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Quantitative and cell type-specific transcriptional regulation of A-type cyclin-dependent kinase in *Arabidopsis thaliana*

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ABSTRACT

A-type cyclin-dependent kinase (CDKA) is an ortholog of yeast Cdc2/Cdc28p, and is assumed to have an essential function in plant growth and organogenesis. Previous studies revealed that its kinase activity is controlled by post-translational modifications, such as binding to cyclins and phosphorylations, but its transcriptional regulation is poorly understood. Here, we generated a promoter dissection series of *Arabidopsis* (*Arabidopsis thaliana*) *CDKA;1*, and used β-glucuronidase (*GUS*) gene-fused reporter constructs for expression analyses *in planta*. The results revealed two types of transcriptional control in shoots: general quantitative regulation and cell type-specific regulation. We identified a promoter region that promotes *CDKA;1* expression in the leaf epidermis, but not in the L1 layer of the shoot apical meristem. This region also directed abaxial side-biased expression, which may be linked to the adaxial/abaxial side specification. Another reporter construct showed that *CDKA;1* expression in the inner layers of leaves is controlled by a distinct regulatory region in the promoter. These results suggest that the transcriptional regulation of *CDKA;1* may play a key role in proper development of leaves by coordinating cell division and differentiation of different cell types.

Introduction

Cell proliferation in multicellular organisms must be temporally and spatially regulated to accomplish proper development. Plant cells are cemented with each other; thus, particularly in plants, the control of cell division plays an essential role in overall growth and organ formation. Since cell number and cell size are the determinants for organ shape and size, the relationship between cell proliferation and cell expansion has been an interesting question that has been under debate thus far. In general, dividing cells are relatively small, and they increase their volume by endoreduplication after exiting the mitotic cycle (Inzé and De Veylder, 2006). Endoreduplication is a modified form of the cell cycle wherein the M phase is skipped, indicating that during plant development, the regulation of cell cycle is vital for both cell proliferation and cell expansion.

The cell cycle is controlled by evolutionary conserved protein kinase complexes, which consist of a cyclin-dependent kinase (CDK) as a catalytic subunit and a cyclin as a regulatory subunit. Although a single CDK (Cdc2 in *Schizosaccharomyces pombe*) controls both the G1-to-S and G2-to-M phase transitions in yeast, distinct CDKs are sequentially associated with different cyclins and regulate cell cycle progression in animals (Morgan, 2006). Plants also have different types of CDKs that are classified into six types, namely, CDKA–CDKF (Vandepoele et al., 2002). Among them, the A-type and B-type CDKs are assumed to be crucial for plant cell cycle progression (Inzé and De Veylder, 2006). A-type CDKs (CDKAs) are functional homologs of the fission yeast Cdc2, and they contain a cyclin-binding domain with the canonical PSTAIRE motif. B-type CDKs (CDKBs) are plant-specific CDKs with a divergent cyclin-binding motif and, in contrast to CDKAs, they are unable to complement *cdc2* mutations in yeast (Imajuku et al., 1992; Fobert et al., 1996). *CDKA* is expressed constitutively throughout the cell cycle, whereas the expression of *CDKB* is restricted to a specific stage, which is from the late S phase to the M phase (Segers et al., 1996; Magyar et al., 1997; Umeda et al., 1999; Sorrell et al., 2001; Menges et al., 2005). Recently, we reported that accumulation of CDKB is regulated not only at the transcriptional level but also through proteasome-mediated protein degradation (Adachi et al., 2006).

The *Arabidopsis* genome encodes a single gene for CDKA, namely, *CDKA;1* (Vandepoele et al., 2002). Several cyclins are known to bind to CDKA;1 and control cell cycle progression; D-type cyclins are assumed to function in mediating internal or external signals to the cell cycle, and A- and B-type cyclins are involved in DNA replication, G2/M transition, and mitotic events (Criqui et al., 2000; Healy et al., 2001; Weingartner et al., 2003; Dewitte et al., 2003; Menges et al., 2006; Kono et al., 2007). The CDK-activating kinase is known to enhance CDKA;1 activity by phosphorylation (Shimotohno et al., 2006), and substitution of the phosphorylated threonine residue with alanine rendered it inactive (Harashima et al., 2007). Hemerly et al. (1995) generated transgenic tobacco plants that overexpressed *CDKA;1* with dominant-negative mutations. Compared to wild-type

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plants, these plants exhibited lower CDKA activities and produced smaller leaves and flowers consisting of larger cells. When an embryo-specific promoter was used to drive the expression of dominant-negative *CDKA;1* in *Arabidopsis*, the phyllotactic pattern and leaf shape were distorted, and some seedlings consisted of one or two cotyledon-like structure(s) (Hemerly et al., 2000). It has been recently reported that the knock-out mutants of *CDKA;1* exhibited defects in male gametogenesis, which resulted in the production of bicellular pollen grains, and failed in double fertilization and embryogenesis (Nowack et al., 2005; Iwakawa et al., 2006). All these results demonstrate the importance of *CDKA;1* for a broad range of developmental processes during the plant life cycle.

Previous reports have shown that the *CDKA;1* transcripts were accumulated in various tissues of actively dividing cells, such as shoot and root apical meristems, developing leaves, floral organs, and pericycle and vascular tissues (Martinez et al., 1992; Hemerly et al., 1993). Its expression is up-regulated by auxin or cytokinin application and wounding stress in leaves, and is inhibited by abscisic acid treatment of root tissues (Hemerly et al., 1993). Using tobacco leaf protoplasts for studying the promoter activity of *CDKA;1*, Hemerly et al. (1993) revealed that expression of this gene was induced in the dedifferentiation process, which was triggered by auxin and cytokinin, but it was also enhanced under conditions wherein cells did not actually divide, such as in media containing either auxin or cytokinin. This result indicates that *CDKA;1* expression may not always be linked to actual cell division but precede it. In fact, *CDKA;1* transcripts were detected in the pericycle of roots, in which cells are capable of restarting cell division and producing lateral root primordia (Martinez et al., 1992; Hemerly et al., 1993; Himanen et al., 2002). Therefore, it is likely that the induction of *CDKA;1* expression may be a prerequisite for the activation of post-embryonic cell division during organ formation.

Imajuku et al. (2001) dissected the *CDKA;1* promoter region and identified the regulatory elements required for expression in developing trichomes by using *glabra* mutants. They also demonstrated that the region downstream of the transcriptional start site is required for *CDKA;1* expression in proliferating tissues. Since *CDKA;1* is the sole gene encoding for the yeast Cdc2 ortholog in *Arabidopsis*, spatial and temporal control of its expression will play a vital role in determining mitotic activity and cell division competency. However, little is known about how its transcription is regulated in different cell types during plant development. Here, to gain more insight into *CDKA;1* expression, we made a dissection series of the *CDKA;1* promoter and analyzed their expression patterns in tissues. Our results revealed tissue-specific regulatory regions as well as general quantitative regulatory regions in the promoter. We also identified cell layer-specific transcriptional regulation, which may be involved in proper development of leaves in *Arabidopsis*.

Materials and methods

Plant material

Arabidopsis plants were grown in Murashige and Skoog (MS) medium (0.5×MS salts, 1×MS vitamins, and 2% (w/v) sucrose (pH 6.3)) under continuous light conditions at 23 °C. To observe the inflorescences and embryos, we transplanted the seedlings into soil and grew them in a greenhouse under a 15-h light/8-h dark cycle at 22 °C. The *phb-1d* mutant (CS3761) was obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH, USA). The *GUS* reporter genes were introduced into *A. thaliana* ecotype Col-0 via *Agrobacterium*-mediated transformation (Clough and Bent, 1998). T2 plants were tested for segregation, and at least three independent T3 lines that showed representative *GUS* expression pattern were subjected to *GUS* expression analyses. For analyzing each construct, more than 15 SAMs were sectioned, and more than 30 individuals were used for observations of other tissues. Two independent and representative lines were used for crossing with *phb-1d*, and at least 10 *phb-1d*/+ individuals in F1 generation were analyzed for each line.

Plasmid construction for GUS expression analysis

The promoter fragments of *CDKA;1* were amplified by PCR using the primers listed in Supplemental Table 1 and cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Then, each fragment was cloned into pGWB3533 (Nakagawa, unpublished data) with LR clonase (Invitrogen) to generate a fusion construct with *GUS*. For construction of 7509F/7R and *proCDKA-CDKA::GUS*, pGWB3 (Nakagawa et al., 2007) was used. Neither of these vectors contains any minimum promoter region. For construction of *proCDKA-CDKA::GUS*, a genomic fragment encompassing the promoter and the coding region was amplified with primers shown in Supplemental Table 1.

GUS staining

Plant tissues were incubated in 90% (v/v) acetone at −20 °C overnight, and washed in 100 mM sodium phosphate buffer (pH 7.0). For GUS staining, samples were incubated in a solution (100 mM sodium phosphate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 0.5 mg mL^{-1} 5-bromo-4-chrolo-3-indolyl-β-D-glucuronide (pH 7.0)) at 37 °C for 1 or 24 h, and mounted in a mixture of chloral hydrate, glycerol, and water (8 g:1 mL:2 mL). To make sections of the shoot apices, the GUS-stained samples were dehydrated with an ethanol series, and then the ethanol was substituted with Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) solution. After solidification with hardener II (Heraeus Kulzer), they were sectioned at a 6 μm or 10 μm thickness, and mounted in 50% (v/v) glycerol.

Results

Expression pattern of CDKA;1 in various tissues

Imajuku et al. (2001) determined the transcription start site of the *CDKA;1* gene to be at position −679 bp (we considered the A of the initiation codon as +1). The 5′ untranslated region (UTR) contains the first intron, the position of which determined to be from −566 bp to −52 bp (The *Arabidopsis* Information Resource (TAIR); www.arabidopsis.org, June 2008). While Y patches were present around the transcriptional start site, the typical TATA box was not found in the 150-bp region proximal to the initiation codon by the program PlantPromoterDB (Yamamoto et al., 2006). This suggests that *CDKA;1* may utilize the Y patches instead of a TATA box as core promoter elements (Yamamoto et al., 2006).

To reveal the regulatory mechanisms underlying *CDKA;1* expression, we first cloned the 2690-bp upstream region from the initiation codon and fused it to the β-glucuronidase (*GUS*) gene to create a reporter construct (Fig. 1A). Seven-day-old seedlings carrying the reporter gene showed strong *GUS* expression, especially in the roots. The root tips, stele, and vascular tissue of the shoots and stipules were stained by incubation in a GUS-staining buffer for 1 h (Figs. 1B, D, E, G). On 24-h incubation, we observed an intense signal in the stomata, which was surrounded by weakly stained mesophyll cells in the leaves (Figs. 1C, F). Vertical sections of GUS-stained tissues showed a uniform signal in shoot apical meristems (SAMs) and developing trichomes (Figs. 1H, I). GUS expression was also observed to be uniform in developing and mature embryos (Figs. 1J–L). In flowers, the reporter gene was highly expressed in the pistils, whereas weaker expressions were detected in the petals, sepals, pollen grains, and filaments (Figs. 1M–O). In summary, the strongest promoter activity was observed in root tips, and the second strongest activity was found in root steles

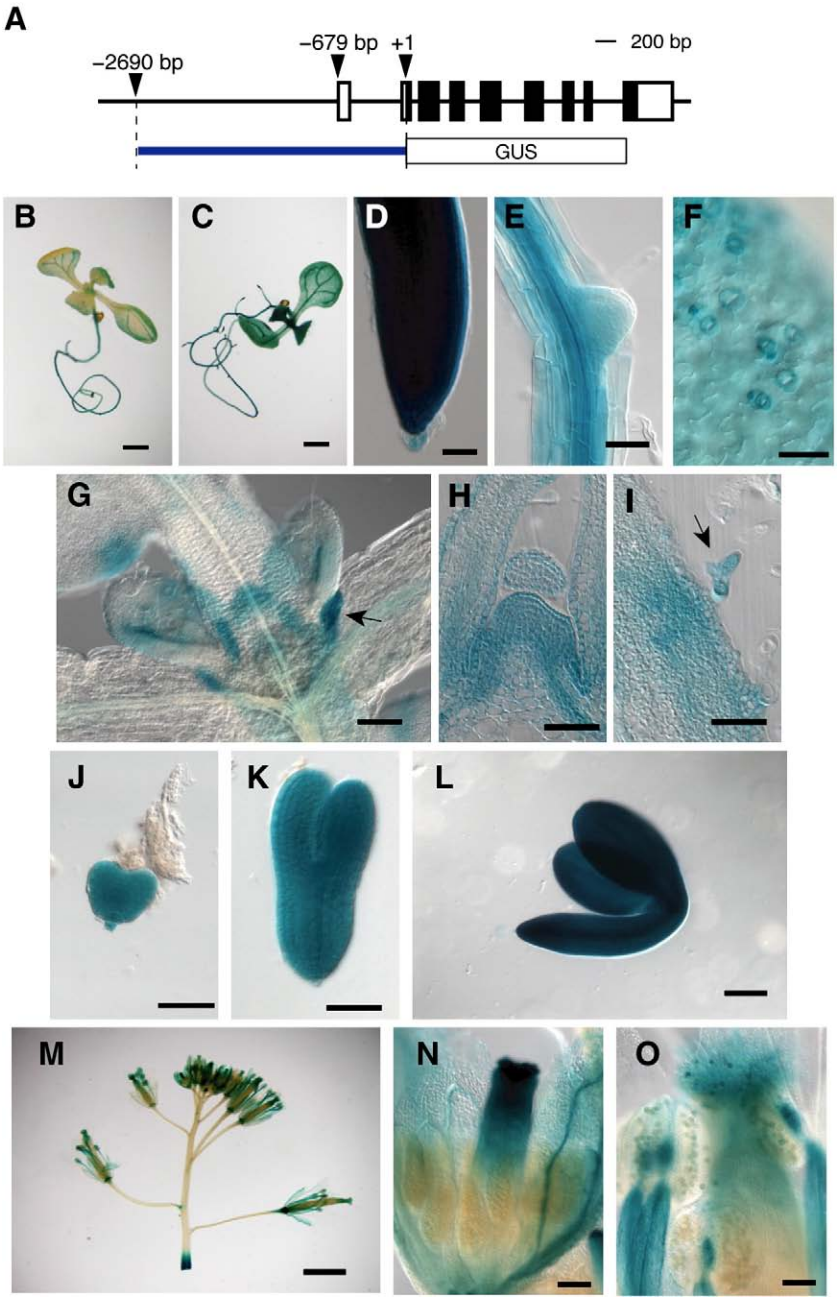

Fig. 1. *CDKA;1* expression in various tissues. (A) The promoter region from −2690 bp to −1 bp (blue bar) was fused to *GUS* and introduced into *Arabidopsis* plants. The white and black boxes indicate UTRs and protein-coding regions of *CDKA;1*, respectively. (B–G) Seven-day-old seedlings were stained for 1 h (B, D, E, G) or 24 h (C, F) at 37 °C: root tip, (D); lateral root primordia, (E); cotyledon, (F); and shoot apex, (G). The arrow indicates a stipule. Shoot apices of 10-day-old seedlings were sectioned and stained for 24 h (H, I). The arrow indicates a developing trichome. Embryos (J–L) and inflorescences (M–O) were stained for 24 h. (N) and (O) show immature and mature flowers, respectively. Bars = 50 μm (D–F, H–K), 1 mm (B, C, M), 100 μm (G, L), and 200 μm (N, O).

and stipules. A weaker but significant level of *GUS* expression was also detected in the SAMs, vascular tissues, developing trichomes, and in the mesophyll and guard cells of leaves.

Regulatory elements involved in quantitative control of CDKA;1 expression

We then dissected the *CDKA;1* promoter and observed *GUS* expression in tissues. Since Imajuku et al. (2001) have reported that the promoter region from −1268 bp to the initiation codon was enough to reproduce the expression pattern driven by longer promoter fragments, we generated a deletion series of the 1290-bp region as shown in Fig. 2. The constructs, which were termed 7505, 7506, 7507, 7508, 7509, and 7510, differed with regard to the length of the 5′ region at 100-bp intervals. Seven-day-old seedlings harboring each reporter gene were subjected to GUS staining. We could not find any significant difference in the level and tissue specificity of the expressions of this gene among transgenic lines transformed with the constructs of 7501, 7505, 7506, 7507, 7508, and 7509 (Fig. 3). A shorter incubation in a GUS-staining buffer at a lower temperature (20 °C, 10 min) also showed no significant difference among these lines (Supplemental Fig. 1). However, *GUS* expression in the 7510 seedlings was lower in both the shoots and roots as compared to that in the above-mentioned lines, although no obvious change was observed in the expression pattern (Fig. 3). We could observe similar quantitative differences in the embryos and inflorescences (Supplemental Fig. 2).

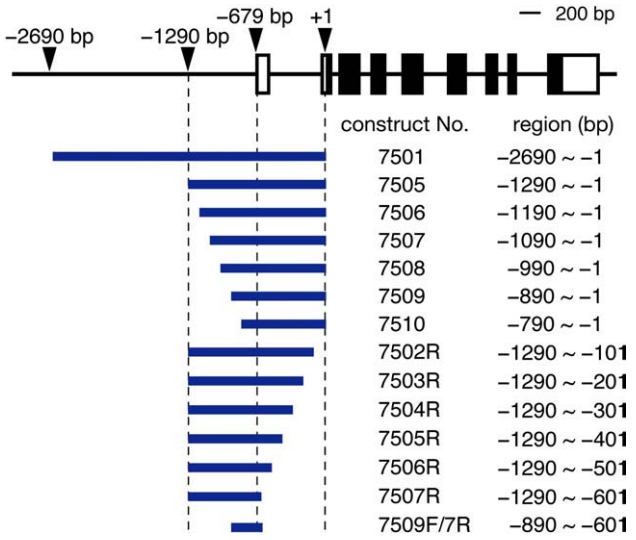

Fig. 2. Promoter dissection series of *CDKA;1*. The blue bars indicate promoter regions cloned into the expression vector. In the schematic diagram of the *CDKA;1* genomic region, the white and black boxes indicate UTRs and protein-coding regions, respectively. The construct 7501 is the same as that shown in Fig. 1A.

These results suggest that the region from −890 bp to −791 bp contains *cis*-regulatory element(s) that promote *CDKA;1* expression independently of tissues.

It has been described that the region between the transcriptional start site and the initiation codon contains a regulatory element that functions in proliferating cells (Imajuku et al., 2001). Therefore, we constructed *GUS* reporter genes with 3′-end truncations of the 1290-bp region. The 7505, 7502R, 7503R, 7504R, 7505R, 7506R, and 7507R constructs differed with regard to the length of the 3′ region at 100-bp intervals (Fig. 2). In the 7-day-old seedlings, we found a significant decrease in the level of *GUS* expression between the 7502R and 7503R constructs (Fig. 4A). A similar reduction was also noted in embryos (Supplemental Fig. 3), indicating the presence of another *cis*-regulatory element between −200 and −101 bp. In the 7503R–7505R lines, the expression in the roots was gradually reduced as the 3′ end became shorter and, in the roots of the 7506R or 7507R plants, we could not detect any *GUS* expression (Fig. 4B). Despite the disappearance of *GUS* expression in roots, we could observe GUS signals in young leaves of the 7506R and 7507R lines (Fig. 4B), indicating the presence of root-specific regulatory elements that promote *CDKA;1* expression.

Presence of a promoter region enhancing CDKA;1 expression in the leaf epidermis

To examine cell type-specific regulation of *CDKA;1* expression, we made sections of GUS-stained shoot apices. When the 5′-deletion series (7505–7510) were investigated, no difference was found in the expression patterns as compared to that of the full-length construct 7501; *GUS* expression was observed in the SAM, leaf primordia, and developing leaves (Fig. 5A). In order to examine whether post-transcriptional regulation is engaged in the *CDKA;1* expression, we generated transgenic lines expressing the CDKA;1::GUS fusion protein under the 2690 bp promoter region (*proCDKA-CDKA::GUS*). The expression pattern in the SAM was almost the same as that of the transcriptional fusion line 7501; namely, the fusion protein was accumulated uniformly in the meristem and leaf primordia, and the leaf epidermis showed slightly stronger expression (Fig. 5B). This result indicates that regulatory mechanisms associated with translation or protein stability do not play an important role in the control of spatial expression pattern of *CDKA;1*.

In contrast, we found differential expression patterns in the dissection series with 3′ truncations (Fig. 5C). While the 7505 and 7502R lines did not show any difference, 7503R exhibited lower *GUS* expression in the SAM and no expression in the inner layers of leaves. The results showed that the epidermis of leaves and trichomes were prominently GUS-stained (Fig. 5C). This indicates that the region between −200 bp and −101 bp is associated with *CDKA;1* expression in the SAM and in leaves except for the epidermis. The lack of this region in the 7503R line might cause a significant reduction of GUS staining in seedlings due to the loss of expression in the inner layers of leaves (Fig. 4). The epidermis-specific expression pattern was also observed in 7504R–7507R. However, in the SAM, *GUS* expression was much lower in the 7504R line, and almost no expression was detected in the 7505R–7507R lines (Fig. 5C). It should be noted that the GUS signal disappeared not only in the inner layers but also in the L1 layer of the SAM, although the epidermis-specific expression persisted in the leaves of these transgenic lines. *In situ* hybridization using probes for transcripts of *GUS* showed the similar epidermis-specific expression in leaves of the 7503R and 7507R lines (Supplemental Fig. 4), confirming the results of GUS staining as described above.

The 7510 construct with a deletion in the region before −790 bp displayed *GUS* expression in any cell type of the leaves (Fig. 5A), and 7507R lacking the region after −601 bp still showed epidermal expression of this gene (Fig. 5C). Therefore, it is likely that the region between −790 bp and −601 bp contains another regulatory element that promotes *CDKA;1* expression in the epidermis. To examine this idea, we created another reporter construct, 7509F/7R, which carries the region between −890 bp and −601 bp (Fig. 2). Although we included the region from −890 bp to −791 bp that promotes *CDKA;1* expression as mentioned above, the GUS signal was very weak as compared to that in 7507R. However, we could

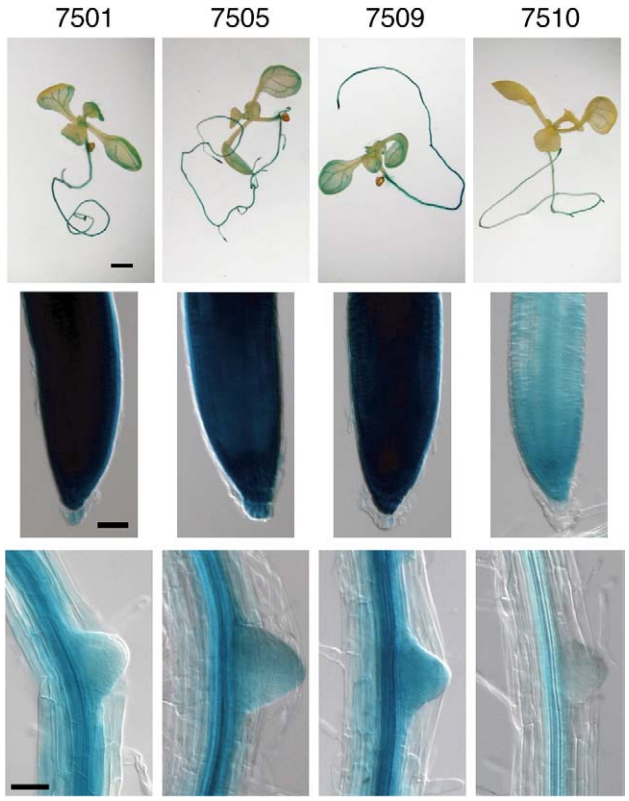

Fig. 3. *GUS* expression of the 5′-end dissection series. Seven-day-old seedlings were stained for 1 h at 37 °C. Top row, whole seedlings; middle row, root tips; bottom row, lateral root primordia. Bars for the roots or lateral root primordia equal 50 μm, and that for the whole seedlings equals 1 mm.

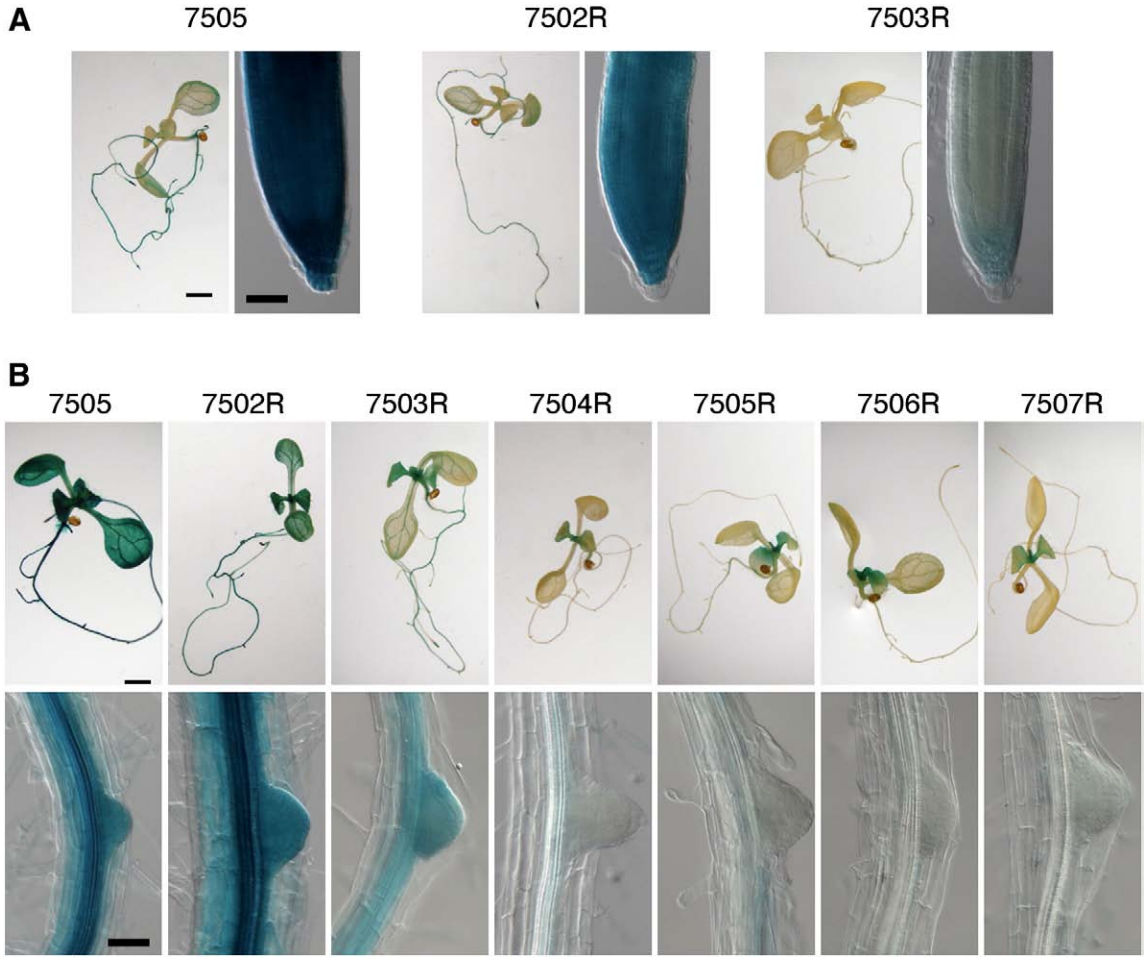

Fig. 4. *GUS* expression of the 3′-end dissection series. Seven-day-old seedlings were stained for 1 h (A) or 24 h (B) at 37 °C. (A) Left panel, whole seedlings; right panel, root tips. (B) Upper row, whole seedlings; lower row, lateral root primordia. Bars for the roots or lateral root primordia equal 50 μm, and those for the whole seedlings equal 1 mm.

again identify epidermis-specific expression (Fig. 5D), suggesting that this region contains enough information to promote epidermal expression in leaves.

We then conducted a detailed analysis of the epidermal expression of this gene by using the 7507R reporter gene. Since the longitudinal sections of shoot apices showed biased GUS staining on the abaxial side of the leaf epidermis (Fig. 5C), we also created transverse sections of the SAM and leaves. As expected, *GUS* expression was higher on the abaxial side of young leaves, and only a trace level of expression was observed on the adaxial side (Fig. 6B). In the SAM, we could not detect *GUS* expression. We then introduced the 7507R reporter gene into the *phbulosa-1d* (*phb-1d*) mutants. The *phb-1d* mutant is known to develop filamentous leaves that lose the abaxial identity (McConnell and Barton, 1998). Although the stipules were stained in a similar manner to the wild-type background, the *GUS* expression in the leaf epidermis was severely suppressed, and almost no expression was detected in any cell type (Figs. 6C, D). This result supports the idea that a regulatory mechanism functions to enhance the *CDKA;1* expression on the abaxial side of the leaf epidermis.

Discussion

Quantitative regulation of CDKA;1 expression

Our analyses of the promoter dissection series identified a region from −890 bp to −791 bp, which is involved in quantitative up-regulation of *CDKA;1* expression (Fig. 7). The promoter-*GUS* reporter gene lacking the 100-bp region showed much lower expression as compared to the intact promoter construct, and no tissue specificity was found in the reduction of GUS staining in both shoots and roots. This suggests that the 100-bp region may contain a general regulatory element that functions independent of cell type. In the database of PlantPromoterDB (Yamamoto et al., 2006), two *cis*-regulatory elements are annotated in the region between −890 bp and −791 bp—AACCCGGT and CCGGTATA—overlapping sequences of which is known as an abscisic acid (ABA) responsive element (Nelson et al., 1994). However, Hemerly et al. (1993) have reported that *CDKA;1* expression is suppressed by ABA treatment; thus, it is unlikely that these elements function in response to the ABA signal and activate cell division.

A transcription factor termed TEOSINTE-BRANCHED1, CYCLOIDEA, PCF20 (TCP20) is known to bind *in vivo* to a GCCCR motif in the promoter region of Cyclin B1;1 and enable a high level of Cyclin B1;1 expression at G2/M. (Li et al., 2005). It is also required for high-level expression of ribosomal protein genes by its binding to the GCCCR element in their promoters, suggesting a mechanistic link between the regulation of cell proliferation and cell growth. The *TCP20* gene belongs to class I TCP genes that positively regulate gene expression (Kosugi and Ohashi, 1997); while class II TCP genes, however, negatively control proliferation (Nath et al., 2003). There is no GCCCR element in the promoter region of *CDKA;1*. However, since the amounts of protein such as mitotic cyclins and CDKA;1 may be coordinately regulated in the process of cell division and differentiation, other class I TCP proteins may bind to the region between −890 bp and −791 bp and quantitatively control *CDKA;1* expression in response to environmental conditions.

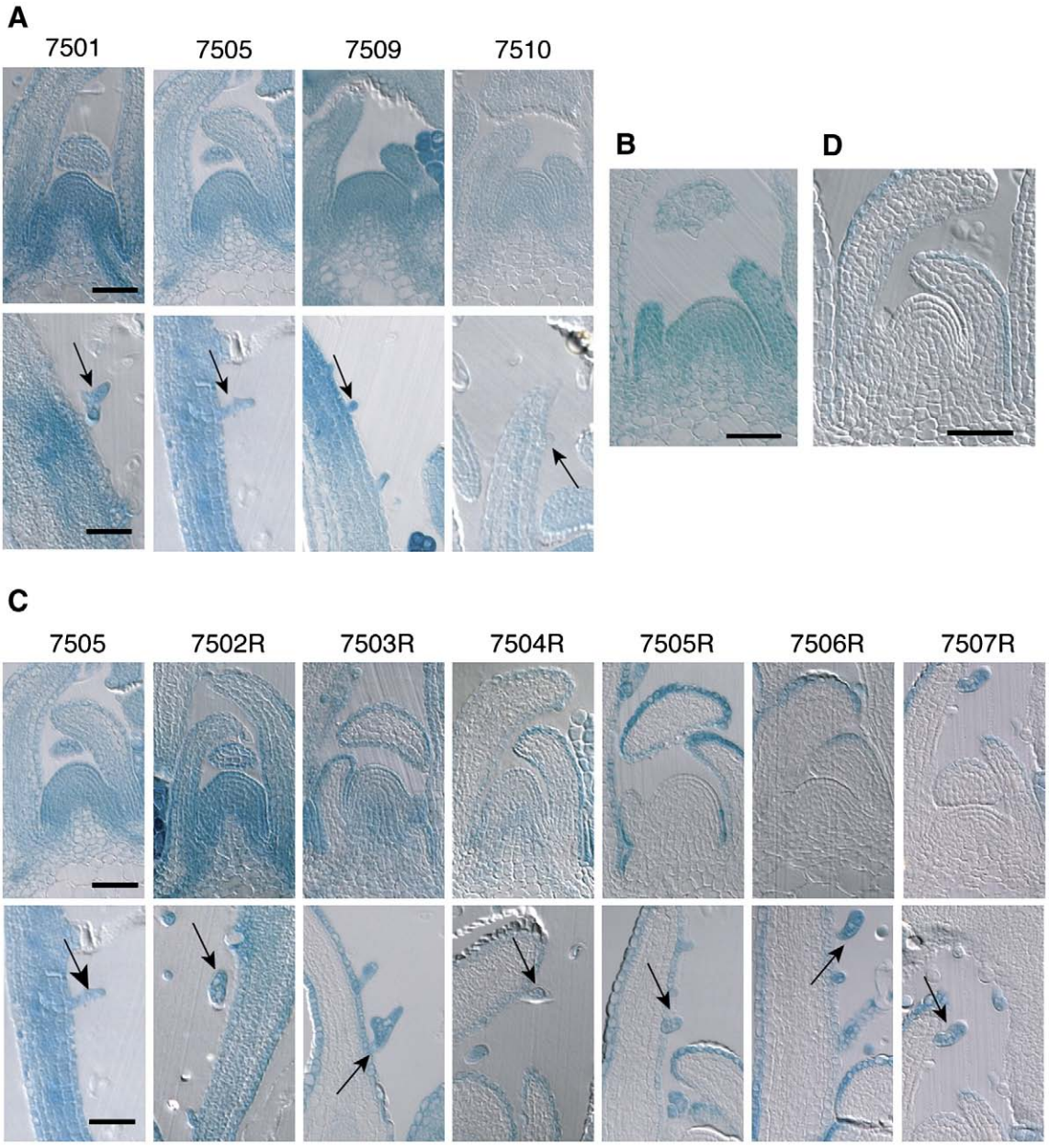

Fig. 5. *GUS* expression pattern in shoot apices. Shoot apices of 10-day-old seedlings were stained for 24 h at 37 °C and sectioned at a 6 μm (A–C) or 10 μm (D) thickness. (A, C) Upper row, SAMs and leaf primordia; lower row, developing trichomes (indicated by arrows) on young leaves. (B) The SAM of *proCDKA-CDKA::GUS*. (D) The SAM of 7509F/7R. Bars = 50 μm.

Cell layer-specific regulation of CDKA;1 expression

Although *CDKA;1* is expressed in various tissues, this study revealed differential control of its expression in the leaf epidermis. The promoter region from −200 bp to −101 bp seems to enhance *CDKA;1* expression in the SAM and root apical meristems (RAMs), but also in inner layers of the leaf primordia (Supplemental Fig. 5, Fig. 7). The 7502R and the 7503R lines lack one of the splicing sites of the first intron (Fig. 2), thus we cannot deny the possibility of unsplicing, alternative splicing or the use of alternative translational start sites in the 5′ UTR. However, since it is unlikely that splicing occurs differentially in distinct cell types, we assume that the region from −200 bp to −101 bp has a regulatory function in inner layers of leaves. In contrast, the region from −890 bp to −601 bp was assumed to function in elevating the expression in the leaf epidermis (Fig. 7). This region contains no splicing site, suggesting that the possibility of alternative splicing can be ignored. These complementary expression patterns further indicate that *CDKA;1* expression in leaves depends on at least two regulatory elements in the 5′ UTR. L1- and epidermis-specific expressions are known to be regulated by a *cis*-regulatory element, namely, the L1 box, which exists in the promoter of *Arabidopsis thaliana* MERISTEM LAYER 1 (*AtML1*) (Sessions et al., 1999; Abe et al., 2001). In the region from −890 bp to −601 bp, however, we could not find any L1 box-like sequence, suggesting that the mechanisms underlying up-regulation of *CDKA;1* expression in the leaf epidermis are independent of L1 box-mediated signaling.

Recently a few reports indicated that cell division in distinct cell layers is differentially regulated during shoot development. Desvoyes et al. (2006) inactivated RETINOBLASTOMA-RELATED (RBR) protein in an inducible manner in *Arabidopsis*. The retinoblastoma protein (pRb) is known to bind to the transcription factor E2F and, by suppressing E2F function, it blocks progression from the G1 to the S phase (Weinberg, 1995). As expected, RBR inactivation caused excess cell divisions and decreased cell size in the leaf epidermis. However, in mesophyll cells, no alteration in cell number or cell size was observed in response to RBR inactivation. This result implies the

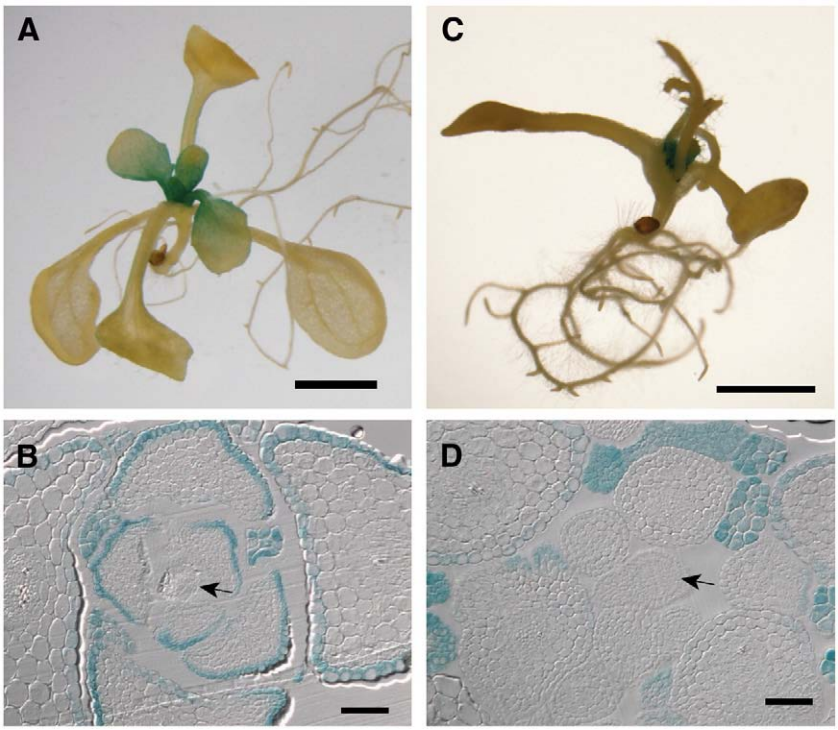

Fig. 6. *GUS* expression pattern in the *phb-1d* mutant. Ten-day-old seedlings of wild-type (A, B) and *phb-1d* mutant (C, D) plants harboring the 7507R construct were stained for 24 h at 37 °C. Cross-sections were created from shoot apices (B, D). Arrows indicate SAMs. Bars = 2 mm (A, C) and 50 μm (B, D).

difference and independence of cell cycle regulation between epidermal and mesophyll cells in leaves. Bemis and Torii (2007) investigated transgenic *Arabidopsis* plants that ectopically express genes for CDK inhibitors, such as *KIP-RELATED PROTEIN1* and *KIP-RELATED PROTEIN4*. When the expression was driven by the *AtML1* promoter, epidermal cell division was severely inhibited with compensatory cell size enlargement, but normal cell numbers were maintained in the mesophyll and cortex layers. On the other hand, a recent report indicated that the dwarf phenotype of *Arabidopsis* mutants, which exhibit defects in brassinosteroid biosynthesis or signaling, was rescued by the expression of responsible genes with the *AtML1* promoter (Savaldi-Goldstein et al., 2007). The epidermis-specific expression restored the cell size not only in the epidermis but also in the inner mesophyll layers, suggesting the relay of a non-autonomous signal from the epidermis to the inner tissues. These results indicate that cell division is differentially controlled in the epidermis and underlying tissues, but inter-layer communications in terms of regulation of cell elongation or cell differentiation are present. Our present data showed that two distinct *cis*-regulatory elements in the promoter control *CDKA;1* expression in the epidermis and inner layers of leaves. This suggests that CDKA;1 may play a key role not only in cell division but also in coordinating

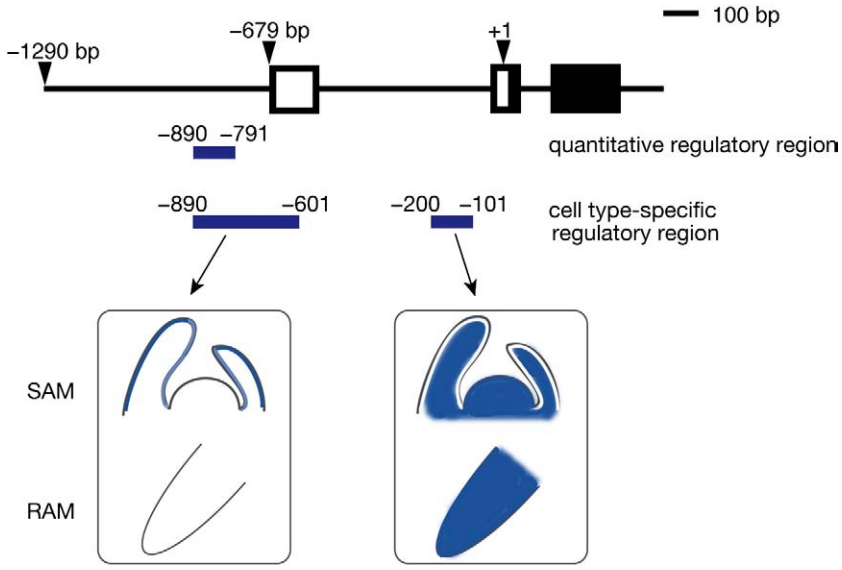

Fig. 7. Regulatory regions in the *CDKA;1* promoter. The blue bars indicate the transcriptional regulatory regions identified in this study. In the diagram of the *CDKA;1* genomic region, the white and black boxes indicate UTRs and protein-coding regions, respectively. Tissues or cell layers in which each regulatory region functions in activating *CDKA;1* expression are schematically shown in blue in the diagrams.

cell differentiation between different cell layers of leaves. This assumption is supported by our previous observation that differentiation of root stem cells was controlled by CDK activities in *Arabidopsis* (Umeda et al., 2000).

In transgenic plants carrying the 7507R construct, abaxial side-biased *GUS* expression was observed in young leaf primordia. However, when the same reporter construct was introduced into the *phb-1d* mutants, *GUS* expression was suppressed on both sides of the leaves. In wild-type plants, *PHB* is expressed in the adaxial domain, and the expansion of the expression domain in the gain-of-function mutant *phb-1d* leads to spreading of the adaxial domain and generates radially symmetric leaves lacking abaxial cell types (McConnell and Barton, 1998). Therefore, our result demonstrates that the *CDKA;1* promoter region between −890 bp and −601 bp contains a *cis*-regulatory element that up-regulates the expression on the abaxial side. Since this region is overlapping with the transcription start site, further dissection of the fragment will need careful investigation. *Arabidopsis* possesses other genetic programs that differentially regulate cell division on each side to guarantee proper leaf expansion. For example, the *ASYMMETRIC LEAVES2* (*AS2*) gene is expressed in the adaxial domain of leaf primordia and represses cell division. The loss-of-function mutants develop downward-curled leaves due to excess cell division on the adaxial side (Iwakawa et al., 2007). Although it remains unknown as to whether the differential regulation of *CDKA;1* expression is linked to the *AS2*-associated network, it is likely that such regulatory mechanisms are involved in adjusting the cell division rate on both sides of epidermal cells and fine-tuning shoot growth and leaf expansion.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.03.002.

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