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

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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 shotward 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
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 (Azavedo 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
A previous report demonstrated that 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 (Biliou 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

![Image](https://example.com/image.png)

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

<table>
<thead>
<tr>
<th></th>
<th>No. of ground tissue cell files (n)*</th>
<th>No. of CEI/CEIDs in mature embryos (n) and seedlings (n)</th>
<th>No. of columella layers in mature embryos (n) and seedlings (n)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Mature embryo</td>
<td>Seedling</td>
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<tr>
<td><strong>WT (Col)</strong></td>
<td>8±0 (10)</td>
<td>0.7±0.1 (38)</td>
<td>0.5±0.1 (8)*</td>
</tr>
<tr>
<td><strong>pub4-1</strong></td>
<td>9.0±0.4 (8)</td>
<td>3.6±0.2 (28)</td>
<td>2.1±0.2 (11)*</td>
</tr>
<tr>
<td><strong>pub4-4</strong></td>
<td>8.2±0.1 (10)</td>
<td>1.7±0.1 (28)</td>
<td>1.2±0.2 (11)*</td>
</tr>
<tr>
<td><strong>WT (Utr)</strong></td>
<td>8±0 (8)</td>
<td>0.8±0.1 (9)</td>
<td>0.2±0.1 (25)*</td>
</tr>
<tr>
<td><strong>pub4-5</strong></td>
<td>8±0 (10)</td>
<td>6.1±0.3 (13)</td>
<td>2.5±0.3 (28)*</td>
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Data represented are means±s.e.m. Values in parentheses represent sample size.

*Three days after germination. ‡Seven days after germination. ‡‡Four days after germination.
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...
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). The accumulation of 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 pCYCD6;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). 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). These data suggest that the single ground tissue cells in pub4 have properties of CEI/CEID.

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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,F) or pub4-4 (E,H) 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.

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.

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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 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
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 pericllinal 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-Ramirez 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-Ramirez 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
hypoctlateral 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-Ramirez 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-Ramirez 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-Ramirez 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; Matsuizaki 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). cliz/ 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/m²). MCLV3 was synthesized and applied to the media as described previously (Kondo et al., 2006).

Map-based cloning and NGS of cliz/sol3

To map the CLIZ locus, the cliz mutant in Col-0 background was crossed to Landsberg erecta (Ler) 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.
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 (Bouguerd et al., 2000; ten Hove et al., 2010; Truemet 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 a RNeasy Plant Mini Kit (Qiagen) and subjected to 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 p69-4a.

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.

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Competing interests
The authors declare no competing or financial interests.

Author contributions

References


Fig. S1 The timing and orientation of cell division are disrupted in pub4 roots
Transverse sections for two independent samples of 4-day-old pub4-1. (A-C) In sample 1, the number of CEI/CEID is increased immediately above of QC (A), which suggests that an additional longitudinal anticlinal division occurs during embryogenesis. Further additional longitudinal anticlinal divisions give rise to in total eleven ground tissue cell files per ring (B), and some longitudinal periclinal divisions (black asterisks) are observed in the later developmental stages (C). (D-F) In sample 2, the number of cell files per ring is normal in the early developmental stages (D), and the longitudinal periclinal division is delayed in some CEI/CEIDs (E, black asterisks). A spontaneous longitudinal anticlinal cell division (red asterisks) gives rise to an additional cell file (F, G) in the middle of RM.
Fig. S2 The effects of MCLV3 and MG132 on Arabidopsis root growth

Wild type plants were grown on the media with/without 5nM MCLV3 and 10µM MG132 for 7 days. The inhibitory effect of MCLV3 on the root elongation was not recovered by MG132 treatment.
Fig. S3 pPUB4:GFP-PUB4 expression pattern

(A) 11 different lines showed similar expression patterns, with weaker localization in the QC. (B) Expression pattern of pPUB4:GFP-PUB4; Col-0 stained with DAPI. GFP localizes in the cytoplasm and in nuclei (arrowheads). (C-E) Complementation of pub4-4 phenotypes by introducing pPUB4:GFP-PUB4 construct. Root length (C), number of columella layers (D) and root width (E) were examined 7 days after germination. n>8, SE is indicated. Scale bars; 50 µm (A), 20 µm (B).
Fig. S4  

*pub4* shows resistance to ectopic columella stem cell differentiation induced by MCLV3

Frequency and number of roots carrying starch granules in the previously designated domains (Stahl et al., 2009).
Fig. S5 Variety of pCYCD6;1::GFP expression in pub4 roots and embryos
RAM of 3-day-old main root (A-F) and mature embryos (G-I) in WT (A, D, G), pub4-1 (B, E, H) and pub4-5 (C, F, I) background. Asterisks; QC, arrowheads; CEI(D)s, double arrowheads; cortex and endodermis. White arrowheads indicate GFP-positive cells. Scale bars; 20 µm (A-F), 40 µm (G-I).
Fig. S6 PUB4 does not interact with known CYCD6;1-related or CLV-related components in yeast

(A) Yeast cells expressing SHR and RBR in the pDEST22 and SCR in the pDEST32. (B, C) Yeast cells expressing either PUB4ΔUbox protein or full size of PUB4 cloned in the pDEST32 bait vector were cotransformed either with full length of SHR, SCR, and RBR (B), or kinase domains of CLV1, SOL2, RPK2, ACR4 and BAM1 (C) in the pDEST22 vector. Yeast growth on SD –Leu/-Trp (+H) confirms the presence of both vectors. Growth on SD –Leu/-Trp/-His (-H) indicates protein-protein interaction.
Fig. S7 Potential models for PUB4 functions in root development

(A) PUB4 promotes the asymmetric cell division in columella stem cells (CSC) to produce the third differentiated columella layer during embryogenesis. (B) PUB4 promotes asymmetric periclinal divisions in CEI(D) and endodermis, via CYCD6;1 expression. (C) During embryogenesis, PUB4 regulates (1) the division in the CSC; (2) the asymmetric cell division (ACD) in CEI(D) and; (3) the asymmetric periclinal division in the endodermis to form 2nd-cortex layers. A part of its function is mediated by CYCD6;1 expression. The formation of 2nd-cortex layer can also be regulated by CLV2 and RPK2. The delayed asymmetric division in the CSC and endodermis in pub4 could lead to the decreased number of columella layers and the increased number of meristematic cells in the mature root, respectively. In root development, PUB4 functions downstream of exogenous CLV3/CLE peptide to regulate root meristem size and CSC fate. The contribution of CLV3/CLE signaling for meristem maintenance is relatively weaker and masked by other signaling pathways, for example, cytokinin.
### Table S1. List of primers used in this study.

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