The epidermis-specific cyclin CYCP3;1 is involved in the excess brassinosteroid signaling-inhibited root meristem cell division

Yuxiao Chen¹, Shiyong Sun² and Xuelu Wang²*

1. State Key Laboratory of Genetic Engineering, Department of Genetics, School of Life Sciences, Fudan University, Shanghai 200433, China
2. State Key Laboratory of Crop Stress Adaptation and Improvement, Henan University, Kaifeng 475001, China
*Correspondence: Xuelu Wang (xueluw@henu.edu.cn)
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Abstract

Cell division is precisely regulated and highly tissue-specific; studies have suggested that diverse signals in the epidermis, especially the epidermal brassinosteroids (BRs), can regulate root growth. However, the underlying molecular mechanisms that integrate hormonal cues such as BR signaling with other endogenous, tissue-specific developmental programs to regulate epidermal cell proliferation remain unclear. In this study, we used molecular and biochemical approaches, microscopic imaging and genetic analysis to investigate the function and mechanisms of a P-type cyclin in root growth regulation. We found that CYCP3;1, specifically expressed in the root meristem epidermis and lateral root cap, can regulate meristem cell division. Mitotic analyses and biochemical studies demonstrated that CYCP3;1 promotes cell division at the G2-M duration by associating and activating cyclin-dependent kinase B2;1 (CDKB2;1). Furthermore, we found that CYCP3;1 expression was inhibited by BR signaling through BRI1-EMS-SUPPRESSOR1 (BES1), a positive downstream transcription factor in the BR signaling pathway. These findings not only provide a mechanism of how root epidermal-specific regulators modulate root growth, but also reveal why the excess of BRs or enhanced BR signaling inhibits cell division in the meristem to negatively regulate root growth.

INTRODUCTION

In multicellular organisms, growth and development depends on precise cooperation among cells, tissues and organs (Howe et al. 1998; Petricka et al. 2012). The outmost layer of plant organs, the epidermis, is thought to control the growth rate of the entire organs. Biophysical concept favored that epidermis controls the rate and direction of plant growth because of the different thicknesses of the outer and inner cell walls and the high responsiveness to auxin-induced wall extensibility and elongation (Green 1980; Kutschera 1992). In addition, the importance of epidermal cell proliferation for root growth has received increased interest in recent years. A study on brassinosteroid (BR)-regulated plant development suggests that other than the BR signaling in inner cell layers, epidermal BR signaling is sufficient to promote root cell proliferation (Hacham et al. 2011). However, the cellular and molecular mechanisms by which the epidermis drives root cell proliferation and growth, and the roles of signaling in this process, are largely unknown.

Tissue-specific cell proliferation is tightly controlled by cell cycle regulators. The root contains meristem, elongation, and differentiation zones (Dolan et al. 1993). In the meristem zone, cells undergo rounds of mitotic cell division to achieve cell proliferation; in the elongation zone, cells elongate; as soon as reaching a threshold size, cells stop elongation and start differentiating to enter into the differentiation zone (Pavelescu et al. 2018). A reduced cell number in the root meristem is usually associated with decreased mitotic activity or premature cell differentiation.
(Petricka et al. 2012). Perturbing the expression of core cell cycle regulators, such as cyclin-dependent kinase A-1 (CDKA;1) or cyclin-dependent kinase B2 (CDKB2), causes a significant decrease of cell number in the meristem, leading to a severe root growth defect (Andersen et al. 2008; Nowack et al. 2012). Cyclins are primary regulators of cyclin-dependent kinases (CDKs) via activating CDKs or determining their substrate specificity (Loog and Morgan 2005; Pagliuca et al. 2011). In Arabidopsis, there are more genes encoding cyclins than encoding CDKs, which may benefit plants to specifically adapt to a variety of developmental processes (Wang et al. 2004; Pagliuca et al. 2011). Many cyclins are specific to tissues or developmental stages (Wang et al. 2004). For instance, CYCD6;1 is specifically expressed in the cortex/endodermis initial (CEI)/CEI-daughter cells to ensure formative division and proper pattern formation (Sozzani et al. 2010). Similarly, CYCD3;3 expression is specifically detected in the root cap of developing embryos from heart to torpedo stages (Forzani et al. 2014). However, most tissue-specific cyclins have not been investigated, especially in roots.

P-type cyclins (CYCPs) are a novel class of cyclins, including seven genes in Arabidopsis. They were first isolated and characterized as PHO80-like proteins (Torres Acosta et al. 2004). All CYCPs contain cyclin box, a conserved central region of 100 amino acids, except that most of CYCPs contain a conserved Y(L/A)E(A/R)(F/A)(R/K)(Y/F) motif at the positions of the A-type LVEVxEEY motif, which may be considered as a signature of this family. CYCPs were mostly expressed in proliferating cells (Wang et al. 2004). In Arabidopsis, CYCP2;1 integrates genetic and nutritional information to promote meristem cell division (Peng et al. 2014). CYCU4;1 and CYCU2 (CYCP are also named as CYCU) were identified to control cell proliferation in the abaxial sclerenchyma and mesocotyl in rice, respectively (Sun et al. 2015, 2018). Additionally, CYCPs are shown to associate with nutrient signaling because CYCP4;2 is able to partially re-establish the phosphate-dependent expression of the PHOS gene in a pho80 mutant strain of yeast (Torres Acosta et al. 2004), and recently, OsCYCP4;5 were identified to coordinate phosphate starvation signaling with cell cycle progression in rice (Xu et al. 2019). Further, CYCP2;1 is a direct target of STIMPY transcriptional activation and an early responder to sugar signals (Peng et al. 2014).

BRs are a class of indispensable phytohormones that regulate various aspects of plant growth and development, such as hypocotyl and stem elongation, cell expansion, root hair initiation, vascular differentiation, and male fertility (Ye et al. 2010; Xie et al. 2011; Cheng et al. 2014), and many of these processes occur by regulating cell proliferation (Yang et al. 2011; Vilarrasa-Blasi et al. 2014; Sun et al. 2015, 2018). In Arabidopsis, a suitable level of BR signaling is required for optimal mitotic activity because either the BR signaling-reduced or enhanced mutants exhibit fewer cells and cell division defects in root meristems, that negatively impact on the meristem size (Gonzalez-Garcia et al. 2011; Hacham et al. 2011). BR signaling is initially proposed to promote the transition from G1 to S phases via activating CYCD3;1 in suspension cells of the BR-deficient mutant de-etiolated2 (det2) (Hu et al. 2000), and overexpression of CYCD3;1 driven by the 35S promoter rescues the mitotic activity defect of BR insensitive mutant bri1-116 (Gonzalez-Garcia et al. 2011), implying that the root cell cycle defect in BR signaling-reduced mutants may result from the G1-S disruption. However, the mechanisms of how the enhanced BR signaling causes root growth defect is unknown. In addition, ectopic expression of BRASSI-NOSTEROID INSENSITIVE 1 (BR1) in the epidermis of bri1-116 or bri1 brl1 brl3, which is the triple mutant of BR1, BR1-Like1 (BRL1), and BR1-Like3 (BRL3), suggests that epidermal BR perception is sufficient to promote root cell proliferation and root growth, while BR perception in the stele restricts root growth (Hacham et al. 2011; Vragovic et al. 2015). Further studies revealed that the outer and inner tissues undergo opposing responses to BRs because the genes activated and repressed by BR signaling are primarily observed in the basal domain of outer tissues and the apical domain of inner tissues, respectively. And the positive effect of epidermal BR signaling on cell proliferation may be primarily via auxin signaling (Vragovic et al. 2015). However, the cell cycle regulators involved in the BR-regulated root growth are still unclear, and the mechanisms of why enhanced BR signaling inhibits root growth are to be explored.

In this study, in order to initially identify the root epidermis-specific cell cycle regulators, we searched the publicly available transcriptional data and then focused on the P-type cyclin family. Through expression pattern and phenotypic analyses, we found
and confirmed that CYCP3;1, specifically expressed in root meristem epidermis and lateral root cap (LRC), promotes root meristem cell proliferation. Biochemical experiments indicated that CYCP3;1 interacted with CDKB2;1 both in vitro and in vivo, and was able to enhance the kinase activity of CDKB2;1. Furthermore, we found that CYCP3;1 expression was directly suppressed by BR signaling via BRI1-EMS-SUPPRESSOR1 (BES1), a positive downstream transcription factor in the BR signaling pathway. Finally, our physiological experiments and genetic analyses confirmed the excess BRs/BR signaling inhibits root meristem cell division through CYCP3;1. Therefore, our findings provide important insights into how an epidermis-specific cyclin controls cell proliferation in root meristems, and also explain why optimal strength of BR signaling is required for normal root growth.

RESULTS

CYCP3;1 is specifically expressed in the root meristem epidermis and LRC

The epidermis is vital in determining root growth. Given that cyclins are important tissue-specific cell cycle regulators, we searched for cyclin genes specifically expressed in the root epidermis. Previous studies indicated that CYCP4;2 is highly enriched in root hair cells (Deal and Henikoff 2010; Simon et al. 2013). Therefore, we measured the relative expression of these cyclin P genes in root tips, differentiation zones, and aboveground tissues of developing seedlings via reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Our data revealed that, except for CYCP2;1, all of the other cyclin P genes are expressed in the root, and CYCP3;1 has the highest expression level in the root tip compared to that in the aboveground or differentiation zone (Figures S1A, B). Furthermore, we constructed transgenic plants CYCPpro:GUS carrying a β-glucuronidase (GUS) reporter driven by their native promoters. Our GUS staining demonstrated that most of the cyclin P genes were diversely expressed in the root (Figure S1C). In accord with the RT-qPCR result, CYCP3;1 was highly expressed in actively dividing meristem zone (Figures 1B, S1C). The cross-sections indicated that it was more specifically expressed in the root epidermis and LRC, while it was very weakly expressed in the cortex cell layer (Figure 1A). We then created a CYCP3;1-GFP (green fluorescent protein) construct driven by its native promoter, gCYCP3;1-GFP, and observed that CYCP3;1-GFP was mainly localized in the LRC and epidermis of root meristem zones, with the strongest in the apical meristem. The GFP signal was gradually weakened along the transition zone and was quite faint in cells around the quiescent center (QC), but not observed in cortical cells (Figures 1C, S2). Moreover, CYCP3;1-GFP was localized in both the nuclei and cytoplasm (Figure 1D). Therefore, CYCP3;1 is a cyclin specifically expressed in the root epidermis and LRC, which provides a unique start point to study the mechanism by which cell division is regulated in the root epidermis.

CYCP3;1 controls root growth by regulating cell proliferation in the root meristem

To explore the function of CYCP3;1, we created CYCP3D-RNAi lines driven by the 35S promoter with reduced expression of both CYCP3;1 and CYCP3;2, which share 86% identity at the amino acid level. Using two independent knockdown lines (Figure S3A), we found knocking-down of CYCP3 results in fewer cells in root meristems, and shorter cells and meristems compared to the wild type (Figures 1I–K, S3B–D), and furthermore, we found roots of CYCP3D-RNAi -1 (referred to as CYCP3D-RNAi in the paper) lines are shorter than that in the wild type (Figure 1E–H). We also observed the phenotype of gCYCP3;1-GFP lines, and found they produced more cells in root meristem than the wild type, despite having shorter cells, root meristems and roots than the wild type at the 5th d after germination (Figures 1E–K, S3E–H) (data in Figure 1 show the phenotype of gCYCP3;1-GFP). Therefore, these indicated that CYCP3;1 promotes cell proliferation in the epidermis of the root meristem.

CYCP3;1 regulates cell division in the root meristem

To elucidate how CYCP3;1 promotes cell proliferation, we analyzed the cell cycle progression in the root meristem. We counted the number of meristem cells that are present at the G2-M phases by using a pCYCB1;1:GUS reporter line. CYCB1;1 is expressed at G2-M transition, and the pCYCB1;1:GUS line is used to visualize the patterns of mitotic activity (Colon-Carmona et al. 1999). Since the meristem cell number of both the CYCP3D-RNAi and the gCYCP3;1-GFP
lines were different from that of the wild type, we normalized the number of cells with the pCYCB1;1:GUS expression to the total number of meristematic cells to determine the proportion of cells undergoing G2-M phases. We found that the gCYCP3;1-GFP lines contained more cells with GUS staining but the CYCP3D-RNAi lines had fewer cells with GUS staining than the wild type (Figure 2A, B), suggesting that CYCP3;1 regulates cell division.

CYCP3;1 interacts with and activates CDKB2;1

CYCP3;1 possesses a typical cyclin box domain, which is usually involved in interaction with CDKs. Therefore, we tested whether it can interact with the five main mitotic CDKs in Arabidopsis (Francis 2007) via in vitro pulldown assays and found that CYCP3;1 had the highest affinity for CDKB2;1 among all of the tested CDKs (Figure 2C). Using a semi-in vivo pulldown assay, we found that CDKB2;1 pulled down CYCP3;1-GFP in protein extracts from the transgenic gCYCP3;1-GFP seedlings (Figure 2D). The interaction between CYCP3;1 and CDKB2;1 was further determined in vivo by bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (Co-IP) assays (Figure 2E, F). Because CDKB2 is specifically expressed during the G2 and M phases (Andersen et al. 2008), we detected the protein level of CYCP3;1-GFP at different phases of cell cycle with 4′,6-diamidino-2-phenylindole (DAPI) staining in the root epidermal cells. Except during the G0, G1, and S phases, CYCP3;1-GFP was also expressed during cytokinesis (Figure 2G). Therefore, it is likely that CYCP3;1 affects the G2 and M phases by regulating CDKB2;1.

Figure 1. CYCP3;1 is specifically expressed in the root meristem epidermis and lateral root cap (LRC) to regulate root growth

(A, B) CYCP3;1pro:GUS expression in the root meristem of the 5-d-old seedlings. Shown are cross-sections (A) and epidermal layers (B); scale bars, 100 μm. (C, D) Confocal images of the CYCP3;1 expression pattern in the root tip. The white line indicates the meristem zone (C). The subcellular localization of CYCP3;1-GFP in the epidermal cells (D). Scale bars, 100 μm (C) and 30 μm (D). (E) Five-d-old seedlings. Scale bar, 1 cm. (F) Confocal images of root tip of the 5-d-old seedlings. The lower and upper white arrows indicate the quiescent center (QC) and the boundary between the meristem and elongation zones, respectively. Scale bar, 75 μm. (G) Root length measurements. Data are means ± SD (n ≥ 40). (H–K) Statistical analyses of the root meristem index. Data are means ± SD (n = 35, 33, and 35). Significance was determined by Student's t-test. ***P < 0.001.
Figure 2. CYCP3;1 interacts with CDKB2;1 to promote mitosis in the root meristem

(A) pCYCB1;1:GUS expression in root tips. Scale bar, 100 μm. (B) Statistical analysis of the relative percentage of G2-M cells in the root meristem (see details in Methods); n = 35 (Col-0), 29 (CYCP3D-RNAi), and 27 (gCYCP3;1-GFP). Significance was determined by Student's t-test. *P < 0.05, ***P < 0.001. Error bars are SD. (C) Interaction of CYCP3;1 with glutathione S-transferase-cyclin-dependent kinases (GST-CDKs) by in vitro pull-down assays. The upper panel shows an immunoblot with anti-His antibody; the lower panel indicates the amount of GST and GST-CDKs stained by Ponceau-S. (D) Interaction of CYCP3;1-GFP with GST-CDKB2;1 as assessed by semi-in vivo pull-down assays. (E) Interaction of CYCP3;1 and CDKB2;1 in bimolecular fluorescence complementation (BiFC) assays. Gel blots were probed with anti-Flag and anti-HA antibodies. (G) Localization of CYCP3;1-GFP at different mitotic stages. White arrows point to cells undergoing cytokinesis. (H) The phosphorylation activity of CDKB2;1 on histone H1. Histone H1 was incubated with the CDKB2;1-Flag complex with or without CYCP3;1-Myc from Arabidopsis. The arrow indicates CYCP3;1-Myc. Quantifications of the relative phosphorylation level of Histone H1 and CDKB2;1-Flag are shown below. The phosphorylation levels of Histone H1 and CDKB2;1-Flag in line 2 are defined as “1”, respectively.
detect whether the phosphorylation activity of CDKB2;1 was regulated by CYCP3;1. We observed that histone H1 was phosphorylated by CDKB2;1, and additional CYCP3;1 strongly enhanced this phosphorylation of H1 from 1.0 to 1.56-fold higher even with less (0.4 times) CDKB2;1 (Figure 2H), indicating that CYCP3;1 can effectively enhance the kinase activity of CDKB2;1.

**CYCP3;1 expression is inhibited by BRs**

On account of BRs’ important role and tissue-specific regulation in root meristem cell division (Gonzalez-García et al. 2011; Hacham et al. 2011), we tested if the expression of CYCP3;1 is regulated by BRs. First, we treated the 5-d-old seedlings with epibrassinolide (eBL), and found that eBL strongly inhibited CYCP3;1 expression (Figure 3A, B). Furthermore, in a BR biosynthetic mutant, det2-1, CYCP3;1 expression was strongly up-regulated compared to that in the wild type. In various plants with altered BR signaling, CYCP3;1 expression was increased in the BR signaling-reduced bri1-116 mutant and BES1-RNAi lines and decreased in the BR signaling-enhanced bin2-3 bii1 bil2 and best-D mutants compared to the wild type (Figure 3C). Treatment with bikinin, an inhibitor of glycogen synthase kinase 3 (GSK3) kinases, also inhibited CYCP3;1 expression (Figure 3D). Moreover, the applied eBL strongly suppressed the GUS expression in the CYCP3;1pro:GUS seedlings (Figures 3E, S5A, S5B). Finally, in gCYCP3;1-GFP seedlings, the GFP signal was significantly weakened upon eBL treatment (Figure 3F). Thus, it was demonstrated that BR signaling inhibits CYCP3;1 expression and might do so through the downstream transcription factor BES1.

**BES1 directly binds to the promoter of CYCP3;1 to inhibit its expression**

Next, we checked whether BES1 could regulate CYCP3;1 expression by directly binding to its promoter. First, we synthesized DNA fragments containing 5’ biotin for amplification of the CYCP3;1 promoter and purified the maltose-binding protein (MBP)-BES1 protein expressed in Escherichia coli. Then, using streptavidin magnetic beads to recruit the biotin-labeled promoter, we conducted DNA pull-down assays and found that BES1 strongly bound to the CYCP3;1 promoter (Figure 3G). Moreover, chromatin immunoprecipitation qPCR (ChIP-qPCR) assays using BES1-FLAG transgenic seedlings with anti-FLAG antibody showed that BES1 binding was enriched primarily at positions 3, 4, and 7 in the CYCP3;1 promoter, which contains the known BES1 binding motif (Figure 3H). Finally, we tested the impact of BES1 binding on the CYCP3;1 promoter using a dual-luciferase assay in Nicotiana benthamiana leaves (Cheng et al. 2014). The luciferase gene driven by the CYCP3;1 promoter was used as a reporter, and Myc, BES1-Myc, and CYCP3;1-Myc were used as effectors (Figure 3I). We found that, unlike Myc or CYCP3;1-Myc, BES1-Myc significantly inhibited CYCP3;1 expression (Figure 3J). Furthermore, we found the expression of CYCP3;1 in the root meristem was increased in BES1-RNAi lines (Figure S5C). Taken together, BES1 can inhibit the expression of CYCP3;1 by direct binding to its promoter.

**Enhanced BR signaling inhibits root meristem cell division through CYCP3;1**

To address whether BRs regulate CYCP3;1 to modulate cell cycle in the root meristem, we treated the Columbia-0 (Col-0) and gCYCP3;1-GFP with 1 nmol/L and 10 nmol/L eBL for 5 d, respectively. The number of meristematic cells in the Col-0 is reduced to about 89% and 44% as compared with the mock, respectively (Figure 4A, B); and for the gCYCP3;1-GFP, the effect of 1 nmol/L eBL on the meristematic cell number was similar to that in Col-0, while 10 nmol/L eBL treatment only reduced the root meristematic cell number to 64% (Figure 4A, B). It is suggested that the cell number in the root meristem of gCYCP3;1-GFP lines is less sensitive to BRs than that in Col-0. Otherwise, we applied eBL on CYCP3D-RNAi seedlings, and we found that the meristematic cell number in CYCP3D-RNAi with 10 nmol/L eBL treatment is reduced to 37%, less than 44% in Col-0 (Figure 4A, B), indicating that CYCP3D-RNAi is slightly more sensitive to BRs in root meristem cell division. These data illustrate that the enhanced BR signaling inhibits root meristem cell division through CYCP3;1 (Figure 5).

In addition, BR signaling-reduced mutants also display decreased mitotic activity (possibly caused by the disruption of G1-S transition) in the root meristem because overexpression of CYCD3;1 can rescue the reduced number of root meristem cells in the bri1-116 (Gonzalez-García et al. 2011). Consistently, we found the number of meristematic cells was weakly decreased in the 5-d-old root of BES1-RNAi lines (Figure 4C–F).
Figure 3. Brassinosteroid (BR) signaling regulates CYCP3;1 expression through BRI1-EMS-SUPPRESSOR1 (BES1) (A, B) Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) analysis of CYCP3;1 expression with epibrassinolide (eBL) treatment in the wild type (Col-0). Data are mean ± SD. In (B), 1 μmol/L eBL was used, ***P < 0.001, **P < 0.01, ns, non-significant. (C) RT-qPCR analysis of CYCP3;1 expression in the BR-related mutants and transgenic lines. Data are means ± SD. ***P < 0.001, **P < 0.01. (D) RT-qPCR analysis of CYCP3;1 expression with 40 μmol/L bikinin treatment. ***P < 0.001, **P < 0.01. (E) β-glucuronidase (GUS) staining of root tips of the 5-d-old seedlings. CYCP3;1pro:GUS seedlings were treated with or without 1 μmol/L eBL for 4 h. Scale bar, 100 μm. (F) Three-dimensional stacked images of CYCP3;1-GFP signal in gCYCP3;1-GFP transgenic plants. The contour of the root tip is depicted with yellow dashed lines. Scale bar, 75 μm. Values below the images represent the means of fluorescence density (n > 15) ± SD; ***P < 0.001. (G) The CYCP3;1 promoter interacts with BES1. The immunoblot was conducted with anti-MBP antibody. (H) Chromatin immunoprecipitation (ChIP) assays of BES1 binding to the CYCP3;1 promoter. Gray circles show the E-box. Gray rectangles show the BES1/BZR1 binding site. Regions analyzed by quantitative reverse-transcription polymerase chain reaction (RT-qPCR) are indicated by short lines marked with numbers (1–9). The fold enrichment represents binding efficiency ratio of antibody/no antibody. Data are means ± SD (n = 3). (I) Schematic of the dual-luciferase reporters and effectors. Firefly luciferase driven by the CYCP3;1 promoter was used as the reporter. The Renilla luciferase (REN) reporter was controlled by the CaMV promoter (35S). BES1 and CYCP3;1 fused with Myc tag were used as effectors. (J) Transient gene expression assays in Nicotiana benthamiana mesophyll cells. The LUC reporter was co-transfected with Myc, BES1-Myc, or CYCP3;1-Myc. Data are means ± SD (n = 3). **P < 0.01; ns, non-significant.
We constructed the gCYCP3;1-GFP/BES1-RNAi line and observed that the root meristematic cells in it are still fewer than that in the wild type and gCYCP3;1-GFP, indicating that overexpression of CYCP3;1 cannot rescue the phenotype of BES1-RNAi, and the cell cycle defect (possibly caused by the G1-S disruption) in the BES1-RNAi roots cannot be rescued by CYCP3;1, the G2-M regulator in the cell cycle (Figure 4C, D). As CYCP3;1 is up-regulated in BES1-RNAi lines (Figure S5C), we further knocked down CYCP3 in BES1-RNAi, and found that the cell number in the root meristem of the BES1-RNAi/CYCP3D-RNAi lines was much lower than that in the single transgenic lines (Figure 4E, F), indicating that down-regulation of CYCP3 can further depress cell division in the root meristem of BES1-RNAi, the BR signaling-reduced plants.

**DISCUSSION**

The function of CYCP3;1 in root growth

We provide several lines of evidence to demonstrate that the epidermal-localized CYCP3;1 is vital for root meristem cell cycle and root growth. First, unlike other reported cyclin genes in Arabidopsis (Polyn et al. 2015), CYCP3;1 is specifically expressed in the meristem epidermal and LRC cells. Second, the number of root meristem cells is reduced in CYCP3D-RNAi lines, but increased in gCYCP3;1-GFP lines. Third, the number of G2-M cells is increased in the gCYCP3;1-GFP lines and reduced in the CYCP3D-RNAi lines. Finally, CYCP3;1 directly interacts with CDKB2;1 and enhances its kinase activity. Although CDKB2;1 is widely expressed throughout all cell layers (Andersen et al. 2008), and
previous data indicate that CYCB2 regulates CDKB2;1 in rice (Lee et al. 2003), we found CDKB2;1 is regulated by the epidermis-specific CYCP3;1 to elaborately regulate root mitotic cell division in Arabidopsis. Besides regulating cell proliferation, CYCP3;1 may also regulate other aspects of root development. We noticed the gCYCP3;1-GFP lines display short meristems and roots, albeit cell division is accelerated. By measuring cell length, we found the gCYCP3;1-GFP lines produced shorter cells in the differentiation zone than the wild type (Figure S6). Interestingly, we found cells in the differentiation zone of CYCP3D-RNAi lines are also shorter than that of the wild type (Figure S6). Therefore, CYCP3;1 seems to impact on cell elongation with an unknown mechanism. Smaller yeast cells were also observed in the Schizosaccharomyces pombe mutant of WEE1, which showed accelerated entry into mitosis and abolished checkpoint control in G2 (Enoch and Nurse 1990).

A homolog gene of CYCP3;1, CYCP3;2 may function similarly to CYCP3;1. First, the amino acids sequence of CYCP3;2 shares 86% similarity with CYCP3;1. Second, CYCP3;2 was also expressed in root meristem by RT-qPCR. Third, CYCP3;2 can interact with CDKB2;1 (Figure S8A), similar to CYCP3;1. Finally, we constructed CYCP3;2 overexpression lines driven by its native promoter termed gCYCP3;2-GFP, and found that CYCP3;2-GFP is expressed in the root meristem epidermis (Figure S9A). Moreover, gCYCP3;2-GFP transgenic lines consist of more cells in meristems, and also exhibit shorter cells and shorter root meristems than that in the wild type (Col-0) (Figure S9B–E), indicating CYCP3;1 and CYCP3;2 may function redundantly in root growth and development.

Figure 5. A proposed model to illustrate the mechanisms of CYCP3;1 in root meristem cell division

(A) Schematic diagram of the expression pattern of CYCP3;1 in the root tip. Purple indicated the CYCP3;1. (B) A proposed model to illustrate the mechanisms of CYCP3;1 in regulating cell division in the root meristem. CYCP3;1 mainly promotes the G2-M duration by activating CDKB2;1, leading to meristematic cell proliferation. In the excess of brassinosteroids (BRs) or the BR signaling-enhanced seedlings, BR signaling can severely inhibit CYCP3;1 expression to disrupt cell division in the root meristem, leading to reduction in the number of meristematic cells. In the wild type plants, the balanced BR signaling guarantees the optimal cell proliferation and root growth via different pathways. In the BR-deficient or the BR signaling-reduced plants, CYCP3;1 is able to promote cell division without the inhibition of BRs. However, deficient BR signaling fails to activate cell division through other pathways, resulting in cell division defect in the root meristem. The solid lines represent the direct regulation, gray lines indicate the regulation at the transcription level, dotted lines represent the indirect regulation.
gCYCP3;1-GFP and CYCP3D-RNAi exhibit less and slightly more sensitivity to eBL in root meristem cell division, respectively, providing an explanation for the cell division defect in the excess of BRs or the BR signaling-enhanced mutants. BRs promote cell proliferation by regulating a series of cell cycle regulators, including CYCD3;1, ETHYLENE RESPONSE FACTOR 115 (ERF115) and BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER (BRAVO) in Arabidopsis roots, and CYC U2 in rice mesocotyls (Hu et al. 2000; Heyman et al. 2013; Vilarrasa-Blasi et al. 2014; Sun et al. 2018). However, the positive role of BR signaling in the mitotic cell cycle seems to conflict with the reduced mitotic activity in the excess BRs or BR signaling-enhanced mutants (Gonzalez-Garcia et al. 2011). Here, we found that the expression of CYCP3;1 is directly inhibited by BES1, indicating that BR signaling can negatively regulate the G2-M duration through CYCP3;1. Meanwhile, overexpression of CYCP3;1 partially rescued the meristematic cell number reduction in the eBL-treated seedlings, suggesting the cell division defect in the eBL-treated seedlings is partially resulting from the perturbation of G2-M phase. This negative regulation of BR signaling on cell proliferation via CYCP3;1 is somewhat similar to the inhibitory effect of BR signaling on abaxial sclerenchyma cell proliferation through CYCU4;1 in rice lamina joints (Sun et al. 2015). Interestingly, overexpression of CYCP3;1 with its own promoter failed to rescue the reduced number of root meristem cells in BES1-RNAi, and CYCP3D-RNAi lines further reduce meristem cell number in BES1-RNAi lines, suggesting that the cell cycle defect in the BR-deficient or BR signaling-reduced mutants may result from other pathways, which is consistent with the phenomenon that overexpression of CYCD3;1 could rescue the root cell proliferation of bri1-116 (Gonzalez-Garcia et al. 2011).

We propose a model to illustrate the mechanism of an epidermal-specific CYCP3;1 to regulate root growth. As an epidermal-specific cell cycle regulator, CYCP3;1 mainly promotes the G2-M duration by associating with and activating CDKB2;1, leading to meristematic cell proliferation in the epidermis and inner cell layers. In addition, BR signaling can severely inhibit CYCP3;1 expression through BES1 in vivo. Therefore, in the excess BRs or the BR signaling-enhanced seedlings, down-regulation of CYCP3;1 causes G2-M duration disruption, leading to cell number reduction in the root meristem.

MATERIALS AND METHODS

Plant materials and growth conditions
The det2-1, BES1-RNAi and pCYCB1;1:GUS lines are in the Col-0 background. The gCYCP3;1-GFP, CYCP3D-RNAi, CYCPpro:GUS, CYCP3;1-Myc/CDKB2;1-Flag and CDKB2;1-Flag lines were generated in Col-0 by Agrobacterium tumefaciens-mediated transformation. For phenotype analysis, seeds were sterilized in 75% EtOH and then grown in oriented plates containing half-strength Murashige and Skoog (MS) medium, 1% sucrose, and 0.8% agar. After vernalization at 4 °C for 3 d, plants were transferred to 23 °C/21 °C (d/night) temperatures under long-d conditions (16 h light/8 h dark cycle). Phenotypes of roots were observed and analyzed at 5 d after germination.

Constructs and protein purification
To construct plasmids for the transgenic gCYCP3;1-GFP plants, the genomic sequence of CYCP3;1 containing the 3.1 kb promoter and the 753-bp complementary DNA (cDNA) was inserted into the modified binary vector pCAMBIA1302 (GFP-tagged). For the CYCP3D-RNAi construct, we used the artificial microRNA cloning system to target to the sequence “TAAACTCTGAACACAGTCAC”. All the GUS reporter lines of CYCP family genes were produced by fusing the promoter into pCAMBIA1391Z (GUS-tagged). All the primers are listed in Table S1. The materials, CYCP3;1-Myc/CDKB2;1-Flag and CDKB2;1-Flag lines, used in kinase assays are constructed by inserting the full-length coding sequences of CYCP3;1 or CDKB2;1 into the pCAMBIA2300 (Myc-tagged) and pCAMBIA1306 (Flag-tagged) driven by the 35S promoter, respectively.

The full-length coding sequence of CYCP3;1 was cloned into the pET28a(+) plasmid. The full-length coding sequences of each of the five CDKs (CDKA, CDKB1;1, CDKB1;2, CDKB2;1, CDKB2;2) tested were introduced into the pGEX4T-1 plasmid. Plasmids were transformed into the BL21 strains. After being expressed, proteins were purified with glutathione resin (GenScript) or Talon metal affinity resin (Clontech).

RNA extraction and gene expression analysis by RT-qPCR
For the expression pattern analysis of CYCP family genes, we sampled different parts of vertically grown 5-d-old seedlings or observed them with microscopy (Leica).
To detect CYCP3 expression levels, we collected the different parts of the 5-d-old seedlings. The collected materials were ground to a fine powder in liquid nitrogen. A Tiangen RNAprep Plant Kit (Tiangen) was used to extract total RNA. The first-strand cDNA, which was synthesized using a Takara PrimeScript First-Strand cDNA synthesis kit (TaKaRa), was combined with SYBR Master Mix for PCR (Invitrogen). A U-Box gene (At5g54000) was used to normalize the data. Quantitative PCR reactions were performed in triplicate with an Eppendorf iCycler (Eppendorf). Data were collected and analyzed with the Eppendorf real-time PCR detection system.

Phytohormone treatments
Five-d-old seedlings were floated in liquid half-strength MS liquid medium (see Plant Materials and Growth Conditions) with or without eBL or 40 µmol/L bikinin. For the long eBL treatment, 1 nmol/L, 10 nmol/L eBL or DMSO was added to the solid medium for seedling growth.

Histochemical analysis and root sectioning
Histochemical analysis of GUS expression and root sectioning was performed as described (Cheng et al. 2014). The sections were observed under a light microscope (Olympus, Japan).

Confocal microscopy and image analysis
All confocal images were taken with a Leica SP8 confocal microscope. For meristem index analysis, the roots of seedlings were stained in 10 µg/mL propidium iodide for 2–5 min and then used for sectioning. The excitation wavelengths were 561 nm for propidium iodide or mCherry signal, 488 nm for GFP signal, and 340 nm for DAPI signal. The lengths of the root and meristem were measured with Image J software. The meristem was defined as the region of isodiametric cells from the QC up to the cell that was twice the length of the last cell. At least three independent experiments were applied. Student's t-tests were performed in GraphPad software for comparisons.

In vitro pull-down assay and semi-in vivo pull-down assay
Proteins with glutathione S-transferase (GST), His tags were expressed in E. coli and affinity purified. GST-CDKs or GST alone were incubated with glutathione resin for 1 h and then CYCP3;1-His was coincubated with GST-CDK- or GST-containing beads for another 2 h. After washing several times, the beads were resuspended with 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, separated by 12% SDS-PAGE gel, transferred to nitrocellulose membrane (Amersham Biosciences) and detected with anti-His antibody (Abmart). For the semi-in vivo pull-down assay, gCYCP3;1-GFP seedlings were ground to a fine powder in liquid nitrogen and total proteins were extracted with 2× extraction buffer (100 mmol/L Tris-HCl (pH 7.5), 300 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0), 1% Triton X-100, 10% glycerol, and a protease inhibitor cocktail). Protein extracts were centrifuged at 12,000 rpm for 10 min twice, and the supernatant was incubated with pretreated GST- or GST-CDKB2;1-containing beads for 2 h. After washing several times, the beads were resuspended with 1× SDS-PAGE loading buffer and analyzed by SDS-PAGE and anti-GFP antibody (Roche).

Transient expression assays in N. benthamiana leaves
For BiFC assays and subcellular localization observation, the nYFP-CDKB2;1, CYCP3;1-cYFP, CDKB2;1-mCherry, and CYCP3;1-GFP constructs or their corresponding empty vectors were transformed into Agrobacterium tumefaciens strain GV3101 and co-injected into young leaves of N. benthamiana. The plants were grown for 2 d under long-d conditions. Then, injected leaves were clipped and stained with 2 µg/mL DAPI. The fluorescence was observed by confocal microscopy (Leica SP8).

Co-immunoprecipitation
The leaves of N. benthamiana expressing the proteins were ground to a fine powder in liquid nitrogen and solubilized with 2× extraction buffer. The extracts were centrifuged at 12,000 rpm for 10 min twice, and the supernatant was incubated with prewashed anti-FLAG M2 agarose gel (Sigma#M8823) for 2 h. The immunoprecipitated proteins were resuspended with 1× SDS-PAGE loading buffer. Then, the protein samples were separated on a 12% SDS-PAGE gel, transferred to a nitrocellulose membrane (Amersham Biosciences), and detected with Flag (Abmart #M2008) and HA (Sigma #PLA0001) antibodies. Photos were captured with a Chemiscope (Clinx).

Kinase assays for histone H1
Total protein was extracted from the CDKB2;1-Flag/CYCP3;1-Myc or CDKB2;1-Flag lines. The protein extracts were
immunoprecipitated with the FLAG affinity gel; the gel containing the immunoprecipitates (IPs) was washed three times and then incubated with histone H1 in kinase buffer (Sun et al. 2015). Finally, the IPs was examined by immunoblotting with anti-phosphorylated histone H1 (Sigma #05-1324), anti-Flag (Abmart #M2008), and anti-Myc antibodies (Sigma #PLA0001).

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AUTHOR CONTRIBUTIONS

Y.X. conception and design, acquisition of data, analysis and interpretation of data, drafting or revising the article; S.Y. provided advice for the experiments and the project, drafting or revising the article; X.W. conception and design, interpretation of data, drafting or revising the article. All authors read and approved the contents of this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.12975/suppinfo

Figure S1. Expression patterns of the CYCLIN P gene family in Arabidopsis
(A) Schematic diagram of the different tissues analyzed in (B). (B) Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) analysis of gene expression of the CYCLIN P family in 5-day-old seedlings. Data are means ± SD. ***P < 0.001, **P < 0.01, *P < 0.05, ns, non-significant. (C) β-glucuronidase (GUS) staining of the 5-day-old seedlings. Scale bar, 1 mm.

Figure S2. Localization of the CYCP3;1-GFP in Arabidopsis
(A) Localization of CYCP3;1-GFP. Yellow lines indicate the quiescent center. (B) 3D scanning figures of the root tip of 5-day-old gCYCP3;1-GFP.

Figure S3. CYCP3;1 regulates cell number in the root meristem
(A) Relative expression levels of CYCP3;1 and CYCP3;2 in CYCP3D-RNAi seedlings. Error bars are SD. ***P < 0.001, **P < 0.01, (B, F) Root meristem length, ***P < 0.001, **P < 0.01, ns, non-significant. (C, G) Cell number in root meristems. ***P < 0.001, **P < 0.01. (D, H) Cell length in root meristems. ***P < 0.001, (E) Relative expression levels of CYCP3;1 in gCYCP3;1-GFP seedlings. Error bars are SD. ***P < 0.001.

Figure S4. Colocalization of CYCP3;1-GFP and CDKB2;1-mCherry
(A) Colocalization of CYCP3;1 and CDKB2;1 in Arabidopsis root protoplasts. (B) Colocalization of CYCP3;1 and CDKB2;1 in Nicotiana benthamiana pavement cells.

Figure S5. Brassinosteroids (BRs) inhibit CYCP3;1 expression in root meristems
(A, B) β-glucuronidase (GUS) staining of CYCP3;1pro:GUS roots with epibrassinolide (eBL) treatment; 1 μmol/L eBL is used in (B). Scale bars, 100 μm. (C) GUS staining of roots of CYCP3;1pro:GUS and BES1-RNAi/CYCP3;1pro:GUS lines. Scale bars, 100 μm.
Figure S6. CYCP3;1 affects root cell length
(A) Statistic analysis of cell length in differentiation zones. Data are means ± SD (n ≥ 30). ***P < 0.001.

Figure S7. The root phenotype of 35S:CYCP3;1-GFP overexpression lines
(A) CYCP3;1-GFP expression in the 35S:CYCP3;1-GFP transgenic lines. Roots are scanned from the middle layer to the epidermal layer of roots from left to right. Scale bars, 75 μm.
(B) Root tips of Columbia-0 (Col-0) and 35S:CYCP3;1-GFP transgenic lines. White lines indicate meristem zones. Scale bars, 75 μm.
(C–E) Statistical analyses of root meristem index. Data are means ± SD (n ≥ 30). Significance was determined by Student's t-test. ***P < 0.001, ns, no significance.

Figure S8. CYCP3;2 interacts with CDKB2;1 in bimolecular fluorescence complementation (BiFC) assays
(A) CYCP3;2 interacts with CDKB2;1 in BiFC assays.

Figure S9. Expression pattern of CYCP3;2 in root meristems and the root phenotype of gCYCP3;2-GFP transgenic plants
(A) Three-dimensional stacked images of the CYCP3;2-GFP signal in the gCYCP3;2-GFP transgenic plants. Arrows point to meristem zones. Scale bars, 100 μm.
(B) Confocal images of root tips of the 5-d-old seedlings. Arrows point to meristem zones. Scale bar, 100 μm.
(C–E) Statistical analyses of the root meristem index. Data are means ± SD (n = 28 and 35). Significance was determined by Student's t-test. ***P < 0.001, **P < 0.01.

Table S1. Primers used in this study