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Cytokinin-Mediated Cell Cycling Arrest of Pericycle Founder Cells in Lateral Root Initiation of *Arabidopsis*

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In *Arabidopsis*, lateral root formation is a post-embryonic developmental event, which is regulated by hormones and environmental signals. In this study, via analyzing the expression of cyclin genes during lateral root (LR) formation, we report that cytokinins (CTKs) inhibit the initiation of LR through blocking the pericycle founder cells cycling at the G_2 to M transition phase, while the promotion by CTK of LR elongation is due to the stimulation of the G_1 to S transition. No significant difference was detected in the inhibitory effect of CTK on LR formation between wild-type plants and mutants defective in auxin response or transport. In addition, exogenously applied auxin at different concentrations could not rescue the CTK-mediated inhibition of LR initiation. Our data suggest that CTK and auxin might control LR initiation through two separate signaling pathways in *Arabidopsis*. The CTK-mediated repression of LR initiation is transmitted through the two-component signal system and mediated by the receptor CRE1.

Keywords: *Arabidopsis* — Cell cycle — Cytokinin — Lateral root initiation.

Abbreviations: AG, after germination; 6-BA, 6-benzylaminopurine; CDK, cyclin-dependent kinase; CTK, cytokinin; DAG, days after germination; GFP, green fluorescent protein; GUS, β-glucuronidase; HU, hydroxyurea; KT, kinetin; LR, lateral root; LRP, lateral root primordium; NAA, α-naphthaleneacetic acid; NPA, *N*-1-naphthylphthalamic acid; PI, propidium iodide; QRT–PCR, quantitative real-time PCR; ZT, trans-zeatin.

Introduction

Cytokinins (CTKs) have long been recognized as essential plant hormones that influence almost all aspects of plant development and physiology (Mok 1994). The inhibitory effect of CTK on root growth was first reported by Skoog and Miller (1957). It was found that callus placed on medium with a low ratio of CTK to auxin usually produces few shoots and more roots, and vice versa. Applying exogenous CTKs or changing the endogenous CTK levels in mutants or transgenic plants results in the alteration of plant root development (Baskin et al. 1995, Beemster and Baskin 2000, Werner et al. 2001, Werner et al. 2003). Two aspects of inhibitory effects of CTKs on root growth have been reported, including the impeding of primary root elongation and the regulation of lateral root (LR) initiation (Baskin 1995, Beemster and Baskin 2000, Werner et al. 2001, Debi et al. 2005, Werner et al. 2003).

In *Arabidopsis*, LRs arise from asymmetric transverse divisions of pericycle founder cells, pericycle cells in cell files adjacent to the two xylem poles (Dubrovsky et al. 2001). Some of the newly divided cells undergo further periclinal divisions to form a dome-shaped lateral root primordium (LRP) gradually. The LRP then grows through the epidermis of the primary root to form a functional LR meristem (Malamy and Benfey 1997). Overexpression of *AtCKX* genes in transgenic *Arabidopsis* seedlings leads to a reduced level of endogenous CTK and increased numbers of branched roots, suggesting that CTK acts as a negative regulator in LRP initiation (Werner et al. 2003). Similarly, CTK was shown to be a negative regulator of LR initiation in rice, while it promotes the elongation of LR (Debi et al. 2005).

Auxin plays an essential role in LR formation and development (Celenza et al. 1995, Casimiro et al. 2001, Bhalerao et al. 2002). Several lines of evidence indicate that auxin regulates LR development in at least three phases: LR initiation, LRP establishment and the reactivation of LR meristem. Basipetal polar auxin transport is required for the first formative divisions in the pericycle, and the shoot-derived auxin regulates the outgrowth of LRs (Casimiro et al. 2001, Bhalerao et al. 2002). It has been reported that the frequency and position of LR initiation are determined by the amount and direction of auxin flow in the root. Auxin helps more pericycle cells re-entering the cell cycle to pass the G_1 to S transition through down-regulating the transcription of *Kip related protein (KRP)2*. Therefore, the G_1 to S checkpoint is the target of auxin in the case of LR initiation (Himanen et al. 2002).

Previous studies have demonstrated the existence of synergistic, antagonistic and additive interactions between CTK and auxin (Coenen and Lomax 1997). The inhibitory role of auxin in the activity of axillary buds in the shoot (Cline 1991) and the stimulatory role in LR and

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adventitious root formation (Casimiro et al. 2001, Falasca et al. 2004) can be antagonized by CTKs (Lloret and Casero 2002). It is well known that exogenous auxin or CTK can inhibit root elongation in plants (Eliasson et al. 1989, Baskin et al. 1995, Beemster and Baskin 2000). In root vascular development, only in the presence of IAA, CTK could stimulate vascular differentiation and regeneration (Aloni 1995). The roles of the two hormones in the control of the cell cycle in undifferentiated cells are synergistic due to both of them having positive regulatory effects on the expression of the Cdc2 kinase. In LRP, on the other hand, the roles of the two hormones are antagonistic. While auxin up-regulates the Cdc2 kinase, CTK acts as a negative regulator (Coenen and Lomax 1997).

Although it has been known that CTKs inhibit LR initiation, the specific cell cycle stage of CTK-induced repression and the pathway mediating the inhibitory signal, however, remain unknown. Here we report that CTK impedes early LR initiation by blocking the transition from G_2 to M phase in the pericycle founder cells. Our data reveal that the inhibition by CTK of LR initiation is mediated by *CRE1* via a two-component system of CTK signaling, which is not directly dependent on the auxin signaling pathway in *Arabidopsis*.

Results

Exogenous CTK inhibits LR formation and primary root elongation but promotes root hair growth

To investigate the effect of exogenous CTK on the root structures, three types of exogenous CTKs, i.e. 0.08 μM 6-benzylaminopurine (6-BA), 0.1 μM trans-zeatin (ZT) and 0.2 μM kinetin (KT) were applied to the growth medium. Three genotypes of *Arabidopsis* seedlings, i.e. Columbia (Col), Landsbergerecta (Ler) and Wassilewskija (Ws), were used in the experiment. As shown in Fig. 1A–D, compared with the seedlings growing on control (6-BA-free) medium, the application of all types of CTKs repressed the formation of LR and adventitious root and the elongation of primary roots, while it enhanced root hair development in the seedlings of Col, as well as the other genotypes (data not shown). To assess the dosage effect of exogenous CTKs on primary root and LR development, a range of 6-BA concentrations of 0, 0.02, 0.05, 0.06, 0.08 and 0.1 μM were tested. The length of primary roots was decreased along with the increase in 6-BA concentration (Fig. 1E). The formation and growth of LRs were completely repressed at an CTK concentration of 0.08 μM (Fig. 1E, F).

Exogenous CTK inhibits the divisions of pericycle founder cells

To determine which stage of LR formation was blocked by the CTKs, we examined the β-glucuronidase (GUS) expression pattern of transgenic lines harboring *CYCB1;1::GUS* (*CYCB1;1* promoter fusion with the GUS reporter line) under 6-BA treatment. This line was reported to be a good marker to visualize the first round of cell division in LR founder cells and to determine the site of LRP initiation and development (Beeckman et al. 2001, Himanen et al. 2002). While under normal growth condition, GUS was expressed in the root apical meristem and LRPs (Fig. 2A–C), no GUS staining was detected in the region around the pericycle cells (Fig. 2E, F) other than the faint staining in apical meristem of the primary root after 6-BA treatment (Fig. 2D). This suggests that the inhibition of the formation of LRs by CTKs is at the first divisions of pericycle founder cells.

The *IAA2::GUS* reporter line, which uses the auxin-inducible promoter *IAA2* to drive the GUS reporter gene (Swarup et al. 2001), was used to detect the accumulation of auxin signaling in root and the developmental phases of LRP through GUS staining. Under 6-BA-free conditions, GUS expression could be detected in the entire vascular cylinder and in the apical meristems of primary root and LRPs (Fig. 2G–I). A longitudinal section of the GUS-stained LRPs of the reporter line clearly showed the first anticlinal division (Fig. 2M) and the developmental process of LRPs (Fig. 2M–O). No LRP formation and cell division in pericycle were detected in the roots of 6-BA-treated seedlings (Fig. 2K, L). As expected, the GUS staining in the root meristem of *IAA2::GUS* seedlings was reduced by exogenously applied CTK due to the antagonistic interaction between these two phytohormones (Fig. 2G, J). The GUS expression was mainly in the apical part of the primary root stele (Fig. 2L) but not in the basal part in the 6-BA-treated *IAA2::GUS* seedlings (Fig. 2K).

To determine whether the CTK-induced block is caused by re-specifying the identity of the founder cells in xylem pole pericycle tissues, an *Arabidopsis* GAL4–GFP (green fluorescent protein) enhancer trap line, J0121, was used. In J0121 lines, GFP was expressed specifically in the files of pericycle cells competent to become LR founder cells but is no longer expressed once LRP enters the dividing stage I (Laplaze et al., 2005). In this case, the GFP expression could be easily detected in the pericycle cells of the differentiation region when the J0121 seedlings were grown on either 6-BA-free (Fig. 2P) or 6-BA-containing medium (Fig. 2Q). The lengths of the cortex cells, however, were decreased when grown on 6-BA-containing medium (Fig. 2P, Q), which is in agreement with the previous observation that 6-BA treatment reduced primary root length. Taken together, the results show that 6-BA treatment blocked the cells from undegoing cell division rather than re-specifying the identity of pericycle founder cells.

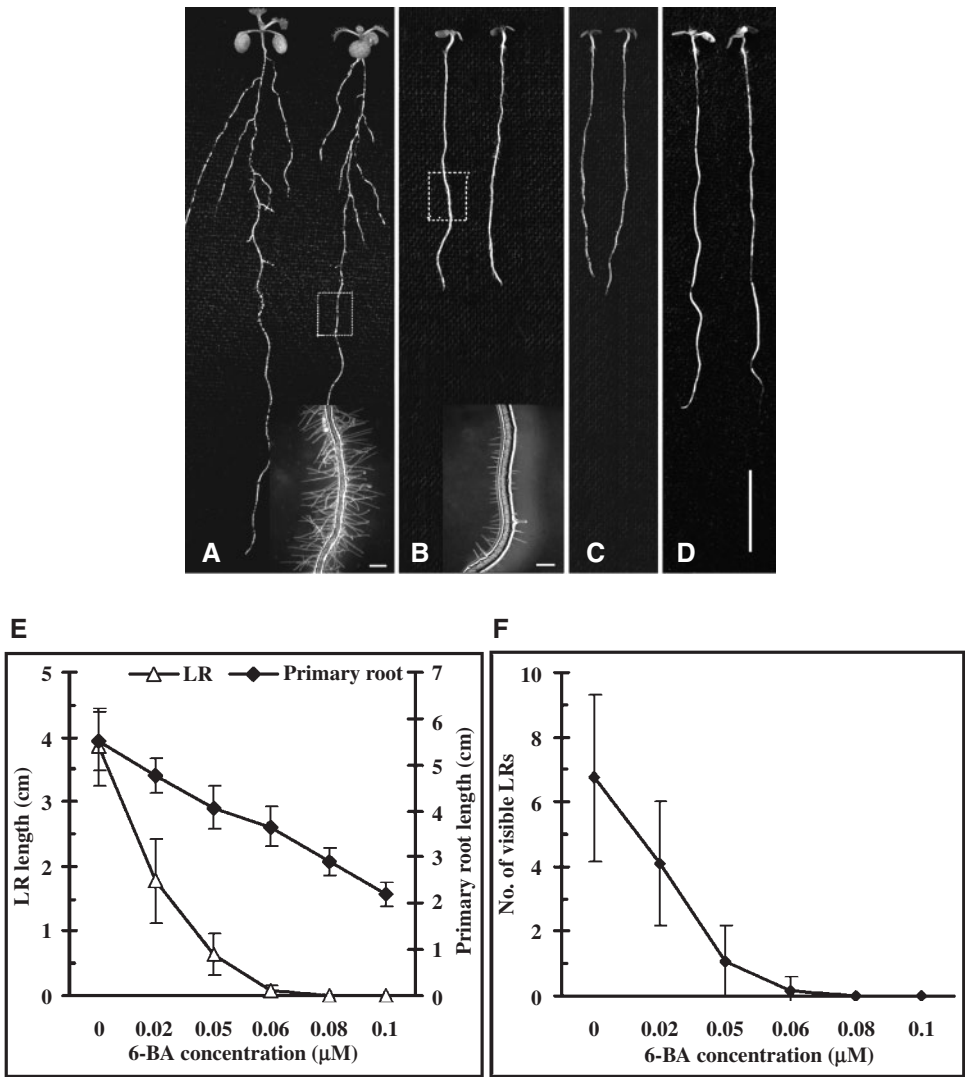


Fig. 1 The inhibitory effects of CTK on primary root elongation and LR formation, and the stimulatory effect on root hair growth. (A–D) Wild-type (Col) seedlings were grown on control (CTK-free) medium (A), and medium containing 0.08 μM 6-BA (B), 0.1 μM trans-zeatin (C) or 0.2 μM kinetin (D) at 10 DAG. Dashed line boxes in (A) and (B) indicate the region of the primary roots from which the close-up images were obtained. Bars in (D) = 1.0 cm for (A–D) and 0.5 mm for the close-up images. (E, F) The dosage inhibitory effect of CTK was shown by testing the effect of different 6-BA concentrations on the length of primary root (E, filled point), the number of LRs (F) and the total length of visible LRs (E, open point) in Col seedlings at 10 DAG. Each data point represents an average of 18 seedlings. Error bar = SD.

Exogenous CTK has no inhibitory effect on the development of initiated LRPs and promotes LR elongation

To determine whether the presence of 6-BA affects the development of initiated LRP into mature LR, the *IAA2::GUS* seedlings were transferred from the 6-BA-free medium at 0, 4, 6 and 14 days after germination (DAG) to the 6-BA-containing medium. As shown in Fig. 3A, the CTK treatment inhibited the initiation of LR. The earlier the exogenous CTK was applied, the fewer was the number of LRs. The numbers of LRPs and LRs in the treatments were calculated before and after the seedlings were transferred to 6-BA-containing medium. The results showed that all initiated LRPs developed into mature LRs regardless of the 6-BA treatment (Fig. 3B), indicating that exogenous CTK had no inhibitory effect on the development of initiated LRPs.

It was also found that the LR length was longer in the seedlings transferred to the 6-BA-containing medium at 4 DAG than those without CTK treatment (Fig. 3A). As shown in Fig. 3C, the LR length increased steadily with increasing concentration of applied 6-BA or ZT. The LR length was greatest at 0.08 μM 6-BA, indicating that exogenous CTK could stimulate LR elongation.

Fig. 2

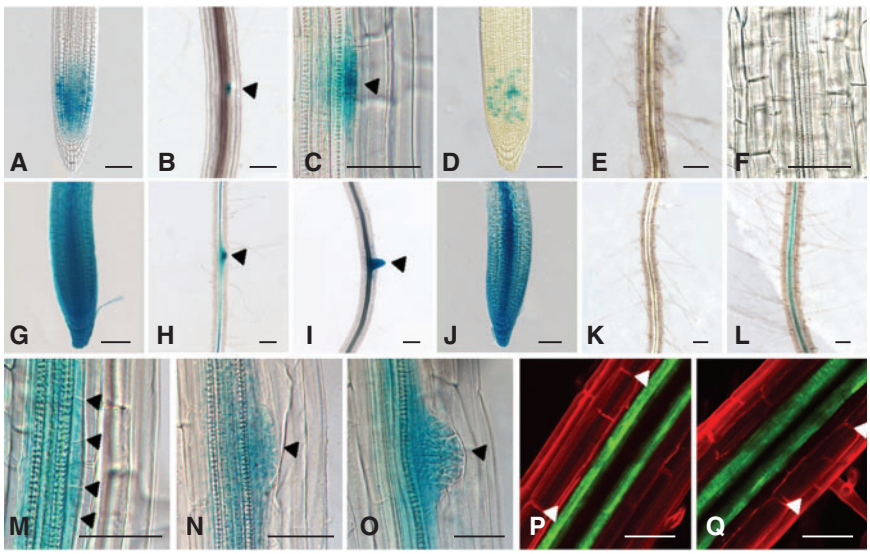
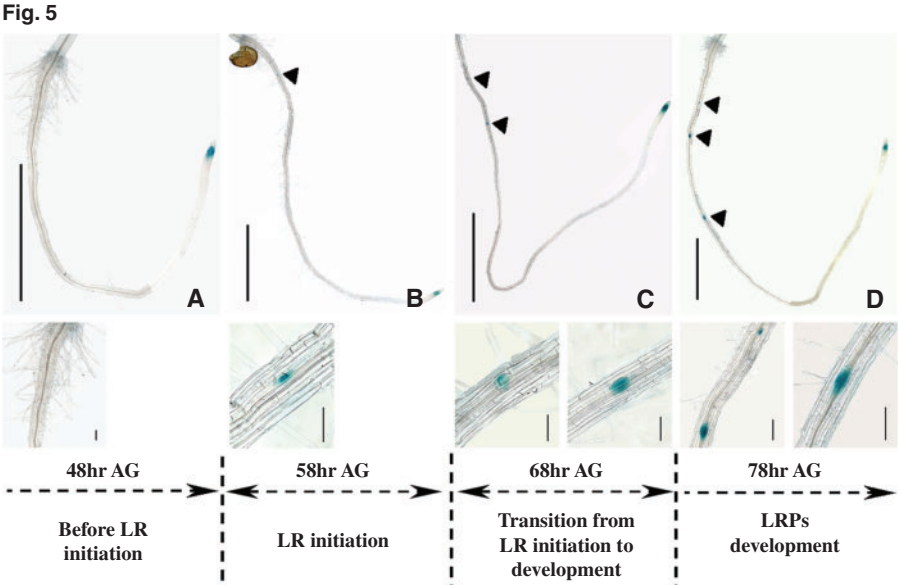


Fig. 5



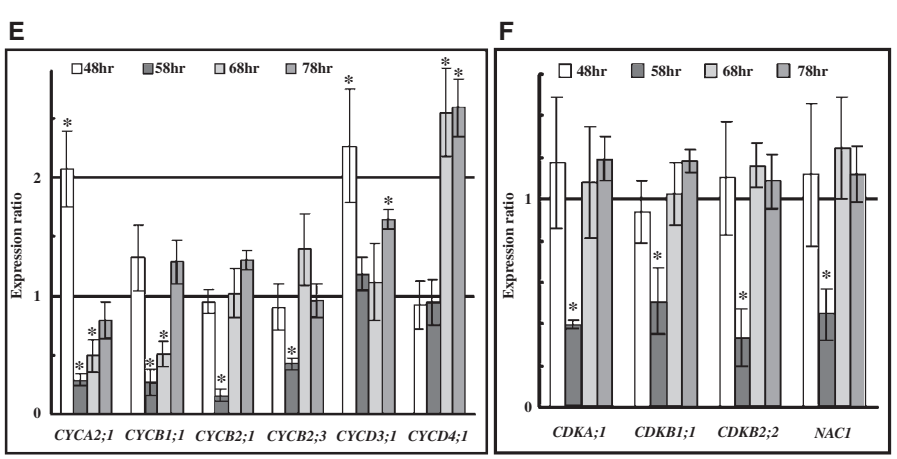


Fig. 2 Exogenous CTK blocks the pericycle founder cells from undergoing cell divisions during LR initiation and alters the distribution of endogenous auxin in the root. (A–F) *CYCB1;1::GUS* expression in roots of 10 DAG seedlings on 6-BA-free and 6-BA-containing medium. (A) Root tip of *CYCB1;1::GUS* on the 6-BA-free medium. (B) The GUS activity (arrowhead) of an LRP in a *CYCB1;1::GUS* root on 6-BA-free medium. (C) A detailed view of (B). The arrowhead indicates a developing LRP. (D) Root tip of *CYCB1;1::GUS* on 6-BA-containing medium. (E) No GUS staining in a *CYCB1;1::GUS* root after application of 6-BA. (F) A detailed view of (E). (G–O) The expression of marker line *IAA2::GUS* in roots of 10 DAG seedlings on the 6-BA-free and 6-BA-containing medium. (G) Root tip of the *IAA2::GUS* line on 6-BA-free medium. (H, I) GUS expression in the entire vascular cylinder and LRPs (arrowhead) of the *IAA2::GUS* line on 6-BA-free medium. (J) Root tip of *IAA2::GUS* on 6-BA-containing medium. (K) No GUS staining in the basal part of the primary root stele in the 6-BA-treated *IAA2::GUS* line. (L) Strong GUS staining in the apical part of the primary root stele in the 6-BA-treated *IAA2::GUS* line. (M) An LRP undergoing the first anticlinal divisions in the pericycle founder cells from a seedling grown on 6-BA-free medium. Arrowheads indicate the cell intervals by the division. (N) An LRP having three cell layers from a seedling on 6-BA-free medium. The arrowhead indicates the site of the LRP. (O) An LRP having five cell layers from a seedling on 6-BA-free medium. The arrowhead indicates the site of LRP. (P, Q) The expression of J0121 line on 6-BA-free (P) and 6-BA-containing medium (Q). J0121 seedlings at 10 DAG were stained with 10 μM PI solution and imaged by a confocal scanning system using double channels. The portion between two arrowheads shows a cortical cell. Bars = 100 μm in (A, B, D, E, G–L), and 50 μm in (C, F, M–Q).

Fig. 5 Histochemical GUS staining pattern of *CYCB1;1::GUS* at different developmental stages and examination of the response of genes to exogenous CTK. (A–D) Roots of *CYCB1;1::GUS* (grown on 6-BA-free medium) at four developmental time points, including 48 h AG (A), 58 h AG (B), 68 h AG (C) and 78 h AG (D). Arrowheads indicate the LRP formation sites in the basal half of the root. The detailed views of these sites are under the corresponding images (A–D). The developmental stages as predicted from LRP formation profiles in (A–D) are indicated by arrowheads as: 'Before LR initiation', 'LR initiation', 'Transition from LR initiation to development' and 'LR development'. Bars = 1 mm in (A–D) and 50 μm in the detailed views. (E, F) Results are the ratio of the relative expression after 6-BA treatment of the corresponding control. These analyses are based on QRT–PCR, and normalized to the *ACTIN* transcript level. The asterisks indicate significant expression responses compared with control. (E) The responses of cyclins expressed specifically at the G_1 to S (*CYCD3;1* and *CYCD4;1*) and G_2 to M transition (*CYCA2;1*, *CYCB1;1*, *CYCB2;1* and *CYCB2;3*) to exogenous CTK at four time points. (F) The responses of three CDKs (*CDKA;1*, *CDKB1;1* and *CDKB2;2*) and *NAC1* to exogenous CTK at four time points. Error bar = SD.

CTK blocks the G_2 to M transition in the founder cell activation

The transitions from G_1 to S and G_2 to M are the major checkpoints of the eukaryotic mitosis cell division cycle. Hydroxyurea (HU) was reported to block the cell cycle during the G_1 to S transition by inhibiting ribonucleotide diphosphate reductase (Planchais et al. 2000). Also, the *CYCB1;1* gene is expressed specifically at the G_2 to M transition during the cell cycle (Doerner et al. 1996). Once the cell division in the pericycle founder cells has occurred, the cell cycle activity could be visualized by the *CYCB1;1::GUS* line. To investigate the point of arrest in the cell cycle due to exogenous CTK, seedlings of the *CYCB1;1::GUS* line were germinated and grown for 3 d on 0.08 μM 6-BA, then transferred to medium with or without (as control) 100 mM HU for 48 or 72 h. GUS expression of the seedlings was monitored.

In the basal part of the roots of *CYCB1;1::GUS* seedlings from controls, GUS expression could be detected in LRPs (Fig. 4A, B), indicating the normal initiation of LRP. In the seedlings treated with HU for 48 or 72 h, no LRP was found although a few GUS sectors were detected in LR initiation sites (Fig. 4C). Closer examination of these sectors revealed that the GUS staining was restricted in or adjacent to a few founder cells, indicating that the founder cells had undergone a division (Fig. 4D, E). In contrast to the control plants, no more developed LRPs were detected. These results suggest that the exogenous CTK blocked the cell cycle in pericycle founder cells at the transition from G_2 to M.

CTK regulates the cell cycle via reducing cyclins expressed at the G_2 to M transition during LR initiation and inducing D-type cyclins during LR development

The *CYCB1;1::GUS* line was used to visualize cell cycling activity in the pericycle and determine the appropriate sampling time point. As shown in Fig. 5, the LRP has not initiated until 58 h after germination (AG) (Fig. 5A, B). At 58 h AG, one LRP in about 65% of seedlings was undergoing the first anticlinal division (Fig. 5B). At 68 h AG, two LRPs were being initiated in about 75% of seedlings (Fig. 5C). At 78 h AG, about 80% of seedlings had developed three LRPs (Fig. 5D) or two LRPs including one ready to emerge (data not shown). At ~58 h AG, cell divisions mainly contributed to pericycle cell de-differentiation to initiate LRPs, to be the 'LR initiation' stage. At ~78 h AG, more cells entered divisions to produce manifold LRPs. This stage, therefore, is the stage of 'LR development'.

The effect of CTK on the expression of cyclin and cyclin-dependent kinase (CDK) genes in the maturation region of roots was analyzed using quantitative real-time reverse transcription–PCR (QRT–PCR). QRT–PCR was performed on the RNA extracted from the seedlings treated or not with 6-BA at the above four time points. As shown in Fig. 5E, the 6-BA treatment at 48 h AG did not cause a

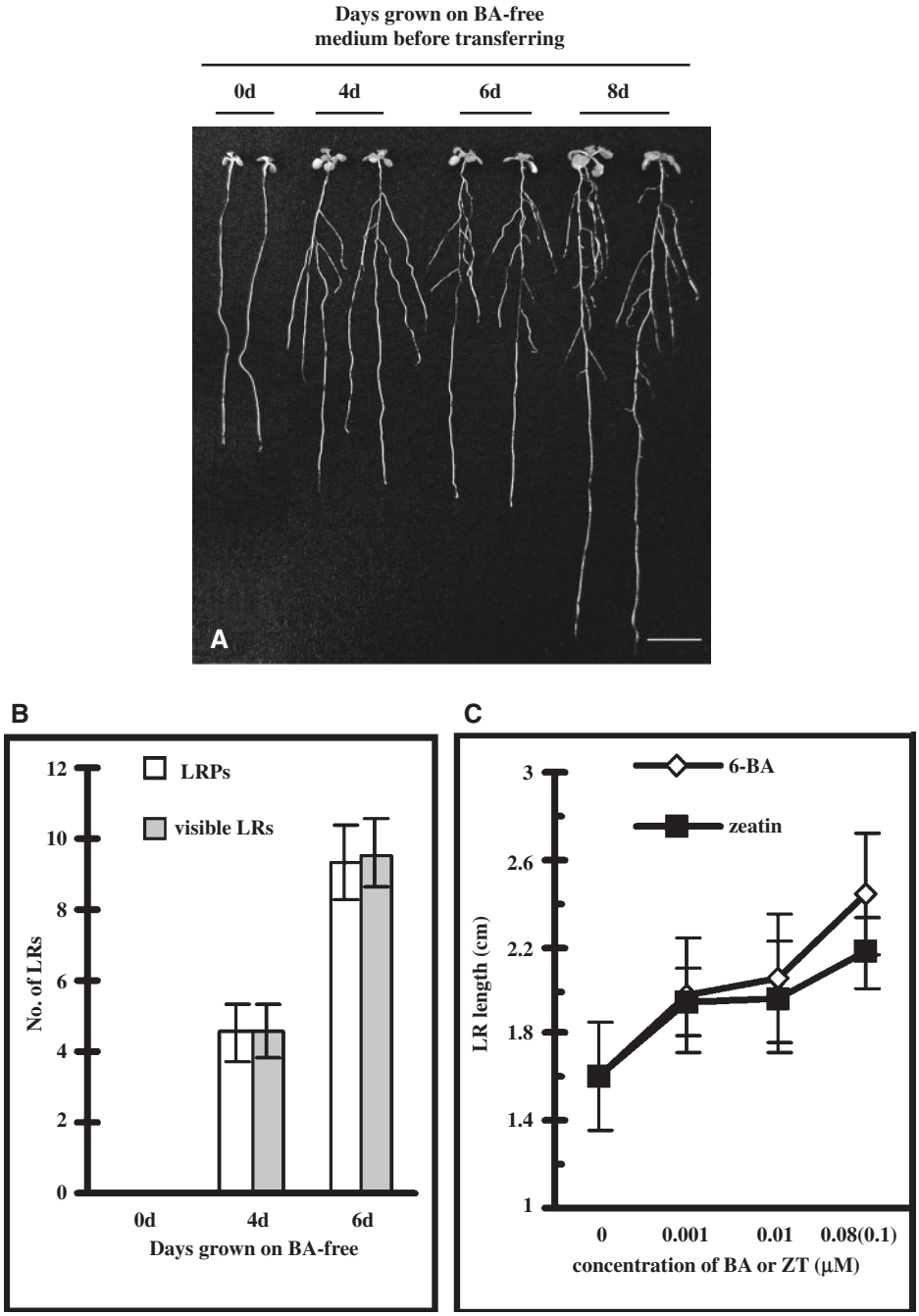


Fig. 3 CTK has no inhibitory effect on the development of the initiated LRPs and has a stimulatory effect on LR elongation. (A) *IAA2::GUS* marker line seedlings grown on 6-BA-free medium for 0, 4, 6 and 14 DAG were transferred to medium containing 0.08 μM 6-BA for an additional 14, 10, 8 and 0 d. Bar = 1.0 cm. (B) Comparison of the number of LRPs and LRs in the *IAA2::GUS* line before and after the seedlings were transferred from 6-BA-free medium into the medium containing 0.08 μM 6-BA at 0, 4 and 6 DAG. Each data point represents the average of 18 seedlings. Error bar = SD. (C) Col seedlings grown on 6-BA-free medium for 4 d were transferred to different concentrations of 6-BA (0, 0.001, 0.01 and 0.08 μM) or ZT (0, 0.001, 0.01 and 0.1 μM) for an additional 10 d. Then the two longest LRs in each seedling were measured. Each data point represents the average of 12 seedlings. Error bar = SD.

significant change in the expression of most of the G_2 to M transition phase-specific cyclins, including *CYCB1;1, CYCB2;1* and *CYCB2;3* (Mironov et al. 1999; Himanen et al. 2002; De Veylder et al. 2003). The A-type G_2 to M transition phase-specific cyclin, *CYCA2;1* (Mironov et al. 1999, De Veylder et al. 2003) was induced at 48 h AG but was down-regulated at the other time points by exogenous 6-BA treatment. Interestingly, the application of 6-BA at 58 h AG significantly reduced the expression of *CYCA2;1*, *CYCB1;1*, *CYCB2;1* and *CYCB2;3*. At 68 h AG, 6-BA treatment did not change the expression of *CYCB2;1* and *CYCB2;3*, while it repressed the expression of *CYCB1;1*. At the 78 h seedling stage, expression of these *CYC* genes was not changed by the 6-BA treatment (Fig. 5E). Similarly, the effect of 6-BA on the expression of G_1 to S and S phase-specific D-type cyclins (Mironov et al. 1999, Himanen et al. 2002, De Veylder et al. 2003), *CYCD3;1* and *CYCD4;1* was analyzed.

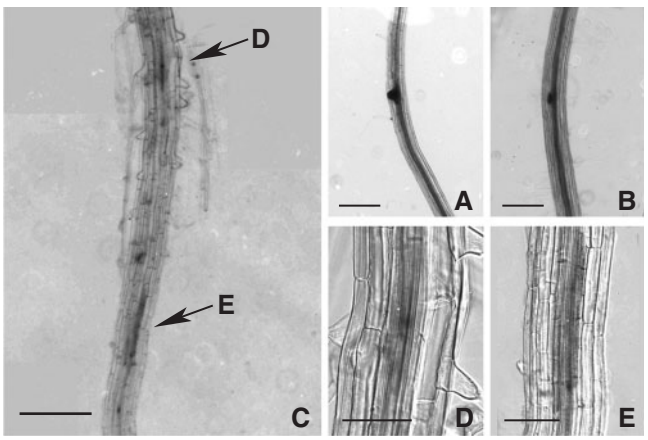


Fig. 4 Exogenous CTK blocks the cell cycle in the pericycle founder cells at the G_2 to M transition. *CYCB1::GUS* lines grown on medium containing 0.08 μM 6-BA for 3 DAG were transferred to medium containing 100 mM HU and control (HU-free) for 48 or 72 h. (A, B) LRP of control plants, indicating normal cell cycle progression after shifting. (C–E) The cell divisions in the pericycle cells of the root of HU-treated plants. Arrows in (C) indicate the sites of cell division depicted in the close-up image in (D) and (E), respectively. Bars = 100 μm in (A–C) and 50 μm in (D and E).

The expression of *CYCD3;1* and *CYCD4;1* was induced by the application of 6-BA at 48 and 78 h AG, and at 68 and 78 h AG, respectively, while the expression remained constant when 6-BA was applied at the other two points (Fig. 5E).

Expression of three CDKs, *CDKA;1*, *CDKB1;1* and *CDKB2;2*, was down-regulated about 50–70% by 6-BA treatment at the seedling stage of 58 h AG, while 6-BA treatment at the other three points did not alter their expression (Fig. 5F). The expression pattern was similar to that of the transcription activator, *NAC1* (Xie et al. 2000) (Fig. 5F). We also found that at 58 h AG, two C-type *CDKs*, *CDKC;1* and *CDKC;2*, were not regulated by CTK treatment, the same as *ALF4* and *TIR1*, two genes involved in LR initiation (Ruegger et al. 1998, DiDonato et al. 2004) (data not shown).

CTK-mediated inhibition of LR initiation is not directly dependent on the auxin signaling pathway

To investigate whether the CTK-mediated repression of LR initiation can be rescued by auxin, seedlings of *Arabidopsis* (Col) were grown on medium containing 0.08 μM 6-BA for 10 d, and then transferred to medium containing either 0.1 μM α-naphthaleneacetic acid (NAA), 0.1 μM IAA or 0.08 μM 6-BA for an additional 10 d. No LRs were found on the primary roots grown within the first 10 d under 6-BA treatment, while LRs developed on hormone-free medium or medium containing NAA or IAA after transfer (Fig. 6A). Moreover, we carried out an experiment using medium containing 0.1 μM auxin (IAA or NAA), with or without 0.08 μM 6-BA. When 0.1 μM auxin (IAA or NAA) was added to the growth medium, the development of the LRs was promoted compared with those on hormone-free medium (Fig. 6B–D), while no visible LRs were found in the seedlings in the medium containing 6-BA or 6-BA plus NAA or IAA (Fig. 6E–G). Increasing the NAA (or IAA) concentration up to 1 μM in the BA-containing medium (0.08 μM 6-BA) showed a similar inhibition of LR initiation (data not shown).

To determine whether the CTK-induced block of LR initiation is caused by the defect in response to the auxin signal, four mutants defective in auxin response or transport, i.e. *tir1-1*, *axr4-2*, *eir1-1* and *aux1-7* (Hobbie and Estelle 1995, Luschnig et al. 1998, Ruegger et al. 1998, Marchant et al. 2002), were grown on either 6-BA-free or 6-BA-containing medium. It is known that mutation of the auxin receptor gene *TIR1* resulted in the reduction of LR proliferation (Ruegger et al. 1998, Dharmasiri et al. 2005, Kepinski and Leyser 2005). The mutation in the *AXR4* gene, which regulates auxin polar transport via the accessory protein of influx facilitator AUX1, results in a reduced number of LRs and a defect in root gravitropism (Hobbie and Estelle 1995, Dharmasiri et al. 2006). In this experiment, none of the above mutants produced LRs on the medium containing 0.08 μM 6-BA at 10 DAG (Fig. 6I, K, M, O), while clearly visible LRs on 6-BA-free medium were detected (Fig. 6H, J, L, N), suggesting that the genes which are mutated in the above lines are not involved in the CTK-mediated inhibition of LR formation. Taken together, our observations suggest that CTK and auxin might control LR initiation through two separate signaling pathways in *Arabidopsis*.

CTK-induced LR initiation arrest is mediated by CRE1 (AHK4)

The CTK signaling pathway in *Arabidopsis* has been well documented as a two-component signaling system (Lohrmann and Harter 2002). The T-DNA insertion mutants on three CTK receptors, *cre1-12*, *ahk2-2* and *ahk3-3*, and the corresponding double mutants (*cre1-12*/*ahk2-2*, *cre1-12*/*ahk3-3* and *ahk2-2*/*ahk3-3*) (Higuchi et al. 2004) were used to determine whether the impeding effect of CTK on LR initiation is mediated by the two-component signaling pathway. The *ahk2-2* and *ahk3-3* mutants, and the *ahk2-2*/*ahk3-3* double mutant showed a similar phenotype of no visible LRs as the wild type on 0.08 μM 6-BA at 10 DAG, suggesting that these two genes are not involved in the CTK-induced inhibitory effect (Fig. 7A, C, D, G). In contrast, the *cre1-12* single mutant (Fig. 7B), and the

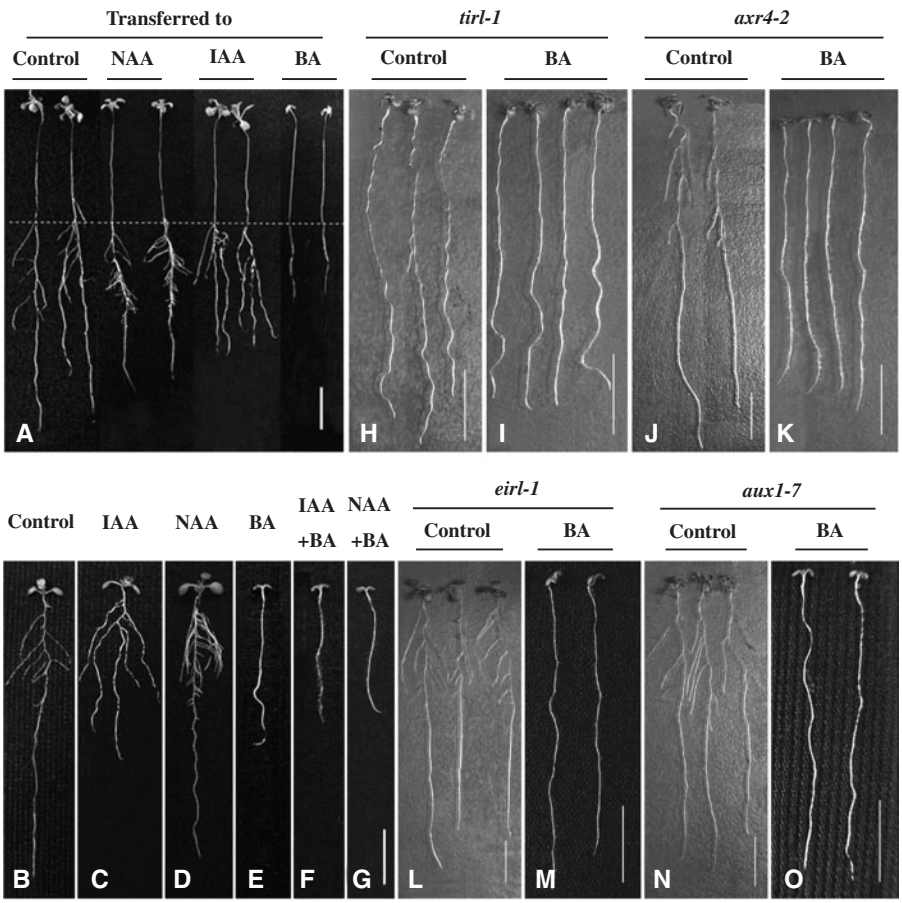


Fig. 6 CTK-induced inhibition of LR initiation is not directly dependent on auxin signaling. (A) The inhibition by CTK of LR initiation is irreversible, even if IAA or NAA is added to the medium. Col seedlings grown on medium containing 0.08 μM 6-BA for 10 DAG were transferred to control (hormone-free) medium, or medium containing 0.1 μM NAA or 0.1 μM IAA as well as 0.08 μM 6-BA for an additional 10 d. The dashed line indicates the root segment before (above the line) and after (below the line) shifting. (B–G) The phenotypes of Col seedlings grown on control medium (B) or medium containing 0.1 μM IAA (C), 0.1 μM NAA (D), 0.08 μM 6-BA (E), 0.1 μM IAA + 0.08 μM BA (F) and 0.1 μM NAA + 0.08 μM BA (G) at 10 DAG. (H–O) CTK-induced inhibitory effect on the mutants defective in auxin response or transport. (H, I) Morphology of *tir1-1* seedlings at 10 DAG on 6-BA-free medium (H) and medium containing 0.08 μM 6-BA (I). (J, K) Phenotypes of *axr4-2* seedlings at 10 DAG on 6-BA-free medium (J) and medium containing 0.08 μM 6-BA (K). (L, M) Morphology of *eir1-1* seedlings at 10 DAG on 6-BA-free medium (L) and medium containing 0.08 μM 6-BA (M). (N, O) Phenotypes of *aux1-7* seedlings at 10 DAG on 6-BA-free medium (N) and medium containing 0.08 μM 6-BA (O). Bars = 1.0 cm.

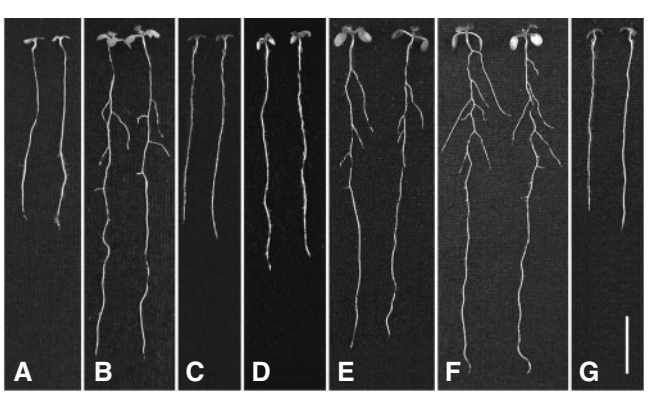


Fig. 7 Morphology of CTK receptor mutants and their double mutants, showing that the signal of CTK-induced inhibition is transduced via receptor CRE1. Seedlings of wild-type Col (A), single mutants *cre1-12* (B), *ahk2-2* (C) and *ahk3-3* (D), and double mutants *cre1-12/ahk2-2* (E), *cre1-12/ahk3-3* (F) and *ahk2-2/ahk3-3* (G) grown on medium containing 0.08 μM 6-BA at 10 DAG. Bar = 1.0 cm for (A–G).

cre1-12/ahk2-2 and *cre1-12/ahk3-3* double mutants (Fig. 7E, F) showed normal LRs and primary root development in the presence of 6-BA at 10 DAG, indicating the crucial role of *CRE1*. The insensitive phenotype of *cre1-12* to the CTK treatment is similar to that of a loss-of-function mutant *cre1-1* and a T-DNA insertion line *cre1-2* (data not shown). Compared with *cre1-12/ahk2-2*, the double mutant *cre1-12/ahk3-3* showed an increased number of LRs and length of primary roots (Fig. 7E, F), suggesting that AHK3 is synergistic to the CRE1-mediated CTK inhibition of LR initiation. Taken together, the results reveal that the CTK-induced inhibition of LR initiation is mediated by CTK receptor CRE1.

Discussion

As LR initiation is the first pivotal checkpoint in LR development, it is conceivable that this process is rigorously modulated by hormones and environmental factors. Using the auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA), Casimiro et al. (2001) concluded that the basipetal movement of auxin activates the LR initiation of the first formative divisions in the root xylem pericycle founder cells. The data presented in this case show the effect of CTK on this key developmental checkpoint. The unchanged competence of xylem pole pericycle cells and the fact that no divided cells were detected in

the pericycle under CTK treatment indicate that CTK impedes the founder cells from re-entering division. In addition, using HU which blocks the cell cycle at the G1 phase (Planchais et al. 2000), we found that exogenous CTK blocks the G2 to M transition in pericycle founder cell activation.

It has been documented that elevated CTK levels inhibit the root elongation of *Arabidopsis* seedlings, because of a decrease in the number of dividing cells and the size of the meristem (Beemster and Baskin 2000, Werner et al. 2003). In this study, 6-BA treatment repressed GUS expression in the root apical meristem of the *CYCB1;1::GUS* marker line, indicating that the reduced cell divisions in the meristems, with a decrease of cortex cell size in 6-BA-treated seedlings, may cause the inhibition by CTK of primary root elongation.

It has been reported that plant hormones affect the cell cycle directly and mainly at the transcriptional level (Stals and Inzé 2001). QRT–PCR results show that CTK inhibits LR initiation through down-regulating genes involved in the G_2 to M transition, including *CYCA2;1*, *CYCB1;1*, *CYCB2;1*, *CYCB2;3*, *CDKB1;1* and *CDKB2;2* (Mironov et al. 1999, Himanen et al. 2002, De Veylder et al. 2003). In plants, the G_2 to M transition in the cell cycle is activated and regulated by the complex of CDKs (A or B) and A- or B- cyclins (Magyar et al. 1997, Porceddu et al. 2001). Although the expression of the above cyclins and CDKs is not completely repressed by exogenous CTK, the synchronous down-regulation in the expression of cyclins and CDKs probably inhibited the formation of CDK–cyclin complex and the transition of the cell cycle from G_2 to M phase. The exogenously applied CTK did not affect the expression of the G_1 toS transition- and S phase-specific cyclins, *CYCD3;1* and *CYCD4;1*, during LR initiation. The CTK application, however, remarkably induced the expression of the above cyclins during LRP development, supporting the earlier report that CTK activates cell division by stimulating the G_1 to S cell cycle transition (Riou-Khamlichi et al. 1999). *NAC1*, as a transcription factor, functions downstream of the auxin signal cascade, and is involved in the control of LR initiation in the process of LR development (Xie et al. 2000). Our data suggest that during LR initiation it is down-regulated by exogenous CTK. A hypothesis is drawn up that NAC*1* may be a factor mediating the CTK-induced inhibitory signal. Further specific physiological and biochemical evidence is needed to reveal the regulation system.

CTK is a well known phytohormone antagonistic to auxin in many aspects of plant growth. In this study, through the *IAA2::GUS* marker line, we observed that *IAA2* expression was reduced by treatment with CTK. However, the following three lines of evidence showed that auxin might be not directly involved in the CTK-mediated LR inhibition. First, different concentrations of exogenous auxins could not rescue the CTK-induced inhibitory effect on LR initiation. Secondly, mutants defective in auxin transport (*eir1-1 aux1-7* and *axr4-2*) and in auxin response (*tir1-1*) maintained the sensitivity to CTK. Thirdly, CTK blocked the cell cycle in pericycle founder cells at the transition of G_2 to M. It is a different checkpoint from the G_1 to S transition that auxin promotes in the process of LR initiation (Himanen et al. 2002). In rice, the inhibitory effect of CTK on LR initiation could be effectively counteracted by auxin (Debi et al. 2005). The inconsistency in the effect of these two hormones on LR initiation may imply different regulation mechanisms in LR initiation between monocots and dicots.

Recently, it has been suggested that in root development, CTK and auxin have antagonistic roles, especially in LR formation (Aloni et al. 2006). However, our results show that auxin could not rescue the CTK-induced inhibitory effect on LR initiation. The CTK-induced inhibition and the auxin-induced stimulation of LR initiation regulate two different cell cycle checkpoints. Although the application of CTK and auxin results in the opposite phenotype, the two hormones might mediate the two separate regulatory processes during LR initiation. Moreover, our data indicate the stimulatory role of CTK in LR elongation and root hair development, and its repressive role in the elongation of primary root, which are similar to those of the auxin (Eliasson et al. 1989, Masucci and Schiefelbein 1994, Malamy and Benfey 1997, Pitts et al. 1998, Casimiro et al. 2001). In regulating the other processes of root development, such as root gravitropism and root vascular differentiation, these two hormones have synergistic and dependent effects (Aloni 1995, Aloni et al. 2006). Taken together, CTK and auxin may interact differently during root system development.

The visible LR development in the CTK receptor mutant, *cre1*, in the presence of exogenous CTK indicates the participation of a two-component signaling system and the crucial role of CRE1 in the CTK-induced repression of LR initiation. The additive effect observed in the double mutant *cre1-12*/*ahk3-3*, indicating that AHK3 has an accessorial role to CRE1-mediated CTK repression of LR initiation, suggests the redundant roles of the CTK receptors (Nishimura et al. 2004). Using the single, double and triple mutants produced by T-DNA insertion in *CRE1*, *AHK2* and *AHK3*, Higuchi et al. (2004) suggest that receptor CRE1 plays a pivotal role in CTK-induced inhibition of root development, while AHK2 or AHK3 have only minor effects, and the functions of the three receptors are redundant in the root. Our results on the functions of the CRE family genes in LR initiation are in agreement with this.

Materials and Methods

Plant materials

The genetic backgrounds of the marker lines and mutants used in the present study are as follows: *CYCB1*;1::*GUS*(C24), *IAA2::GUS*(Ws), J0121(C24), *tir1-1*(Col), *axr4-2*(Col), *aux1-7*(Col), *eir1-1*(Col), *cre1-1*(Ler), *cre1-12*(Col), *ahk2-2*(Col), *ahk3-3*(Col), *cre1-12*/*ahk2-2*(Col), *cre1-12*/*ahk3-3*(Col) and *ahk2-2*/*ahk3-3*(Col).

Plant growth conditions

Unless otherwise stated, the standard basic mineral compositions of all the growth media used in this study consisted of 200 mM KCl, 80 mM $MgSO_4$, 40 mM $CaCl_2$, 44 mM NaH_2PO_4, 2.3 mM $MnSO_4$, 1 mM KNO_3, 0.18 mM KI, 1.94 mM H_3BO_4, 0.28 mM $ZnSO_4$, 6 nM $CuSO_4$, 41.2 nM Na_2MoO_4, 4.3 nM $CoCl_2$, 3.6 mM Fe-EDTA and 0.5 g l^{-1} MES and 5 g l^{-1} sucrose. The standard BA + medium contained all the components of the control medium plus 0.08 μM 6-BA, or the indicated concentration. All growth media are adjusted to pH 5.7 using 1.0 M KOH, solidified with 1% agar–agar (Fisher Chemicals, Pittsburgh, PA, USA) and autoclaved at 121°C for 20 min. All phytohormones (6-BA, ZT, KT, IAA and NAA), NPA and HU used in this study were added to the medium when the medium temperature was about 50–60°C after autoclaving. The 6-BA was dissolved in dimethylsulfoxide (DMSO) to make 10 mM stock and then diluted using sterile ddH_2O to 0.1 mM. The HU stock of 2 M was dissolved in ddH_2O and sterilized through a filter film. Seedlings were grown vertically oriented on the surface of the agar-solidified medium in a plant growth cabinet (Percival Scientific, Perry, IA, USA) at 20°C with a 16 h/8 h light/dark regime and overhead lighting with an intensity of 300 μmol m^{-2} s^{-1}. The primary root and LR lengths were determined using the RHIZO program (Version 5.0A, Canada).

Seeds were surface sterilized with 10% sodium hypochlorite diluted with 95% ethanol for 10 min, then rinsed with 95% ethanol five times. Finally, they were dried in the flowhood for 20–30 min.

Histochemical analysis

The histochemical stain for GUS activity was performed as described (Jefferson et al. 1987). Briefly, seedlings were incubated in the staining buffer for 2 h at 37°C, then cleared and whole mounted in 50% glycerol prior to photography under

Table 1 Gene-specific primers used for QRT–PCR analysis

Gene	Accession No.	Primers
CYCA2;1	NM122447	F: 5′-GCTCCAGCTACTTGGTATCACTTG-3′ R: 5′-CCGCTGAAGCGGCAATTAGGGATGG-3′
CYCB1;1	NM119913	F: 5′-CGAGACGCCCCCACTACTTAGACTT-3′ R: 5′-CGGGTTTAGCTCGAATCGGACATGC-3′
CYCB2;1	NM127316	F: 5′-GCTGAAGCCCTCAGTTCCAAGTGCT-3′ R: 5′-CCTGTCGCCGCCCTCTGATGCAGAC-3′
CYCB2;3	NM101912	F: 5′-AGAAACTTAGTTGCGTGCCT-3′ R: 5′-AGCTTCTCCATATCTAGCACTTC-3′
CYCD3;1	NM119579	F: 5′-CCGTCTCCTCCTCTCTGTAATCTC-3′ R: 5′-CGTGACTCTTGCGTTTCTTG-3′
CYCD4;1	NM125940	F: 5′-GATTGTTGACGAAACTCCGAT-3′ R: 5′-ATTCTTTGGACTGATTTAGCCTC-3′
CDKA;1	NM114734	F: 5′-GCCACTCTCATAGGGTTCTCCATCG-3′ R: 5′-GGCATGCCTCCAAGATCCTTGAAGT-3′
CDKB1;1	NM115278	F: 5′-CATCAACCATCAACCAAATCTC-3′ R: 5′-CAGTCACGCAGTGTGGAAAC-3′
CDKB2;2	NM101946	F: 5′-GCGAAGGAAGAAGAAGAGTTGTC-3′ R: 5′-CGGTCCATCAAGAGATTGTG-3′
CDKC;1	NM121065	F: 5′-ACCATCCCACTAACAACGCACCAC-3′ R: 5′-GCTCTTTACTGTTGCCATCCGTATT-3′
CDKC;2	NM125895	F: 5′-GTAATTTTCAGATTCGGTTGA-3′ R: 5′-CCGAGATGATTTCATTTGGTTATAC-3′
ALF4	NM121141	F: 5′-AATAGATTGTTCGGACTACACGC-3′ R: 5′-CGACTTTGCTTCTCTCTTTACCTGC-3′
NAC1	NM202225	F: 5′-TCAATCACGCTGAAACTTCT-3′ R: 5′-GTTCTGTTCACTGACCCATCTA-3′
TIR1	NM116163	F: 5′-AAGTGCGACCAGATGTTTACTCT-3′ R:5′-ACAAACGGAAGCGAGTCAT-3′
ACTIN		F: 5′-TCTCTATGCCAGTGGTCGTA-3′ R:5′-CCTCAGGACAACGGAATC-3′

a light microscope (Zeiss AxioCam HRC, Oberkochen, Germany). For confocal scanning, roots were mounted in 10 μM propidium iodide (PI) solution for 5–10 min. The fluorescence excitation and image acquisitions were monitored by the LSM 510 Carl Zeiss Laser Scanning System (Carl Zeiss Co., Germany).

RNA preparation and quantitative real-time PCR (QRT–PCR)

In the analysis of the response of cell cycle-regulatory genes to exogenous CTK during LR formation, Columbia seedlings at 43, 53, 63 and 73 h AG were transferred to the regular growth medium (containing DMSO) and the medium containing 10 μM 6-BA for 5 h. The sampling time points were 48, 58, 68 and 78 h AG. Total RNAs were extracted specifically from the region of root competent to form LR (maturation region of root segments, the region above the root apical meristem and below the root–hypocotyl junction) for the QRT–PCR analysis. Each RNA sample was extracted with Trizol from about 500 excised root segments. cDNA was synthesized using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA).

QRT–PCR was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the SYBR green I master mix produced by the same company. Each 20 μl reaction system consists of 2× SYBR green I master mix, 2 μl of cDNA and 0.2 μl of forward and reverse primers (10 μM). PCR cycling conditions are as follows: 95°C for 2 min and 40 cycles of 95°C for 15 s, 58°C for 20 s and 72°C for 50 s. Fluorescence data are collected during the 72°C step and analyzed with Sequence Detector version 1.7 software (Applied Biosystems, Foster City, CA, USA). The forward (F) and reverse (R) primers used in this analysis are displayed in Table 1.

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