© 2015. Published by The Company of Biologists Ltd | Development (2015) 142, 444-453 doi:10.1242/dev.113167

RESEARCH ARTICLE

STEM CELLS AND REGENERATION

A plant U-box protein, PUB4, regulates asymmetric cell division and cell proliferation in the root meristem

Atsuko Kinoshita[1,*], Colette A. ten Hove[2,3,*], Ryo Tabata[4], Masashi Yamada[5], Noriko Shimizu[4], Takashi Ishida[4], Katsushi Yamaguchi[6], Shuji Shigenobu[6,7], Yumiko Takebayashi[1], Satoshi Iuchi[8], Masatomo Kobayashi[8], Tetsuya Kurata[9], Takuji Wada[10], Mitsunori Seo[1], Mitsuyasu Hasebe[7,11], Ikram Blilou[2,12], Hiroo Fukuda[13], Ben Scheres[2,12], Renze Heidstra[2,12], Yuji Kamiya[1] and Shinichiro Sawa[4,‡]

ABSTRACT

The root meristem (RM) is a fundamental structure that is responsible for postembryonic root growth. The RM contains the quiescent center (QC), stem cells and frequently dividing meristematic cells, in which the timing and the frequency of cell division are tightly regulated. In *Arabidopsis thaliana*, several gain-of-function analyses have demonstrated that peptide ligands of the CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION-RELATED (CLE) family are important for maintaining RM size. Here, we demonstrate that a plant U-box E3 ubiquitin ligase, PUB4, is a novel downstream component of CLV3/CLE signaling in the RM. Mutations in *PUB4* reduced the inhibitory effect of exogenous CLV3/CLE peptide on root cell proliferation and columella stem cell maintenance. Moreover, *pub4* mutants grown without exogenous CLV3/CLE peptide exhibited characteristic phenotypes in the RM, such as enhanced root growth, increased number of cortex/endodermis stem cells and decreased number of columella layers. Our phenotypic and gene expression analyses indicated that PUB4 promotes expression of a cell cycle regulatory gene, *CYCD6;1*, and regulates formative periclinal asymmetric cell divisions in endodermis and cortex/endodermis initial daughters. These data suggest that PUB4 functions as a global regulator of cell proliferation and the timing of asymmetric cell division that are important for final root architecture.

KEY WORDS: *Arabidopsis* development, Root meristem, Asymmetric cell division, Cell proliferation

INTRODUCTION

In multicellular organisms, the proper balance between cell proliferation and differentiation is crucial for development. Plants, in which rigid cell walls prevent cell migration, require precise coordination of asymmetric cell division and cell proliferation for optimal growth and development (Gallagher and Smith, 1997; Scheres and Benfey, 1999). The model organism *Arabidopsis thaliana* possesses a simple and well-organized root meristem (RM) structure, where the quiescent center (QC) maintains adjacent cells as stem cells (van den Berg et al., 1997). The QC represents the organizer cells in the RM stem cell niche. The stem cells divide asymmetrically to produce self-renewing stem cells and progeny cells that are displaced into the meristematic zone (MZ), where they divide frequently to provide source cells for root elongation before ultimately undergoing differentiation (Baum and Rost, 1996; Dolan et al., 1993; Heidstra and Sabatini, 2014). At the rootward tip of the RM, stem cell daughters differentiate into columella (Col), lateral root cap (LRC) and epidermis (Ep). Columella and LRC cells together form the root cap. Stem cells shootward of the QC give rise to tissue layers containing cortex (C), endodermis (E) and the centrally located stele (Fig. 1A). Among these, cortex and endodermis, collectively referred to as ground tissue, derive from shared stem cells, the cortex/endodermis initials (CEI) (Dolan et al., 1993).

The CEI first divides anticlinally to regenerate the CEI-daughter (CEID) cell, which subsequently undergoes a periclinal asymmetric cell division to produce the cortex and the endodermis. It has been demonstrated that the periclinal asymmetric division in the CEID is tightly regulated under the control of two GRAS family transcription factors, SHORT-ROOT (SHR) and SCARECROW (SCR). *SHR* is expressed in the stele and the protein moves to the endodermis, the CEID and QC, to promote the expression of *SCR* (Di Laurenzio et al., 1996; Helariutta et al., 2000; Nakajima et al., 2001). In the CEID, SHR and SCR directly activate a D-type cyclin, *CYCD6;1*, which, by feedback inhibition of an inhibitory SCR-RBR (for RETINOBLASTOMA-RELATED) complex, promotes the formative cell divisions (Sozzani et al., 2010; Cruz-Ramírez et al., 2012). Consequently, *shr* and *scr* mutants fail to divide periclinally in CEID and exhibit a single ground tissue cell layer with cortex or mixed cortex/endodermis identities, respectively, whereas a loss-of-function mutation in *CYCD6;1* increases the population of undivided CEID in mature and developing embryos (Benfey et al., 1993; Di Laurenzio et al., 1996; Sozzani et al., 2010).

The columella stem cells divide anticlinally to generate self-renewing stem cells and differentiating columella cells, which accumulate starch granules. The identity of the columella stem cells is maintained non-cell autonomously by a QC-specific homeodomain transcription factor, *WUSCHEL-RELATED HOMEOBOX* 5 (*WOX5*) (Sarkar et al., 2007). In addition, recent studies have highlighted the role of a small peptide ligand, CLE40 (for CLAVATA3/EMBRYO SURROUNDING REGION-RELATED), in columella stem cell maintenance. CLE40 is transcribed in the differentiated columella cells and acts through a leucine-rich repeat (LRR) and a non-LRR

[1]RIKEN Center for Sustainable Resource Science, Tsurumi, Yokohama 230-0045, Japan. [2]Molecular Genetics, Department of Biology, Utrecht University, Utrecht 3584 CH, The Netherlands. [3]Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, Wageningen 6703HA, The Netherlands. [4]Graduate School of Science and Technology, Kumamoto University, Kumamoto 860-8555, Japan. [5]Department of Biology and Institute for Genome Science and Policy Center for Systems Biology, Duke University, Durham, NC 27708, USA. [6]Functional Genomics Facility, National Institute for Basic Biology, Okazaki 444-8585, Japan. [7]School of Life Science, The Graduate University for Advanced Studies, Okazaki 444-8585, Japan. [8]RIKEN BioResource Center, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan. [9]Graduate School of Biological Sciences, NAIST, Ikoma 630-0192, Japan. [10]Graduate School of Biosphere Sciences, Hiroshima University, Higashi-Hiroshima 739-8528, Japan. [11]Division of Evolutionary Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan. [12]Plant Developmental Biology, Wageningen University, Droevendaalsesteeg 1, Wageningen 6700AP, The Netherlands. [13]Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan.
*These authors contributed equally to this work

[‡]Author for correspondence (sawa@sci.kumamoto-u.ac.jp)

Received 27 May 2014; Accepted 30 November 2014

Development (2015) 142, 444-453 doi:10.1242/dev.113167

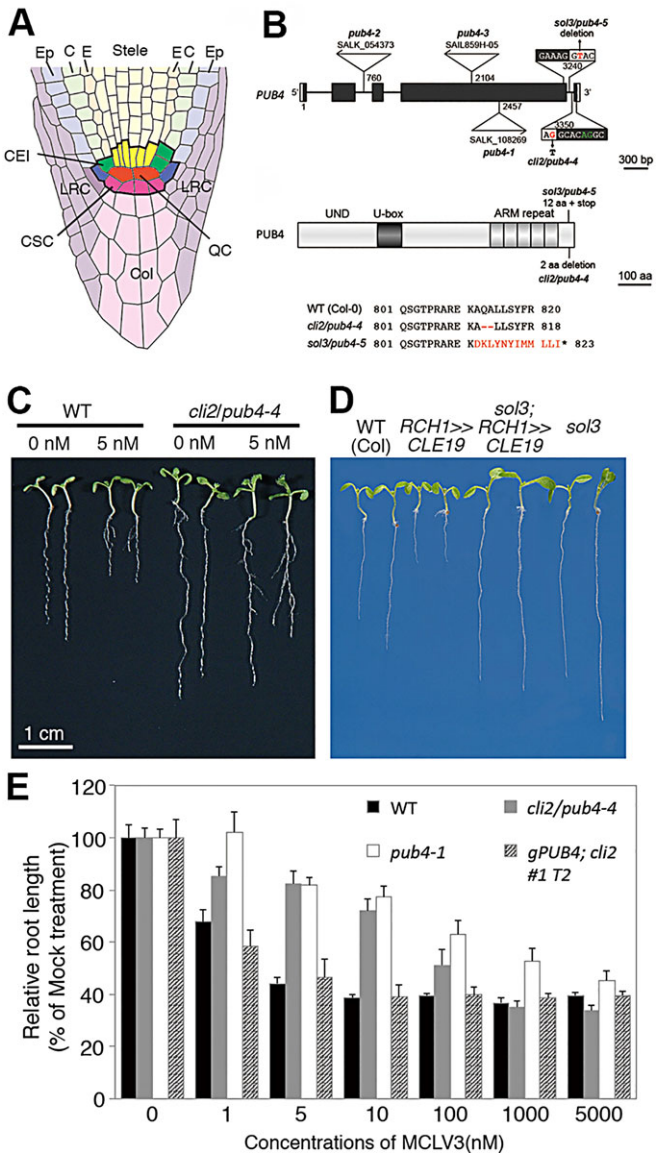

Fig. 1. *pub4* is resistant to the root elongation inhibitory effect caused by MCLV3 application or mis-expression of *CLE19*. (A) Schematic model of the *Arabidopsis* RAM. Ep, epidermis; C, cortex; E, endodermis; LRC, lateral root cap; Col, columella. Quiescent center (QC, red) and stem cells are enclosed by a heavy line; cortex/endodermis initial (CEI; green), Ep/LRC stem cell (blue), Col stem cell (CSC, pink). (B) Gene and protein structures of PUB4. Red characters in the *PUB4* gene structure indicate the mutation site in *cli2*/*pub4-4* and *sol3*/*pub4-5*. Green characters are the predicted splicing acceptor sites in *cli2*/*pub4-4*. (C) 7-day-old seedlings of WT (Col-0) and *cli2*/*pub4-4* grown with or without 5 nM MCLV3. (D) 5-day-old WT (Utr), *RCH1≫CLE19*, *sol3;RCH1≫CLE19* and *sol3* seedlings. (E) Relative root lengths of WT, *pub4-1*, *cli2*/*pub4-4* and *gPUB4; cli2* in different concentrations of MCLV3, compared with mock treatment. s.e.m. is indicated.

receptor-like kinase, CLAVATA 1 (CLV1) and ARABIDOPSIS CRINKLY 4 (ACR4), respectively, to regulate both *WOX5* expression and columella stem cell fate (Stahl et al., 2013, 2009). Although little is known about this mechanism to date, the essential components share high homology with those of the CLV3-WUSCHEL (WUS) pathway in the shoot apical meristem (SAM), leading to the suggestion that the SAM and the RM utilize a common mechanism for the stem cell maintenance (Mitchum et al., 2008).

Besides a role in columella stem cell regulation, the peptide ligands are also proposed to regulate root cell proliferation, as overexpression of several *CLE* genes or exogenous application of synthetic CLE peptides leads to reduced RM activity (Casamitjana-Martinez et al., 2003; Fiers et al., 2005; Kinoshita et al., 2007; Strabala et al., 2006). Furthermore, the inhibitory effect of CLE peptides on root elongation is diminished by mutations either in known CLV receptors, CLV2, SUPPRESSOR OF LLP1 2 (SOL2)/CORYNE (CRN) or in RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2), supporting the idea that CLE ligands are perceived by CLV receptors to regulate root cell proliferation (Casamitjana-Martinez et al., 2003; Fiers et al., 2005; Kinoshita et al., 2010; Miwa et al., 2008; Muller et al., 2008). However, *clv1* mutants exhibit shorter root length upon treatment with CLV3/CLE peptides, suggesting that at least two distinct pathways exist downstream of CLV3/CLE peptide in the RM to regulate columella stem cells and RM activity (Fiers et al., 2005; Stahl et al., 2009). Nevertheless, the presence of an endogenous CLE-mediated regulatory mechanism for root cell proliferation is still under debate, as none of the three CLE-resistant mutants, *clv2*, *crn* and *rpk2*, are shown to exhibit obvious root phenotypes in the absence of exogenous CLE peptide(s) (Fiers et al., 2005; Kinoshita et al., 2010; Miwa et al., 2008; Muller et al., 2008).

Here, we report the identification of a plant U-box (PUB) protein, PUB4, as a novel component that regulates the RM downstream of CLV3/CLE19 peptide activity. PUB4 has been previously demonstrated to have an E3 ubiquitin ligase activity and function in the pollen development (Wang et al., 2013). Notably, loss-of-function mutants of *PUB4* exhibited overproliferated root meristematic cells, suggesting that endogenous PUB4 plays an additional role in the regulation of root cell proliferation. Moreover, the asymmetric divisions in the CEID, endodermis and columella stem cells are delayed in *pub4*, indicating that *PUB4* controls the timing of asymmetric cell division in these cells. Our phenotypic analyses revealed that PUB4 is a global regulator, which governs both asymmetric cell divisions and cell proliferation in the RM.

RESULTS

PUB4 regulates root cell proliferation downstream of CLV3/CLE19

To find new genes involved in RM maintenance, we performed two independent suppressor screens. *clv3 peptide insensitive* (*cli*) *2* was isolated as one of 19 candidate suppressors from ~11,300 T3 lines of FOX *Arabidopsis* mutant lines (RIKEN) for insensitivity to a synthetic CLV3 peptide, MCLV3, in both SAM and RM maintenance (Fig. 1C). *suppressor of llp1* (*sol*) *3* was identified among a T-DNA activation population as a mutant that suppresses the short root phenotype conferred by root-specific overexpression of *CLE19* (Fig. 1D). Complementation analysis showed that *cli2* and *sol3* are recessive alleles of the same gene. Map-based cloning located *CLI2* in a 230 kb region on chromosome 2, and subsequent next-generation sequencing (NGS) analysis identified a nucleotide substitution at a splice donor site of *At2g23140*, which causes a two-amino acid deletion in the protein of *cli2* (Fig. 1B). The *sol3* allele contains a single nucleotide deletion within the same gene, which results in a frameshift and production of a truncated protein (Fig. 1B). *At2g23140* encodes a member of the plant U-box (PUB) family and has been previously described as *PUB4* (Azevedo et al., 2001; Wang et al., 2013). *pub4-1*, a T-DNA insertion null allele (Wang et al., 2013), showed resistance to MCLV3, as roots displayed longer roots when compared with WT even in presence of higher concentration of the peptide (Fig. 1E). In addition, the *cli2* phenotypes were complemented by the introduction of a 5.9 kb

Development (2015) 142, 444-453 doi:10.1242/dev.113167

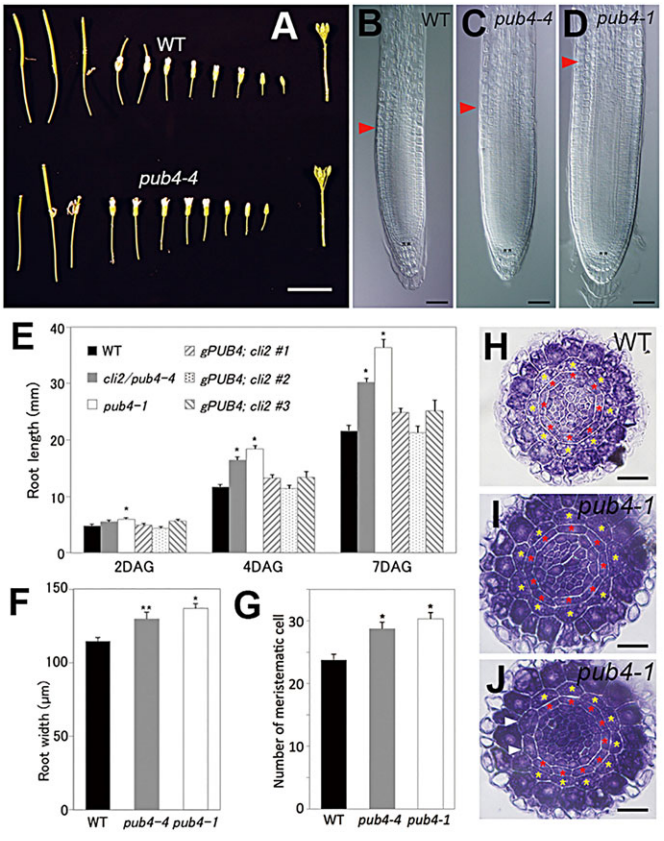

Fig. 2. *pub4* shows enhanced root cell proliferation. (A) Fruits and inflorescence of WT and *pub4*. *pub4-4* shows reduced fertility. (B-D) 7-day-old root meristems of WT (B), *pub4-4* (C) and *pub4-1* (D). Asterisks, QC; arrowheads, end of the MZ. (E) Root lengths of WT, *pub4-1*, *pub4-4* and three independent *gPUB4; cli2* lines (T3) 2, 4 and 7 days after germination (DAG). s.e.m. is indicated; $n \geq 9$; **P*<0.01. (F,G) Root width and meristematic cell number of 7-day-old WT, *pub4-4* and *pub4-1* roots. Error bars indicate s.e.m. $n \geq 10$, **P*<0.01, ***P*<0.02. (H-J) Transverse sections of 4-day-old WT (H) and *pub4-1* roots (I,J). Yellow asterisks, cortical cells; red asterisks, endodermal cells; arrowheads, ground tissue cells. Scale bars: 1 cm in A; 50 μm in B-D; 20 μm in H-J.

genomic *PUB4* fragment (*gPUB4; cli2*), corroborating that *PUB4* is the causal gene for the *cli2*/*sol3* mutation (Fig. 1E). Accordingly, we renamed our mutants *cli2* and *sol3* as *pub4-4* and *pub4-5*, respectively.

A previous report demonstrated that *PUB4* is involved in male fertility by regulating development of tapetal cells (Wang et al., 2013). Our *pub4* alleles, *pub4-4* and *pub4-5*, also showed a strong reduction in fertility, suggesting that the function of PUB4 protein is impaired in these mutant alleles (Fig. 2A). In addition, *pub4* showed enhanced root growth and increased root width in the absence of MCLV3 (Fig. 1C,D, Fig. 2B-F). Associated with these phenotypes, *pub4* roots exhibited an increased number of meristematic cells and resulted in an increased number of cortical and endodermal cell files, which are typically eight files in wild type (Fig. 2G-J; Table 1). Further observation of serial cross-sections revealed that the extra cell files can originate from longitudinal anticlinal division of CEIDs as well as by longitudinal anticlinal division of meristematic cortex and endodermal cells, suggesting that these extra longitudinal anticlinal divisions occur at various developmental stages in *pub4* root (supplementary material Fig. S1). Theses observations indicate that *PUB4* regulates root cell proliferation in both longitudinal and radial axes. Although PUB4 was shown to mediate protein ubiquitylation (Wang et al., 2013), the application of the proteasome inhibitor MG132 did not affect wild-type (WT) response to addition of MCLV3, suggesting that the response is not mediated by the MG132 pathway (supplementary material Fig. S2).

A previous report demonstrated *pPUB4::GUS* expression in various developmental tissues, including roots, vascular tissues, guard cells and anthers (Wang et al., 2013). To further confirm the spatial pattern of *PUB4* expression in the RM, we generated transgenic plants expressing a GFP-PUB4 fusion protein under the control of the *PUB4* promoter. *pub4-4* root phenotypes were restored by expressing *pPUB4:GFP-PUB4*, suggesting that the GFP-PUB4 fusion protein is functional *in vivo* (supplementary material Fig. S3). In seven-day-old roots, GFP-PUB4 protein was detected in meristematic cells (Fig. 3A) and in the vasculature of the differentiation zone (Fig. 3B). Notably, the fluorescence signal was present in undifferentiated columella stem cells (D1) and the outermost columella layer (D5), whereas the signal was weaker in the QC and the middle three columella layers (D2, D3, D4) (Fig. 3C,D; supplementary material Fig. S3A). GFP-PUB4 is localized in the cytoplasm as reported previously (Wang et al., 2013) and partially in nuclei (Fig. 3C; supplementary material Fig. S3B).

PUB4 regulates root cell proliferation in a cytokinin-independent manner

Previous studies have demonstrated that RM size is determined by the antagonistic interaction between two plant hormones: auxin as a promoter of cell division and cytokinin as a regulator for cell differentiation (Blilou et al., 2005; Dello Ioio et al., 2007, 2008). To examine whether the longer root phenotype of *pub4* has resulted from impaired cytokinin signaling, we tested the sensitivity of *pub4* to exogenous cytokinin. As a result, cytokinin treatment induced a decrease in the number of meristematic cells down to 63% and 68% of mock treatment in *pub4-4* and *pub4-1*, respectively. Although these rates are higher than those in wild type (52%), they are

Table 1. Number of root cell layers in *pub4*

	Number of ground tissue cell files (*n*)*	No. of CEI/CEIDs in mature embryos (*n*) and seedlings (*n*)		No. of columella layers in mature embryos (*n*) and seedlings (*n*)	
		Mature embryo	Seedling	Mature embryo	Seedling
WT (Col)	8±0 (10)	0.7±0.1 (38)	0.5±0.1 (8)*	4±0 (40)	4.3±0.1 (34)‡
pub4-1	9.0±0.4 (8)	3.6±0.2 (28)	2.1±0.2 (11)*	3.2±0.1 (28)	3.0±0.1 (28)‡
pub4-4	8.2±0.1 (10)	1.7±0.1 (28)	1.2±0.2 (11)*	3.1±0.1 (29)	3.3±0.1 (31)‡
WT (Utr)	8±0 (8)	0.8±0.1 (9)	0.2±0.1 (25)§	4±0 (9)	5.6±0.1 (19)§
pub4-5	8.6±0.2 (10)	6.1±0.3 (13)	2.5±0.3 (28)§	3±0 (13)	3.6±0.1 (29)§

Data represented are mean±s.e.m. Values in parentheses represent sample size.
*Three days after germination. ‡Seven days after germination. §Four days after germination.

Development (2015) 142, 444-453 doi:10.1242/dev.113167

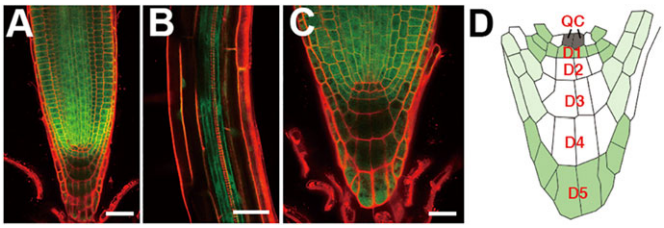

Fig. 3. *PUB4* is expressed in the RAM. (A-C) Main roots of *pPUB4:GFP-PUB4* transgenic plant were observed 7 days after germination. GFP fluorescent signal was detected in the RM (A,C) and vascular tissue (B). (D) Schematic model of *PUB4* expression in the QC and root cap. Scale bars: 40 μm in A,B; 20 μm in C.

significantly lower than those of the cytokinin signaling mutant, *arr1-3 arr12-1* (90%), suggesting that cytokinin signaling is still mostly active in *pub4* roots (Fig. 4A). In addition, the *pub4* mutation enhanced the longer root phenotype of *ahk3-3*, another cytokinin signaling mutant (Fig. 4B). The levels of two major bioactive cytokinins, isopentenyladenine (iP) and trans-zeatin (tZ), were indistinguishable between wild-type and *pub4* roots, indicating that cytokinin biosynthesis is not disrupted in *pub4* (Fig. 4D,E). Furthermore, *ipt3 ipt5 ipt7* and *arr1-3 arr12-1*, cytokinin biosynthesis and signaling mutants, respectively, showed similar sensitivity to MCLV3 peptide treatment as wild type (Fig. 4C). These data suggest that cytokinin and MCLV3 regulate proliferation of RM cells independently of each other.

We next examined the concentration of Indole-3-acetic acid (IAA) in *pub4* roots. The *pub4* roots contained 30% higher IAA level than wild-type roots (Fig. 4F). Furthermore, to monitor the response to IAA, we inspected the effect of auxin on root elongations. In wild type, lower concentrations (1 or 10 nM) of exogenous IAA enhance primary root elongation, whereas higher concentrations (1000 or 10,000 nM) of IAA in turn inhibit root growth (Fig. 4G). Roots treated with 10 nM IAA are not enhanced in *pub4* and the inhibitory effect of 100 or 1000 nM IAA was stronger on *pub4* than on wild type (Fig. 4G). These results suggest that the accumulation of auxin, but not cytokinin, causes larger RM and enhanced root growth in *pub4*.

PUB4 regulates the timing of columella stem cell division

In addition to the longer root phenotype, *pub4* exhibited a distinct defect in the number of differentiated columella cells. Wild-type roots contain on average four tiers of differentiated cell layers, which accumulate starch granules, whereas the *pub4* mutant has three differentiated columella layers (Fig. 5A-C, Table 1). Although previous studies have presented examples that enhanced distal stem cell identity can affect the number of differentiated columella layers (Sarkar et al., 2007), *pub4* roots consistently exhibit one layer of undifferentiated columella stem cells (Fig. 5D-F; supplementary material Fig. S4). The decreased number of columella layers in *pub4* mature embryos suggests that the columella stem cells do not divide as frequently as in WT, leading to a decreased number of columella layers in *pub4* (Fig. 6A-C, arrow; Table 1). These results imply a possibility that *PUB4* promotes the cell division in columella stem cells.

The identity of the columella stem cells is regulated by *WOX5*, the expression of which is restricted in the QC under the control of CLE40-mediated signaling (Sarkar et al., 2007; Stahl et al., 2009). It has been reported that treatment of synthetic CLE40 peptide or CLV3 peptide induces proximal shift of *WOX5* expression and differentiation of columella stem cells (Stahl et al., 2009). In order to examine the involvement of *PUB4* in this pathway, we observed *pWOX5::erGFP* expression upon MCLV3 treatment. Prior to MCLV3 treatment, *pWOX5::erGFP* signal is confined to the QC, both in wild-type and *pub4* background (Fig. 7C,G). In wild type, *pWOX5::erGFP* expression was detected in the stele cells 2 and 3 days after MCLV3 treatment (Fig. 5J-L). By contrast, the GFP signal was restricted in the QC in *pub4* background (Fig. 5M-O), suggesting that the proximal shift of *WOX5* expression is mediated

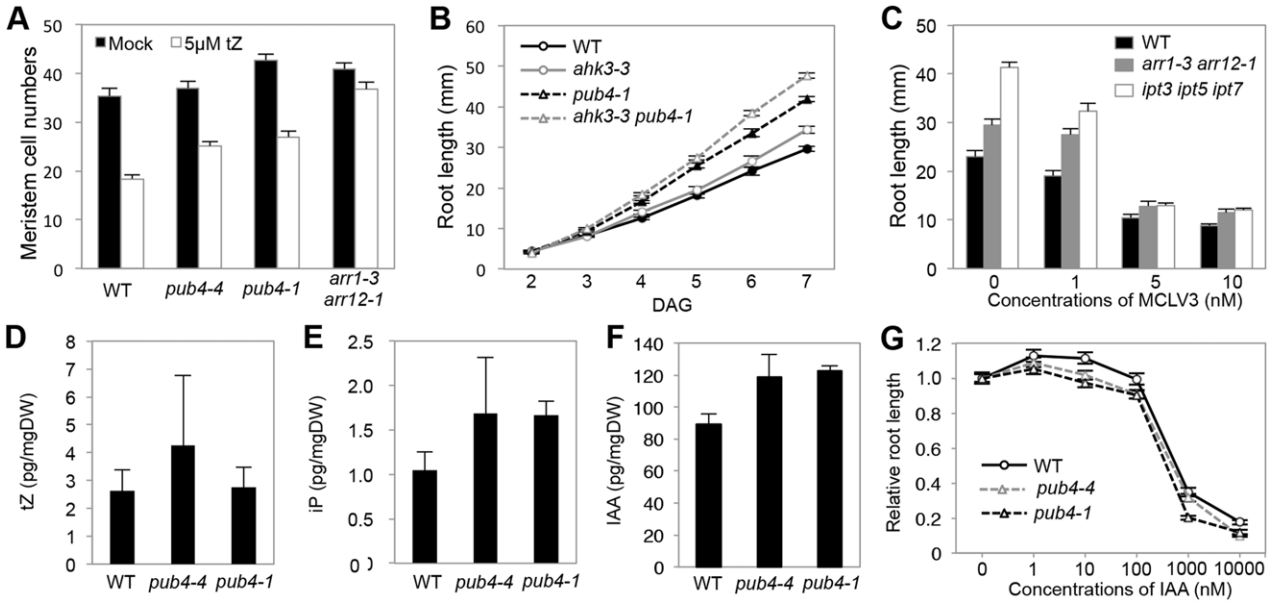

Fig. 4. *PUB4* regulates RM size independently of cytokinin. (A) Numbers of meristematic cells of 6-day-old wild-type, *pub4-4*, *pub4-1* and *arr1-3 arr12-1* roots, treated with or without 5 μM tZ for 24 h. s.e.m. is indicated. (B) Root lengths of WT, *ahk3-3*, *pub4-1* single and *ahk3-3 pub4-1* double mutants 2-7 days after germination (DAG). s.e.m. is indicated. (C) Root lengths of 7-day-old wild-type, *arr1-3 arr12-1, ipt3 ipt5 ipt7* seedlings, grown on the media containing different concentrations of MCLV3. s.e.m. is indicated. (D-F) Concentrations of tZ (D), iP (E) and IAA (F) in 4-day-old seedling roots. s.d. is indicated. (G) Relative root lengths of wild-type, *pub4-4* and *pub4-1* seedlings, grown on the media containing different concentrations of IAA. s.e.m. is indicated.

Development (2015) 142, 444-453 doi:10.1242/dev.113167

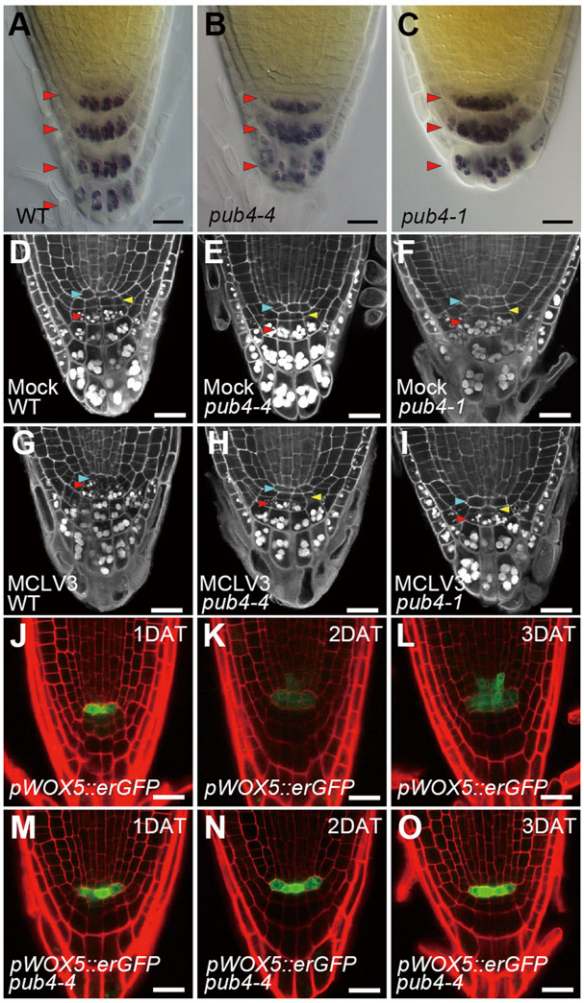

Fig. 5. *pub4* shows resistance to columella stem cell differentiation induced by MCLV3. (A-C) RM of 7-day-old wild-type (A), *pub4-4* (B) and *pub4-1* (C) seedlings stained with Lugol solution. (D-I) 4-day-old RAM of wild-type (D,G), *pub4-4* (E,H) and *pub4-1* (F,I) seedlings grown on media with (G-I) or without (D-F) 1 µM MCLV3. (J-O) Expression patterns of *pWOX5::erGFP* in wild-type (J-L) or *pub4-4* (M-O) background. 4-day-old seedlings were transferred to media containing 1 µM MCLV3 and observed 1 (J,M), 2 (K,N) and 3 (L,O) days after treatment (DAT). Arrowheads: QC position (blue), columella stem cell (yellow), differentiated columella cell (red). Scale bars: 20 µm.

by *PUB4*. Accordingly, no ectopic accumulation of starch granules in the QC and D1 cells was observed in MCLV3-treated *pub4* roots, indicating that columella stem cells are maintained in *pub4* (Fig. 5G-I; supplementary material Fig. S4). These data indicate that *PUB4* is required for ectopic columella stem cell differentiation caused by exogenous CLV3/CLE peptide.

PUB4 regulates the formative periclinal division in cortex/endodermis initial daughter cells

To gain more insight into the possible role of *PUB4* in root stem cell divisions, we observed the ground tissue stem cells in *pub4* mutants. In wild-type embryos, we found 0.7±0.1 CEI/CEID cells below a single set of cortex and endodermal cells, whereas this number was increased to 3.6±0.2 and 1.7±0.1 in *pub4-1* and *pub4-4* embryos, respectively (Fig. 6A-C, Table 1). Similarly, the CEI/CEID-like single ground tissue cells were observed in 3- or 4-day-old *pub4* seedlings (Fig. 2J and Fig. 6D-F; Table 1). The accumulation of

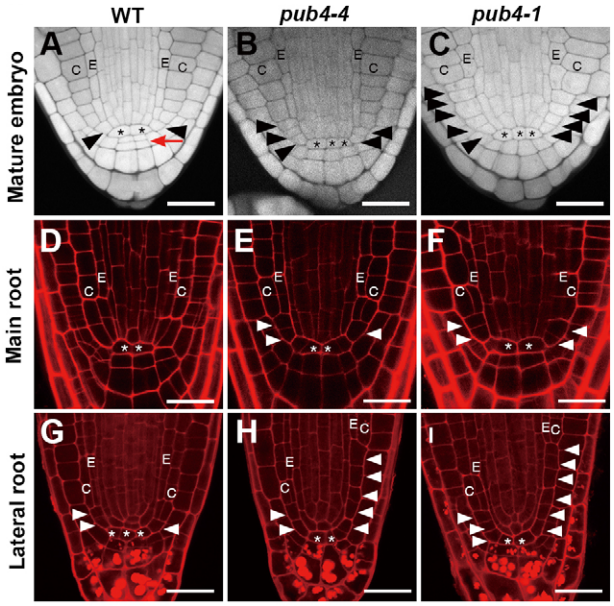

Fig. 6. *pub4* exhibits increased number of CEIDs. (A-C) Wild-type (A), *pub4-4* (B) and *pub4-1* (C) mutant mature embryos. (D-I) RM of 3-day-old main root and 10-day-old lateral roots in WT, *pub4-4* and *pub4-1*. Asterisks, QC; arrow, division in the columella stem cell; arrowheads, CEIDs; C, cortex; E, endodermis. Scale bars: 20 µm.

CEI/CEID-like cells was even more pronounced in *pub4* lateral roots (Fig. 6G-I). These result show that *PUB4* also functions during postembryonic development to regulate ground tissue cell division.

To investigate the identity of the accumulating undivided ground tissue cells, we observed the expression of several marker genes in *pub4* background. In wild-type roots, *pEn7::H2B-YFP* is expressed in the endodermis and CEID, whereas *pCo2::H2B-YFP* is highly expressed in the cortex, but is excluded from the CEI and CEID (Fig. 7A,B) (Heidstra et al., 2004). In *pub4*, *pEn7::H2B-YFP* is expressed in the CEID-like cells, whereas *pCo2::H2B-YFP* expression is absent (Fig. 7E,F). In addition, the expression of *pWOX5::erGFP*, is excluded in those cells in *pub4* (Fig. 7C,G). These data suggest that the single ground tissue cells in *pub4* have properties of CEI/CEID.

To further inspect how these cells are generated, we observed the expression of *pCYCD6;1::GFP* in *pub4* roots. It has been reported that *CYCD6;1* is expressed in the CEI/CEID and promotes the formative periclinal cell division to generate the endodermis and cortex (Fig. 7D) (Sozzani et al., 2010). The specific expression of *CYCD6;1-GFP* is prominent in early developmental stage, before day 5 (Koizumi and Gallagher, 2013; Koizumi et al., 2012; Sozzani et al., 2010); therefore, we observed its expression in *pub4* background at day 3 and 4. *pCYCD6;1::GFP* expression shifted proximally in *pub4*, with the strongest expression around the point of the formative periclinal cell division (Fig. 7H; supplementary material Fig. S5). Notably, the *pCYCD6;1::GFP* expression was frequently absent in the cells adjacent to QC, and the ratio of GFP-negative CEI/CEID was significantly higher in *pub4-1* (38%) and *pub4-5* (52%) than in wild type (19%) on day 4 (Fig. 7H,O). These data suggest that the *CYCD6;1* expression is downregulated in the stem cells and/or delayed in the CEI/CEID in *pub4*, which causes delays in the formative cell divisions resulting in columns of single ground tissue cells displaying CEI/CEID identity. The similar misexpression pattern of *pCYCD6;1::GFP* in *pub4* seedling roots was also seen in mature embryos (supplementary material Fig. S5),

Development (2015) 142, 444-453 doi:10.1242/dev.113167

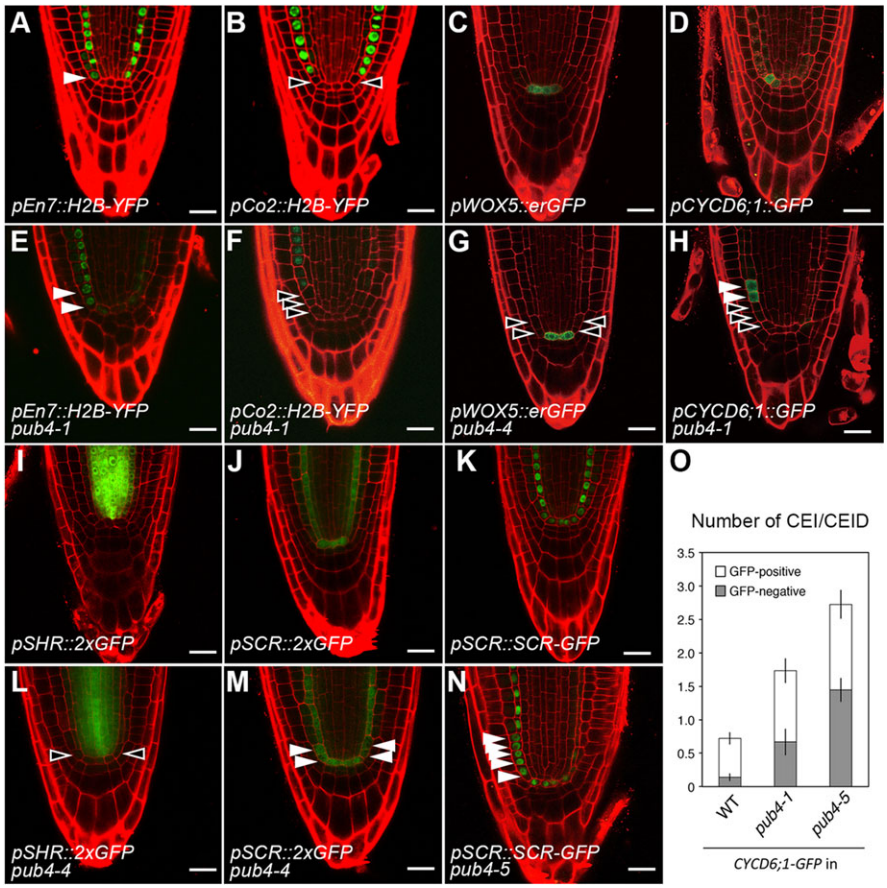

Fig. 7. Marker-line expressions in wild-type and *pub4* roots. (A-N) Expression patterns of *pEn7:H2B-YFP* (A,E), *pCo2:H2B-YFP* (B,F), *pWOX5::erGFP* (C,G) *pCYCD6;1::GFP* (D,H) *pSHR::2xGFP* (I,L), *pSCR::2xGFP* (J,M) and *pSCR::SCR-GFP* (K,N) in wild-type (A-D,I-K) and *pub4* (E-H,L-N) background. Main roots were stained with PI and observed 3 days after germination (A-N). Arrowheads; CEI/CEIDs. White and black arrowheads indicate GFP/YFP-positive and -negative cells, respectively. Scale bars: 20 µm. (O) Number of CEI/CEID with/without *CYCD6;1-GFP* signal in 4-day-old WT, *pub4-1* and *pub4-5*. s.e.m. is indicated. $n \geq 30$.

consistent with our observation of accumulated CEI/CEID in *pub4* mature embryos (Fig. 6A-C, Table 1).

It has been demonstrated that *CYCD6;1* is directly activated by two GRAS family transcription factors, SHR and SCR. In wild type, *SHR* is expressed in the stele and the protein moves to the endodermis, the CEI, CEID and QC, where it activates *SCR* expression (Di Laurenzio et al., 1996; Helariutta et al., 2000; Nakajima et al., 2001). To examine whether the impaired expression of *CYCD6;1* in *pub4* is caused by defects in SHR and/or SCR expression, we observed *pSHR::2xGFP* and *pSCR::2xGFP* marker lines in *pub4* background. However, no obvious differences in these gene expression patterns were observed in *pub4* (Fig. 7I,J,L,M). On the other hand, although it has also been reported that SCR is subject to proteasome-mediated degradation (Cruz-Ramírez et al., 2012), our observation of *pSCR::SCR-GFP* did not detect any changes in its expression pattern in *pub4* background and in wild type (Fig. 7K,N). These data suggest that *CYCD6;1* expression is downregulated in *pub4*, independently of SHR and SCR expression. Consistently, the transcript level of *CYCD6;1* is specifically reduced in 4-day-old *pub4* roots, whereas the mRNA levels of *SHR*, *SCR* and *WOX5* are indistinguishable from the wild type (Fig. 8).

PUB4 regulates secondary formative ground tissue cell divisions during embryogenesis

It has been demonstrated that *CYCD6;1* is also expressed at the point of secondary formative divisions of endodermal cells that form an additional cortex layer during embryogenesis and root development (Sozzani et al., 2010). In *Arabidopsis* embryogenesis, the second cortex layer is generated during torpedo stage (Scheres et al., 1994). Our observations of mature embryos indicated that all wild-type mature embryos exhibited the second cortex layer (n=29), at the point where the lateral root caps ends (Fig. 9A). The position of the second formative division is shifted shootward in *pub4-4*, and, strikingly, four out of twelve embryos did not exhibit a second cortex layer in the *pub4-1* null allele (Fig. 9B,C). Accordingly, the number of cells between QC and the point of second formative division was significantly increased in *pub4* (Fig. 9D,E). Interestingly, this number was also increased in two other MCLV3-resistant mutants, *clv2-101* and *rpk2-5*, but not in the

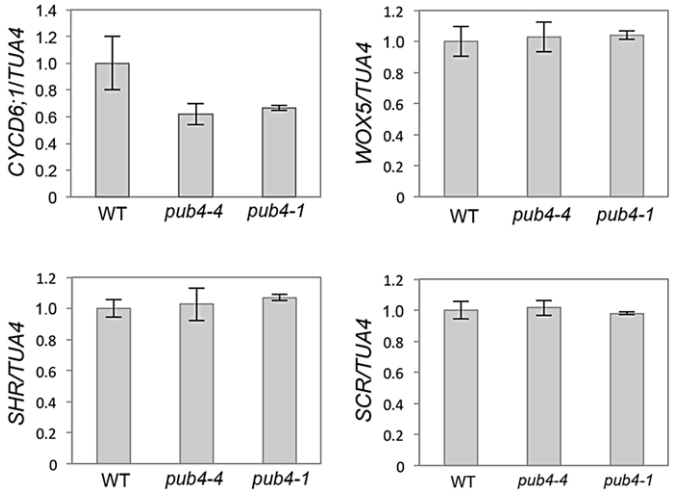

Fig. 8. *CYCD6;1* expression levels are altered in *pub4*. Real-time quantitative RT-PCR analysis of *CYCD6;1*, *WOX5*, *SHR* and *SCR* against *TUA4* in 4-day-old whole roots. s.d. is indicated.

Development (2015) 142, 444-453 doi:10.1242/dev.113167

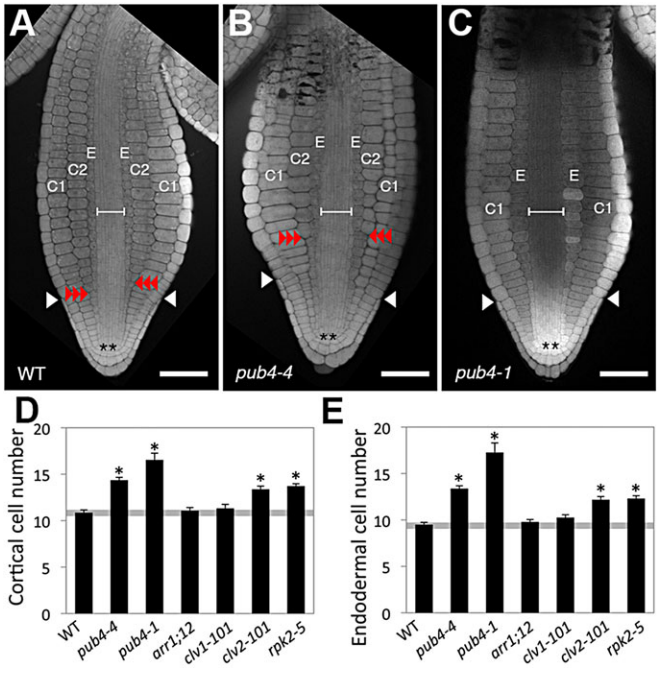

Fig. 9. *pub4* shows a defect in secondary periclinal endodermis division during embryogenesis. (A-C) Wild-type (A), *pub4-4* (B) and *pub4-1* (C) mutant mature embryos. (D,E) Number of cortical (D) and endodermal (E) cells between QC and the point of second formative periclinal division in WT and in *pub4-4*, *pub4-1*, *arr1-3 arr12-1*, *clv1-101*, *clv2-101* and *rpk2-5* mutants. The number of cells between asterisks and triple arrowheads were counted. As for *pub4-1* and *pub4-4*, only those with second cortex layers were counted. The ranges of the average±s.e.m. in WT are shaded. Asterisks, QC; arrowheads, lateral root cap end; triple arrowheads, point of the second periclinal division; C1, first cortex; C2, second cortex; E, endodermis; vertical bars, stele. Scale bars: 50 µm. s.e.m. is indicated. $n \geq 10$, $^*P<0.01$ (D,E).

MCLV3-sensitive *clv1-101* mutant, or the *arr1-3 arr12-1* cytokinin signaling mutant (Fig. 9D,E). These observations imply that *PUB4* not only regulates the formative periclinal division in CEID but also the second formative division in endodermis during embryogenesis, which together give rise to an additional cortex layer in the hypocotyl of seedlings.

PUB4 does not bind putative interactors in yeast

It has been demonstrated that *CYCD6;1* is directly activated by SHR and SCR, the function of which is negatively regulated by RBR (Cruz-Ramírez et al., 2012; Sozzani et al., 2010). As these proteins were shown to form complexes, we tested whether PUB4 interacts with any of these components. We performed yeast two-hybrid analyses, using the full size of PUB4 (PUB4 full) and the armadillo (ARM) domain without the U-box of PUB4 (PUB4ΔU-box) as baits, as previous studies have demonstrated that the ARM domain in other PUBs is responsible for the interaction with target proteins (Lu et al., 2011; Mbengue et al., 2010; Stegmann et al., 2012). However, none of SHR, SCR and RBR interacts with PUB4 in yeast, whereas SCR directly interacts both with SHR and RBR in yeast as reported previously (supplementary material Fig. S6A,B) (Cruz-Ramírez et al., 2012; Cui et al., 2007). We also examined whether PUB4 interacts with known CLV-related LRR-RLKs, CLV1, SOL2, RPK2, ACR4 and BAM1, as previous reports demonstrated that several U-box E3 ubiquitin ligases associate with receptor-like kinases *in vivo* (Gu et al., 1998; Kim et al., 2003; Lu et al., 2011; Mbengue et al., 2010; Samuel et al., 2008; Yee and Goring, 2009). However, we did not detect interaction of PUB4 with any of these kinases (supplementary material Fig. S6C). These data suggest that PUB4 does not directly bind and mediate the degradation of any known components.

DISCUSSION

Whereas the CLV3/CLE functions are well characterized in the SAM and vascular procambium cells, their roles in the RM are less well understood (Betsuyaku et al., 2011; Hirakawa et al., 2010; Yamada and Sawa, 2013). In this study, we have identified *PUB4* as a novel regulatory component that controls RM activity downstream of exogenous CLV3/CLE peptide. Notably, *pub4* displays multiple defects in root morphogenesis in the absence of exogenous CLV3/CLE peptide, suggesting that endogenous *PUB4* is a prominent factor for root development. Our observations have enlightened the importance of *PUB4* and putative hidden roles of CLV3/CLE signaling in the RM.

Previously, gain-of-function analyses have shown that exogenous applications of CLV3/CLE peptides or overexpression of *CLV3/CLE* genes induce RM consumption and suggested a possibility that CLV3/CLE signaling pathway(s) regulate cell proliferation in the RM (Casamitjana-Martinez et al., 2003; Fiers et al., 2005; Kinoshita et al., 2010, 2007; Miwa et al., 2008; Muller et al., 2008; Strabala et al., 2006). However, no mutations in any of the *CLV3/CLE* genes or their putative receptor genes are reported to exhibit obvious defects in RM size in the absence of exogenous CLV3/CLE peptide. Here, we demonstrate that *pub4* not only confers resistance to MCLV3 peptide, but also displays a larger RM size and enhanced primary root elongation in the absence of CLV3/CLE treatment. This observation strengthens the hypothesis that endogenous CLV3/CLE signaling functions to regulate the size of the RM. Notably, our genetic and quantitative analysis revealed that CLV3/CLE signaling regulates RM independently of cytokinin. On the other hand, the endogenous level of auxin is significantly increased in *pub4*. These data suggest that the dominant auxin signaling, compared with normal cytokinin signaling in *pub4* roots, alters the balance between cell division and differentiation in the RM, and results in shootward shift of the transition zone. However, we do not rule out the possibility that the increased auxin concentration might be a result of enlarged RM size. Further research will be needed to understand the relationship between PUB4 and phytohormone signaling crosstalk.

Interestingly, *PUB4* not only controls cell proliferation but also the formative cell divisions in the columella stem cell, CEID and endodermis. We found that the number of columella layers is decreased, whereas that of CEI/CEID cell is increased in *pub4* mutant roots. These apparently opposite phenotypes could be interpreted by a single function of *PUB4*; controlling the timing of asymmetric division in root cells. In this scenario, a delayed asymmetric division in columella stem cells during embryogenesis can result in a decreased number of columella layers (supplementary material Fig. S7A), whereas a shootward shift of asymmetric periclinal division versus regular anticlinal division in CEID cells can cause accumulation of CEID cells (supplementary material Fig. S7B). Similarly, an altered timing for second formative division of hypocotyl endodermis can contribute to expanded single cortex layers in *pub4* (supplementary material Fig. S7B). Notably, our observation revealed that a similar phenotype in the formative hypocotyl ground tissue cell division is also observed in *clv2* and *rpk2* embryos, the known MCLV3-resistant mutants, but not in *clv1* and *arr1 arr12*, the mutants that show sensitivity to the inhibitory effect of MCLV3 on root elongation. These data imply that endogenous CLV3/CLE signaling functions during embryogenesis to regulate the timing of periclinal division in

Development (2015) 142, 444-453 doi:10.1242/dev.113167

hypocotyl endodermis via CLV2, RPK2 and PUB4. The exogenous CLV3/CLE peptides function through the same components to regulate the cell proliferation in the RM in later developmental stages (supplementary material Fig. S7C).

Although the CLV3/CLE peptide function in the asymmetric cell division has not been fully assessed to date, recent studies have provided hints on their possible roles in the root stem cell maintenance. For example, CLE40 is shown to restrict formative cell divisions and differentiations in columella stem cell daughters (Stahl et al., 2013, 2009). In another example, exogenous treatments of CLV3/CLE peptide were shown to generate a column of single ground tissue cells, which resemble CEI/CEID cell accumulation in *pub4*, but express the *pCo2::H2B-YFP* differentiation marker normally absent in CEI/CEID (Fiers et al., 2005). These data imply that CLV3/CLE signaling might regulate divisions and differentiations in columella stem cells and CEID. *PUB4* could function downstream of such signaling.

We found that the expression of *CYCD6;1* is downregulated and thus the number of cells possessing CEI/CEID identity was increased in *pub4*, which suggests that *PUB4* regulates the timing of asymmetric division in the ground tissue cells at least partially via *CYCD6;1*. Notably, the expression levels and patterns of its direct regulators, *SHR* and *SCR*, are not altered in *pub4*, indicating that *PUB4* functions independently of these genes. As PUB4 has shown to have E3 ligase activity (Wang et al., 2013), it is highly plausible that PUB4 mediates the ubiquitylation and degradation of specific target protein(s) via the 26S proteasome. It is reported that the treatment of a 26S proteasome inhibitor, MG132, stabilizes SCR and cell cycle components, such as CYCD6, CDKB1 and RBR (Cruz-Ramírez et al., 2012). Our microscopic observation of *pCYCD6;1::GFP*, however, suggests that *PUB4* acts on the transcription of *CYCD6;1* rather than its protein stability. In addition, our yeast-two hybrid analysis showed that PUB4 did not interact with RBR nor SHR or SCR in yeast. Indeed, our microscopic observation of *pSCR::SCR-GFP* did not detect any ectopic signals in *pub4*, suggesting that PUB4 probably neither recognizes SCR as a target for ubiquitylation. Interestingly, recent mathematical modeling has proposed that the nested-feedback circuit among SHR, SCR, RBR and CYCD6;1 generates a bistable patterning switch, which defines the precise position of asymmetric periclinal cell division in the CEID (Cruz-Ramírez et al., 2012). PUB4 might not target any of those proteins themselves, but possibly modulate the robustness of the bistable switch. However, the abnormal expression of *CYCD6;1* in *pub4* cannot fully explain PUB4 function in CEID, as *pub4*, with only 35% reduction of *CYCD6;1*, shows more severe defects in CEI/CEID accumulation than *cycd6;1* (Sozzani et al., 2010). Notably, our observations showed that ground tissue cells in the *pub4* RM occasionally divide in a longitudinal and anticlinal direction to produce additional cell files. This finding suggests that *PUB4* regulates not only the timing but also the orientation of cell division in the CEI, CEID and root meristematic cells. Future studies should identify the target(s) and partner of PUB4, and uncover the precise mechanism of PUB4 function on the timing and orientation of cell division in the RM.

Our microscopic observation of *pPUB4::GFP-PUB4* showed its protein localization in both cytoplasm and nuclei throughout the RM with a limited accumulation in the QC and differentiated columella layers. The previous study observed *PUB4* promoter activity in the root cap (Wang et al., 2013). This might hint at the possibility that PUB4 protein moves *in vivo*. Alternatively, the slightly longer promoter and/or gene-coding region used in this study might more accurately reflect the expression pattern of *PUB4* in the root. In addition, as PUB4 does not have any obvious nuclear localization signals (NLS), its protein localization in nuclei might result from the interaction with its target protein(s).

Here, we have reported the unique features of *pub4* and have discussed the possible function of *PUB4* in the RM. In addition, PUB4 is found to be expressed in diverse tissues in the plant and functions in pollen development (Wang et al., 2013). Thus, PUB4 is likely to have a global function rather than to act on a specific event. One possible explanation for this is the abundance of CLV3/CLE peptides in plant tissues. In *Arabidopsis*, 32 *CLE* genes are expressed in various tissues, including root tips and stamens, and a high degree of functional redundancy is suggested to exist among those genes (Jun et al., 2010). *PUB4* might be a common downstream component of such redundant CLV3/CLE peptides in these various tissues. Another possibility is that PUB4 could modulate other global regulatory pathways, such as phytohormone signaling. For example, auxin has been shown to regulate RM activity, columella stem cell differentiation and the asymmetric cell division of the CEID (Cruz-Ramírez et al., 2012; Dello Ioio et al., 2008; Ding and Friml, 2010). Hence, it is conceivable that PUB4 has a role to fine-tune such signaling pathway(s). Indeed, recent studies have provided several examples of possible crosstalk between peptide and phytohormone signaling (Kondo et al., 2011, 2014; Matsuzaki et al., 2010). In addition, the CLV3 signaling pathway has shown to control *WUS* expression and cell division in the SAM antagonistically with cytokinin (Chickarmane et al., 2012; Gordon et al., 2009; Leibfried et al., 2005). Future studies should reveal whether such cross-talk mediates precise coordination of RM development, illustrating an overall feature of complex, but well-organized signaling networks.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis wild-type Columbia-0 (Col-0) and *pub4-1* (SALK_108269) seeds were obtained from the Arabidopsis Biological Resource Center (www.abrc.osu.edu) at Ohio State University, USA. *ahk3-3*, *arr1-3 arr12-1*, *clv1-101*, *clv2-101*, *ipt3;5;7* and *rpk2-5* have been described previously (Higuchi et al., 2004; Kinoshita et al., 2010; Replogle et al., 2013). *cli2/pub4-4* and *sol3/pub4-5* have been isolated in this study, among the collection of FOX *Arabidopsis* mutant lines (Ichikawa et al., 2006) and a T-DNA activation population, respectively. All lines used in this paper are in the Col-0 background, except for *clv1-101* and *sol3/pub4-5*, which are in Col-2 and Utrecht (Utr) background, respectively.

Surface-sterilized seeds were plated on growth medium containing Murashige and Skoog basal salts, 1% (w/v) sucrose, 0.05% (w/v) MES (pH 5.7) and 1.5% (w/v) agar. After cold treatment in the dark for two days, seeds were transferred to a growth room at 22°C under continuous white light (~10 W/m^{-2}). MCLV3 was synthesized and applied to the media as described previously (Kondo et al., 2006).

Map-based cloning and NGS of *cli2*/*sol3*

To map the *CLI2* locus, the *cli2* mutant in Col-0 background was crossed to Landsberg *erecta* (L*er*) and homozygous mutants were selected in the F2 population for segregation analyses. NGS was performed as reported previously (Tabata et al., 2013).

Construction of transgenic plants

For complementation analysis, the 5.9 kb fragment of genomic *PUB4* was amplified by PCR. For *PUB4* expression analysis, the *GFP*-coding region was combined with the genomic *PUB4* fragment using PCR with overlapping primers. Each fragment was cloned into the gateway entry vector pENTR-D/TOPO, and subsequently transferred to the gateway-compatible binary vector pGWB1 using LR clonase (Invitrogen). These vectors were introduced into *Agrobacterium tumefaciens* strain GV3101:: pMP90 and then into either Col-0 or *pub4-4* plants using the floral dip

Development (2015) 142, 444-453 doi:10.1242/dev.113167

method (Clough and Bent, 1998). All primer sets used for this study are listed in supplementary material Table S1.

Microscopy analyses

Starch granule staining, mPS-PI staining of roots and Aniline Blue staining of mature embryos were performed as described previously (Bougourd et al., 2000; ten Hove et al., 2010; Truernit et al., 2008). For differential interference contrast (DIC) optics, roots were cleared with chloral hydrate and observed using an Olympus BX53 microscope. For confocal microscopy, roots were mounted in propidium iodide (PI, 10 µg/ml in distilled water) and visualized using a Zeiss LSM700 confocal microscope. Histological sections were prepared as described previously (Kinoshita et al., 2010).

Gene expression analysis

Total RNA for quantitative RT-PCR (qRT-PCR) was isolated from whole roots of 4-day-old seedlings using an RNeasy Plant Mini Kit (Qiagen) and subjected to on-column DNA digestion with a RNase-free DNase set (Qiagen). First-strand cDNA was synthesized using a Superscript III First Strand Synthesis System (Invitrogen). qRT-PCR analysis was performed on a Stratagene Mx3000P machine (Agilent Technologies) according to the manufacturer's instructions, using THUNDERBIRD SYBR qPCR MIX (Toyobo). Three independent biological replicates were performed for each genotype and transcript levels were normalized to *TUA4*.

Yeast two-hybrid assay

The yeast two-hybrid assay was performed as reported previously (Welch et al., 2007). The coding sequences for amino acids 301-829 of PUB4 (PUB4ΔU-box) and the full size of PUB4 (PUB4 full) were cloned into the pDEST32 vector (Invitrogen). The full sizes of SHR, SCR and RBR, and kinase domains of CLV1, SOL2/CRN, RPK2, ACR4 and BAM1 were cloned into the pDEST22 vector (Invitrogen) and co-transformed with PUB4ΔU-box/pDEST32 into the yeast strain pJ69-4a.

Quantification of cytokinins and auxin

Whole roots of *Arabidopsis* grown for 4 days were frozen with liquid nitrogen, ground with 3-mm ceramic beads, and extracted with 1 ml of 80% (v/v) acetonitrile, containing 1% (v/v) acetic acid and internal standards, for 1 h. Purification and quantification of cytokinins and auxin were performed as reported previously (Yoshimoto et al., 2009), except that liquid chromatography was performed with a gradient of 3-22% of acetonitrile, containing 0.05% acetic acid, over 27 min for cytokinin measurement.

Acknowledgements

We thank Prof. Tsuyoshi Nakagawa (Department of Molecular and Functional Genomics, Center for Integrated Research in Science, Shimane University, Japan) for the pGWB1 binary vector, Prof. Tatsuo Kakimoto for *ahk3-3* and *ipt3;5;7* seeds (Department of Biological Sciences, Osaka University, Japan), Prof. Hitoshi Sakakibara for *arr1-3 arr12-1* seeds (Center for Sustainable Resource Science, RIKEN, Japan), Prof. Philip N. Benfey (Department of Biology and IGSP Center for Systems Biology, Duke University, USA) for *pCYCD6;1::GFP* and *pSCR::SCR-GFP* seeds, Prof. Thomas Laux (Institute of Biology III, University of Freiburg, Germany) for *pWOX5::erGFP* seeds, Dr Minami Matsui (RIKEN, Center for Sustainable Resource Science, Japan) for FOX line seed. We appreciate TAIR, the University of Wisconsin-Madison and GABI-Kat for their valuable resources. We also thank Dr Yusuke Jikumaru and Ms Maki Umegae for their technical assistance.

Competing interests

The authors declare no competing or financial interests.

Author contributions

C.A.t.H., N.S. and I.B. performed *sol3* analysis. R.T., M.Y., K.Y., S.Shigenobu, S.I., M.K. and M.H. performed PUB4/SOL3 mapping and genome sequencing. T.K. and T.W. produced marker lines. A.K., T.I., Y.T., M.S. and Y.K. carried out *pub4* mutant analysis. A.K., H.F., B.S., R.H. and S.Sawa designed research, analyzed the data and wrote the article.

Funding

This work was supported by Grant-in-Aid for Scientific Research (KAKENHI) [23119517, 23012034, 24114001, 24114009, 24370024, 24657035, 24658032 and 23870040 to A.K. and S.S.], an International Research Experience for Students and Young Researchers grant by the Japan Society for the Promotion of Science (JSPS), the RIKEN Special Postdoctoral Researchers Program (to A.K.), and by a Centre for BioSystems Genomics (CBSG) and Horizon grant, originating from the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research (to C.A.t.H. and R.H.). NGS was performed under the National Institute for Basic Biology (NIBB) cooperative research program [#12-103].

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.113167/-/DC1

References

Azevedo, C., Santos-Rosa, M. J. and Shirasu, K. (2001). The U-box protein family in plants. *Trends Plant Sci.* **6**, 354-358.

Baum, S. F. and Rost, T. L. (1996). Root apical organization in Arabidopsis thaliana 1. Root cap and protoderm. *Protoplasma* **192**, 178-188.

Benfey, P. N., Linstead, P. J., Roberts, K., Schiefelbein, J. W., Hauser, M. T. and Aeschbacher, R. A. (1993). Root development in Arabidopsis: four mutants with dramatically altered root morphogenesis. *Development* **119**, 57-70.

Betsuyaku, S., Sawa, S. and Yamada, M. (2011). The function of the CLE peptides in plant development and plant-microbe interactions. *Arabidopsis Book* **9**, e0149.

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K. and Scheres, B. (2005). The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. *Nature* **433**, 39-44.

Bougourd, S., Marrison, J. and Haseloff, J. (2000). Technical advance: an aniline blue staining procedure for confocal microscopy and 3D imaging of normal and perturbed cellular phenotypes in mature Arabidopsis embryos. *Plant J.* **24**, 543-550.

Casamitjana-Martínez, E., Hofhuis, H. F., Xu, J., Liu, C.-M., Heidstra, R. and Scheres, B. (2003). Root-specific CLE19 overexpression and the sol1/2 suppressors implicate a CLV-like pathway in the control of Arabidopsis root meristem maintenance. *Curr. Biol.* **13**, 1435-1441.

Chickarmane, V. S., Gordon, S. P., Tarr, P. T., Heisler, M. G. and Meyerowitz, E. M. (2012). Cytokinin signaling as a positional cue for patterning the apical-basal axis of the growing Arabidopsis shoot meristem. *Proc. Natl. Acad. Sci. USA* **109**, 4002-4007.

Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J.* **16**, 735-743.

Cruz-Ramírez, A., Díaz-Triviño, S., Blilou, I., Grieneisen, V. A., Sozzani, R., Zamioudis, C., Miskolczi, P., Nieuwland, J., Benjamins, R., Dhonukshe, P. et al. (2012). A bistable circuit involving SCARECROW-RETINOBLASTOMA integrates cues to inform asymmetric stem cell division. *Cell* **150**, 1002-1015.

Cui, H., Levesque, M. P., Vernoux, T., Jung, J. W., Paquette, A. J., Gallagher, K. L., Wang, J. Y., Blilou, I., Scheres, B. and Benfey, P. N. (2007). An evolutionarily conserved mechanism delimiting SHR movement defines a single layer of endodermis in plants. *Science* **316**, 421-425.

Dello Ioio, R., Linhares, F. S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P. and Sabatini, S. (2007). Cytokinins determine Arabidopsis root-meristem size by controlling cell differentiation. *Curr. Biol.* **17**, 678-682.

Dello Ioio, R., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M. T., Aoyama, T., Costantino, P. and Sabatini, S. (2008). A genetic framework for the control of cell division and differentiation in the root meristem. *Science* **322**, 1380-1384.

Di Laurenzio, L., Wysocka-Diller, J., Malamy, J. E., Pysh, L., Helariutta, Y., Freshour, G., Hahn, M. G., Feldmann, K. A. and Benfey, P. N. (1996). The SCARECROW gene regulates an asymmetric cell division that is essential for generating the radial organization of the Arabidopsis root. *Cell* **86**, 423-433.

Ding, Z. and Friml, J. (2010). Auxin regulates distal stem cell differentiation in Arabidopsis roots. *Proc. Natl. Acad. Sci. USA* **107**, 12046-12051.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K. and Scheres, B. (1993). Cellular organisation of the Arabidopsis thaliana root. *Development* **119**, 71-84.

Fiers, M., Golemiec, E., Xu, J., van der Geest, L., Heidstra, R., Stiekema, W. and Liu, C.-M. (2005). The 14-amino acid CLV3, CLE19, and CLE40 peptides trigger consumption of the root meristem in Arabidopsis through a CLAVATA2-dependent pathway. *Plant Cell* **17**, 2542-2553.

Gallagher, K. and Smith, L. G. (1997). Asymmetric cell division and cell fate in plants. *Curr. Opin. Cell Biol.* **9**, 842-848.

Gordon, S. P., Chickarmane, V. S., Ohno, C. and Meyerowitz, E. M. (2009). Multiple feedback loops through cytokinin signaling control stem cell number within the Arabidopsis shoot meristem. *Proc. Natl. Acad. Sci. USA* **106**, 16529-16534.

Gu, T., Mazzurco, M., Sulaman, W., Matias, D. D. and Goring, D. R. (1998). Binding of an arm repeat protein to the kinase domain of the S-locus receptor kinase. *Proc. Natl. Acad. Sci. USA* **95**, 382-387.

Heidstra, R. and Sabatini, S. (2014). Plant and animal stem cells: similar yet different. *Nat. Rev. Mol. Cell Biol.* **15**, 301-312.

Heidstra, R., Welch, D. and Scheres, B. (2004). Mosaic analyses using marked activation and deletion clones dissect Arabidopsis SCARECROW action in asymmetric cell division. *Genes Dev.* **18**, 1964-1969.
Helariutta, Y., Fukaki, H., Wysocka-Diller, J., Nakajima, K., Jung, J., Sena, G., Hauser, M.-T. and Benfey, P. N. (2000). The SHORT-ROOT gene controls radial patterning of the Arabidopsis root through radial signaling. *Cell* **101**, 555-567.
Higuchi, M., Pischke, M. S., Mahonen, A. P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S. et al. (2004). In planta functions of the Arabidopsis cytokinin receptor family. *Proc. Natl. Acad. Sci. USA* **101**, 8821-8826.
Hirakawa, Y., Kondo, Y. and Fukuda, H. (2010). Regulation of vascular development by CLE peptide-receptor systems. *J. Integr. Plant Biol.* **52**, 8-16.
Ichikawa, T., Nakazawa, M., Kawashima, M., Iizumi, H., Kuroda, H., Kondou, Y., Tsuhara, Y., Suzuki, K., Ishikawa, A., Seki, M. et al. (2006). The FOX hunting system: an alternative gain-of-function gene hunting technique. *Plant J.* **48**, 974-985.
Jun, J., Fiume, E., Roeder, A. H. K., Meng, L., Sharma, V. K., Osmont, K. S., Baker, C., Ha, C. M., Meyerowitz, E. M., Feldman, L. J. et al. (2010). Comprehensive analysis of CLE polypeptide signaling gene expression and overexpression activity in Arabidopsis. *Plant Physiol.* **154**, 1721-1736.
Kim, M., Cho, H. S., Kim, D.-M., Lee, J. H. and Pai, H.-S. (2003). CHRK1, a chitinase-related receptor-like kinase, interacts with NtPUB4, an armadillo repeat protein, in tobacco. *Biochim. Biophys. Acta* **1651**, 50-59.
Kinoshita, A., Nakamura, Y., Sasaki, E., Kyozuka, J., Fukuda, H. and Sawa, S. (2007). Gain-of-function phenotypes of chemically synthetic CLAVATA3/ESR-related (CLE) peptides in Arabidopsis thaliana and Oryza sativa. *Plant Cell Physiol.* **48**, 1821-1825.
Kinoshita, A., Betsuyaku, S., Osakabe, Y., Mizuno, S., Nagawa, S., Stahl, Y., Simon, R., Yamaguchi-Shinozaki, K., Fukuda, H. and Sawa, S. (2010). RPK2 is an essential receptor-like kinase that transmits the CLV3 signal in Arabidopsis. *Development* **137**, 3911-3920.
Koizumi, K. and Gallagher, K. L. (2013). Identification of SHRUBBY, a SHORT-ROOT and SCARECROW interacting protein that controls root growth and radial patterning. *Development* **140**, 1292-1300.
Koizumi, K., Hayashi, T., Wu, S. and Gallagher, K. L. (2012). The SHORT-ROOT protein acts as a mobile, dose-dependent signal in patterning the ground tissue. *Proc. Natl. Acad. Sci. USA* **109**, 13010-13015.
Kondo, T., Sawa, S., Kinoshita, A., Mizuno, S., Kakimoto, T., Fukuda, H. and Sakagami, Y. (2006). A plant peptide encoded by CLV3 identified by in situ MALDI-TOF MS analysis. *Science* **313**, 845-848.
Kondo, Y., Hirakawa, Y., Kieber, J. J. and Fukuda, H. (2011). CLE peptides can negatively regulate protoxylem vessel formation via cytokinin signaling. *Plant Cell Physiol.* **52**, 37-48.
Kondo, Y., Ito, T., Nakagami, H., Hirakawa, Y., Saito, M., Tamaki, T., Shirasu, K. and Fukuda, H. (2014). Plant GSK3 proteins regulate xylem cell differentiation downstream of TDIF-TDR signalling. *Nat. Commun.* **5**, 3504.
Leibfried, A., To, J. P. C., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J. J. and Lohmann, J. U. (2005). WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. *Nature* **438**, 1172-1175.
Lu, D., Lin, W., Gao, X., Wu, S., Cheng, C., Avila, J., Heese, A., Devarenne, T. P., He, P. and Shan, L. (2011). Direct ubiquitylation of pattern recognition receptor FLS2 attenuates plant innate immunity. *Science* **332**, 1439-1442.
Matsuzaki, Y., Ogawa-Ohnishi, M., Mori, A. and Matsubayashi, Y. (2010). Secreted peptide signals required for maintenance of root stem cell niche in Arabidopsis. *Science* **329**, 1065-1067.
Mbengue, M., Camut, S., de Carvalho-Niebel, F., Deslandes, L., Froidure, S., Klaus-Heisen, D., Moreau, S., Rivas, S., Timmers, T., Herve, C. et al. (2010). The Medicago truncatula E3 ubiquitin ligase PUB1 interacts with the LYK3 symbiotic receptor and negatively regulates infection and nodulation. *Plant Cell* **22**, 3474-3488.
Mitchum, M. G., Wang, X. and Davis, E. L. (2008). Diverse and conserved roles of CLE peptides. *Curr. Opin. Plant Biol.* **11**, 75-81.
Miwa, H., Betsuyaku, S., Iwamoto, K., Kinoshita, A., Fukuda, H. and Sawa, S. (2008). The receptor-like kinase SOL2 mediates CLE signaling in Arabidopsis. *Plant Cell Physiol.* **49**, 1752-1757.
Muller, R., Bleckmann, A. and Simon, R. (2008). The receptor kinase CORYNE of Arabidopsis transmits the stem cell-limiting signal CLAVATA3 independently of CLAVATA1. *Plant Cell* **20**, 934-946.
Nakajima, K., Sena, G., Nawy, T. and Benfey, P. N. (2001). Intercellular movement of the putative transcription factor SHR in root patterning. *Nature* **413**, 307-311.
Replogle, A., Wang, J., Paolillo, V., Smeda, J., Kinoshita, A., Durbak, A., Tax, F. E., Wang, X., Sawa, S. and Mitchum, M. G. (2013). Synergistic interaction of CLAVATA1, CLAVATA2, and RECEPTOR-LIKE PROTEIN KINASE 2 in cyst nematode parasitism of Arabidopsis. *Mol. Plant Microbe Interact.* **26**, 87-96.
Samuel, M. A., Mudgil, Y., Salt, J. N., Delmas, F., Ramachandran, S., Chilelli, A. and Goring, D. R. (2008). Interactions between the S-domain receptor kinases and AtPUB-ARM E3 ubiquitin ligases suggest a conserved signaling pathway in Arabidopsis. *Plant Physiol.* **147**, 2084-2095.
Sarkar, A. K., Luijten, M., Miyashima, S., Lenhard, M., Hashimoto, T., Nakajima, K., Scheres, B., Heidstra, R. and Laux, T. (2007). Conserved factors regulate signalling in Arabidopsis thaliana shoot and root stem cell organizers. *Nature* **446**, 811-814.
Scheres, B. and Benfey, P. N. (1999). Asymmetric cell division in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 505-537.
Scheres, B., Wolkenfelt, H., Willemsen, V., Terlouw, M., Lawson, E., Dean, C. and Weisbeek, P. (1994). Embryonic origin of the Arabidopsis primary root and root-meristem initials. *Development* **120**, 2475-2487.
Sozzani, R., Cui, H., Moreno-Risueno, M. A., Busch, W., Van Norman, J. M., Vernoux, T., Brady, S. M., Dewitte, W., Murray, J. A. H. and Benfey, P. N. (2010). Spatiotemporal regulation of cell-cycle genes by SHORTROOT links patterning and growth. *Nature* **466**, 128-132.
Stahl, Y., Wink, R. H., Ingram, G. C. and Simon, R. (2009). A signaling module controlling the stem cell niche in Arabidopsis root meristems. *Curr. Biol.* **19**, 909-914.
Stahl, Y., Grabowski, S., Bleckmann, A., Kühnemuth, R., Weidtkamp-Peters, S., Pinto, K. G., Kirschner, G. K., Schmid, J. B., Wink, R. H., Hülsewede, A. et al. (2013). Moderation of Arabidopsis root stemness by CLAVATA1 and ARABIDOPSIS CRINKLY4 receptor kinase complexes. *Curr. Biol.* **23**, 362-371.
Stegmann, M., Anderson, R. G., Ichimura, K., Pecenkova, T., Reuter, P., Zarsky, V., McDowell, J. M., Shirasu, K. and Trujillo, M. (2012). The ubiquitin ligase PUB22 targets a subunit of the exocyst complex required for PAMP-triggered responses in Arabidopsis. *Plant Cell* **24**, 4703-4716.
Strabala, T. J., O'Donnell, P. J., Smit, A.-M., Ampomah-Dwamena, C., Martin, E. J., Netzler, N., Nieuwenhuizen, N. J., Quinn, B. D., Foote, H. C. C. and Hudson, K. R. (2006). Gain-of-function phenotypes of many CLAVATA3/ESR genes, including four new family members, correlate with tandem variations in the conserved CLAVATA3/ESR domain. *Plant Physiol.* **140**, 1331-1344.
Tabata, R., Kamiya, T., Shigenobu, S., Yamaguchi, K., Yamada, M., Hasebe, M., Fujiwara, T. and Sawa, S. (2013). Identification of an EMS-induced causal mutation in a gene required for boron-mediated root development by low-coverage genome re-sequencing in Arabidopsis. *Plant Signal. Behav.* **8**, e22534.
ten Hove, C. A., Willemsen, V., de Vries, W. J., van Dijken, A., Scheres, B. and Heidstra, R. (2010). SCHIZORIZA encodes a nuclear factor regulating asymmetry of stem cell divisions in the Arabidopsis root. *Curr. Biol.* **20**, 452-457.
Truernit, E., Bauby, H., Dubreucq, B., Grandjean, O., Runions, J., Barthelemy, J. and Palauqui, J.-C. (2008). High-resolution whole-mount imaging of three-dimensional tissue organization and gene expression enables the study of Phloem development and structure in Arabidopsis. *Plant Cell* **20**, 1494-1503.
van den Berg, C., Willemsen, V., Hendriks, G., Weisbeek, P. and Scheres, B. (1997). Short-range control of cell differentiation in the Arabidopsis root meristem. *Nature* **390**, 287-289.
Wang, H., Lu, Y., Jiang, T., Berg, H., Li, C. and Xia, Y. (2013). The Arabidopsis U-box/ARM repeat E3 ligase AtPUB4 influences growth and degeneration of tapetal cells, and its mutation leads to conditional male sterility. *Plant J.* **74**, 511-523.
Welch, D., Hassan, H., Blilou, I., Immink, R., Heidstra, R. and Scheres, B. (2007). Arabidopsis JACKDAW and MAGPIE zinc finger proteins delimit asymmetric cell division and stabilize tissue boundaries by restricting SHORT-ROOT action. *Genes Dev.* **21**, 2196-2204.
Yamada, M. and Sawa, S. (2013). The roles of peptide hormones during plant root development. *Curr. Opin. Plant Biol.* **16**, 56-61.
Yee, D. and Goring, D. R. (2009). The diversity of plant U-box E3 ubiquitin ligases: from upstream activators to downstream target substrates. *J. Exp. Bot.* **60**, 1109-1121.
Yoshimoto, K., Jikumaru, Y., Kamiya, Y., Kusano, M., Consonni, C., Panstruga, R., Ohsumi, Y. and Shirasu, K. (2009). Autophagy negatively regulates cell death by controlling NPR1-dependent salicylic acid signaling during senescence and the innate immune response in Arabidopsis. *Plant Cell* **21**, 2914-2927.