The D-type cyclin CYCD4;1 modulates lateral root density in *Arabidopsis* by affecting the basal meristem region

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Root cell division occurs primarily in the apical meristem, from which cells are displaced into the basal meristem, where division decreases and cell length increases before the final differentiation zone. The organization of the root in concentric files implies coordinated division and differentiation of cell types, including the xylem pole pericycle cells, which uniquely can resume division to initiate lateral roots (LR). Here, we show that D-type cyclin *CYCD4;1* is expressed in meristematic pericycle protoxylem poles and is required for normal LR density. *Cycd4;1* mutants also show a displacement of the apical/basal meristem boundary in the pericycle and longer pericycle basal meristem cells, whereas other cell layers and overall meristem size and root growth are unaffected. Auxin is proposed to separately prepattern and stimulate LR initiation. Stimulation is unimpaired in *cycd4;1*, suggesting *CYCD4;1* requirement for normal spacing but not initiation. Both pericycle cell length and LR density phenotypes of *cycd4;1* are rescued by low concentrations of applied auxin, suggesting that the basal meristem has a role in determining LR density. We further show *CYCD4;1* is rate-limiting for sucrose-dependent LR formation, since *CYCD4;1* expression is sucrose-dependent and wild-type roots fully phenocopy *cycd4;1* in sucrose absence. We conclude that *CYCD4;1* links meristem pericycle cell behavior to LR density consistent with a basal meristem prepatterning model and that D-type cyclins can confer division potential of defined cell types through cell-specific expression patterns.

cell cycle | cell division | plant development

Postembryonic plant development, in contrast to that of animals, is characterized by de novo formation and growth of secondary organs. The activation and maintenance of these developmental programs require integration of cell division and cellular differentiation and is influenced by environmental factors. The root has a broadly cylindrical structure composed of concentric files of cells of distinct identity, whose origin can be traced back to initials adjacent to a small group of rarely dividing cells known as the quiescent center (QC) (1). During root growth, the initials divide, and their progeny are therefore displaced from the QC (2). These cells undergo further rounds of divisions with the result that in the root apical meristem (RAM), cell division is the main contributor to growth, and cell lengths are relatively uniform, since cell division occurs once a critical cell size has been achieved. Subsequently, cells more distal from the QC increase in average length but continue to divide albeit at a lower frequency. This region of combined cell division and elongation has been called the basal meristem (3). Cells exit the basal meristem and enter a zone of rapid elongation where cell division ceases, and cell differentiation is externally observed in the formation of root hairs on specific epidermal cell files.

Lateral roots (LR) result from the reactivation of cell division within the rapid elongation/differentiation zone in specific files of cells within the layer known as the pericycle, which lie adjacent to the xylem poles of the root internal vasculature. Division of these cells then leads to the formation of a primordium, the establishment of a new LR apical meristem, and eventual emergence of the growing LR (4).

Auxin has long been linked to the initiation of LR, since mutants in the auxin response pathway such as *iaa14/solitary root* lack LR (5), and addition of ectopic auxin at high levels can induce the activation of all xylem pole pericycle cells (6). The specification of xylem pole pericycle cells is dependent on a localized activation of auxin response (7, 8). Two broad models have been proposed to explain the normal patterning and initiation of LR. Recently it has been proposed that a local auxin maximum in pericycle cells of the mature root is sufficient for LR formation (9). A second model suggests that pericycle "founder" cells in the basal meristem are primed by an oscillating auxin response, whereby they are prepatterned to be responsive to a second, independent auxin-requiring (and *iaa14/slr*-dependent) initiation event in the differentiation zone (8, 10). As mentioned, in addition to this potential priming mechanism involved in normal LR spacing, which appears to be responsible for the responsiveness of a limited number of xylem pole pericycle cells, all such cells have the ability to form LR, since exogenous application of auxin activates pericycle cells to form LR (6, 11–13).

Cell flux through the basal meristem during root growth is determined by the rate of cell division and may have an effect on the number of LR founder cells. As in all eukaryotes, the cell cycle is controlled by cyclin-dependent kinases (CDK) that associate with positive regulators called cyclins (14). The activated cyclin-CDK complexes phosphorylate the retinoblastoma-related protein (RBR), allowing transcription of genes regulated by E2F transcription factors that promote in turn DNA synthesis and commitment to the cell cycle. Cell division in the root meristem has been shown to be dependent on the RBR pathway (15), but as RBR is expressed throughout the root, it is likely that other factors regulate the coordinated division rates of specific cell types or files. Good candidates are the D-type cyclins (CYCDs), which are rate-limiting components for activation of the RBR-E2F pathway, and their expression is under the control of intrinsic and extrinsic signals (16, 17), making them potential developmentally relevant cell cycle activators. Combined loss-of-function mutants in the three *CYCD3* genes of *Arabidopsis* indeed show more rapid exit of cells from the

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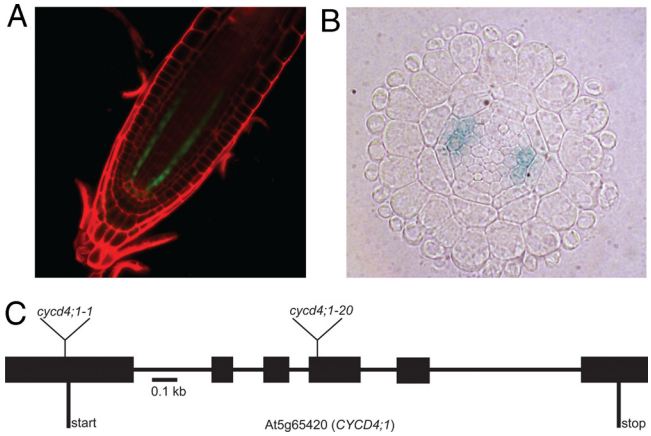

Fig. 1. Expression and gene structure of *CYCD4;1*. (*A*) The *CYCD4;1* promoter is active in the pericycle cells of the root meristem as shown by GFP signal. (*B*) *CYCD4;1* is expressed in the xylem poles of the pericycle in the apical meristem (*CYCD4;1* promoter driving GUS reporter). (*C*) Gene structure of *CYCD4;1*. Boxes indicate exons, and the position of the T-DNA insertions in the *CYCD4;1* mutants are shown. PCR amplicons from homozygote mutant plants were purified and sequenced using insert specific primers, showing that both mutants had tandem head-to-head insertion events within the *CYCD4;1* locus. The *cycd4;1-1* has a T-DNA insertion at position −25 bp from the initiation codon and *cycd4;1-20* in the fourth exon.

mitotic phase of leaf development, linked to impaired cytokinin responses (16), but the relatively broad expression of these genes in young developing leaves does not indicate whether CYCDs may provide separate control of division of adjacent cell types. Similarly, several CYCDs, including *CYCD4;1* are expressed early during germination, and loss of these early expressed CYCD leads to decreased cell division and germination rate (18). *Cycd4;1* mutants also have a reduced number of stomata in the hypocotyl epidermis (19), but no other root phenotype has been previously observed. Notably, available microarray data based on cell sorting suggest that the *CYCD* genes show discrete tissue-specific expression patterns in the root, especially in the root meristem (20, 21), suggesting that individual CYCDs could have specific roles in root growth and development.

Here, we analyze the role of *CYCD4;1* in root growth and show that it is expressed in pericycle cells adjacent to the xylem poles. Loss of *CYCD4;1* causes an increase in pericycle cell length in the basal meristem zone consistent with reduced cell division rates and a shift toward the root apex of the point at which average pericycle cell lengths start to increase, representing the apical/basal meristem boundary, showing that specific control of the cell cycle in pericycle cells is conferred by *CYCD4;1*. *Cycd4;1* mutants have a reduced LR density, but high levels of ectopic auxin still induce supernumerary LR, and *CYCD4;1* expression levels are not affected in *slr-1* plants, which together suggest that *CYCD4;1* is not involved in the IAA14 pathway of LR initiation but rather in the acquisition of founder cell identity in the basal meristem.

Results

Examining multiple lines carrying *CYCD* promoters driving a GUS-GFP reporter protein (18), we identified the *CYCD4;1* promoter as a pericycle-specific element within the root meristem (Fig. 1*A*). Cross-sections through the meristem revealed that the *CYCD4;1* promoter is not active in all pericycle cell files, but is restricted to the files adjacent to the xylem poles (Fig. 1*B*). Maximum expression was associated with the cells closest to the QC, and a gradual decline of signal intensity as cells progressed through the meristem was observed. In the maturation zone and older root parts, including the early stages of LR initiation, no *CYCD4;1* expression was detected (Fig. 1*A*).

To investigate the role of *CYCD4;1*, two independent lines containing T-DNA insertions in the CYCD4;1 gene were used. *Cycd4;1-1* (18) has an insert just before the start codon, and *cycd4;1-20* is an allele with a T-DNA inserted in the fourth exon (Fig. 1*C*). In neither allele are full-length *CYCD4;1* transcripts detected by PCR on cDNA using primers spanning the coding region (data not shown). Using quantitative real-time PCR, *cyc4;1-1* and *cycd4;1-20* showed 0.8% and 0.4%, respectively, of *CYCD4;1* 3′ mRNA levels compared to wild-type (WT) levels. *Cycd4;1* mutants did not show obvious growth defects, were fully fertile, and in vertical plate assays the primary root growth rate of WT and both *cycd4;1* alleles were not significantly different (Fig. S1). However, compared to WT, both *cycd4;1* mutants displayed a 30% reduction in LR number (Fig. 2*A*). Careful examination revealed that this was a consequence of a reduced level of LR primordium formation and not a failure of outgrowth of formed primordia. Complementation tests confirmed that both mutations are allelic and rescued by a genomic fragment encoding the *CYCD4;1* gene.

Since CYCDs regulate the G1-S transition and can be rate limiting for cell division in plants (16, 18), we compared cell division in the root meristems of *cycd4;1-1* and WT using the mitotic CYCB1;1-GUS marker (22), but neither the overall pattern of cell division nor meristem length (Fig. S2) were affected as determined from the overall distribution of mitotic cells.

We therefore analyzed the sizes and numbers of root cells in different root cell files in more detail, allowing us to determine the lengths of epidermal, cortical, endodermal, and pericycle cells from the initials adjacent to the QC up into the rapid elongation/differentiation zone. We found no significant differences in cell lengths in epidermis, cortex, and endodermis between *cycd4;1-1*, *cycd4;1-20*, and WT (Fig. 2*B*). The pericycle cells in *cycd4;1-1* and WT apical meristems were also equal in size, but in *cycd4;1-1*, we found that elongation in *cycd4;1-1* initiates closer to the QC, corresponding to a shift of the boundary of the apical-basal meristem (Fig. 2*B*). In the mutant, average cell lengths starts to increase from cell number 15 (150 μm from the QC), whereas in WT, the average cell length starts to increase around cell number 20 (200 μm; Fig. 2*B*). The total number of cells between the QC and the start of the rapid elongation zone in the pericycle layer is unchanged as the inflection point on the cell length curve marking the transition into the rapid elongation zone in both *cycd4;1-1* and WT occurs at cell 35 (Fig. 2*B*). However, this basal meristem-rapid elongation zone transition occurs further in physical distance from the QC (736 μm in *cycd4;1-1* and 623 μm in WT), because the average length of pericycle cells across the basal meristem region is larger in *cycd4;1-1*. This phenotype was also found in *cycd4;1-20* and could be rescued in tandem with the LR reduction by complementation of *cycd4;1* by the genomic fragment spanning the *CYCD4;1* gene (Fig. S3). Taken together, in *cycd4;1-1* there are less pericycle cells present in the basal meristem region per unit length relative to the other cell files (Fig. 2*C*), pericycle cells exit more rapidly from the purely mitotic zone of the RAM, and the length of the pericycle basal meristem is increased because it is composed of larger cells.

Hence, *CYCD4;1* appears to control cell length in the pericycle of the basal meristem and affects the formation of LR, and we sought to establish whether these phenotypes are causally linked. It has recently been proposed that particular pericycle cells in the basal meristem become primed as LR founder cells as a consequence of local oscillations in auxin or auxin responses, and that this mechanism controls normal LR specification and spacing (8). Additionally, high levels of applied auxin promote ectopic LR formation (5, 6). Given the effect of loss of *CYCD4;1* in the basal meristem, we investigated the relationship between auxin and the *cycd4;1* phenotype using a range of concentrations.

1-Napthaleneacetic acid (NAA) was included in the growth medium at concentrations between 0 and 100 nM, and LR density was measured after 10 days of vertical growth (Fig. 2*D*). At 0–1 nM

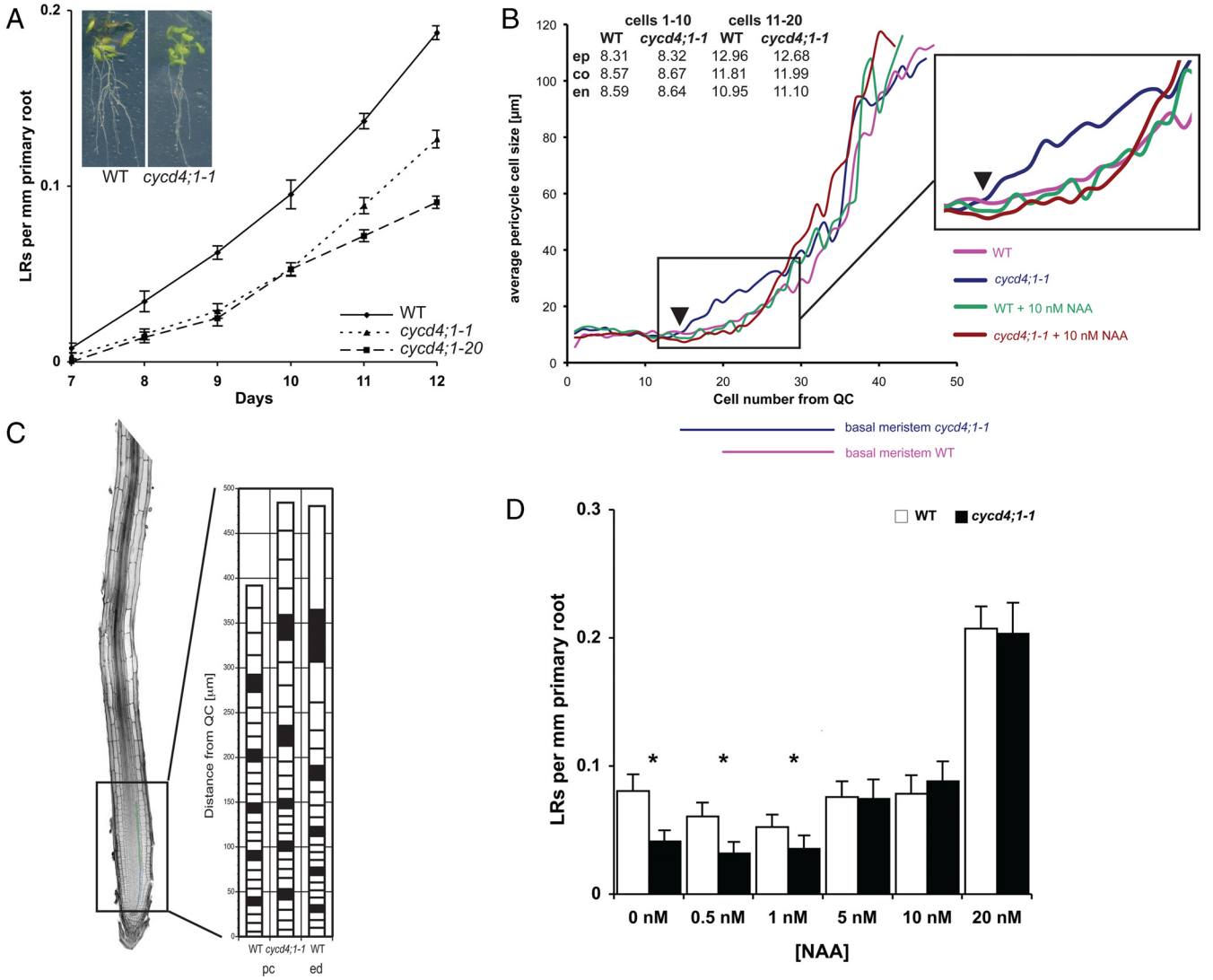

Fig. 2. The phenotype of the *cycd4;1* null-mutants. (*A*) *cycd4;1* show a decreased number of LR. (*B*) Cell lengths in different zones of the root meristem. Average WT and *cycd4;1-1* cell lengths in epidermis (ep), cortex (co), and endodermis (en) for cells 1–10 and 11–20 counted from the QC are shown in upper left table. Main figure: Average pericycle cell lengths are shown for cells 1->40 from QC for WT and *cycd4;1* mutants grown on 0 nM and 10 nM exogenous auxin. Note (*Inset*) that cells of the basal meristem in *cycd4;1-1* are enlarged only in the absence of auxin and that the start of net elongation takes place closer to the QC (arrowhead) than in WT. Significant (*t* test $P < 0.05$) changes were observed between *cycd4;1-1* without NAA and the other condition for cell number 17 to 25. Lines below the *x*-axis indicate basal meristem regions of WT and *cycd4;1-1*. The apical meristem and rapid elongation zones lie to the left and right of the basal meristem region, respectively. (*C*) Cell length and position from the QC of the pericycle cells (pc) in the *cycd4;1-1* and WT and epidermis cells in the WT (ed) as comparison. (*D*) LR density at 10 DAG in WT and *cycd4;1-1* upon treatment of increasing levels of auxin. Asterisks indicate differences are significant (*t* test $P < 0.05$).

NAA, the *cycd4;1-1* LR density phenotype was manifest. However, at slightly higher concentrations (5–10 nM NAA), LR density of *cycd4;1* mutants was restored to WT levels, but no response was observed in WT. Within this range of auxin concentrations, primary root growth was not affected (Fig. S1). As expected, addition of higher levels of auxin increased LR density in WT roots, and a similar total number of LR were also observed in *cycd4;1* mutants, demonstrating that auxin responses are not impaired in *cycd4;1*.

Since LR number was restored in the *cycd4;1-1* by the presence of 10 nM NAA, we examined whether this coincided with a restoration of cell length in the mutant's pericycle to WT lengths (Fig. 2*B*). Cell length analysis of *cycd4;1-1* revealed that the distal meristem pericycle cell length was also restored, and this further supports the link between the LR and basal meristem cell length phenotypes of *cycd4;1*.

It has been proposed that AUX1 is involved in LR priming through its function as an auxin influx carrier protein (8), and the *aux1* mutant has a 30% reduction in LR density (8, 23), similar to that observed in *cycd4;1-1*. We therefore measured the cell lengths in the meristem of *aux1-7* mutant roots and found that pericycle cells also start to elongate prematurely and more rapidly (Fig. 3*A*). However, unlike *cycd4;1* meristems, *aux1-7* meristems are greatly reduced in overall length throughout all cell files (data not shown), and pericycle cell expansion appears to be impaired in the rapid elongation zone (Fig. 3*A*). Although it has been reported that the *aux1* LR phenotype can be restored by exogenous application of 10 nM NAA (23), an observation we reconfirmed (data not shown), pericycle cell lengths are not affected in *aux1-7* grown on 10 nM NAA, consistent with a lack of responsiveness of the *aux1* meristem cells (Fig. 3*A*). Overall *CYCD4;1* transcript levels were also found to be unchanged in *aux1* roots (Fig. S3).

IAA14/SLR is not proposed to be involved in priming mechanisms, but is required in the root differentiation zone for LR initiation in response to auxin, and *slr* roots are thus devoid of

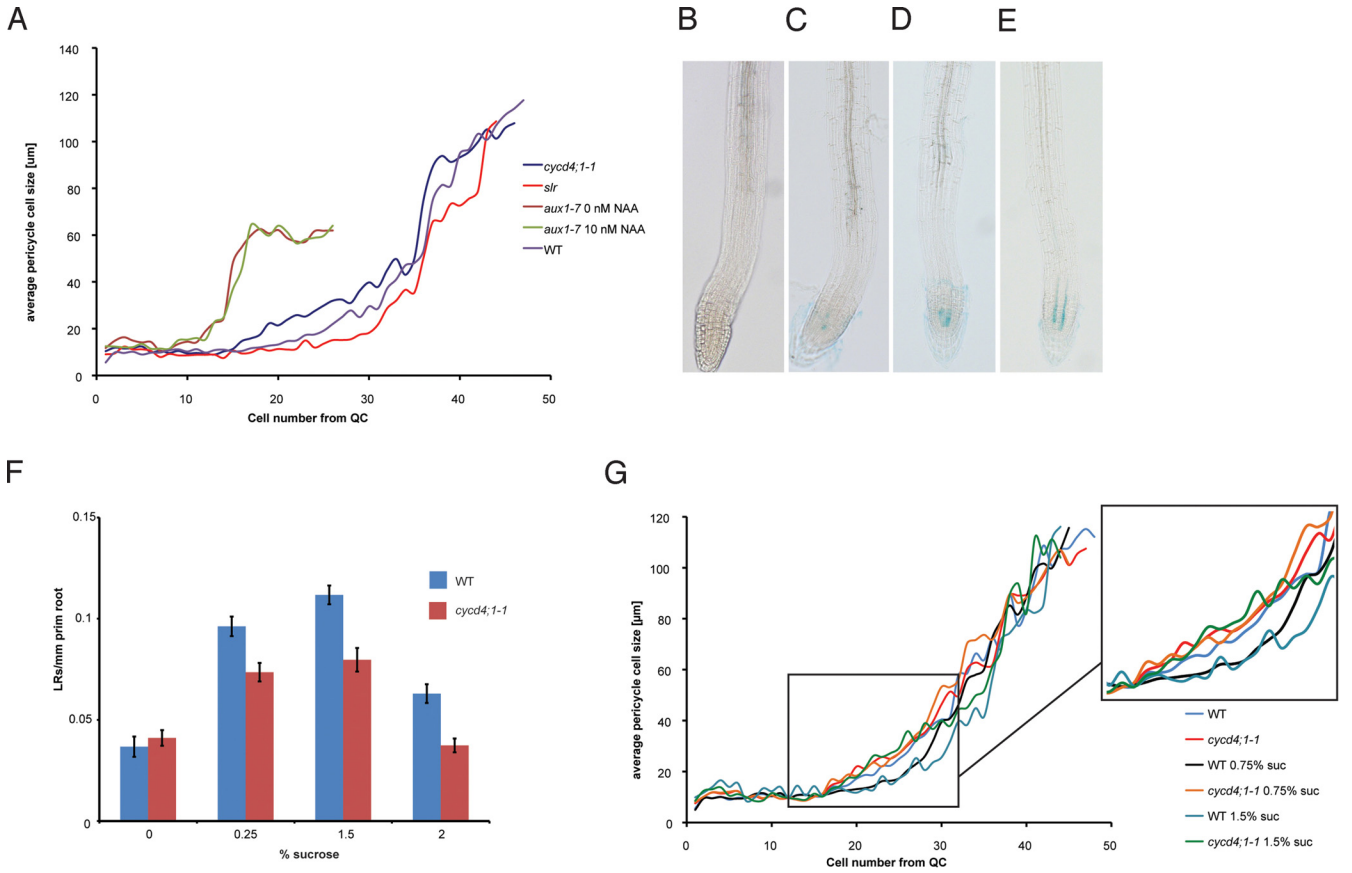

Fig. 3. Effect of auxin and sucrose on the *cycd4;1-1* phenotype. (*A*) Pericycle cell lengths in the *aux1* and *slr* mutants compared to WT and *cycd4;1-1*. *Aux1-7* plants were treated were grown without and with 10 nM NAA. Cell lengths are significantly different (*t* test $P < 0.05$) compared to WT for cells 14–26 (both *aux1*), 17–26 (*cycd4;1-1*), and 25–30 (*slr*). (*B–E*) *CYCD4;1* promoter driving GUS-GFP reporter in 10-day-old roots on different sucrose and light conditions. (*B*) Roots grown for 9 days in the dark (−suc) transferred to the dark (−suc). (*C*) Roots grown for 9 days in the dark (−suc) transferred to the light (−suc). (*D*) Roots grown for 9 days in the dark (−suc) transferred to the dark (+suc). (*E*) Roots grown in the light (+suc) transferred to the light (+suc). (*F*) LR density at 10 DAG in WT and *cycd4;1-1* grown on media containing different sucrose concentrations. (*G*) Average pericycle cell lengths in WT and *cycd4;1-1* grown on media containing different sucrose concentrations. *Inset* shows enlargement of the basal meristem region of the curves. No significant differences were found between WT on 0.75% and 1.5% sucrose, except for cell numbers 29–31 (*t* test $P < 0.05$). No significant changes (*t* test $P < 0.05$) were found between WT on 0% sucrose and the *cycd4;1-1* conditions. Cell lengths are significantly different (*t* test $P < 0.05$) between WT on sucrose and WT without sucrose or the *cycd4;1-1* conditions between cells 21–27.

laterals (8). To further investigate the role of basal meristem cell length in LR initiation, we examined pericycle cell length in an *slr* mutant. Consistent with the proposed action of *IAA14/SLR* in the differentiation zone and not in the basal meristem, *slr-1* roots showed a similar cell length profile to WT in the basal meristem region. We also found that *CYCD4;1* transcript levels were unaffected in *slr-1* (Fig. S4). Taking together, the lack of *CYCD4;1* induction by auxin or alterations in auxin flux and response mutants, and the production of WT levels of LR on addition of higher auxin levels, we conclude that *CYCD4;1* is not directly involved in auxin-mediated LR formation, but rather influences LR density through affecting basal meristem pericycle cell length.

LR density also responds to the carbon:nitrate balance (24), and *CYCD4;1* mRNA levels in *Arabidopsis* suspension cultures are dependent on the presence of sucrose (25). To test whether sucrose regulates *CYCD4;1* expression in planta, we examined expression in the p*CYCD4;1*::GUS-GFP reporter line in response to sucrose induction assay. Dark grown plants can be induced to produce sucrose through photosynthesis by shifting plants from dark to light conditions. In dark grown plants without exogenous sucrose, no p*CYCD4;1*::GUS activity was detected in the root meristem (Fig. 3*B*). Transfer of the plants to the light, inducing sucrose synthesis, led to detectable *CYCD4;1* expression (Fig. 3*C*). Plants grown continuously in light showed higher levels of *CYCD4;1* expression (Fig. 3*E*). We also tested our reporter line in a comparable assay where sucrose availability was controlled by transferring dark grown plants from media containing no sucrose to media supplemented with sucrose. The transfer onto sucrose containing media induced *CYCD4;1* expression in the root meristem (Fig. 3*D*). Continuous light with exogenous sucrose resulted in strongest expression of *CYCD4;1* (Fig. 3*E*). We conclude that expression from the *CYCD4;1* promoter is dependent on sucrose availability in roots.

The dependence of *CYCD4;1* expression on sucrose suggested that dark grown plants lacking sucrose may phenocopy *cycd4;1*. WT and *cycd4;1-1* plants were grown on different levels of sucrose and scored for LR emergence after 10 days (Fig. 3*F*). In WT plants, LR density increases with sucrose levels up to 1.5%, but declines at 2% sucrose. In *cycd4;1-1* plants, a similar trend is seen, although overall LR density is reduced compared to WT, except in the absence of sucrose, when LR densities of WT and *cycd4;1-1* are equal. Analysis of meristem cell lengths showed that absence of sucrose affects pericycle cell length in the basal meristem, and under these conditions, both WT and *cycd4;1* mutants show equivalent cell lengths and meristem boundary positions (Fig. 3*G*). Whereas WT cell lengths decrease in response to sucrose presence correlating with induction of *CYCD4;1* expression and an increased LR density, in the *cycd4;1-1* mutant, the distal meristem pericycle cell lengths do

not respond to sucrose, and LR density does not respond to the same extent as WT (Fig. 3*G*). We conclude that sucrose presence is required for both aspects of the *cycd4;1* phenotype to be manifest and that the effect of sucrose on pericycle cell length and about half of the sucrose effect on LR density is dependent of *CYCD4;1*.

Discussion

Root growth depends on the combination of cell production and subsequent cell elongation, processes that are spatially separated in the root meristem. Cell division predominates in the apical meristem, and average cell lengths are constant. Rapid cell expansion occurs with final cell differentiation, but between the apical meristem and rapid elongation/differentiation zones lies an intermediate region known as the basal meristem (8), where average cell lengths increase while divisions still occur. The mechanisms that control and integrate these changes in different cell types of the growing root in response to intrinsic and external signals are mostly unclear, although gravitropism is one well-known example of response to an external signal that causes a temporal and asymmetric change in the cell division-elongation balance, leading to a macroscopic change in root morphology (26). Here, we show that an intrinsic factor, the D-type cyclin, *CYCD4;1*, affects pericycle cell length independently of other cell layers and influences LR density.

We find that *CYCD4;1* is expressed in the RAM within pericycle cells adjacent to the xylem poles. Previous in situ hybridization of roots of *Raphanus sativus* (radish) showed expression hybridizing to an *Arabidopsis CYCD4;1* probe associated with the LR primordium itself (25, 27), but we could not detect any activity of the *CYCD4;1* promoter in this region in *Arabidopsis*, suggesting possible species differences. The expression pattern we describe here and previously (18) is in agreement with the low level of expression and distribution reported for *CYCD4;1* from cell sorting and microarray experiments ("digital in situ"), including its expression in aerial tissues (20, 21). Hence, loss of *cycd4;1* appears to influence LR density through its expression in the meristem region and not during the subsequent initiation of the LR primordium in the differentiation zone.

Loss of *CYCD4;1* function results in an earlier transition of pericycle cells from apical to basal meristem region and a larger average length of pericycle cells in the basal meristem, therefore reducing pericycle cell density in this region. Correlating with these changes in the pericycle basal meristem of *cycd4;1* plants, fewer LR are initiated, suggesting that loss of *CYCD4;1* expression in the root meristem could interfere with the priming of founder cells in the pericycle (8). Although recent modeling of LR initiation and the role of auxin therein suggests that LR formation can also take place without pericycle cell priming (9) and that the creation of a stable auxin maximum in the mature pericycle is sufficient for LR formation, our observed correlation between pericycle cell length in the basal meristem and normal LR density is consistent with a prepatterning model. We saw a consistent correlation between LR density and basal meristem pericycle cell length in all experiments and treatments, strongly suggesting that these aspects are causally linked. In this case, the prepatterning of LR in the basal meristem would be influenced by the size of the pericycle cells in this region, consistent with a possible cell counting mechanism for LR spacing. It is also possible that loss of *CYCD4;1* in the meristem has a permanent effect on pericycle cells that could render them less likely to initiate LR later on.

For example, it has been proposed that xylem pole pericycle cells progress through S phase to a G2 state, whereas other pericycle cells remain in G1 phase (28), and the loss of *CYCD4;1* could therefore affect this progression. However, this might be expected to be seen as a subsequent reduced sensitivity to auxin in the stimulation assay, and this was not observed in our analysis of response to exogenous auxin across a range of concentrations. We propose based on our data that the two models of prepatterning versus auxin maxima in mature pericycle are not mutually inconsistent and that both may contribute to the observed pattern of LR in normal plant growth.

The plasticity of plant roots is intimately linked to their functional role as the primary site of nutrient uptake. Thus, changes in nutrient availability have also been noted as key mediators of division and root architecture (11). For instance recent data shows that sucrose availability influences the initiation of LR in response to nitrate levels (29). We show that root expression of *CYCD4;1* is dependent on sucrose, as previously reported for the related *CYCD2;1* and *CYCD3;1* (6, 30) and for *CYCD4;1* in suspension cells (25), and that this has important effects within the root meristem in determining the transition boundary between apical and basal meristem with consequential effects on LR density. Moreover we show that the absence of sucrose causes WT roots to phenocopy *cycd4;1*. Taken together, these data uncover a mechanism of nutrient-regulated LR density whereby sucrose deprivation apparently limits LR initiation through effects on the flux of pericycle cells through the basal meristem.

In addition to the interaction of the *cycd4;1* phenotype with sucrose levels, we also observed that the cellular and LR phenotypes of *cycd4;1* were completely restored by addition of very low concentrations (5 nM) applied exogenous auxin. This effect may be due to transcriptional responses of other *CYCD* genes to auxin (31) or to the reported stabilization by auxin of E2Fb protein, the downstream positive effector of *CYCD* action (32). Taken together, the interplay of the *cycd4;1* phenotype with subtle changes in auxin or sucrose levels suggest that it may form part of a network of responses tuning LR density to environmental conditions.

We show here that the analysis of cell lengths through the meristem can reveal information on the position of meristem boundaries in individual cell files. In the *cycd4;1* mutant root, only pericycle cells are affected, whereas in the *aux1* mutant, which bears several superficial phenotypic similarities, there is a large reduction in overall meristem size and cell expansion is not responsive to 10 nM NAA, suggesting that *aux1* effects on LR density are likely to be distinct from those of *cycd4;1*.

Loss of *CYCD4;1* affects cell length in the basal meristem and the boundary between apical and basal meristem of the pericycle, as we detected an earlier transition to the basal meristem in *cycd4;1*. Despite the significant expression of *CYCD4;1* in the apical meristem pericycle cells, we could not detect a change in cell length in this region of *cycd4;1*, indicating that *CYCD4;1* is not rate-limiting for cell division in the apical meristem nor does it directly control cell length. D-type cyclins are described as positive regulators of cell division (33), and we suggest that *CYCD4;1* is a rate-limiting cell division regulator specifically in the pericycle of the root basal meristem. Previous analysis of a triple loss-of-function mutant of the *CYCD3* family, showed a related phenotype in leaf development with a decrease in cell number and an increase in cell size in lateral organs of the shoot (16). However, in the shoot, the three *CYCD3* genes have relatively broad expression in primordia and young developing leaves, whereas the expression and phenotype of *CYCD4;1* is highly cell-specific. As the overall rate of root growth in *cycd4;1* is unaffected, a lower frequency of cell division in the mutant requires the cells to be larger. Our results show that the boundary between apical and basal meristem can be different and dynamic in different cell types and that division rates and cell sizes of distinct cell files can be autonomously regulated by differential CYCD expression.

Materials and Methods

Plant Material. WT *Arabidopsis thaliana* ecotype Columbia (Col-0) were obtained from the Nottingham *Arabidopsis* Stock Centre. The loss-of-function mutant lines *cycd4;1-1* (TAIR accession SALK_015525) and *cycd4;1-20* (TAIR accession GK-344D08-016232) were recovered from the SALK (34) and GABI-Kat (35) collections, respectively. Both mutants were backcrossed, and at least 100 F2 progeny were assayed for segregation of the insert using herbicide resistance as a marker (kanamycin resistance was scored for *cycd4;1-1* and

sulfadiazine resistance for *cycd4;1-20*). In both F2 populations the marker segregated in a Mendelian manner (three resistant:one sensitive) as expected for a single dominant insertion. Genomic DNA was extracted from resistant plants, and PCRs were performed using insert-specific and flanking gene-specific primers. This genotyping confirmed that the insert was present in all resistant plants analyzed. Identified mutants homozygous for the insertion were then backcrossed at least twice more to create our homozygous lines used in all experiments. Segregating WT lines from the backcross populations were used as WT controls in all growth experiments. Total RNA was extracted to confirm a reduction in the 3′ region of *CYCD4;1* transcripts using quantitative real-time PCR. For allelic complementation, homozygous *cycd4;1-1* and *cycd4;1-20* plants were crossed in both directions. Since each of the mutants contributes a different herbicide resistance, some F1 seed from each cross was scored on media containing both kanamycin and sulfadiazine to test for successful crosses. All F1 progeny tested were resistant to both herbicides, while appropriate controls were all sensitive. Remaining F1 seeds from these crosses were scored for decreases in LR. All F1 plants had a significant decrease in LR number confirming that *cycd4;1-1* and *cycd4;1-20* describe two independent *CYCD4;1* null alleles. *Cycd4;1-1 COMP* was generated by transforming *cycd4;1-1* with a construct carrying the genomic fragment of the *CYCD4;1* gene. The start of the *CYCD4;1* promoter was chosen from the end of the 3′ UTR from the gene upstream (AT5G65430) using primer ACCAGTTGTTTTCAAGAATTTGCT. The 3′ end of the genomic fragment is until the STOP codon of *CYCD4;1* using primer AGAAAGATGTGTATAAGAAGAAGA. *aux1-7* (36) and *slr* (5) were kindly provided by Malcolm Bennett and Ben Scheres, respectively.

Growth Conditions. Seeds were sterilized in 10% sodium hypochloride and placed on square Petri plates containing Murashige and Skoog (MS) mixture, 1.5% sucrose, 0.5 g/L 2-(N-morpholino) ethanesulfonic acid (Mes), pH 5.8, in 1% agar. Seeds were imbibed in the dark at 4 °C for 3 days, and plates were incubated vertically in Conviron TC30 (Controlled Environments) in a cycle of 8 h dark/16 h white light (170–200 μL $m^{-2}s^{-1}$) at 22 °C, 70% humidity. For auxin and sucrose treatments, seeds were plated as described above on medium containing NAA or sucrose, respectively.

LR Density Measurements. Plants were grown as described above and scanned at 1,500 dpi. Emerged LR were counted, and primary root length was measured using the software analysis package, ImageJ.

Expression Analyses. Plants carrying the pCYCD4;1::GUS-GFP construct were grown as described above. Roots were stained histochemically essentially as described in ref. 37, embedded in Technovit 7100 (Hereaus Kulzer), and 6-μm sections were cut on a Leica RM2145 microtome and examined with a Nikon Optiphot microscope.

Real-Time PCR. Transcripts were measured using quantitative real-time PCR as described in ref. 38. Total RNA (2 μg) was used to generate cDNA, and relative expression was calculated using the 2-delta-delta CT method (39) using ACTIN2 as reference transcript. CycD4;1 RT primer pair: GCCAGCACAACCAAAGGTAT and CCCATTGGG TGTTTGTGAAC. CDKA;1 RT primer pair: CCGAGCACCAGAGATACTCC and GTTACCCCACGCCATGTATC. ACTIN2 RT primer pair: ACATTGTGCTCAGTGGTGGA and CTGAGGGAAGCAAGAATGGA.

Root Cell Length Measurements. Root sections were fixed in 50% methanol, 10% acetic acid. After fixation, roots were incubated for 20 min in 1% periodic acid and then for 1 h in 0.18 M sodium bisulfite, 0.15 N hydrochloric acid, and 100 μg/mL propidium iodide. The roots were cleared in chloral hydrate (265 g in 100 mL water). Confocal stacks of roots were obtained with a Zeiss LSM 510 Meta or Zeiss LSM 710 Meta confocal microscope using filter settings for propidium iodide staining, and cell lengths were measured in ImageJ. A minimal of 15 roots per genotype were analyzed.

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