A cyclin-dependent kinase-activating kinase regulates differentiation of root initial cells in *Arabidopsis*

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Cell division and differentiation continue throughout the plant life cycle without significant loss of control. However, little is known about the mechanisms that allow the continuous development of meristems. Cell division is controlled by a family of cyclin-dependent kinases (CDKs). CDK-activating kinases (CAKs) are known to phosphorylate and activate almost all CDKs and thus may have a crucial role in controlling CDK activities in each cell of the meristems. Here, we show that overexpression of sense or antisense gene for Cak1At in *Arabidopsis* by using the glucocorticoid-mediated transcriptional induction system resulted in a reduction of CDK activities. After 14–24 h of glucocorticoid treatment, starch granules appeared in columellar initials in the root meristem, and cortical initials were periclinally divided into cortical and endodermal cells. Accumulation of the cyclin::β-glucuronidase fusion protein ceased after 72 h of glucocorticoid treatment. Our results indicate that a change of Cak1At activity leads to differentiation of initial cells, followed by cessation of cell division. Therefore, we propose that differentiation of initial cells is controlled by Cak1At but is maintained independent of cell division.

In the root apical meristem of plants, new cells are added by the activity of initials and their daughters. In *Arabidopsis*, each initial cell has a stereotyped pattern of cell division that leads to each column in the root meristem (1–3). The initial cells surround the four mitotically inactive central cells of the quiescent center (QC), onto which the cylindrical layers of dividing cells converge. Complete laser ablation of the four central cells leads to replacement of the QC by vascular initials, and ablation of cortical and epidermal initials results in replacement of the QC by pericycle and cortical cells, respectively (2). Therefore, positional control plays an important role in the determination of the cell fate in the root meristem.

For the continuous operation of such meristematic organization, cell division activity must be tightly controlled by machinery that regulates the cell cycle. A family of cyclin-dependent serine/threonine protein kinases (CDKs) is involved in the activation of cell division and the transitions between different phases of the cell cycle (4). In *Arabidopsis*, overexpression of p34[cdc2aAt], a CDK with the conserved PSTAIRE motif, results in only a twofold increase in kinase activity (5). However, this is not surprising, considering that kinase activation is mediated by posttranslational activation of CDKs at multiple levels to control normal development of plant tissues.

In addition to cyclins, the activation of CDKs requires phosphorylation of a threonine residue within the T-loop of kinase subdomain VIII by CDK-activating kinases (CAKs). Vertebrate CAKs are composed of a catalytic kinase subunit p40[MO15]/Cdk7 (6–8), a regulatory cyclin H subunit (9, 10), and an assembly factor, MAT1 (11–13). They are also identified as subunits of the transcription factor IIH and are known to phosphorylate the carboxyl-terminal domain of the largest subunit of RNA polymerase II (14–16). We have recently identified an *Arabidopsis* cDNA, named *cak1At*, as a suppresser of temperature-sensitive *cak* mutant of budding yeast (17). However, Cak1At only slightly resembles vertebrate-type CAKs and phosphorylated threonine 160 of human CDK2 but not *Arabidopsis* carboxyl-terminal domain (17). Therefore, Cak1At is involved in activation of

CDKs but not in transcriptional regulation. Because CAK is an upstream kinase that activates almost all CDKs, up- or down-regulation of Cak1At might have a crucial effect on total CDK activity in each cell of the meristems.

Here, we show that overexpression of sense or antisense gene for Cak1At in *Arabidopsis* results in a gradual reduction of CDK activity and differentiation of initial cells in the root meristem. Our data indicate that progression of cell differentiation precedes cessation of cell division, thus the differentiation state of initial cells is determined independent of cell division. We propose that Cak1At regulates the indeterminate state of initial cells to guarantee continuous development of the root meristem.

Materials and Methods

Plant Transformation. The coding region of *cak1At* cDNA (17) was cloned into *Xho*I–*Spe*I sites of the pTA7002 vector (18) in both sense and antisense orientations and introduced into *Arabidopsis thaliana* ecotype Col-0 via *Agrobacterium*-mediated root transformation (19). Transgenic seedlings (T0 generation) were selected on MS plates (20) containing 20 μg/ml hygromycin. Assays were performed on homozygous T2 progeny. Plants were grown at 23°C under continuous light conditions.

Biochemical Analyses. Total protein was extracted from the root tip (average 1 cm). Immunoblotting was performed using an ECL Western blotting detection system (Amersham Pharmacia). Anti-Cak1At antibody was described previously (17). Anti-p34[cdc2aAt] antibody was raised against the carboxyl-terminal FKDLGGMP peptide of *Arabidopsis* p34[cdc2aAt] (21–23). CDK2 and histone H1 kinase assays were performed as described previously (17, 24). Phosphatase treatment was performed with 200 units of lambda protein phosphatase (New England Biolabs) in a buffer (50 mM Tris-HCl/0.1 mM Na$_2$EDTA/5 mM DTT/0.01% polyoxyethylene lauryl ether, pH 7.5) at 30°C for 1 h.

Histological Analysis. For the β-glucuronidase (GUS) staining, seedlings heterozygous for both *cak1At* and *cyc1aAt*::GUS were washed twice in a buffer [50 mM PO$_4$-Na, pH 7.0/5 mM K$_3$Fe(CN)$_6$/3 mM EDTA] for 5 min and stained with 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in the same buffer overnight at 37°C. Samples were mounted in chloral hydrate solution [74% (wt/wt) chloral hydrate, 7.4% (wt/wt) glycerol]. For visualization of starch granules, roots were incubated for 3 min in Lugol solution (Sigma) and mounted in chloral hydrate solution. For the hydroxyurea treatment, seeds germinated on MS medium were transferred onto a medium containing 100 mM hydroxyurea.
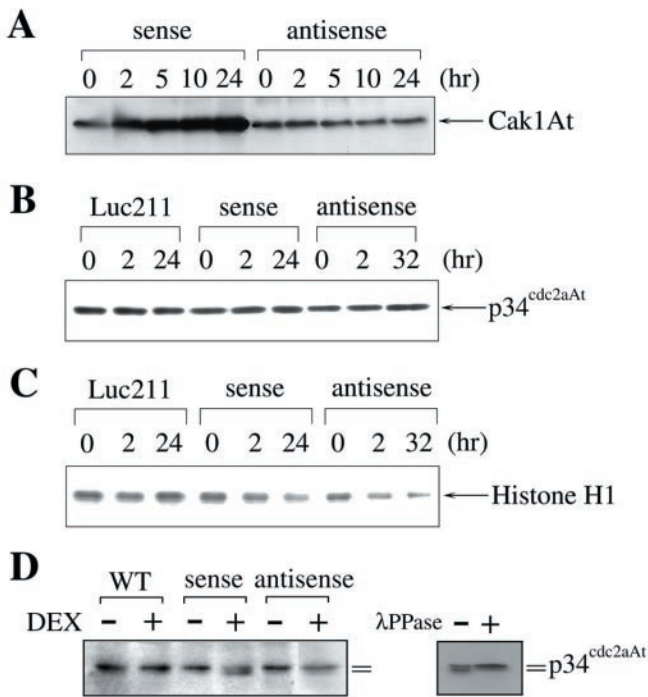

Fig. 1. Overexpression of sense or antisense *cak1At* reduces CDK activity in the root meristem. Representative results of transgenic sense line S8 (sense) and antisense line A31 (antisense) together with the luciferase line 211 (Luc211) and wild-type plants (WT). Seedlings were treated with 1 µM DEX for *cak1At* transgenic lines and with 10 µM DEX for Luc211 for indicated hours. Fifteen micrograms of proteins extracted from roots was immunoblotted with (*A*) anti-Cak1At antibody and (*B*) anti-p34^{cdc2aAt} antibody. (*C*) Thirty-five micrograms of proteins was immunoprecipitated with anti-p34^{cdc2aAt} antibody, and immunoprecipitates were assayed for histone H1 kinase activity. (*D*) Fifteen micrograms of proteins extracted from roots that were grown in the absence (−) or presence (+) of 1 µM DEX for 3 days was immunoblotted with anti-p34^{cdc2aAt} antibody. Ten micrograms of proteins extracted from DEX-treated sense plants was reacted with (+) or without (−) lambda protein phosphatase (λPPase) and immunoblotted with anti-p34^{cdc2aAt} antibody.

Results

Reduction in CDK Activities in *cak1At* Transgenic Plants. Using the glucocorticoid-mediated transcriptional induction system (18), we generated transgenic *Arabidopsis* plants that overexpressed sense or antisense mRNA of *cak1At*. In the above system, a glucocorticoid derivative, dexamethasone (DEX), activates a transcription factor called GVG, which in turn induces *cak1At* expression. Fifteen independent lines were obtained for each sense and antisense plant, and assays were performed on homozygous T2 progeny. Seeds were germinated on MS medium and transferred onto 1.5% (wt/vol) agar plates (25), which were stacked in a vertical position to elongate roots straight downward on the agar surface.

To perform biochemical analyses, total protein was extracted from the root tip on agar plates. In sense lines, treatment with 1 µM DEX resulted in a significant increase in Cak1At protein within 2 h, while the protein level gradually decreased in antisense lines (Fig. 1*A*). In parallel with changes in the protein level, the phosphorylation activity on human CDK2 was significantly increased in the protein extract from sense plants (data not shown). In contrast, the protein level of p34^{cdc2aAt}, a PSTAIRE-type CDK of *Arabidopsis*, did not change by DEX treatment (Fig. 1*B*). When p34^{cdc2aAt} was immunoprecipitated and subjected to kinase assay using histone H1 as substrate, a lower phosphorylation activity was observed. As shown in Fig.

1*C*, the reduction had already been observed after 2 h of DEX treatment, and the kinase activity continued to decrease afterward in both sense and antisense plants.

After 3 days of DEX treatment, the p34^{cdc2aAt} protein in sense plants turned into a doublet on immunoblotting, whereas antisense and wild-type plants did not show such pattern (Fig. 1*D*). The highly migrating protein disappeared by incubation of the protein extract with lambda protein phosphatase (Fig. 1*D*), indicating that the lower band represented a phosphorylated form of p34^{cdc2aAt}. These results suggest that a higher Cak1At activity in the sense plants causes accumulation of the phosphorylated form of p34^{cdc2aAt} but results in a reduction of CDK activities.

Arrest of Cell Division in the Transformants. In the next step, we investigated the phenotype of transgenic plants by focusing on root development. First, seeds germinated on MS medium were transferred onto a DEX-containing medium, followed by measurement of the root growth. As shown in Fig. 2 *A* and *B*, root growth of each of five lines of sense and antisense plants was inhibited on a medium containing 0.1 µM DEX, and more profound inhibition was observed in the presence of 1 µM DEX. The control line 321 transformed with the luciferase gene on the same vector showed no growth inhibition under the same conditions (Fig. 2 *A* and *B*). In contrast, root growth of another luciferase line 211 was inhibited in the presence of a high dose (1 and 10 µM) of DEX (Fig. 2*B*). In such a case, however, we could not detect any change in the protein level and kinase activity of p34^{cdc2aAt}, whereas CDK activity was reduced in *cak1At* transgenic plants with 1 µM DEX (Fig. 1*C*). When seedlings pretreated with 1 µM DEX for 4 days were transferred onto a DEX-free medium, elongation of roots after a lag of 2 days was noted in both sense and antisense plants (Fig. 2*C*), indicating that induced growth defect of roots was not attributable to cell death but rather to arrest of cell division.

The transgenic plants were crossed with a plant line carrying a GUS reporter in which the *Arabidopsis* cyclin (*cyc1aAt*) (26) promoter region and 5′ portion of the cyclin coding region were fused in-frame to the GUS gene (27). As Cyc1aAt contains a destruction box at the N-terminal region, which is involved in the degradation of cyclins during mitosis (28), the cyclin::GUS chimeric protein was degraded in every M phase of the cell cycle, thus showing a patchy pattern in the meristem (Fig. 3*A*). Transgenic roots grown on a DEX-containing media for 48 h still expressed the reporter gene (Fig. 3*B*), but they ceased to accumulate the fusion protein within 72 h (Fig. 3*D*). A similar pattern of the GUS expression was observed in both sense and antisense plants. When plants pretreated with 1 µM DEX for 4 days were transferred onto DEX-free media, expression of the reporter gene resumed (data not shown). Considering that *cyc1aAt* is preferentially expressed during the G2 to M phase (29), cells overexpressing sense or antisense *cak1At* might stop the cell cycle at a stage other than G2 to M, possibly in the G1 to S phase.

Differentiation of Initial Cells in the Root Meristem. After induction of sense or antisense *cak1At* expression, the vascular bundle gradually extended from the upper to the bottom parts of roots (Fig. 3 *C* and *D*) and finally reached the central cells, which constitute the QC (Fig. 3*E*). During the differentiation of vascular cells, root hair emerged from epidermal cells in the apical region (Fig. 3 *D* and *E*), but in contrast, such hair was not usually formed in wild-type plant. This suggests that cell differentiation progressed in the root meristem after induction of the transgene expression.

We then followed columella cells in the root cap by analyzing a columella-specific marker. Mature columella cells contain starch granules that are never found in columellar initials
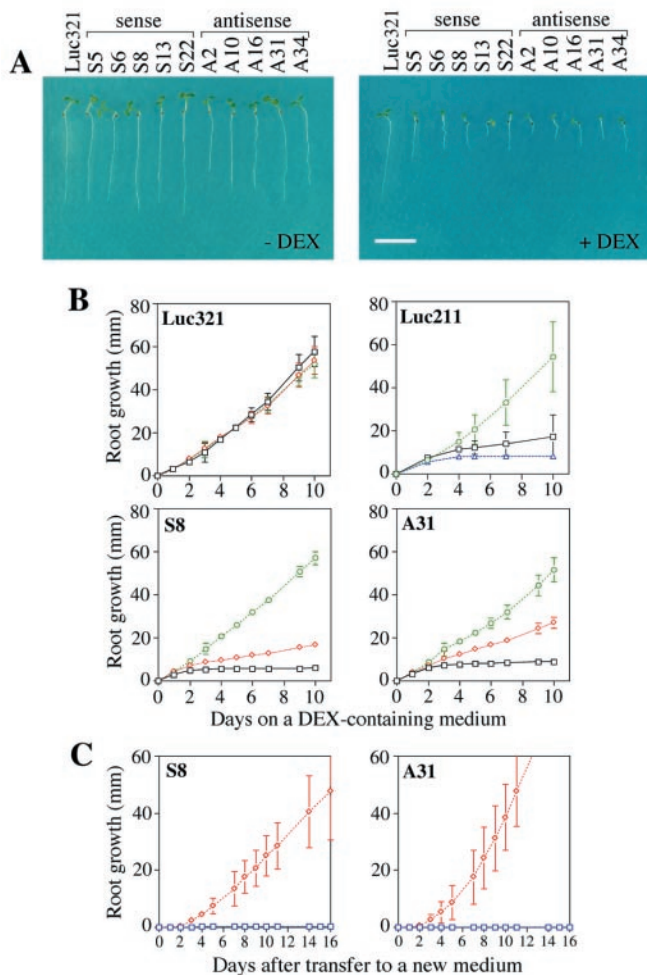
Fig. 3. Arrest of cell division in roots of transgenic plants. A representative result of GUS staining of transgenic line S8 carrying the *cyc1aAt*::GUS reporter gene. Seedlings were grown on agar plates containing 1 μM DEX for 0 h (*A*), 48 h (*B*), 60 h (*C*), 72 h (*D*), and 12 days (*E*). Arrows indicate the vascular bundle. After 12 days (*E*), the vascular bundle reached the central cells of the quiescent centers, which are shown by arrowheads. (Bar = 50 μm.)

Fig. 2. Inhibition of root growth by overexpression of sense or antisense *cak1At*. (*A*) Seedlings of each of five lines of sense and antisense plants and the luciferase line 321 (Luc321) were grown on a medium with or without 1 μM DEX for 5 days. (Bar = 1 cm.) (*B*) Root growth was plotted after transfer onto a DEX-containing medium. DEX concentration: 0 μM (green), 0.1 μM (red), 1 μM (black), and 10 μM (purple). Representative results of sense (S8) and antisense (A31) lines together with the luciferase lines (Luc321 and Luc211). (*C*) Seedlings were grown on a medium containing 1 μM DEX for 4 days and then transferred onto a new DEX-free medium (red) or containing 1 μM DEX (purple). Root growth was plotted after transfer to a new medium.

underlying the QC (Fig. 4*A*). However, after 14–24 h of DEX treatment, starch granules appeared in transgenic plants in the elongated initial cells (Fig. 4*B*), suggesting that columellar initials were differentiated as daughter cells. After that, starch granules started to fade away and almost disappeared after 48 h (Fig. 4*C*). To determine whether other initial cells were differentiated, we examined cortical initials that also established a direct contact with the QC. Cortical initials normally generate cortical daughters by anticlinal division, whereas daughter cells undergo a longitudinal asymmetric division to generate an inner layer of endodermal cells and an outer layer of cortical cells (1). As shown in Fig. 4*B*, cortical initials of plants treated with 1 μM DEX for 24 h were periclinally divided into cortical and endodermal cells, suggesting that cortical initials were also differentiated. After 24 h of DEX treatment, division of these cells was still present as represented by the cyclin::GUS expression (Fig. 3), indicating that the cortical initials could divide once differentiating into daughter cells. These results were obtained in both
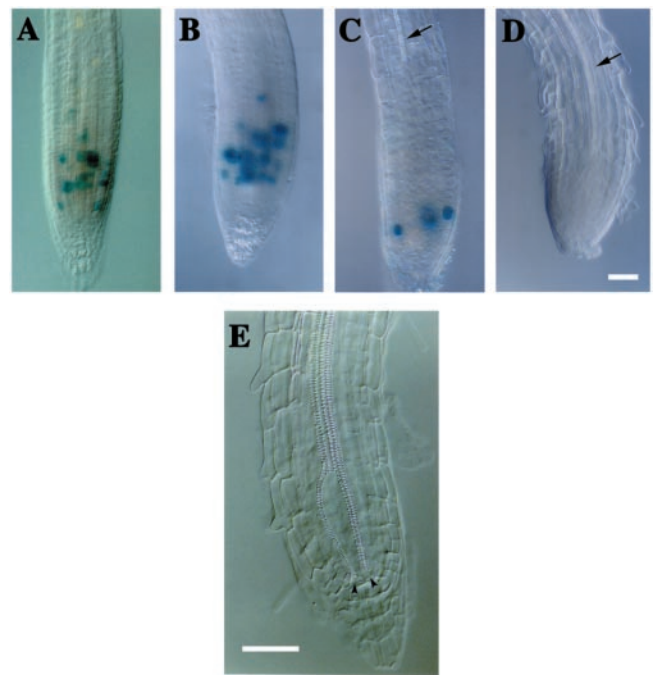
sense and antisense plants. On the other hand, in the control line 211, no differentiation was observed in initial cells, and starch granules persisted in mature columella cells even after 53 h of 10 μM DEX treatment (Fig. 4*D*).

Discussion

Kang *et al.* (30) have reported that the glucocorticoid-inducible system causes growth defects in plants that highly express the transcriptional activator GVG. However, based on the following reasons, we concluded that the phenotype of our transgenic plants was not derived from the system itself. First, all transgenic plants investigated in our study showed the same phenotype in a DEX-dependent manner. One line (line A2) showed growth inhibition of roots at a lower concentration of DEX (0.1 μM), but it also exhibited the same phenotype. Second, in the luciferase line 211, which showed a severe defect in root growth by 10 μM DEX treatment, the kinase activity of p34^{cdc2aAt} was not reduced, whereas *cak1At* transgenic plants showed a decrease of the enzyme activity by 1 μM DEX treatment. Third, in luciferase lines including line 211, we never observed differentiation of initial cells, and starch granules were persistently detected in mature columella cells during inhibition of root growth.

We showed that overexpression of sense or antisense *cak1At* caused reduction in the kinase activity of p34^{cdc2aAt} but not in the amount of protein. Within 2 h of DEX treatment, the phosphorylation activity started to decrease and continued to decrease at least for 3 days (data not shown). In the antisense plants, a reduction of Cak1At activity would result in a decrease of CDK activities. In the sense plants, a phosphorylated form of p34^{cdc2aAt} appeared on immunoblotting, indicating that higher expression of *cak1At* caused extensive phosphorylation of p34^{cdc2aAt}. Nonetheless, the CDK activity was reduced in these plants. Because Cak1At specifically phosphorylates threonine 160 of human CDK2 (17), the reduction of CDK activity is not
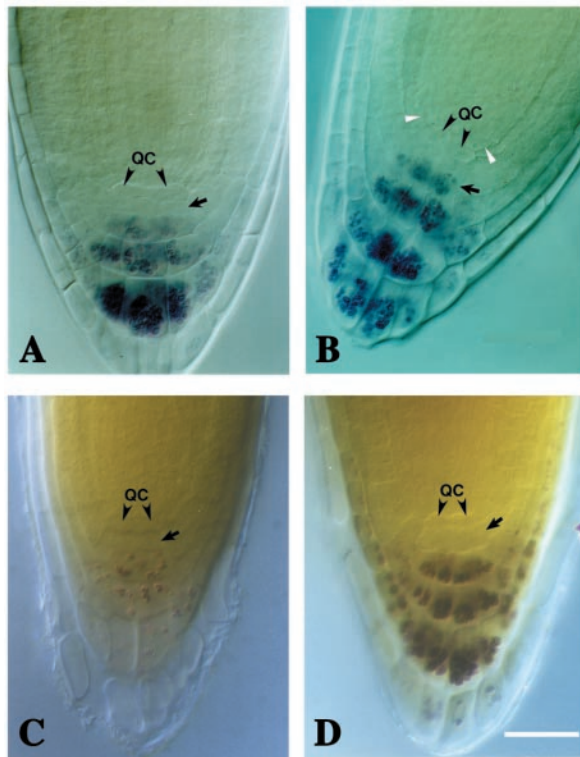
Fig. 4. Differentiation of initial cells in the root meristem. Seedlings of wild-type (*A*) and transgenic line S8 grown on a medium containing 1 μM DEX for 24 h (*B*) and 48 h (*C*). The luciferase line (Luc211) treated with 10 μM DEX for 53 h (*D*) is also shown. Starch granules were visualized in columella cells. Arrows indicate the columella initials abutting the QC. The cortical initials of transgenic plant (*B*) were periclinally divided as indicated by white arrowheads. (Bar = 25 μm.)

Fig. 5. Schematic diagram of sections through the central zone of the root meristem. In the sense and antisense plants treated with 1 μM DEX, the columellar initials acquire markers (starch granules) normally only observed in the daughter cells. The cortical initials also behave like a cortical daughter by dividing longitudinally to give rise to the first cells of the endodermal and cortical files.

attributable to the extensive phosphorylation of p34^{cdc2aAt}. It is known that CDK activities are controlled at multiple levels, such as cyclin binding, phosphorylation, and interaction with CDK inhibitors (31). Therefore, *cak1At* overexpression in the sense plants might promote phosphorylation of another regulatory protein that caused reduction of CDK activities. In mammals, CAK is involved in phosphorylation of the tumor suppressor protein p53 (32) and one of the nuclear receptors, the retinoic acid receptor α (33). Thus, it is intriguing to identify other substrates of Cak1At to reveal its distinct function in *Arabidopsis*.

Reduction of CDK activities was subsequently followed by differentiation of initial cells, which was never observed in wild-type roots (Fig. 5). Accumulation of the cyclin::GUS fusion protein ceased after the columellar and cortical initials were differentiated, indicating that cessation of cell division was not the cause of progression of cell differentiation. To support this idea, the cortical initials were dividing after 24 h of DEX treatment to differentiate into daughter cells (Fig. 4*B*). Moreover, we could not observe differentiation of initial cells in wild-type roots treated with hydroxyurea, which blocks cycling cells at the onset of the S phase (data not shown). Therefore, we propose that the indeterminate state of initial cells is controlled independent of cell division. Because the vascular bundle had extended to the bottom part of roots before cessation of the cyclin::GUS expression (Fig. 3*C*), it is likely that not only initials
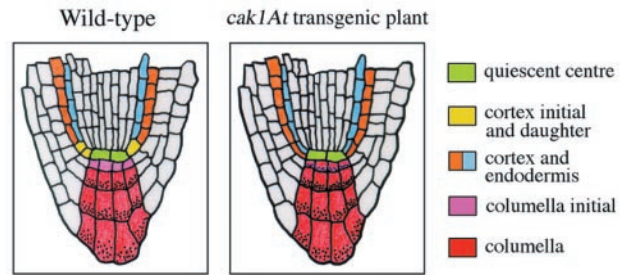
but also other cells in the meristem determine their differentiation state independent of cell division.

The present results showed that Cak1At is involved in the control of differentiation of initial cells in the root meristem. Considering that both sense and antisense plants showed the same outcome, it is likely that a decrease in CDK activities leads to differentiation of initial cells. This assumption is supported by the evidence that starch granules appeared in the columellar initials, and the cortical initials were periclinally divided when roots were treated with roscovitine, a specific inhibitor to CDKs (ref. 34 and unpublished data). In this case, however, starch granules were detected in a small proportion of the initials, probably because of a sudden reduction in CDK activities in contrast to our DEX-treated plants. On the other hand, our results do not rule out that overexpression of *cak1At* changed activities of some other regulatory proteins that are involved in the control of cell differentiation. Therefore, whether Cak1At regulates differentiation of initial cells through CDKs or other regulatory proteins is a crucial question that requires further investigation.

van den Berg *et al.* (35) showed that laser ablation of one QC cell resulted in differentiation of abutting columellar and cortical initials. In their experiment, cortical initials contacting the dead QC cell behaved as cortical daughters and divided asymmetrically into cortical and endodermal cells, and epidermal initials divided normally. This is consistent with our hypothesis that differentiation of initial cells is controlled independent of cell division. Although the mitotically inactive central cells of the QC remained intact in our system, the columellar and cortical initials were differentiated. This suggests that QC prevents the abutting initial cells from differentiation, via unknown signal, which may control the Cak1At activity. Further studies on the shoot meristem using our *cak1At* transgenic plants may help clarify the *in vivo* functions of Cak1At in cell division and differentiation.

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