

Figure 7. Schematic diagram describing how OBP4 regulates root hair growth. ABA application induces OBP4 expression and consequently leads to reduced levels of RSL2, causing root hair growth retardation. OBP4 directly binds the RSL2 promoter to repress its expression. The OBP4-RSL2 pathway may fine-tune the root hair developmental program governed by RHD6 and RSL1 to determine the final length of root hairs. Similarly, RSL4, a close homolog of RSL2, also is regulated by developmental signaling, via RHD6 and RSL1, and fine-tuned by phosphate signaling.

development, as proposed for many DOF family transcription factors (Le Hir and Bellini, 2013; Taylor-Teeples et al., 2015).

Despite our extensive efforts, we have not yet identified a complete loss-of-function mutant for OBP4, and *obp4-1* to *obp4-4* mutant alleles, which show 50% to 80% reduction in transcript levels, do not show obvious root hair phenotypes under normal or ABA conditions (Supplemental Fig. S2). We also tried several abiotic stress conditions, such as sodium chloride and mannitol treatments known to affect root hair growth (Wang et al., 2008), but *obp4-1* to *obp4-4* root hairs appear to respond like wild-type root hairs (B. Rymen, M. Shibata, and K. Sugimoto, unpublished data), suggesting that the residual OBP4 level is sufficient to fulfill its function in vivo. It is also possible that other DOF transcription factors play overlapping functions with OBP4 and, thus, that the mutation in OBP4 alone does not result in strong growth retardation. We have indeed identified five more DOF transcription factors, *OBP2*, *CYCLIC DOF FACTOR2*, *DOF AFFECTING GERMINATION1*, *ARABIDOPSIS DOF ZINC FINGER PROTEIN1* (*ADOF1*), and *ADOF2*, as genes preferentially expressed during cell growth (group IV in Fig. 1 and Supplemental Table S1). These are the best candidates to have redundant functions with OBP4 in terms of cell growth regulation, although, phylogenetically speaking, they are not most strongly related to OBP4 (Moreno-Risueno et al., 2007). Generating the complete loss-of-function allele of OBP4 and simultaneously mutating other DOF transcription factors will be essential to reveal its function in planta and regulatory interaction with other DOFs.

RSL Genes as Integrators of Developmental and Environmental Signals

Root hairs are well known for their role in the uptake of minerals, including Ca^{2+}, K^+, NH_4^+, NO_3^-, Mn^{2+}, Zn^{2+}, Cl^-, and $H_2PO_4^-$ (Nye, 1966; Gilroy and Jones, 2000). The impaired responses of various root hairless mutants to altered nutrient conditions indicate the necessity for proper adjustment of root hair development for optimum interaction with the environment (Tanaka et al., 2014). Consequently, root hair length and density are constantly optimized to current environmental demands (Kochian, 1995; López-Bucio et al., 2003; Wang et al., 2008; Giehl and von Wirén, 2014). Previous studies that compared the responses of various Arabidopsis mutants defective in several hormonal pathways revealed that different hormone signaling pathways are employed to adjust root hair development to different stresses (Schmidt and Schikora, 2001; Chandrika et al., 2013). This study strongly suggests that the OBP4-RSL2 pathway controls root hair growth in response to ABA (Fig. 7). Previous studies revealed that RSL2 controls root hair growth together with RSL4 and that they both act downstream of RHD6 and RSL1, which define the developmental pattern of root hair formation (Yi et al., 2010). It is also reported that only *RSL4* expression increases upon phosphate starvation, while the expression of *RSL2* remains unchanged (Yi et al., 2010). Interestingly, we found exactly the opposite situation after ABA application (i.e. increased expression of *RSL2* and no detectable difference in *RSL4* expression). We also show that the *rsl2-1* mutants, but not the *rsl4-1* mutants, are insensitive to ABA (Fig. 6; Supplemental Fig. S6). This suggests that only RSL2 acts in the ABA-dependent repression of root hair growth. These observations, therefore, provide further evidence that RHD6/RSL transcription factors play central roles in integrating developmental and environmental signals to fine-tune root hair growth.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana* ecotype Columbia-0) seeds, *obp4-1* (SALK_116433), *obp4-2* (SALKseq_085101), *obp4-3* (SALKseq_108296), and *rsl3-1* (SALK_064296), were obtained from the Arabidopsis Biological Resource Center (Ohio State University). The *obp4-4* mutant was isolated from the T-DNA insertion collection of the Max-Planck-Institut für Züchtungsforschung (Ríos et al., 2002). Liam Dolan (Oxford University) provided *rsl2-1*, *rsl4-1*, and *pRSL2-GFP-RSL2* (all in the Columbia-0 background; Yi et al., 2010). Małgorzata D. Gaj provided the previously published plants expressing the *p35S-LEC2-GR* fusion proteins (Ledwoń and Gaj, 2009). Primers used for genotyping are listed in Supplemental Table S3. Plants were grown on plates containing Murashige and Skoog salts, 1% (w/v) Suc, and 0.6% (w/v) phytagel at 22°C under a photoperiod of 16 h of light and 8 h of dark. For the DEX and ABA treatments, the same Murashige and Skoog medium was supplemented with DEX (D1756; Sigma) or ABA (A4906; Sigma) after autoclaving at a concentration of 1 μM, unless stated differently.

In Silico Identification of Cell Growth Regulators

Transcription factors differentially expressed over the developmental gradient were identified based on the selections made by Birnbaum et al. (2003), Beemster et al. (2005), and Andriankaja et al. (2012). Selected genes were subsequently clustered with K means, for which the optimal number of clusters was estimated with the figure of merit calculations in the Multiple Experiment Viewer 2.2 of The Institute for Genome Research (Saeed et al., 2003). The Pearson correlation for coexpression was determined with ATTED-II software (Obayashi et al., 2009).

Vector Construction and Arabidopsis Transformation

For overexpression analysis, the coding sequence of the *OBP4* gene was amplified using a PRIMESTAR MAX polymerase (Takara) and the primers listed in Supplemental Table S3 with genomic DNA as a template according to the manufacturer's protocol (Qiagen). The blunt-end PCR product was introduced into the entry vector pDONR207 and recombined into the destination vector pBI35S-GW-GR in frame with a GR domain using the Gateway cloning system (Invitrogen, Life Technologies). To construct the pBI35S-GW-GR vector, the GUS gene of pBI121 was replaced with the GR fragment of a pMON721 derivative, provided by Dr. T. Aoyama (Kyoto University), and the reading frame B cassette for the Gateway cloning system was inserted between the p35S promoter and the GR of pBI121 (Aoyama and Chua, 1997).

For expression analysis, entry clones containing a 3-kb promoter of OBP4 and the coding sequence of OBP4 were generated using pDONRp4-p1R and pDONR207 (Invitrogen). The promoter and coding sequence were amplified from genomic DNA and cloned in the respective pDONR vectors by BP reaction of the Gateway technology (Invitrogen). Next, the entry clones were recombined using the Multisite LR reaction of the Gateway cloning system (Invitrogen) into the R4L1pGWB550 destination vector (Nakagawa et al., 2007) in frame with the GFP, resulting in pOBP4:OBP4-GFP.

The verified vectors were used to transform *Agrobacterium tumefaciens* strain GV3101 (pPM90) and to generate transgenic lines. Homozygous lines for p35S:OBP4-GR and pOBP4:OBP4-GFP were selected based on their resistance to kanamycin and hygromycin, respectively. All stable plant transformations were generated by the floral dip method (Clough and Bent, 1998).

Microscopy and Growth Analysis

Fluorescent marker lines were imaged using an SP5 confocal laser scanning microscope (Leica), and their signal intensities were quantified using an associated software (Leica). To visualize root cortical cells, cell membranes were stained with 10 μM propidium iodide. The length of cortical cells was quantified from optical longitudinal sections of propidium iodide-stained roots using ImageJ software (Abràmoff et al., 2004). For the interpolation of cell length profiles, smoothing, based on fitting local polynomial, was performed using the LocPoly function in the KernSmooth library of the R statistical software. Average cell length was interpolated at a 2-μm interval, and mature cell length was estimated based on the cell length profile. The position where cell length was equal to or larger then the cell width was defined as the end of the proliferation zone. The rate of cell production was calculated as described by Rymen et al. (2010).

RNA Sequencing

Seven-day-old seedlings of p35S:OBP4-GR were treated with DEX or ethanol for 12 and 24 h. Root tips from 300 seedlings were pooled for each RNA sample and frozen in liquid nitrogen. Total RNA was extracted using the plant RNeasy kit (Qiagen), and its size, abundance, and integrity were analyzed on an Agilent Bioanalyzer Nanochip (Agilent Technologies). RNA samples were reverse transcribed, and their libraries were constructed with the Illumina TruSeq kit. Deep sequencing for a single 50-bp end using an Illumina HiSeq 2000 generated ~8.5 million raw reads for each sample. Mapping and statistical analysis were performed using the CLC Genomics Workbench version 7.5.1. Using this software, about 70% of the reads were uniquely assigned to a single gene in The Arabidopsis Information Resource 10 annotation of the Arabidopsis genome. Gene expression was normalized using reads per kilobase per million mapped reads (RPKM) values (Mortazavi et al., 2008). Differentially expressed genes were identified based on Baggerley's test and false discovery rate correction (Baggerly et al., 2003). To determine GO categories significantly overrepresented among misregulated genes, the BiNGO plug-in for Cytoscape was used (Maere et al., 2005). To compare different gene lists, statistical analysis was performed with the HYPERGEO.DIST function in Excel (Microsoft).

qRT-PCR

Total RNA (200 ng) isolated with RNeasy (Qiagen) was subjected to first-strand cDNA synthesis with the Primescript RT gDNA eraser reagent kit (Takara). For qRT-PCR, cDNA was amplified using the Thunderbird SYBR qPCR mix (Toyobo) and the Mx3000P QPCR system (Agilent). Primer sets used in this study are listed in Supplemental Table S1. Relative expression values were measured using the ΔΔCt method, and a helicase gene (AT1G58050) and a SERINE/THREONINE PROTEIN PHOSPHATASE 2A (PP2A) subunit (AT1G13320) were used as reference genes (Czechowski et al., 2005). Statistical differences were evaluated with Student's *t* test.

Transactivation Assay

For luciferase (LUC) reporter vector construction, a 3-kb promoter of the *WIND1* gene was PCR amplified and cloned into the pGEM-T Easy vector (Promega). The firefly LUC gene sequence fused with the NOS terminator sequence was amplified from the GAL4GCC-LUC vector (Ohta et al., 2001) and inserted into the *Pst*I site of the pGEM-T Easy vector. The resultant pWIND1:LUC vector was used for pOBP4:LUC construction. The 3-kb OBP4 promoter sequence was PCR amplified and cloned between *Sac*II and *Spe*I sites of the pWIND1:LUC vector. For the construction of p35S:OBP4, the OBP4 coding sequence was amplified by PCR, and the PCR products, phosphorylated by T4 polynucleotide kinase (Toyobo), were cloned into p35SSG (Mitsuda et al., 2005) using the *Sma*I site located between the CaMV p35S promoter-Omega and the NOS terminator sequence of the p35SSG vector.

p35S:OBP4 and p35SSG were used as an effector and control vector, respectively. The pOBP4:LUC vector was used as a reporter. As an internal control, the pPTRL vector, which drives the expression of a *Renilla LUC* gene under the control of the CaMV p35S promoter, was used. Particle bombardment and LUC assays with the Dual-Luciferase Reporter Assay System (Promega) were performed as reported previously (Hiratsu et al., 2002) with some modifications: Arabidopsis MM2D cultured cells were used as host cells (Menges and Murray, 2002), and luciferase activity was measured using a Mithras LB940 microplate luminometer (Berthold Technologies). Statistical differences were evaluated with Student's *t* test.

Chromatin Immunoprecipitation

Approximately 1 g of fresh Arabidopsis roots harvested from 14-d-old wild-type plants or plants harboring *pOBP4:OBP4-GFP* was ground using a bead shocker (Yasui Kikai). Fixation, nuclear extraction, and chromatin shearing and immunoprecipitation were performed according to a previously published protocol (Luo and Lam, 2014) using antibodies against GFP (Abcam; ab290).

Accession Numbers

RNA sequencing data have been submitted to ArrayExpress (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4838. Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *OBP4* (At5g60850), *RHD6* (At1g66470), *RSL1* (At5g37800), *RSL2* (At4g33880), *RSL3* (At2g14760), *RSL4* (At1g27740), and *RSL5* (At5g43175).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Effect of ectopic *OBP4* expression on epidermal cell growth in leaves.

Supplemental Figure S2. Phenotypes of *obp4* loss-of-function mutants.

Supplemental Figure S3. Confirmation of RNA sequencing results.

Supplemental Figure S4. Comparison of OBP4 targets with previously identified root hair genes.

Supplemental Figure S5. Isolation of *rsl3-1* loss-of-function mutants.

Supplemental Figure S6. Effect of ABA on root hairs in the *rsl4-1* mutant.

Supplemental Table S1. Ranking of transcription factors with expression related to cell growth.

Supplemental Table S2. Root hair genes differentially expressed after OBP4 induction.

Supplemental Table S3. Primers used in this study.

Supplemental Data Set S1. Clusters of transcriptional factors differentially expressed along the developmental gradient in roots and leaves.

Supplemental Data Set S2. List of genes up- or down-regulated by ectopic *OBP4* expression.

Supplemental Data Set S3. GO terms enriched among OBP4-regulated genes.

ACKNOWLEDGMENTS

We thank Momoko Ikeuchi, Hirofumi Harashima, and Anna Franciosini for providing comments on the article.

Received December 23, 2016; accepted February 3, 2017; published February 6, 2017.

LITERATURE CITED

Abràmoff MD, Magalhães PJ, Ram SJ (2004) Image processing with ImageJ. Biophoton Int **11:** 36–42

Andriankaja M, Dhondt S, De Bodt S, Vanhaeren H, Coppens F, De Milde L, Mühlenbock P, Skirycz A, Gonzalez N, Beemster GTS, et al (2012) Exit from proliferation during leaf development in Arabidopsis thaliana: a not-so-gradual process. Dev Cell **22:** 64–78

Aoyama T, Chua NH (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. Plant J **11:** 605–612

Baggerly KA, Deng L, Morris JS, Aldaz CM (2003) Differential expression in SAGE: accounting for normal between-library variation. Bioinformatics **19:** 1477–1483

Beemster GT, Baskin TI (1998) Analysis of cell division and elongation underlying the developmental acceleration of root growth in *Arabidopsis thaliana*. Plant Physiol **116:** 1515–1526

Beemster GTS, De Veylder L, Vercruysse S, West G, Rombaut D, Van Hummelen P, Galichet A, Gruissem W, Inzé D, Vuylsteke M (2005) Genome-wide analysis of gene expression profiles associated with cell cycle transitions in growing organs of Arabidopsis. Plant Physiol **138:** 734–743

Bernal AJ, Yoo CM, Mutwil M, Jensen JK, Hou G, Blaukopf C, Sørensen I, Blancaflor EB, Scheller HV, Willats WGT (2008) Functional analysis of the cellulose synthase-like genes CSLD1, CSLD2, and CSLD4 in tip-growing Arabidopsis cells. Plant Physiol **148:** 1238–1253

Birnbaum K, Shasha DE, Wang JY, Jung JW, Lambert GM, Galbraith DW, Benfey PN (2003) A gene expression map of the Arabidopsis root. Science **302:** 1956–1960

Braidwood L, Breuer C, Sugimoto K (2014) My body is a cage: mechanisms and modulation of plant cell growth. New Phytol **201:** 388–402

Breuer C, Kawamura A, Ichikawa T, Tominaga-Wada R, Wada T, Kondou Y, Muto S, Matsui M, Sugimoto K (2009) The trihelix transcription factor GTL1 regulates ploidy-dependent cell growth in the *Arabidopsis* trichome. Plant Cell **21:** 2307–2322

Breuer C, Morohashi K, Kawamura A, Takahashi N, Ishida T, Umeda M, Grotewold E, Sugimoto K (2012) Transcriptional repression of the APC/C activator CCS52A1 promotes active termination of cell growth. EMBO J **31:** 4488–4501

Breuninger H, Thamm A, Streubel S, Sakayama H, Nishiyama T, Dolan L (2016) Diversification of a bHLH transcription factor family in streptophytes led to the evolution of antagonistically acting genes controlling root hair growth. Curr Biol **26:** 920–934

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

Bustos R, Castrillo G, Linhares F, Puga MI, Rubio V, Pérez-Pérez J, Solano R, Leyva A, Paz-Ares J (2010) A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in Arabidopsis. PLoS Genet **6:** e1001102

Capella M, Ribone PA, Arce AL, Chan RL (2015) Arabidopsis thaliana HomeoBox 1 (AtHB1), a Homedomain-Leucine Zipper I (HD-Zip I) transcription factor, is regulated by PHYTOCHROME-INTERACTING FACTOR 1 to promote hypocotyl elongation. New Phytol **207:** 669–682

Carles CC, Choffnes-Inada D, Reville K, Lertpiriyapong K, Fletcher JC (2005) ULTRAPETALA1 encodes a SAND domain putative transcriptional regulator that controls shoot and floral meristem activity in Arabidopsis. Development **132:** 897–911

Chandrika NNP, Sundaravelpandian K, Yu SM, Schmidt W (2013) ALFIN-LIKE 6 is involved in root hair elongation during phosphate deficiency in Arabidopsis. New Phytol **198:** 709–720

Cho HT, Cosgrove DJ (2000) Altered expression of expansin modulates leaf growth and pedicel abscission in Arabidopsis thaliana. Proc Natl Acad Sci USA **97:** 9783–9788

Cho HT, Cosgrove DJ (2002) Regulation of root hair initiation and expansin gene expression in *Arabidopsis*. Plant Cell **14:** 3237–3253

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J **16:** 735–743

Cominelli E, Galbiati M, Albertini A, Fornara F, Conti L, Coupland G, Tonelli C (2011) DOF-binding sites additively contribute to guard cell-specificity of AtMYB60 promoter. BMC Plant Biol **11:** 162

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol **139:** 5–17

Datta S, Prescott H, Dolan L (2015) Intensity of a pulse of RSL4 transcription factor synthesis determines Arabidopsis root hair cell size. Nat Plants **1:** 15138

Favery B, Ryan E, Foreman J, Linstead P, Boudonck K, Steer M, Shaw P, Dolan L (2001) KOJAK encodes a cellulose synthase-like protein required for root hair cell morphogenesis in Arabidopsis. Genes Dev **15:** 79–89

Fornara F, Panigrahi KCS, Gissot L, Sauerbrunn N, Rühl M, Jarillo JA, Coupland G (2009) Arabidopsis DOF transcription factors act redundantly to reduce CONSTANS expression and are essential for a photoperiodic flowering response. Dev Cell **17:** 75–86

Franciosini A, Rymen B, Shibata M, Favero DS, Sugimoto K (2016) Molecular networks orchestrating plant cell growth. Curr Opin Plant Biol **35:** 98–104

Gasch AP, Eisen MB (2002) Exploring the conditional coregulation of yeast gene expression through fuzzy k-means clustering. Genome Biol **3:** RESEARCH0059

Giehl RFH, von Wirén N (2014) Root nutrient foraging. Plant Physiol **166:** 509–517

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

Gonzalez N, De Bodt S, Sulpice R, Jikumaru Y, Chae E, Dhondt S, Van Daele T, De Milde L, Weigel D, Kamiya Y, et al (2010) Increased leaf size: different means to an end. Plant Physiol **153:** 1261–1279

Gonzalez N, Vanhaeren H, Inzé D (2012) Leaf size control: complex coordination of cell division and expansion. Trends Plant Sci **17:** 332–340

Hiratsu K, Ohta M, Matsui K, Ohme-Takagi M (2002) The SUPERMAN protein is an active repressor whose carboxy-terminal repression domain is required for the development of normal flowers. FEBS Lett **514:** 351–354

Hu W, Ma H (2006) Characterization of a novel putative zinc finger gene MIF1: involvement in multiple hormonal regulation of Arabidopsis development. Plant J **45:** 399–422

Ikeuchi M, Sugimoto K, Iwase A (2013) Plant callus: mechanisms of induction and repression. Plant Cell **25:** 3159–3173

Ishida T, Kurata T, Okada K, Wada T (2008) A genetic regulatory network in the development of trichomes and root hairs. Annu Rev Plant Biol **59:** 365–386

Jang G, Yi K, Pires ND, Menand B, Dolan L (2011) RSL genes are sufficient for rhizoid system development in early diverging land plants. Development **138:** 2273–2281

Jones MA, Raymond MJ, Smirnoff N (2006) Analysis of the root-hair morphogenesis transcriptome reveals the molecular identity of six genes with roles in root-hair development in Arabidopsis. Plant J **45:** 83–100

Karas B, Amyot L, Johansen C, Sato S, Tabata S, Kawaguchi M, Szczyglowski K (2009) Conservation of lotus and Arabidopsis basic helix-loop-helix proteins reveals new players in root hair development. Plant Physiol **151:** 1175–1185

Kochian LV (1995) Cellular mechanisms of aluminum toxicity and resistance in plants. Annu Rev Plant Physiol Plant Mol Biol **46:** 237–260

Kwasniewski M, Nowakowska U, Szumera J, Chwialkowska K, Szarejko I (2013) iRootHair: a comprehensive root hair genomics database. Plant Physiol **161:** 28–35

Lammens T, Boudolf V, Kheibarshekan L, Zalmas LP, Gaamouche T, Maes S, Vanstraelen M, Kondorosi E, La Thangue NB, Govaerts W, et al (2008) Atypical E2F activity restrains APC/CCCS52A2 function obligatory for endocycle onset. Proc Natl Acad Sci USA **105:** 14721–14726

Ledwoń A, Gaj MD (2009) LEAFY COTYLEDON2 gene expression and auxin treatment in relation to embryogenic capacity of Arabidopsis somatic cells. Plant Cell Rep **28:** 1677–1688

Lee SY, Hwang EY, Seok HY, Tarte VN, Jeong MS, Jang SB, Moon Y-H (2015) Arabidopsis AtERF71/HRE2 functions as transcriptional activator via cis-acting GCC box or DRE/CRT element and is involved in root development through regulation of root cell expansion. Plant Cell Rep **34:** 223–231

Le Hir R, Bellini C (2013) The plant-specific dof transcription factors family: new players involved in vascular system development and functioning in Arabidopsis. Front Plant Sci **4:** 164–175

Lin Z, Yin K, Zhu D, Chen Z, Gu H, Qu LJ (2007) AtCDC5 regulates the G2 to M transition of the cell cycle and is critical for the function of Arabidopsis shoot apical meristem. Cell Res **17:** 815–828

López-Bucio J, Cruz-Ramírez A, Herrera-Estrella L (2003) The role of nutrient availability in regulating root architecture. Curr Opin Plant Biol **6:** 280–287

Luo C, Lam E (2014) Quantitatively Profiling Genome-Wide Patterns of Histone Modifications in Arabidopsis thaliana Using ChIP-seq. Methods Mol Biol **1112:** 177–193

Maere S, Heymans K, Kuiper M (2005) BiNGO: a Cytoscape plugin to assess overrepresentation of Gene Ontology categories in Biological Networks. Bioinformatics **21:** 3448–3449

Menand B, Yi K, Jouannic S, Hoffmann L, Ryan E, Linstead P, Schaefer DG, Dolan L (2007) An ancient mechanism controls the development of cells with a rooting function in land plants. Science **316:** 1477–1480

Menges M, de Jager SM, Gruissem W, Murray JA (2005) Global analysis of the core cell cycle regulators of Arabidopsis identifies novel genes, reveals multiple and highly specific profiles of expression and provides a coherent model for plant cell cycle control. Plant J **41:** 546–566

Menges M, Murray JAH (2002) Synchronous Arabidopsis suspension cultures for analysis of cell-cycle gene activity. Plant J **30:** 203–212

Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M (2005) The NAC transcription factors NST1 and NST2 of *Arabidopsis* regulate secondary wall thickenings and are required for anther dehiscence. Plant Cell **17:** 2993–3006

Mizukami Y, Fischer RL (2000) Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. Proc Natl Acad Sci USA **97:** 942–947

Moreno-Risueno MÁ, Martínez M, Vicente-Carbajosa J, Carbonero P (2007) The family of DOF transcription factors: from green unicellular algae to vascular plants. Mol Genet Genomics **277:** 379–390

Mortazavi A, Williams BA, Mccue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods **5:** 1–8

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, Kimura T (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J Biosci Bioeng **104:** 34–41

Negi J, Moriwaki K, Konishi M, Yokoyama R, Nakano T, Kusumi K, Hashimoto-Sugimoto M, Schroeder JI, Nishitani K, Yanagisawa S, et al (2013) A Dof transcription factor, SCAP1, is essential for the development of functional stomata in Arabidopsis. Curr Biol **23:** 479–484

Nye PH (1966) The effect of the nutrient intensity and buffering power of a soil and the absorbing power, size and root hairs of a root, on nutrient absorption by diffusion. Plant Soil **15:** 81–105

Obayashi T, Hayashi S, Saeki M, Ohta H, Kinoshita K (2009) ATTED-II provides coexpressed gene networks for Arabidopsis. Nucleic Acids Res **37:** D987–D991

Ohta M, Matsui K, Hiratsu K, Shinshi H, Ohme-Takagi M (2001) Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. Plant Cell **13:** 1959–1968

Pérez-Rodríguez P, Riaño-Pachón DM, Corrêa LGG, Rensing SA, Kersten B, Mueller-Roeber B (2010) PlnTFDB: updated content and new features of the plant transcription factor database. Nucleic Acids Res **38:** D822–D827

Rauf M, Arif M, Fisahn J, Xue GP, Balazadeh S, Mueller-Roeber B (2013) NAC transcription factor speedy hyponastic growth regulates flooding-induced leaf movement in *Arabidopsis*. Plant Cell **25:** 4941–4955

Ríos G, Lossow A, Hertel B, Breuer F, Schaefer S, Broich M, Kleinow T, Jásik J, Winter J, Ferrando A, et al (2002) Rapid identification of Arabidopsis insertion mutants by non-radioactive detection of T-DNA tagged genes. Plant J **32:** 243–253

Rodriguez RE, Mecchia MA, Debernardi JM, Schommer C, Weigel D, Palatnik JF (2010) Control of cell proliferation in Arabidopsis thaliana by microRNA miR396. Development **137:** 103–112

Roudier F, Schindelman G, DeSalle R, Benfey PN (2002) The COBRA family of putative GPI-anchored proteins in Arabidopsis: a new fellowship in expansion. Plant Physiol **130:** 538–548

Rymen B, Coppens F, Dhondt S, Fiorani F, Beemster GTS (2010) Kinematic analysis of cell division and expansion. Plant Dev Biol methods Protoc **655:** 203

Rymen B, Fiorani F, Kartal F, Vandepoele K, Inzé D, Beemster GTS (2007) Cold nights impair leaf growth and cell cycle progression in maize through transcriptional changes of cell cycle genes. Plant Physiol **143:** 1429–1438

Rymen B, Sugimoto K (2012) Tuning growth to the environmental demands. Curr Opin Plant Biol **15:** 683–690

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, et al (2003) TM4: a free, open-source system for microarray data management and analysis. Biotechniques **34:** 374–378

Schlereth A, Möller B, Liu W, Kientz M, Flipse J, Rademacher EH, Schmid M, Jürgens G, Weijers D (2010) MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. Nature **464:** 913–916

Schmidt W, Schikora A (2001) Different pathways are involved in phosphate and iron stress-induced alterations of root epidermal cell development. Plant Physiol **125:** 2078–2084

Schnall JA, Quatrano RS (1992) Abscisic acid elicits the water-stress response in root hairs of *Arabidopsis thaliana*. Plant Physiol **100:** 216–218

Shaul O, Mironov V, Burssens S, Van Montagu M, Inze D (1996) Two Arabidopsis cyclin promoters mediate distinctive transcriptional oscillation in synchronized tobacco BY-2 cells. Proc Natl Acad Sci USA **93:** 4868–4872

Skirycz A, Radziejwoski A, Busch W, Hannah MA, Czeszejko J, Kwaśniewski M, Zanor MI, Lohmann JU, De Veylder L, Witt I, et al (2008) The DOF transcription factor OBP1 is involved in cell cycle regulation in Arabidopsis thaliana. Plant J **56:** 779–792

Tanaka N, Kato M, Tomioka R, Kurata R, Fukao Y, Aoyama T, Maeshima M (2014) Characteristics of a root hair-less line of Arabidopsis thaliana under physiological stresses. J Exp Bot **65:** 1497–1512

Taylor-Teeples M, Lin L, de Lucas M, Turco G, Toal TW, Gaudinier A, Young NF, Trabucco GM, Veling MT, Lamothe R, et al (2015) An Arabidopsis gene regulatory network for secondary cell wall synthesis. Nature **517:** 571–575

Truernit E, Haseloff J (2007) A role for KNAT class II genes in root development. Plant Signal Behav **2:** 10–12

Wang S, Chang Y, Ellis B (2016) Overview of OVATE FAMILY PROTEINS, a novel class of plant-specific growth regulators. Front Plant Sci **7:** 417

Wang S, Chang Y, Guo J, Zeng Q, Ellis BE, Chen JG (2011) Arabidopsis ovate family proteins, a novel transcriptional repressor family, control multiple aspects of plant growth and development. PLoS ONE **6:** e23896

Wang Y, Henriksson E, Söderman E, Henriksson KN, Sundberg E, Engström P (2003) The Arabidopsis homeobox gene, ATHB16, regulates leaf development and the sensitivity to photoperiod in Arabidopsis. Dev Biol **264:** 228–239

Wang Y, Zhang W, Li K, Sun F, Han C, Wang Y, Li X (2008) Salt-induced plasticity of root hair development is caused by ion disequilibrium in Arabidopsis thaliana. J Plant Res **121:** 87–96

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

Yi K, Menand B, Bell E, Dolan L (2010) A basic helix-loop-helix transcription factor controls cell growth and size in root hairs. Nat Genet **42:** 264–267

Zou HF, Zhang YQ, Wei W, Chen HW, Song QX, Liu YF, Zhao MY, Wang F, Zhang BC, Lin Q, et al (2013) The transcription factor AtDOF4.2 regulates shoot branching and seed coat formation in Arabidopsis. Biochem J **449:** 373–388

Zuo J, Chua NH (2000) Chemical-inducible systems for regulated expression of plant genes. Curr Opin Biotechnol **11:** 146–151