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RESEARCH ARTICLE

Auxin-Mediated Cell Cycle Activation during Early Lateral Root Initiation

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Lateral root formation can be divided into two major phases: pericycle activation and meristem establishment. In *Arabidopsis*, the first lateral root initiation event is spatially and temporally asynchronous and involves a limited number of cells in the xylem pericycle. To study the molecular regulation during pericycle activation, we developed a lateral root-inducible system. Successive treatments with an auxin transport inhibitor and exogenous auxin were used to prevent the first formative divisions and then to activate the entire pericycle. Our morphological and molecular data show that, in this inducible system, xylem pericycle activation was synchronized and enhanced to cover the entire length of the root. The results also indicate that the inducible system can be considered a novel *in planta* system for the study of synchronized cell cycle reactivation. In addition, the expression patterns of *Kip-Related Protein2 (KRP2)* in the pericycle and its ectopic expression data revealed that the cyclin-dependent kinase inhibitor plays a significant role in the regulation of lateral root initiation. *KRP2* appears to regulate early lateral root initiation by blocking the G1-to-S transition and to be regulated transcriptionally by auxin.

INTRODUCTION

Lateral root formation plays a crucial role in plant development by permitting the construction of branched root systems. The process of lateral root formation consists of two major steps: cell cycle reactivation in the xylem pericycle and establishment of a new meristem (Celenza et al., 1995; Laskowski et al., 1995; Malamy and Benfey, 1997). In *Arabidopsis*, lateral roots are initiated by the local activation of pericycle cells at the xylem poles. Recently, we proposed a model to describe cell cycle progression that precedes the first formative divisions in lateral root initiation (Beeckman et al., 2001). In the xylem pericycle, basal cells proceed to G2 phase, whereas other pericycle cells remain in G1 phase. The first formative divisions in the pericycle depend on the basipetal transport of auxin, whereas shoot-derived auxin regulates the outgrowth of lateral roots (Casimiro et al., 2001; Bhalerao et al., 2002). It remains largely unknown how plants control the reactivation of the cell cycle during development, but it is generally accepted that plant hormones may play a central role. Plant hor-

mones are known to affect the cell cycle directly, mainly at the transcriptional level (Stals and Inzé, 2001).

In all higher eukaryotes, including plants, activation of a cyclin-dependent kinase (CDK) A/cyclin D complex at the G1-to-S transition leads to hyperphosphorylation of the transcriptional repressor retinoblastoma protein (Soni et al., 1995; Meijer and Murray, 2000; Boniotti and Gutierrez, 2001). Inactivated retinoblastoma releases the transcription factor E2F/DP, which in turn triggers the expression of S phase-specific genes (Magyar et al., 2000; De Veylder et al., 2002; Kosugi and Ohashi, 2002). The next checkpoint, G2 to M, regulates cell cycle progression to the mitotic phase, mainly through B-type CDKs (CDKB1 and CDKB2) (Joubès et al., 2000; Boudolf et al., 2001) and A- and B-type cyclins (Renaudin et al., 1998; Mironov et al., 1999; John et al., 2001). Characteristically, cell cycle-regulatory proteins fluctuate during the cell cycle. Generally, this fluctuation is mediated by stringent transcriptional regulation and controlled proteolysis (Fobert et al., 1994; Shaul et al., 1996; Genschik et al., 1998). In addition, a class of CDK-inhibitory proteins, the Kip-related proteins (KRPs), is involved in inactivating CDK/cyclin complexes (Wang et al., 1997, 1998; De Veylder et al., 2001).

To understand the cell cycle regulation mechanisms that result in the initiation of new lateral roots, detailed molecular studies are required. However, the small number of cells

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involved in the first lateral root initiation events seriously hampers such studies (Taylor and Scheuring, 1994). Also, the lack of synchrony of the initiation events makes it very difficult to efficiently follow the development of lateral root initiation. To overcome these problems, we developed a system that allows the synchronization of the pericycle and enhances the activation of lateral root initiation. The system was based on seed germination in the presence of the auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA) and early transfer to the exogenous auxin 1-naphthalene acetic acid (NAA) to prevent and induce pericycle activation, respectively. With this approach, we were able to monitor synchronous cell cycle reactivation during early lateral root induction. Analysis of auxin distribution patterns in the system showed that auxin determines both the positioning and frequency of lateral root initiation. Our results suggested the G1-to-S checkpoint to be a target for auxin-mediated lateral root initiation. In addition, a CDK-inhibitory protein (KRP2) was shown to be regulated transcriptionally by auxin and to prevent lateral root initiation by blocking the G1-to-S transition.

RESULTS

Induction of Lateral Roots

To visualize cell cycle activity in the pericycle, we used the well-characterized marker line for active cell division, *CYCB1;1::uidA*, whose promoter activity also correlates well with the corresponding mRNA localization (Ferreira et al., 1994a, 1994b). In Arabidopsis, the first lateral root primordium is initiated ~36 h after germination and is positioned in the basal half of the root (Beeckman et al., 2001) (Figures 1A and 1B). However, in a population of seedlings, this initiation event is spatially and temporally asynchronous. Furthermore, spontaneous lateral root initiation involves only a limited number of cells (Figures 1C and 1G), so it is burdensome to analyze in detail with molecular techniques. To study cell cycle progression during pericycle reactivation, we developed a lateral root-inducible system. The system is based on successive treatments with an auxin transport blocker (NPA) and exogenous auxin (NAA).

In the inducible system, seeds were germinated on NPA-containing medium to prevent the formative divisions in the pericycle (Casimiro et al., 2001). The optimal NPA concentration was determined by comparing the lateral root induction rate on 1 and 10 μ M NPA-containing medium with that of seedlings grown on Murashige and Skoog (MS) medium. After 48 h on 1 μ M NPA medium, generally one lateral root was initiated per seedling, whereas on MS medium, three primordia were visualized by *CYCB1;1::uidA* promoter activity. However, on 10 μ M NPA, no lateral root initiation sites were detectable after 72 h of growth (Figure 1D). NPA also prevented the activation of adventitious root primordia at the root-hypocotyl junction. Growth for 72 h on NPA pro-

vided sufficiently long main roots (5 mm) to collect samples but did not induce the changes in pattern and polarity described by Sabatini et al. (1999). The 10 μ M concentration of NPA was used in all experiments described below. For the inducible system, after germination, seedlings were grown on NPA for 3 days (72 h). These 3-day-old seedlings were considered to be at time point 0 for the inducible system. After treatment with NPA, seedlings were transferred to medium containing exogenous auxin (10 μ M NAA) for 12 h until the entire pericycle was activated, as indicated by uniform *CYCB1;1* promoter activity (Figure 1E). On NAA, induction of the formative divisions for lateral root initiation involved both xylem pericycle strands (Figures 1F and 1H). From the first round of anticlinal divisions, the development of the xylem pole pericycle cells proceeded to periclinal divisions (Figure 1I) (Malamy and Benfey, 1997).

Auxin Response in the Inducible System

Because lateral root initiation is known to be regulated by auxin, we tested the distribution kinetics of auxin responsiveness and cell cycle activity during NPA and NAA treatment. If auxin determined the position of lateral root initiation, the sequence of initiation would follow the primary direction of the auxin flow. Also, if the amount of applied auxin affected the frequency of lateral root initiation sites, there would be a difference in lateral root numbers between the different treatments. To test these hypotheses, marker lines for auxin responsiveness (*DR5::uidA*; Ulmasov et al., 1997) and cell division activity (*CYCB1;1::uidA*) were used in a set of transfer experiments. Because NPA accumulates auxin in the root apex (Casimiro et al., 2001), a flow of endogenous auxin is expected to move basipetally from the root tip upon release from the NPA block. In our experiments, the seedlings were transferred from NPA to MS medium or from MS medium to NAA and were stained for β -glucuronidase (GUS) activity every 2 h. To estimate the timing of auxin penetration in the tissues, the *DR5::uidA* line also was transferred from NPA to NAA for durations of 0.5, 1, 1.5, 2, and 4 h and stained for GUS activity.

On NPA (72 h after germination), *DR5::uidA* promoter activity in the roots was restricted to the root apical meristem (Figure 2A). When transferred from NPA to NAA, *DR5::uidA* promoter activity was induced at 1.5 h in the apical region of the root and already covered the entire root length at 3 h (Figures 2B and 2C, respectively). After treatment for 72 h on NPA followed by 6 hr on MS medium, *DR5::uidA* promoter activity was present in the apical half of the root (Figures 2D and 2E) and proceeded basipetally in the root during further incubation on MS medium. When *CYCB1;1::uidA* seedlings were transferred from NPA to MS medium, no clear induction in the pericycle was observed by GUS activity, even after 6 h (Figure 2F). At 10 h, individual lateral root initiation sites were detected in the apical half of the root (Figure 2G). By contrast, in seedlings transferred from MS

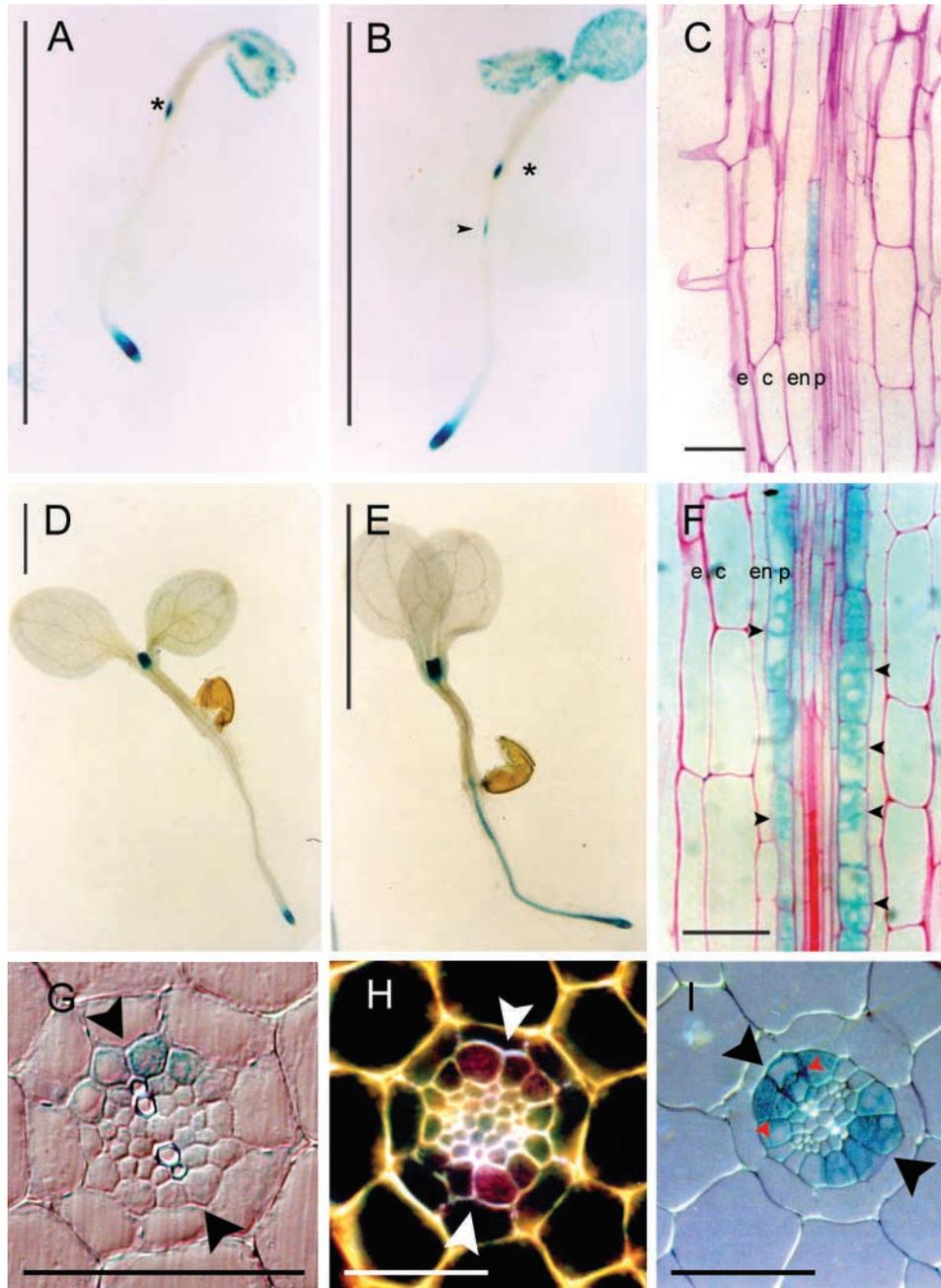


Figure 1. *CYCB1;1* Promoter Activity during Pericycle Activation for Lateral Root Initiation.

- (A) No lateral root initiation sites were visible at 24 h after germination on MS medium.
 (B) Spontaneous lateral root initiation at 36 h after germination on MS medium. The first lateral root initiation site is indicated with an arrowhead.
 (C) Longitudinal anatomical section of the initiation site.
 (D) Pericycle activation prevented on NPA.
 (E) Pericycle activation after transfer from NPA to NAA for 12 h.
 (F) Longitudinal anatomical section of a root after 10 h on NAA. Arrowheads indicate the newly formed anticlinal cell walls.
 (G) Transverse section at 36 h after germination on MS medium.
 (H) Transverse section after 10 h of NAA treatment in a dark field. GUS staining is shown as purple.
 (I) Transverse section after 12 h of NAA treatment. Periclinal divisions are indicated with red arrowheads.
 Asterisks indicate adventitious root primordia. c, cortex; e, epidermis; en, endodermis; p, pericycle. Bars = 1 mm for (A), (B), (D), and (E); 0.1 mm for (C) and (F); and 0.05 mm for (G) to (I). Large arrowheads in (G) to (I) indicate xylem pole pericycle cells.

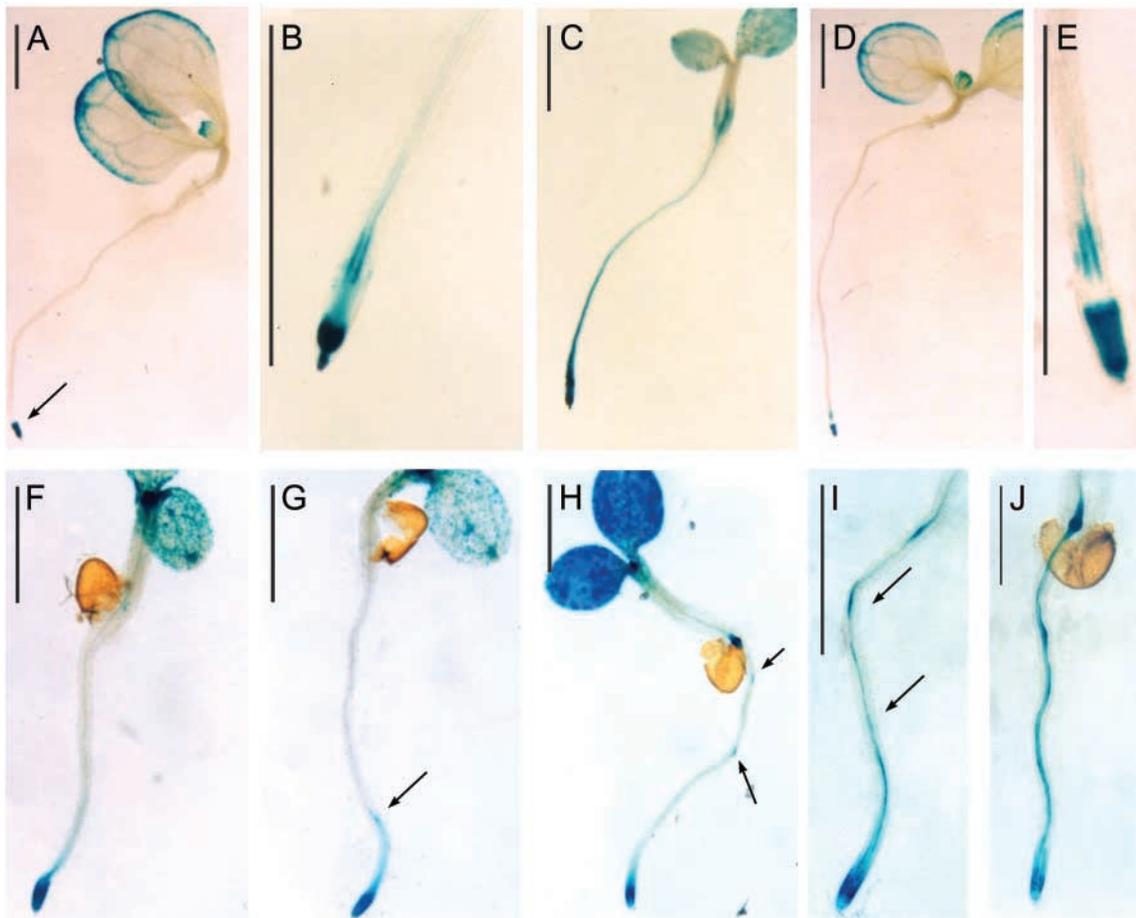


Figure 2. Histochemical GUS Staining Patterns of *DR5::uidA* and *CYCB1;1::uidA* in Transfer Experiments from NPA to NAA, from NPA to MS Medium, and from MS Medium to NAA.

(A) *DR5::uidA* on NPA. The arrow indicates the root apical meristem.

(B) *DR5::uidA* after transfer from NPA to NAA for 1.5 h.

(C) *DR5::uidA* after transfer from NPA to NAA for 3 h.

(D) *DR5::uidA* after transfer from NPA to MS medium for 6 h.

(E) *DR5::uidA* after transfer from NPA to MS medium for 6 h (detailed image).

(F) *CYCB1;1::uidA* after transfer from NPA to MS medium for 6 h.

(G) *CYCB1;1::uidA* after transfer from NPA to MS medium for 10 h. The arrow indicates the first lateral root initiation site at the apical end of the root.

(H) *CYCB1;1::uidA* after transfer from MS medium to NAA for 4 h. Arrows indicate the first initiation sites in the basal half of the root.

(I) *CYCB1;1::uidA* after transfer from MS medium to NAA for 6 h. The lateral root initiation sites are along the entire length of the root. Arrows indicate developing lateral root primordia.

(J) *CYCB1;1::uidA* after transfer from MS medium to NAA for 8 h. The entire pericycle is activated.

Bars = 1 mm.

medium (after 24 h of growth) to NAA, individual lateral root initiation sites at 4 h were observed to be positioned in the basal half of the root (Figure 2H). Only later, after 6 h on NAA, were lateral roots also initiated in the apical half of the root (Figure 2I). From 8 h onward, the entire pericycle

showed GUS activity (Figure 2J). In conclusion, pretreatment with NPA resulted in the relocalization of lateral root initiation sites to the apical half of the root, whereas in the absence of NPA, the first lateral roots initiated in the basal half of the root.

Cell Cycle Activity in the Induced Roots

The fact that the cell cycle reactivation of all xylem pericycle cells of NPA-pretreated roots transferred to NAA medium was rapid and uniform indicated that the entire pericycle was synchronized at the same cell cycle phase on NPA medium. To identify the cell cycle phase in which the xylem pericycle cells were arrested, a cell cycle blocker, hydroxyurea (HU), was applied in the inducible system. HU is an inhibitor of ribonucleotide diphosphate reductase and blocks the cell cycle during the G1-to-S transition (Planchais et al., 2000). If the xylem pericycle cells were blocked at the G1 phase on NPA treatment, no progression to the G2-to-M transition would take place and no *CYCB1;1* promoter activity would be detected when transferred to NAA in the presence of HU. On the other hand, if the pericycle cells were blocked at the G2 phase, a first round of cell divisions could take place, as visualized by *CYCB1;1* promoter activity (Beeckman et al., 2001). To test the effects of HU on cell cycle progression in the inducible system, seedlings of the *CYCB1;1::uidA* line were germinated and grown for 48 h on NPA, then they were transferred to NPA medium containing 100 mM HU. After another 24-h growth period, seedlings were transferred to NAA containing the same concentration of HU. Pericycle activation was evaluated based on *CYCB1;1* promoter activity. On NAA supplemented with 100 mM HU, pericycle activation was prevented, as seen by the absence of induction of *CYCB1;1* promoter activity at 12 h (Figure 3A) compared with 12 h of NAA treatment alone (Figure 1E). This result indicates that on NPA, the xylem pericycle was blocked in the G1 phase. In the root apical meristem, *CYCB1;1* promoter activity was reduced and showed patchy patterns, indicating that the cells were in different cell cycle stages in the meristem when transferred onto HU (data not shown).

To study cell cycle progression during pericycle reactivation, promoter activity in *CDKA;1::uidA*, *CYCA2;1::uidA*, and *CYCB1;1::uidA* fusion lines was evaluated. *CDKA;1* promoter activity reflects the state of competence for cell division activity or an undifferentiated state (Martinez et al., 1992; Hemery et al., 1993). *CYCA2;1* promoter activity was used as a marker for the late S and G2 phases of the cell cycle (Bursens et al., 2000a), whereas *CYCB1;1* promoter activity marked progression through late G2 to M phase (Ferreira et al., 1994b). Whole-mount GUS assay samples were prepared from each line at 0, 4, 6, 8, 10, and 12 h after transfer from NPA to NAA. *CDKA;1* promoter activity in the xylem pericycle was strong at all time points, including in the NPA-treated samples (Figures 3B and 3C). Strong *CDKA;1::uidA* expression also covered the central stele. The two cyclin promoters revealed a defined pattern of cell cycle progression in the pericycle. On NPA, the promoter activity of *CYCA2;1* was limited to the root apical meristem and vascular parenchyma (Figure 3D). After transfer to NAA from 4 to 6 h, *CYCA2;1* promoter activity started to emerge in the xylem pericycle as a sign of progression to G2 phase (Fig-

ures 3E and 3F). At 8 h, the expression marked the first asymmetric divisions in the apical half of the xylem pericycle (Figure 3G), and at 10 h, uniform expression was observed in the small radially expanded xylem pericycle cells (Figure 3H). At 12 h, the apical part of the xylem pericycle approached the two-cell-layer stage, or stage II (Malamy and Benfey, 1997), and *CYCA2;1* promoter activity remained strong in the tissues (data not shown). The expression pattern started from the apical half of the root and gradually covered the basal half of the root (data not shown).

As a marker for the G2-to-M phase of the cell cycle, *CYCB1;1* promoter activity had a delayed pattern compared with that of *CYCA2;1*. On NPA medium, no *CYCB1;1* promoter activity was detected above the root apical meristem (Figure 3I). After seedlings had been transferred to NAA, *CYCB1;1* promoter activity was induced gradually from 6 h on and proceeded basipetally in the xylem pericycle (Figures 3J to 3M). No individual lateral root initiation sites were detected; instead, at 12 h, the whole xylem pericycle showed strong *CYCB1;1* promoter activity as a sign of a fully activated pericycle (Figure 3N). In the whole-mount GUS-stained samples, the *CYCB1;1::uidA* expression is seen as two strands of blue staining, which represent the xylem pole pericycle cell files shown in Figures 1F and 1H. When seedlings remained on NAA for 1 week, lateral roots were induced along the entire length of the root at the two opposite xylem poles of the pericycle (Figure 3O).

Cell Cycle Regulation during Early Lateral Root Initiation

Semiquantitative reverse transcription (RT) PCR was used to study the expression of cell cycle-regulatory genes during pericycle activation in our system. The expression of several well-characterized cell cycle genes corresponded to synchronous cell cycle progression (Figure 4A).

In the inducible system, at 0 h (72 h on NPA), no or low expression of marker genes for the active cell cycle was detected. At 4 h, cell cycle marker genes for the G1-to-S transition, *HISTONE H4* and *E2Fa*, were induced along with the *CYCD3;1* cyclin. Unexpectedly, the B-type CDKs *CDKB1;1* and *CDKB2;1* followed the same pattern. However, the transcript levels showed clear peaks at 8 h, as in other B-type CDKs. The G1-to-S and S phase-specific cyclins *CYCD1;1* and *CYCA2;1* were induced only at 6 h, although their transcripts were seen weakly at 0 and 4 h. The low level of *CYCA2;1* transcripts at these early time points correlates with the constitutive promoter activity observed in the vascular cylinder (Figure 3). At 6 h, genes involved in the G2-to-M transition, *CYCB1;1*, *CYCB2;1*, *CDKB1;1*, and *CDKB2;2*, showed simultaneous induction. *CDKA;1* transcripts were present constitutively from time point 0. Remarkably, three of the CDK inhibitor genes had a pattern opposite that of the other cell cycle-regulatory genes. The transcript levels of *KRP1* and *KRP2* genes were high at 0 h but were downregulated after 4 h on NAA. The *KRP4* gene responded less

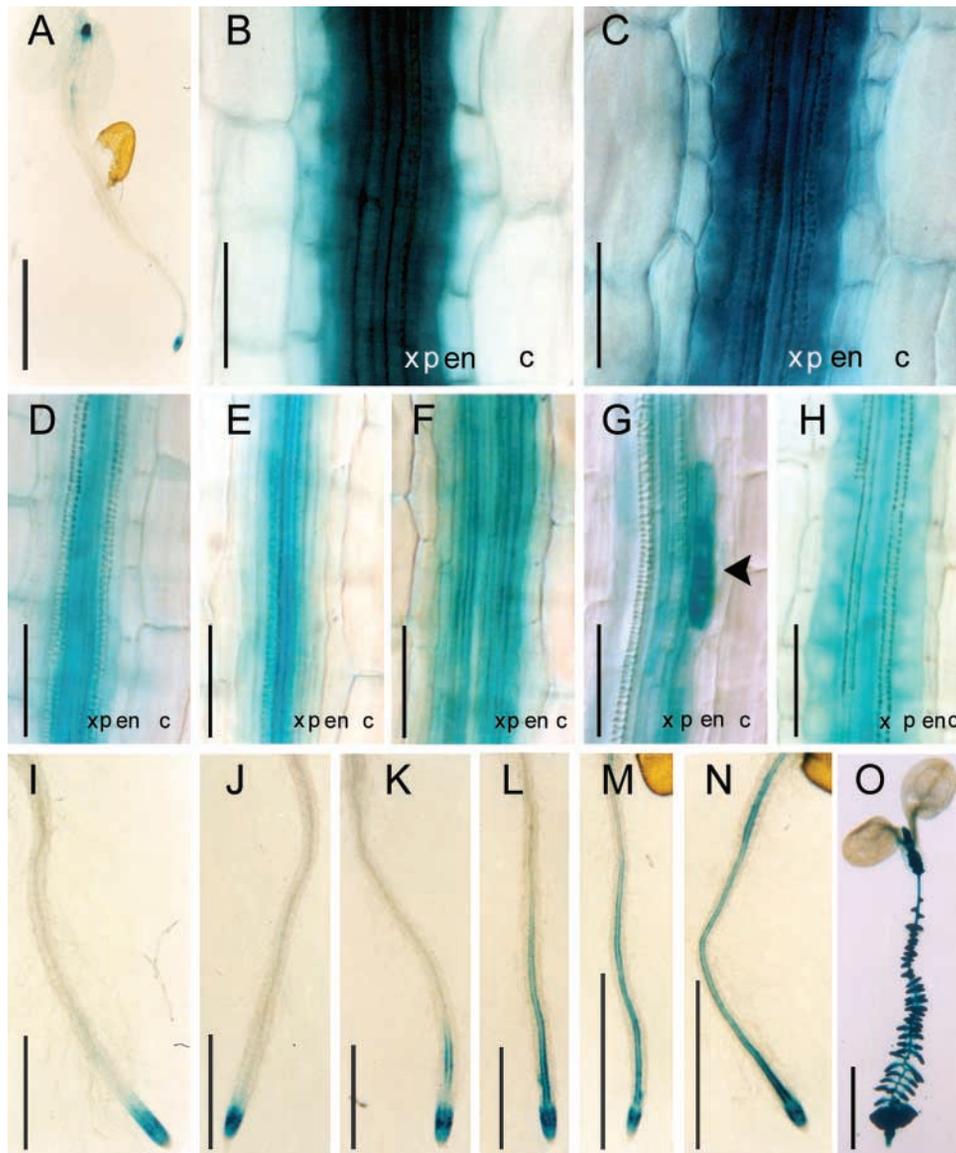


Figure 3. Histochemical GUS Assays of *CDKA;1*, *CYA2;1*, and *CYCB1;1* Promoter Activities in a Time-Course Experiment with NPA and NAA.

(B) to (H) show the promoter activities of *CDKA;1::uidA* and *CYA2;1::uidA* on NPA- and NAA-treated roots with differential interference contrast microscopy. **(A)**, **(I)** to **(N)**, and **(P)** show the activities of *CYCB1;1::uidA* by stereomicroscopy.

(A) *CYCB1;1* promoter activity in roots treated with NAA and 100 mM HU for 12 h (cf. with **[N]**).

(B) *CDKA;1::uidA* promoter activity in the pericycle on NPA.

(C) *CDKA;1::uidA* promoter activity after 12 h on NAA. There was no change compared with **(B)**.

(D) *CYA2;1::uidA* seedlings treated for 72 h with NPA. Strong promoter activity was limited to the central stele. Low staining or diffusion are seen in the pericycle, endodermis, and cortex.

(E) *CYA2;1::uidA* seedlings treated for 4 h with NAA. No expression is seen in the xylem pericycle.

(F) *CYA2;1::uidA* seedlings treated for 6 h with NAA. Expression is emerging in the xylem pericycle.

(G) *CYA2;1::uidA* seedlings treated for 8 h with NAA. The first asymmetric divisions are visualized with strong *CYA2;1::uidA* promoter activity. Arrowhead indicates a newly formed cell wall resulting from an asymmetrical division.

(H) *CYA2;1::uidA* seedlings treated for 10 h with NAA. A fully stained pericycle consisting of small radially expanded cells is seen.

(I) No induction of *CYCB1;1::uidA* activity at 0 h.

(J) No induction of *CYCB1;1::uidA* activity at 4 h.

(K) Induction of *CYCB1;1::uidA* activity at 6 h.

(L) *CYCB1;1::uidA* activity at 8 h.

strongly: the transcripts were present at 0 h but were reduced only slightly at 4 h. The transcript profiles of the *KRP3* genes deviated from those of the other *KRP* genes. The transcript levels were low at 0 h, and clear induction was observed at 4 h. The *actin-2* gene was used to evaluate equal input of RNA in the analysis. Figure 4B highlights the postulated cell cycle progression with arrows, according to the transcript profiles.

In Situ Expression Pattern of *KRP2* mRNA

Because expression of the *KRP1* and *KRP2* genes was high in NPA-treated roots, KRPs may be involved in preventing pericycle activation for lateral root formation. The tissue-specific localization of *KRP2* mRNA was analyzed in wild-type Arabidopsis and radish roots. In sections from Arabidopsis roots treated in the inducible system, strong *KRP2* expression was observed in pericycle cells in the NPA treatment (Figure 5A), whereas it disappeared almost completely by the subsequent NAA treatment (Figure 5B). This result indicates that the transcript accumulation of *KRP2* was affected directly by the application of auxin.

The direct effect of auxin on *KRP2* expression was confirmed further by RT-PCR data from a short-time-course experiment (0, 1, 1.5, 2, 3, and 4 h on NAA after incubation on NPA) in which *KRP2* expression was downregulated already 1.5 h after transfer to NAA (Figure 4C). At that time point, *DR5::uidA* expression revealed that auxin had penetrated the root tissues initially (as described above). These results again indicate that *KRP2* levels are essentially under transcriptional control.

In transverse sections of young parts of radish seedling roots (recognizable by the lack of xylem differentiation in the center of the stele), phloem pericycle-specific expression for the *KRP2* gene was observed (Figure 5D). Interestingly, no transcripts were detected at protoxylem poles (i.e., the sites at which lateral root initiation normally takes place). However, in mature parts of radish roots (with fully differentiated xylem), the expression had more variable patterns. In different sections, the signal was detected at one phloem pole of the pericycle (Figure 5E), at xylem and phloem poles (Figure 5F), or around the entire pericycle (Figure 5G). It is remarkable that *KRP2* expression was seen in pericycle cells opposite developing lateral root primordia (Figure 5C). As such, the

spatial expression pattern of *KRP2* supports the hypothesis that *KRP2* plays a role in regulating cell cycle activity in root pericycle cells.

Lateral Root Initiation in the *35S-KRP2* Line

Recently, the effects on leaf development and cell cycle duration of *KRP2* overexpression have been studied in detail (De Veylder et al., 2001). To evaluate further the postulated involvement of *KRP2* in the regulation of early lateral root initiation, the effects of ectopic expression were studied. The total root length and the number of lateral root primordia of two *35S-KRP2* transgenic lines and wild-type plants were counted after 2 weeks of growth. The number of lateral roots in the *35S-KRP2* line was reduced by >60% compared with that in the wild type, whereas total root length was affected only slightly at this age (Figures 5H and 5I). To exclude the possibility that *KRP2* overexpression still allowed the cell cycle to be induced in the presence of increased auxin concentration, cell cycle activity in the *35S-KRP2* pericycle was analyzed in the inducible system. To visualize the effect of *KRP2* overexpression on pericycle activation, the *CYCB1;1::uidA* marker gene was introduced into the overexpressing line by crossing. In this double transgenic line, no *CYCB1;1::uidA* induction was detected after 12 h of treatment on NAA (Figures 5J and 5K). This result essentially confirms that *KRP2* prevents cell cycle activation for formative divisions in the xylem pericycle.

DISCUSSION

Auxin Determines the Positioning and Frequency of Lateral Root Initiation

In a previous report, unique cell cycle regulation was shown to occur in the xylem pericycle, in which cells proceed to G2 phase, whereas the rest of the pericycle remained at G1 phase (Beeckman et al., 2001). Cell cycle regulation in the xylem pericycle is known to be mediated by auxin because the inhibition of polar auxin transport effectively blocks the first formative divisions for lateral root initiation (Casimiro et

Figure 3. (continued).

(M) *CYCB1;1::uidA* activity at 10 h.

(N) *CYCB1;1::uidA* activity at 12 h.

(O) A seedling kept on NAA for 1 week. Lateral root induction along the entire length of the root at the two opposite xylem poles of the pericycle is seen.

c, cortex; e, epidermis; en, endodermis; p, pericycle; x, xylem. Bars = 0.1 mm for **(B)** to **(H)** and 1 mm for **(A)**, **(I)** to **(N)**, and **(O)**.

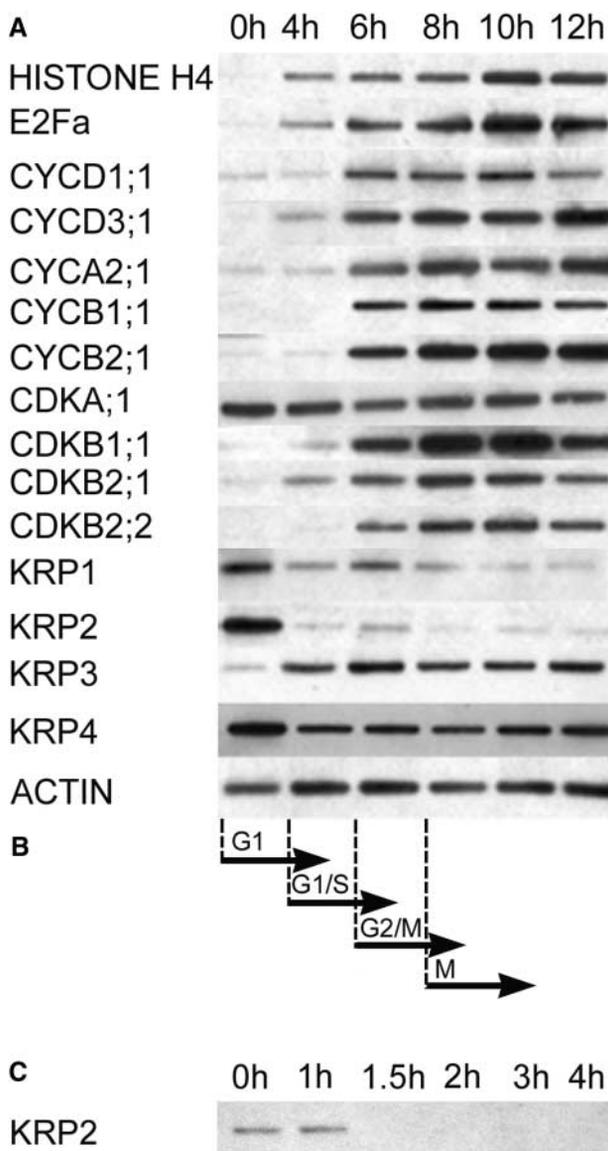


Figure 4. Cell Cycle Gene Expression during Lateral Root Initiation.

(A) RT-PCR gel blots showing the transcript levels of *HISTONE H4*, *E2Fa*, *CYCD1;1*, *CYCD3;1*, *CYCA2;1*, *CYCB1;1*, *CYCB2;1*, *CDKA;1*, *CDKB1;1*, *CDKB2;1*, *CDKB2;2*, *KRP1*, *KRP2*, *KRP3*, *KRP4*, and *ACTIN* from 72 h on NPA (0 h) to 12 h on NAA.

(B) Cell cycle progression as predicted from the transcript profiles in **(A)** and indicated by arrows together with the current cell cycle phase: G1 (gap 1 phase), G1/S (G1-to-S phase transition), G2/M (G2-to-M phase transition), and M (mitosis).

(C) RT-PCR gel blot of *KRP2* transcripts in a short time course.

al., 2001). Auxin also has been reported to affect cell cycle activity (Stals and Inzé, 2001). Our RT-PCR results demonstrate clearly that auxin promotes lateral root initiation by cell cycle stimulation at the G1-to-S transition. This auxin-mediated effect on the cell cycle also has been suggested by previous work in various species and experimental systems (Corsi and Avanzi, 1970; Nougarede and Rondet, 1983; Chriqui, 1985).

In addition, the amount and direction of auxin flow in the roots was shown to determine the frequency and position of lateral root initiation. On NPA, *DR5* promoter activity was restricted to the root apical meristem, where expression was very strong, indicating that NPA blocked auxin transport from the root tip and caused its accumulation in the meristem (Müller et al., 1998; Casimiro et al., 2001). Fast induction of *DR5* promoter activity was observed in the pericycle after release from the NPA block, because the accumulated auxin reserves in the root tips were redistributed rapidly in the root. Auxin redistribution was followed by the induction of *CYCB1;1* promoter activity with a delay. In the two transfer experiments, the number of induced lateral roots depended on low or high concentrations of auxin in the transfer from NPA to MS medium and from NPA to NAA, respectively. Depriving roots of auxin during NPA treatment also appeared to prevent the typical localization of lateral root initiation in the basal half of the root (Beeckman et al., 2001). In both cases (NPA to MS medium and NPA to NAA), the pericycle induction was relocalized to the apical half of the root. By contrast, when transferred from MS medium to NAA, the first lateral root induction sites appeared normally in the basal half of the roots, suggesting that the free endogenous auxin is the only determinative factor for the pericycle to prime the spontaneous lateral root initiation. This finding also implies that lateral root initiation could occur independently of positional control mechanisms from the surrounding tissues.

Pericycle-Specific Expression of *KRP2* mRNA Is Regulated by Auxin

KRP1 and *KRP2* expression were high in the inactive pericycle of NPA-treated roots. Also, during cell cycle arrest caused by sugar starvation, *KRP2* transcript levels are high (Menges and Murray, 2002). Upon transfer to NAA, strong downregulation of *KRP2* and *KRP1* followed. Similarly, the *KRP4* gene also had a weak negative response to the transfer to auxin. Previously, auxin was shown to negatively affect *KRP2* expression in Arabidopsis cell suspensions, whereas *KRP1* did not respond (Richard et al., 2001). In NPA and NAA treatments, the response of *KRP3* was opposite, being induced upon transfer to auxin. *KRP3* also is highly expressed in actively dividing cell suspension cultures (De Veylder et al., 2001; Menges and Murray, 2002), and unlike *KRP1*, it does not respond to the growth-inhibiting hormone abscisic acid (Wang et al., 1998). In conclu-

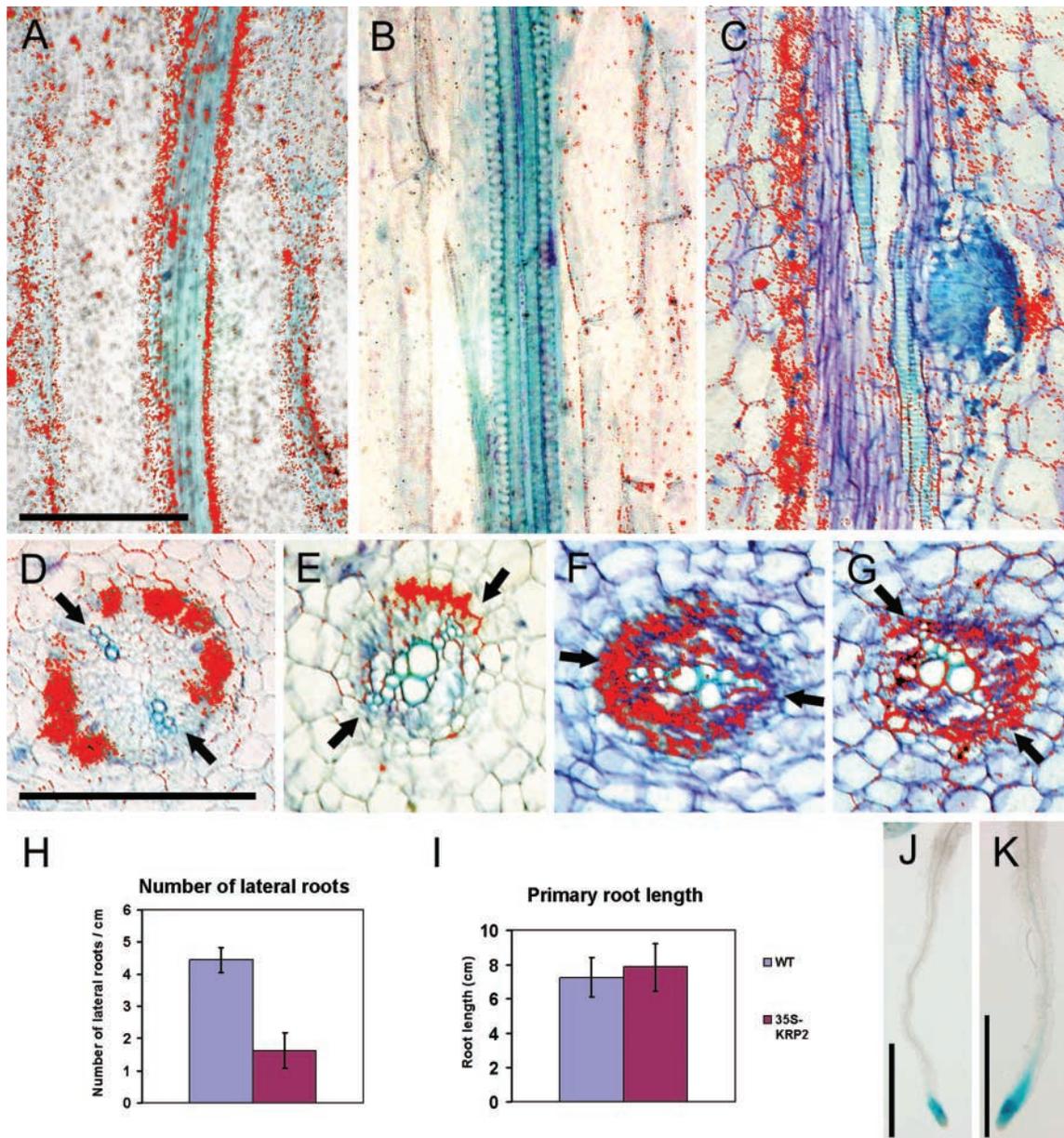


Figure 5. *KRP2* Expression in Root Tissues.

In situ hybridization on Arabidopsis and radish roots performed with a ^{35}S -UTP-labeled *KRP2* antisense riboprobe. Hybridization signals are presented as red dots.

(A) *KRP2* mRNA localization in an Arabidopsis root treated with NPA (72 h).

(B) *KRP2* mRNA localization in an Arabidopsis root treated with NAA (12 h).

(C) Longitudinal section of lateral root primordia in an Arabidopsis root.

(D) Toluidine blue-stained cross-section of young parts of a radish root. *KRP2* expression was found mainly in pericycle cells excluding the xylem poles. Arrows **(D)** to **(G)** indicate protoxylem poles.

(E) *KRP2* expression at the phloem pole of the pericycle.

(F) *KRP2* expression at the xylem and the phloem poles of the pericycle.

(G) *KRP2* expression around the entire pericycle.

(H) Number of lateral roots per centimeter in wild-type and *35S-KRP2* seedlings.

(I) Total root length of wild-type and *35S-KRP2* seedlings.

(J) *35S-KRP2* line 72 h after germination on NPA medium, showing *CYCB1,1::uidA* activity.

(K) *35S-KRP2* line 12 h after transfer from NPA to NAA medium, showing *CYCB1,1::uidA* activity.

WT, wild type. Bars = 0.1 mm for **(A)** to **(C)**, 0.05 mm for **(D)** to **(G)**, and 1 mm for **(J)** and **(K)**.

sion, *KRP3* may play a role in the active cell division cycle, deviating significantly from other *KRPs* analyzed to date.

In *in situ* hybridization of *KRP2* mRNA, the expression patterns showed clearly variable tissue-specific localization depending on the developmental stage of the sample tissue. In young tissues, in which lateral roots are initiated preferentially (Blakely et al., 1982), the expression appeared to be restricted to the phloem pericycle. In older tissues, in which normally no new lateral roots are formed, the expression was seen at xylem poles or around the whole pericycle. Interestingly, *KRP2* expression was observed opposite a developing lateral root primordium. Indeed, under normal conditions, lateral roots are never formed in opposite positions. This variability in the localization of expression may reflect the spatially and temporarily variable competence of the pericycle for lateral root development.

For some of the *KRPs* (*KRP1* and *KRP2*), interaction with *CDKA;1* and inhibition of its kinase activity have been shown (Wang et al., 1998; De Veylder et al., 2001). In Arabidopsis roots, *CDKA;1* also is expressed in tissues that do not divide actively but are competent for cell division (Hemerly et al., 1993). During pericycle activation, *CDKA;1* transcripts and promoter activity were detected on NPA treatment (i.e., in the inactive pericycle), indicating that the pericycle remains competent for cell divisions. The active cell cycle was inhibited, and at the same time, both *KRP1* and *KRP2* were highly expressed. From 4 h onward, *KRP2* transcript levels decreased sharply, whereas those of *CDKA;1* remained high and were accompanied by the accumulation of other marker genes for the active cell cycle. One putative role for *KRPs* might be to keep the constitutively transcribed and possibly translated *CDKA* kinases inactive, because even in the absence of correct cyclin subunits, kinases might be activated by nonspecific cyclins.

In mammalian cells, CDK-inhibiting proteins appear at high levels during the G0 and G1 arrests (Pagano et al., 1995). The levels decrease at entry into the cell cycle, however, without change in the constant levels of transcripts and protein synthesis. This observation indicates that, in mammals, the inhibitory proteins are targeted for proteolysis, mediated by a ubiquitin-dependent pathway (Pagano et al., 1995). In plants, auxin has been shown to be involved in the SCF^{tr}-regulated ubiquitination-dependent proteolytic pathway (Gray et al., 1999). However, no data are available on the accumulation of *KRP* proteins in plants. In contrast to mammalian systems, plant *KRPs* appear to be controlled more at the transcriptional level, which may indicate fundamental differences in the regulation of these inhibitors.

Ectopic Expression of *KRP2* Strongly Affects Pericycle Activation

In animals, CDK inhibitors such as Kip/Cip p27 have been proposed as links between the developmental control of cell proliferation and morphological developments (Chen and

Segil, 1999). In plants, *KRP1* and *KRP2* overexpression causes reduction in organ growth and specific developmental defects in leaves (Wang et al., 2000; De Veylder et al., 2001). In our study, *KRP2* overexpression prevented pericycle activation and reduced the number of lateral roots by >60%. In NAA treatment, the *35S-KRP2* line failed to induce *CYCB1;1::uidA* expression even after 12 h of incubation. These results clearly show that *KRP2* specifically prevents cell cycle induction for formative divisions of lateral roots in the pericycle. During spontaneous lateral root initiation, the xylem pericycle cells are known to proceed to the G2 phase of the cell cycle before lateral root initiation (Blakely et al., 1982; Beeckman et al., 2001). This development takes place in the basal half of the root, where later lateral roots are initiated locally (Beeckman et al., 2001). Based on our results from the inducible system, we postulate that during spontaneous lateral root formation, *KRP2* plays an active role in regulating the G1-to-S transition in the pericycle in an auxin-dependent manner. When the developmental signal, auxin, is absent, the pericycle activation is prevented by *KRP2*, and upon the auxin signal, pericycle activation becomes possible via the downregulation of *KRP2*. During plant development, the pericycle competence for lateral root development appears to vary depending on the maturation state of the root. This variability correlated well with the *KRP2* mRNA expression patterns described previously.

Synchronous Cell Cycle Progression during Pericycle Activation

The analysis of transcript profiles and promoter activities of cell cycle-regulatory genes in this system demonstrated that synchronous cell cycle progression occurred during pericycle activation. The expression profiles are very consistent with those described for a partially synchronized Arabidopsis cell culture (Menges and Murray, 2002). These results suggest that the system could be used as a tool complementary to cell suspension cultures for the analysis of synchronous cell cycle progression in plants.

Extensive research on lateral root initiation has revealed several genes and gene products that are important at various phases of root development. However, we still do not thoroughly understand the regulatory pathways that lead to lateral root initiation. The difficulties may result from the apparent spatial and temporal asynchrony of the initiation events (Malamy and Ryan, 2001). Here, we developed an inducible system in which the pericycle was synchronized for lateral root development in an enhanced manner. The effect of this enhanced synchronization differs essentially from the more arbitrary lateral root induction induced by the exogenous application of auxin alone. This system allowed detailed histological and molecular analysis of early lateral root initiation events. Therefore, we propose this system as a fundamental approach for the study of the early molecular regulation of lateral

root initiation. Currently, we are performing genome-wide expression analysis with this inducible system using microarray (www.microarray.be) and cDNA-amplified fragment length polymorphism techniques. Our preliminary results indicate that the system will allow us to identify and sort regulatory genes involved in the early processes of root branching.

METHODS

Plant Material and Growth Conditions

The transgenic line *CYCB1;1::uidA* of *Arabidopsis thaliana* in a C24 background (Ferreira et al., 1994b) was used for all experiments unless indicated otherwise. For histochemical β -glucuronidase (GUS) assays, the other transgenic lines were *CDKA;1::uidA* (Hemerly et al., 1993), *CYCA2;1::uidA* (Bursens et al., 2000a), and *DR5::uidA* (Ulmasov et al., 1997). Medium containing *N*-1-naphthylphthalamic acid (NPA) and 1-naphthalene acetic acid (NAA) were prepared from Murashige and Skoog (1962) germination medium as described by Valvekens et al. (1988). Seeds were plated on vertically oriented square plates (Greiner Labortechnik, Frickenhausen, Germany). Plants were grown in a growth chamber under continuous light (110 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PAR supplied by cool-white fluorescent tungsten tubes [Osram, München, Germany]) at 22°C. For the time-course experiments, plants were germinated on 10 μM NPA, and the seedlings were grown for 72 h on NPA before they were transferred to NAA-containing medium. Pericycle reactivation was followed every 2 h from 4 to 12 h. For the *KRP2* gene and the *DR5::uidA* line, an earlier time course also was used (0, 0.5, 1, 1.5, 2, and 4 h). For the hydroxyurea experiment, 100 mM hydroxyurea was added to the NPA and NAA media. Sample material was collected at different time points for the various assays.

Histochemical GUS Assays

Complete seedlings or root cuttings were stained in multiwell plates (Falcon 3043; Becton Dickinson, Bedford, MA). GUS assays were performed as described by Beeckman and Engler (1994). Samples mounted in Tris-saline buffer or lactic acid were observed and photographed using a stereomicroscope (Stemi SV11; Zeiss, Jena, Germany) or by a differential interference contrast microscope (Leica, Vienna, Austria).

Microscopy

For anatomical sections, GUS-stained samples were fixed overnight in 1% glutaraldehyde and 4% paraformaldehyde in 50 mM phosphate buffer, pH 7. Samples were dehydrated and embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's protocol. For proper orientation of the samples, transparent strips were used to facilitate tissue alignment (Beeckman and Viane, 2000). Sections of 5- μm samples were cut with a microtome (Minot 1212; Leitz, Wetzlar, Germany), dried on object glasses, and counterstained for cell walls with 0.05% ruthenium red (Fluka Chemica, Buchs, Switzerland) in tap water for 30 s. After drying overnight, the sections were mounted in DePex medium (Brit-

ish Drug House, Poole, UK) and covered with cover slips for analysis and photography.

Reverse Transcription PCR

Endogenous transcript levels of a set of cell cycle-regulatory genes were analyzed during the NPA and NAA treatments by semiquantitative reverse transcription PCR as described by Bursens et al. (2000b). Only the lateral root-inducible segments were used; therefore, the root apical meristems were cut off, and the shoots were removed by cutting below the adventitious root primordia. Total RNA was extracted with Trizol (Invitrogen, Gaithersburg, MD) from \sim 300 excised root segments per sample. cDNA was prepared from 1- μg total RNA samples in a volume of 40 μL using the Superscript RT II first-strand cDNA synthesis kit (Invitrogen). To verify the exponential phase of PCR amplification, 15 and 20 or 18 and 23 cycles were tested for each gene. The primers used in the PCR reactions for *CYCA2;1*, *CYCB1;1*, *CYCB2;1*, *CYCD1;1*, *CYCD3;1*, *CDKA;1*, *KRP1*, *KRP2*, *KRP3*, *KRP4*, and *E2Fb* were as described by Richard et al. (2002); those used for *CDKB1;1*, *CDKB1;2*, *CDKB2;1*, and *CKDB2;2* were as described by Boudolf et al. (2001); and that used for *HISTONE H4* was as described by Magyar et al. (2000). The gene encoding actin-2 was used as a control, with primers 5'-GTTGCACCACTGAAAGGAAG-3' and 5'-CAATGGGACTAAAACGCAAAA-3'.

mRNA in Situ Hybridization

Nearly all steps of the in situ method were performed as described by de Almeida Engler et al. (2001). Radish (*Raphanus sativus*) and *Arabidopsis* seedlings were germinated on K1 medium (Valvekens et al., 1988), and plant material was collected for fixation. Samples were fixed in 2.5% glutaraldehyde, dehydrated, and embedded in paraffin. Sections (10 μm thick) were fixed to 3-aminopropyltriethoxy-silane-coated slides and used during the in situ hybridization procedure. Gene-specific antisense and sense probes of *KRP2* were synthesized from PCR products flanked by T7 and Sp6 promoters, and 15×10^6 cpm/slide was applied (75 ng/mL). Exposure times varied, and slides containing radish tissues always were developed before *Arabidopsis* slides. Images were created using a digital AxioCam (Zeiss) under standard bright-field optics.

Analysis of the *KRP2*-Overexpressing Line

To analyze lateral root development, *KRP2*-overexpressing seedlings ($n = 40$) from two independent transgenic lines were grown for 2 weeks on vertical plates together with wild-type plants ($n = 15$) (both lines were in the Columbia ecotype). Digital images of the root systems were obtained by scanning the plates with a flat-bed scanner. Total root length and number of lateral root primordia were counted per centimeter with Scion Image for Windows (Scion Corp., Frederick, MD). To visualize the effects of *KRP2* overexpression on pericycle activation, the *35S-KRP2* line was crossed with the *CYCB1;1::uidA* line. The F2 population was treated according to the inducible system. Homozygous mutants were selected according to the shoot phenotype (De Veylder et al., 2001) and stained for GUS activity as described above.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any

materials described in this article that would limit their use for non-commercial research purposes.

Accession Number

The accession number for the gene encoding actin-2 is U37281.

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Auxin-Mediated Cell Cycle Activation during Early Lateral Root Initiation

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