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The Plant-Specific Cyclin-Dependent Kinase CDKB1;1 and Transcription Factor E2Fa-DPa Control the Balance of Mitotically Dividing and Endoreduplicating Cells in Arabidopsis

Véronique Boudolf,[a] Kobe Vlieghe,[a] Gerrit T.S. Beemster,[a] Zoltan Magyar,[a,b,1] Juan Antonio Torres Acosta,[a,2] Sara Maes,[a] Els Van Der Schueren,[a] Dirk Inzé,[a,3] and Lieven De Veylder[a]

[a] Department of Plant Systems Biology, Flanders Interuniversity Institute for Biotechnology, Ghent University, B-9052 Gent, Belgium

[b] Biological Research Center, H-6701 Szeged, Hungary

Transgenic *Arabidopsis thaliana* plants overproducing the E2Fa-DPa transcription factor have two distinct cell-specific phenotypes: some cells divide ectopically and others are stimulated to endocycle. The decision of cells to undergo extra mitotic divisions has been postulated to depend on the presence of a mitosis-inducing factor (MIF). Plants possess a unique class of cyclin-dependent kinases (CDKs; B-type) for which no ortholog is found in other kingdoms. The peak of CDKB1;1 activity around the G2-M boundary suggested that it might be part of the MIF. Plants that overexpressed a dominant negative allele of *CDKB1;1* underwent enhanced endoreduplication, demonstrating that CDKB1;1 activity was required to inhibit the endocycle. Moreover, when the mutant *CDKB1;1* allele was overexpressed in an E2Fa-DPa–overproducing background, it enhanced the endoreduplication phenotype, whereas the extra mitotic cell divisions normally induced by E2Fa-DPa were repressed. Surprisingly, *CDKB1;1* transcription was controlled by the E2F pathway, as shown by its upregulation in E2Fa-DPa–overproducing plants and mutational analysis of the E2F binding site in the *CDKB1;1* promoter. These findings illustrate a cross talking mechanism between the G1-S and G2-M transition points.

INTRODUCTION

Mitosis and endoreduplication are two different modes of the cell cycle. The mitotic cell cycle comprises the duplication and subsequent distribution of chromosomes between two daughter cells, whereas endoreduplication involves repetitive chromosomal DNA replication without intervening mitosis or cytokinesis, leading to an increase in the ploidy level (D'Amato, 1964; Brodsky and Uryvaeva, 1977). In plants, endoreduplication is common and assumed to be the most prevalent process for increasing the nuclear ploidy. The level of ploidy varies between species and tissues. In *Arabidopsis thaliana*, up to 32C nuclei have been detected (Galbraith et al., 1991).

Until now, the physiological significance of endoreduplication is poorly understood. A positive correlation commonly exists between the ploidy level of cells and their size, and it was postulated that endoreduplication is required for maintaining an optimal ratio between genome and cell size (Melaragno et al., 1993; Folkers et al., 1997; Traas et al., 1998; Sugimoto-Shirasu and Roberts, 2003). Alternative hypotheses link endoreduplication with increased metabolic activity, maintenance of the optimal ratio between the nuclear and organellar DNA, cellular differentiation, and resistance against irradiation (Traas et al., 1998; Joubès and Chevalier, 2000; Kondorosi et al., 2000; Larkins et al., 2001).

Not only the functional role of endoreduplication is unclear, but also the molecular mechanism triggering the process remains elusive. The continuity of the meristematic zone with the region of endoreduplication suggested that the endocyle is achieved by a modification of the mitotic cell cycle (Jacqmard et al., 1999). In the maize (*Zea mays*) endosperm, the onset of endoreduplication is correlated with the inhibition of M-phase–associated cyclin-dependent kinase (CDK) activity by a yet unidentified component (Grafi and Larkins, 1995). Similarly, in developing tomato (*Lycopersicon esculentum*) fruits, M-phase–specific CDK activity is highly reduced during the endoreduplication process (Joubès et al., 1999). These data indicate that in analogy to what has been found previously in yeast and *Drosophila melanogaster*, an inhibition of the G2-M transition might be sufficient to initiate the endoreduplication program. Several strategies have been proposed by which M-phase CDK activity could be reduced. Currently, the best-documented mechanism involves the CCS52 protein, which activates the anaphase-promoting complex, thereby triggering the destruction of M-phase–specific cyclins. In alfalfa (*Medicago sativa*), *CCS52* expression correlates with the onset of endoreduplication. Moreover, *CCS52* overexpression in yeast transforms the mitotic cycles into endocycles, whereas plants with downregulated levels of *CCS52* display

[1] Current address: School of Biological Sciences, Royal Holloway, University of London, Egham TW20 QEX, UK.

[2] Current address: Center of Applied Genetics, University of Agricultural Sciences, Muhlgasse 18, A-1190 Vienna, Austria.

[3] To whom correspondence should be addressed. E-mail dirk.inze@psb.ugent.be; fax 32-9-3313809.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Lieven De Veylder (lieven.deveylder@psb.ugent.be).

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a decrease in ploidy level (Cebolla et al., 1999). Another likely candidate to inhibit M-phase–specific CDK activity in endoreduplicating cells is the WEE1 kinase, for which transcript levels have been demonstrated to accumulate during the endoreduplication process in maize endosperm (Sun et al., 1999). Nevertheless, the identity of the CDK/cyclin complex to be inhibited to trigger endoreduplication remains to be elucidated.

Recently, we have identified the E2Fa-DPa complex as a positive regulator of the endocycle (De Veylder et al., 2002). E2F-DP transcription factors regulate the onset of S-phase by controlling the expression of genes needed for DNA replication (Trimarchi and Lees, 2002). In *E2Fa-DPa*–overexpressing plants, DNA replication is strongly activated, resulting in a mixed phenotype: some cells are triggered to undergo supernumerous mitotic divisions; in others, the endoreduplication cycle is stimulated, resulting in a dramatic increase in ploidy level and formation of giant nuclei (De Veylder et al., 2002; Kosugi and Ohashi, 2003). The decision of E2Fa-DPa–overproducing cells to undergo extra cell division or endoreduplication depends on the cell type and tissue, as clearly seen in the epidermal cell files of the hypocotyls: stomata forming cell files are stimulated to undergo ectopic divisions, whereas cell files without stomata mainly consist of bulged cells containing huge nuclei (De Veylder et al., 2002). Because the formation of stomata is linked with competence to divide, we have postulated that the decision of E2Fa-DPa–overproducing cells to undergo extra cell divisions depends on the presence of a yet to be identified mitosis-inducing factor (MIF; De Veylder et al., 2002).

As in all eukaryotic organisms, cell division in plants is regulated by CDKs (De Veylder et al., 2003). In the model plant Arabidopsis, 12 CDKs have been annotated to date, grouped into six classes (Vandepoele et al., 2002). Curiously, plants possess a unique class of CDKs (B-type) for which no counterpart is found in other organisms, suggesting that they regulate plant-specific aspects of the cell cycle. A role of CDKB1;1 in skotomorphogenesis has been proposed, based on the observation that dark-grown *CDKB1;1* antisense lines display a short hypocotyl and open cotyledons (Yoshizumi et al., 1999). Recently, CDKB1;1 activity has been demonstrated to be essential for correct stomatal development (Boudolf et al., 2004). Here, we highlight an additional role for CDKB1;1 in plant development: plants with reduced CDKB1;1 activity prematurely exit the mitotic cell cycle and have elevated ploidy levels. Moreover, overexpression of a dominant negative *CDKB1;1* allele in an E2Fa-DPa–overproducing background enhanced the endoreduplication phenotype triggered by E2Fa-DPa, whereas ectopic cell divisions were suppressed, demonstrating that CDKB1;1 determines the fate of *E2Fa-DPa*–expressing cells to divide mitotically or to endoreduplicate. We demonstrate that *CDKB1;1* transcription is regulated through an *E2F cis*-acting element in its promoter, providing a mechanism that links G1-S and G2-M transition points.

RESULTS

CDKB1;1.N161 Overexpression Stimulates Exit from Mitosis

To address the role of CDKB1;1 in plant growth and development, transgenic lines were generated that overproduce

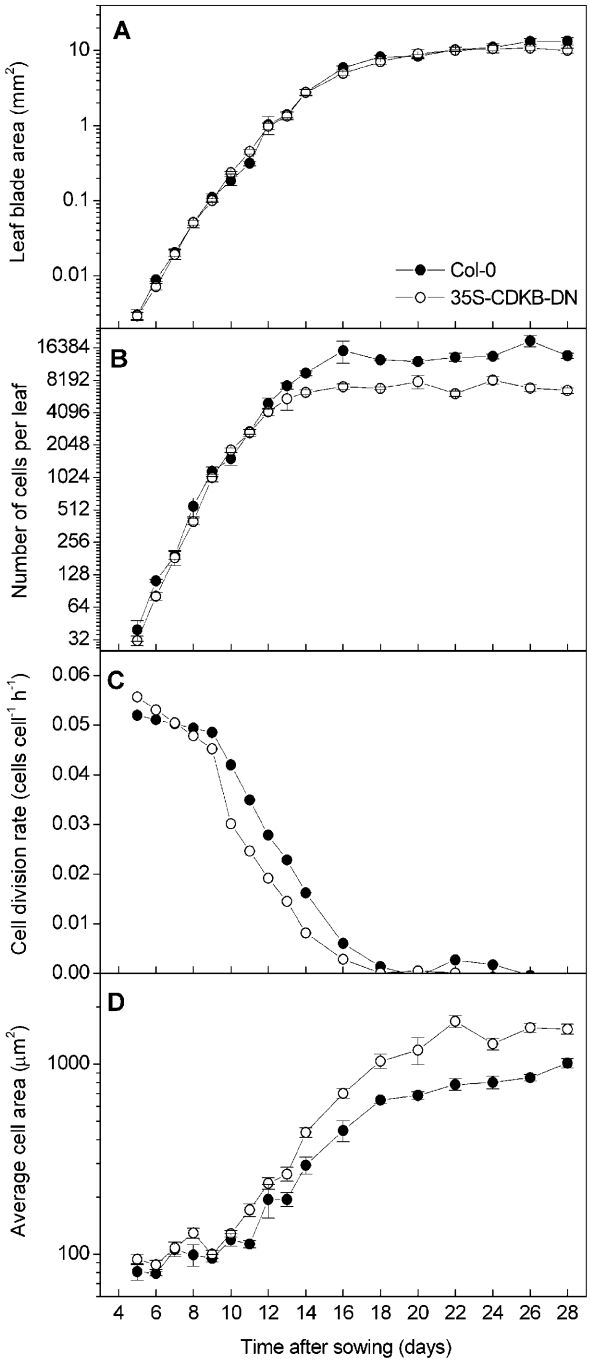

Figure 1. Kinematic Analysis of Leaf Growth of the First Leaf Pair of Wild-Type Columbia-0 and CDKB1;1.N161-Overproducing Plants.

(A) Leaf blade area. Col-0, Columbia-0.
(B) Epidermal cell number on the abaxial side of the leaf.
(C) Average cell division rates of the epidermal cells on the abaxial side of the leaf.
(D) Epidermal cell size on the abaxial side of the leaf. Error bars denote standard errors ($n = 4$ to 10).
Symbols in **(B)**, **(C)**, and **(D)** as in **(A)**.

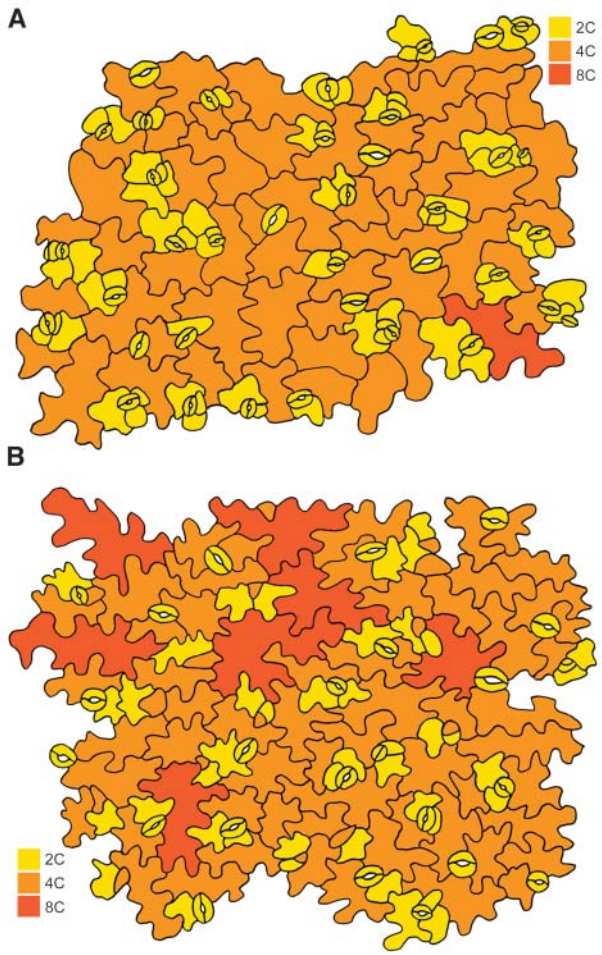

Figure 2. Abaxial Epidermal Peel of the First Leaves 14 d after Germination.

(A) Wild-type plants.
(B) Plants overexpressing *CDKB1;1.N161*.
The ploidy level of the cells is indicated by color according to the legend.

the wild-type or a dominant negative allele of *CDKB1;1* (*CDKB1;1.N161*). The *CDKB1;1.N161* allele encodes a CDK in which the amino acid Asp at position 161 is changed into Asn, resulting into a kinase-dead protein (van den Heuvel and Harlow, 1993; Labib et al., 1995). Previously, we have demonstrated that overexpression of this mutant kinase gene results in a decrease in CDK activity, probably because the mutant and endogenous wild-type proteins compete for the association with rate-limiting interacting proteins such as cyclins (Porceddu et al., 2001; Boudolf et al., 2004).

The effects of overexpression of the wild-type and dominant negative *CDKB1;1* genes on leaf development and cell cycle duration were studied by a kinematic analysis. From day 5 until day 28 after sowing, the first leaf pairs of transgenic and wild-type plants grown side by side under the same conditions were harvested, and the leaf blade area was measured by image analysis (De Veylder et al., 2001). Subsequently, the average cell area of abaxial epidermal cells was determined from drawing-tube images, and total cell number was extrapolated as the ratio of leaf blade and average cell areas. Plants overproducing the native *CDKB1;1* gene did not differ from wild-type plants (data not shown). However, when wild-type and *CDKB1;1.N161*-overexpressing plants were compared, significant differences were noticed (Figure 1). Leaf blade area expansion was similar in the wild-type and *CDKB1;1.N161* transgenic plants: first-leaf size expanded exponentially until day 12, after which expansion rates steadily decreased to zero at day 20, when the leaves reached their mature size (Figure 1A). *CDKB1;1.N161*-overexpressing leaves contained only half the number of epidermal cells at maturity (Figure 1B). The divergence between wild-type and transgenic lines occurred between 10 and 16 d after sowing.

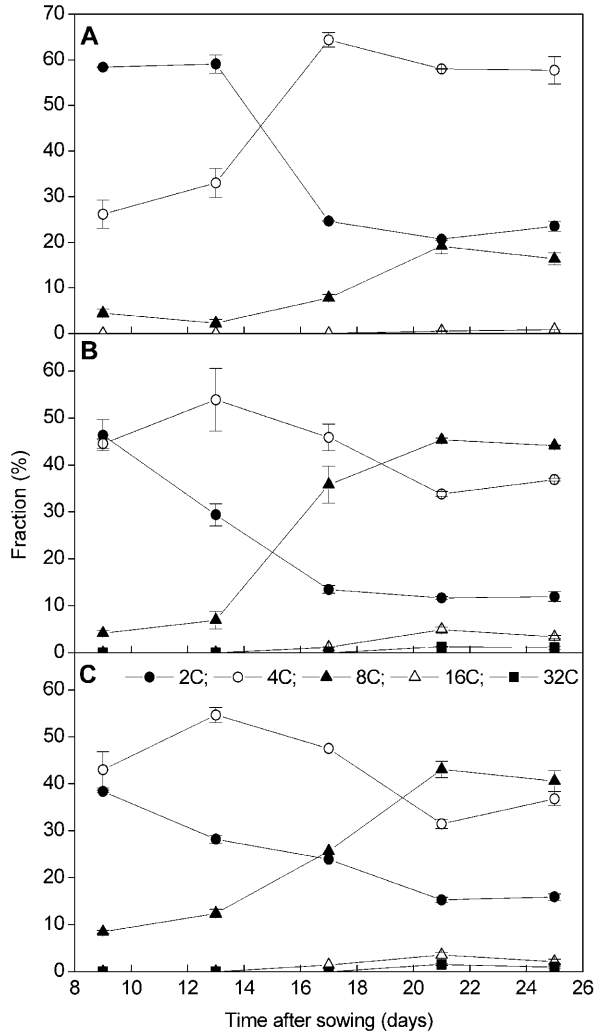

Figure 3. DNA Ploidy Level Distribution of the First Leaves of Wild-Type (Col-0) and CDKB1;1.N161-Overproducing Plants during Development.

(A) Wild type (Col-0).
(B) *CaMV35S:CDKB1;1.N161* line 1.2.
(C) *CaMV35S:CDKB1;1.N161* line 9.2. Leaves were harvested at the indicated time points. Data represent average ± SD.
Symbols in **(A)** and **(B)** as in **(C)**.

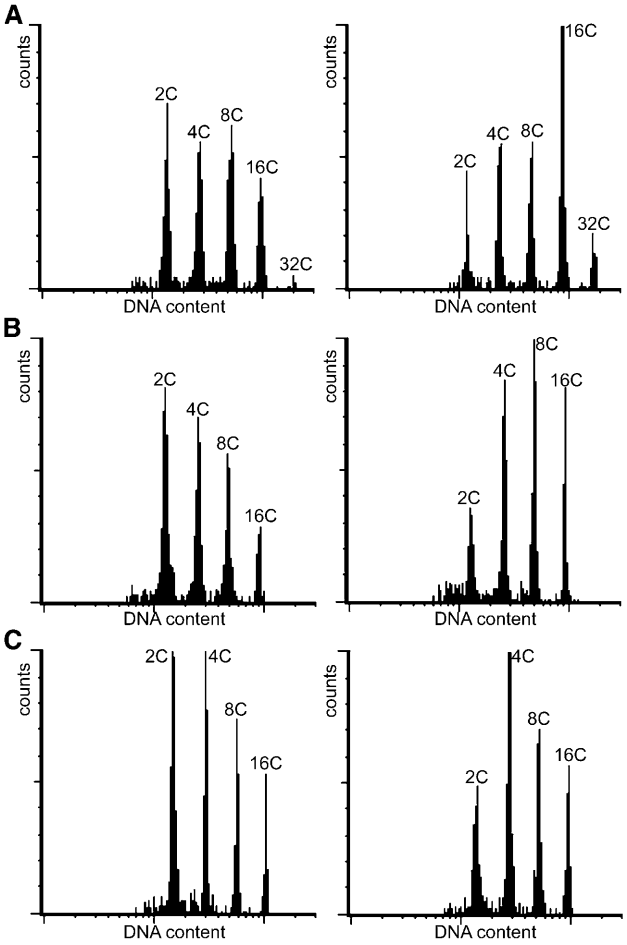

Figure 4. DNA Ploidy Level Distribution of Wild-Type Plants (Col-0; Left) and CDKB1;1.N161-Overproducing Plants (Right) in Different Tissues.

(A) Cotyledons.
(B) Hypocotyls.
(C) Roots.

Supporting this observation, average cell division rates for the whole leaf blade, calculated as exponential increase of the cell number, were approximately constant and comparable in wild-type and *CDKB1;1.N161*-overexpressing leaves until 9 d after sowing. However, from day 10 to day 16, which mark the exit from cell division, cell division rates were constantly lower in the transgenic plants (Figure 1C). Simultaneously, the average cell size, which initially was ~100 μm in both lines, increased significantly faster in the transgenic lines, with cells reaching nearly twice the size of that of wild-type cells (Figure 1D). At day 14 after sowing, the abaxial epidermal pavement cells of the first leaf of *CDKB1;1.N161*-overexpressing plants had the characteristically jigsaw puzzle–shaped appearance. By contrast, wild-type pavement cells of the same age were less lobed, suggesting an advanced differentiation of *CDKB1;1.N161*-overexpressing cells (Figures 2A and 2B). Taken together, these data indicate that cell cycle exit occurred more rapidly in the transgenic plants.

CDKB1;1 Activity Is Required to Suppress Endoreduplication

Previously, we have shown that the exit of the mitotic cell cycle in Arabidopsis is associated with the start of endoreduplication (Jacqmard et al., 1999). To study the onset of endoreduplication in the plants overexpressing *CDKB1;1* and *CDKB1;1.N161*, the ploidy level of the first pair of true leaves was measured by flow cytometry from 9 to 25 d after sowing, harvested at 4-d intervals. Ploidy levels in wild-type and *CDKB1;1*-overexpressing leaves were similar at any stage of leaf development (Figure 3A; data not shown). The 2C and 4C levels remained fairly constant from days 9 to 13. From day 13 onward, the amount of cells with a 2C content decreased steeply, correlated with an increase in the number of cells with a 4C and 8C content until day 21. Afterwards, the ploidy levels remained essentially constant. In two independent *CDKB1;1.N161* lines, the 4C/2C ratio was significantly higher at day 9 than that in wild-type leaves (Figures 3B and 3C). The increase in the number of cells with a 4C DNA content reflects the need for CDKB1;1 activity to progress through mitosis (Porceddu et al., 2001; Boudolf et al., 2004). In contrast with wild-type leaves, the 2C levels in *CDKB1;1.N161* leaves already decreased between day 9 and day 13, which was accompanied by a further increase in 4C and 8C levels (Figures 3B and 3C). These data illustrate that the *CDKB1;1.N161* leaves entered the endocycle earlier than wild-type plants. An advance entry into the endocycle can also clearly been seen at the cellular level. When the ploidy levels were compared of individual abaxial pavement cells in the first leaves 14 d after germination, a higher number of 8C cells could be observed in the transgenic plants (Figures 2A and 2B). In mature leaves, the ploidy levels were overall higher in the transgenic than in the control lines with 8C

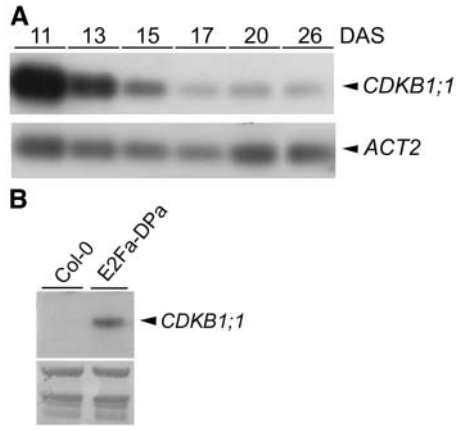

Figure 5. Expression Analysis of the *CDKB1;1* Gene.

(A) Transcript levels of *CDKB1;1* during leaf development. cDNA prepared from the first leaf pair harvested at the indicated time points was subjected to semiquantitative RT-PCR analysis with gene-specific primers. The *actin 2* (*ACT2*) gene was used as loading control. DAS, days after sowing. [AQ14]

(B) RNA gel blot analysis of *CDKB1;1*. RNA was extracted from 12-d-old control (Col-0) and *E2Fa-DPa* seedlings. Equal loading of the gel was confirmed by methylene blue staining of the membrane (bottom panel).

Table 1. DNA Ploidy Levels in 6-d-Old Wild-Type and Transgenic Seedlings

Line	2C (%)	4C (%)	8C (%)	16C (%)	32C (%)	64C (%)
Col-0	26.6 ± 3.3	34.6 ± 1.4	30.2 ± 1.4	6.6 ± 1.6		
CDKB1;1.N161 × Col-0	14.7 ± 1.1	29.1 ± 4.2	35.9 ± 2.7	14.7 ± 0.9		
Col-0 × *E2Fa-DPa*	18.6 ± 2.3	32.6 ± 1.6	23.5 ± 0.7	16.0 ± 1.6	5.4 ± 0.8	0.9 ± 0.2
CDKB1;1.N161 × *E2Fa-DPa*	13.1 ± 1.2	27.6 ± 1.9	25.4 ± 1.7	23.7 ± 2.1	8.0 ± 0.8	1.1 ± 0.2

Data represent average ± SD ($n = 4$ to 6).

levels, reaching up to 45.3% ± 4.4% (35S promoter of *Cauliflower mosaic virus* [*CaMV35S*]:*CDKB1;1.N161* line 1.2) and 43.1% ± 1.7% (*CaMV35S*:*CDKB1;1.N161* line 9.2), compared with 19.2% ± 1.6% in control leaves. In addition, a small but reproducible 32C peak was detected in the transgenic lines but not in the pavement cells of control leaves. This endoreduplication phenotype could not be reverted by the co-overexpression with *CDKA;1* (data not shown), indicating that the effect on the endocycle is specifically because of an inhibition of the B-type CDK activity.

Reduced CDKB1;1 activity also stimulated endoreduplication in other tissues than leaves. In cotyledons, hypocotyls, and roots, the relative abundance of cells with a high ploidy level increased at the cost of cells with a low DNA content (Figure 4). No significant effect on the ploidy level of trichomes was observed, which might be because *CaMV35S* promoter activity in these cells is too low to counteract the endogeneous CDKB1;1 protein.

CDKB1;1 Is Only Expressed during the Mitotic Phase of Leaf Development

The temporal expression pattern of *CDKB1;1* during leaf development was studied by RT-PCR with RNA prepared from the first leaves harvested between 11 and 26 d after sowing. *CDKB1;1* transcript levels were high at day 11 and thereafter decreased to low basal levels (Figure 5A), which coincided with the onset of endoreduplication. By contrast, *CDKA;1* was expressed at an almost constant level during leaf development (data not shown).

CDKB1;1.N161 Suppresses Ectopic Divisions Triggered by E2Fa-DPa

Previously, we have postulated that the decision of cells to undergo a mitotic cell cycle or to endoreduplicate depends on the presence of an MIF. Because of its role in the G2-M transition and the observed increase of endoreduplication upon overexpression of its dominant negative allele, CDKB1;1 is a good candidate to be part of this MIF. To test this hypothesis, *E2Fa-DPa*–overexpressing plants were crossed with a *CDKB1;1.N161*–overexpressing line. As control, *CaMV35*:*CDKB1;1.N161* and *CaMV35S:E2Fa-DPa* lines were crossed separately with wild-type plants. The fidelity of the crosses was confirmed by PCR analysis and RNA gel blots (data not shown). In a first series of experiments, 6-d-old seedlings were analyzed by flow cytometry. As described above for the developing leaves, overexpression of *CDKB1;1.N161* resulted in an increase of the 8C and 16C populations, corresponding with a decrease in the number of cells with a 2C DNA content (Table 1). As reported previously, overexpression of *E2Fa-DPa* resulted in the appearance of two additional endocycles (Table 1; De Veylder et al., 2002). When *CDKB1;1.N161* and *E2Fa-DPa* were co-overexpressed, an additive phenotype was seen, resulting in an even stronger reduction in the amount of 2C cells, whereas the population of 16C and 32C cells increased.

While *CDKB1;1.N161* overexpression stimulated the endocycle phenotype of *E2Fa-DPa* transgenics, it simultaneously suppressed the occurrence of ectopic cell divisions, as can been seen for abaxial epidermal pavement cells of 6-d-old cotyledons (Figure 6, Table 2). Similarly as described above for leaves, overexpression of *CDKB1;1.N161* in cotyledons led to an increase in pavement cell size (Figures 6A and 6B). By contrast, the cotyledon size of *E2Fa-DPa* transgenic plants was considerably smaller, but many more epidermal cells were observed because of ectopic cell division (Figure 6C). However, when *CDKB1;1.N161* was expressed in an *E2Fa-DPa* transgenic

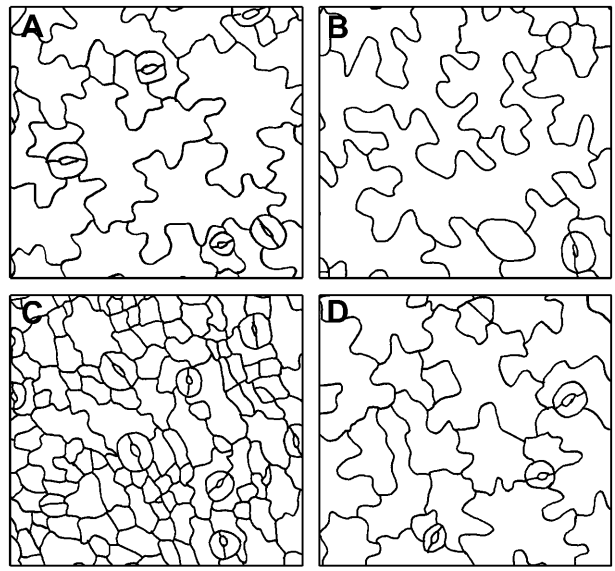

Figure 6. Drawing-Tube Images of Wild-Type and Transgenic Plants.

(A) Wild type (Col-0).
(B) *CaMV35S*:*CDKB1;1N161* × Col-0.
(C) Col-0 × *CaMV35S:E2Fa-DPa*.
(D) *CaMV35S*:*CDKB1;1.N161* × *CaMV35S:E2Fa-DPa*.

Table 2. Abaxial Pavement Cell Size and Cell Number in Cotyledons of *CDKB1;1.N161* and *E2Fa-DPa* Transgenic Lines

		Abaxial Pavement Cells	
Line	Cotyledon Size (mm^2)	Size (μm^2)	Estimated Number
Col-0	2.6 ± 0.2	2082 ± 110	1284 ± 170
CDKB1.1.N161 × Col-0	2.6 ± 0.1	3925 ± 209	664 ± 40
Col-0 × *E2Fa-DPa*	1.6 ± 0.2	232 ± 33	7563 ± 1115
CDKB1;1.N161 × *E2Fa-DPa*	1.8 ± 0.1	2010 ± 110	910 ± 63

All measurements were performed on cotyledons harvested 6 d after sowing. The indicated values are means ± SE (n = 5 to 10).

background, the ectopic cell division phenotype was repressed (Figure 6D), resulting in an average cell size being only slightly smaller than that of wild-type cells.

CDKB1;1 Transcription Is Regulated by E2Fa-DPa

Previously, microarray analysis has revealed that *CDKB1;1* transcripts are upregulated in E2Fa-DPa–overproducing plants (Vlieghe et al., 2003). To validate this result, *CDKB1;1* expression levels were determined in wild-type versus *E2Fa-DPa* transgenic plants via RNA gel blotting. In wild-type control plants, no *CDKB1;1* transcripts were detected, reflecting low expression levels (Figure 5B). By contrast, in the *E2Fa-DPa* transgenic plants a strong *CDKB1;1* hybridization signal was observed, confirming the previous microarray result.

To test the tissue specificity of the *CDKB1;1* induction in *E2Fa-DPa* seedlings, a *CDKB1;1* promoter β-glucuronidase (GUS) construct was crossed into the *E2Fa-DPa* transgenic lines, and the spatial *GUS* expression pattern was compared with that of control plants. In the vegetative part of control plants, *CDKB1;1* promoter activity could be observed in the shoot apical meristem, in the young leaf primordia, and in stomata (Figures 7A and 7C; Boudolf et al., 2004). In the *E2Fa-DPa* transgenic plants, GUS staining was significantly stronger (Figures 7B and 7D). GUS activity was present not only in a much broader region surrounding the meristem, probably in correlation with the occurrence of ectopic cell divisions, but was also stronger at the cellular level, as seen in leaf primordia and stomatal cells. These data suggest that the increased *CDKB1;1* transcript levels observed in the *E2Fa-DPa* transgenic lines are not only the result of the ectopic cell division but also originate by a direct stimulation of *CDKB1;1* promoter activity.

In the promoter of the *CDKB1;1* gene, an E2F-like binding site (TTTCCCGC) can be detected, located 151 bp downstream of the start codon. To investigate how this promoter element contributes to the level of *CDKB1;1* transcription, the E2F binding site was mutated into TTTCCAAC. This mutation has previously been demonstrated to impair E2F-DP binding (Kosugi and Ohashi, 2002). Both wild-type and mutant promoters were transcriptionally fused to the *GUS* reporter gene, and these reporter constructs were used to transform tobacco (*Nicotiana tabacum*) Bright Yellow-2 (BY-2) cells. For each construct, 14 independent calli were isolated, in which the level of GUS protein was quantified by fluorometry. On the average, GUS activity was 29-fold higher in the calli containing the wild-type promoter construct than in those harboring the mutant promoter (Figure 8). These data illustrate that the E2F *cis*-acting element is a major factor in regulating *CDKB1;1* promoter activity.

DISCUSSION

In yeast and Drosophila, downregulation of M-phase–associated CDK activity is sufficient to drive cells into the endoreduplication cycle (Hayles et al., 1994; Sauer et al., 1995). A similar mechanism is probably operational in plants because the onset of endoreduplication in maize endosperm, during tomato fruit development, and Arabidopsis leaf development is correlated with the inhibition of mitotic CDK activity (Grafi and Larkins, 1995; Joubès et al., 1999). Here, we have demonstrated that CDKB1;1 controls the balance between mitotic cell division and endoreduplication in both wild-type and transgenic plants overproducing both E2Fa and DPa. Such a role for CDKB1;1 was hinted by its temporal expression pattern during leaf development. *CDKB1;1* is highly expressed in dividing cells and is downregulated at the onset of endoreduplication. This result is in agreement with previously published data that demonstrate that *CDKB1;1* transcription occurs only in the mitotically dividing cells of the shoot apex (Segers et al., 1996). Additionally, we found that by decreasing the CDKB1;1 activity, cells exit the mitotic cell cycle earlier, accompanied with an early onset of the endocycle.

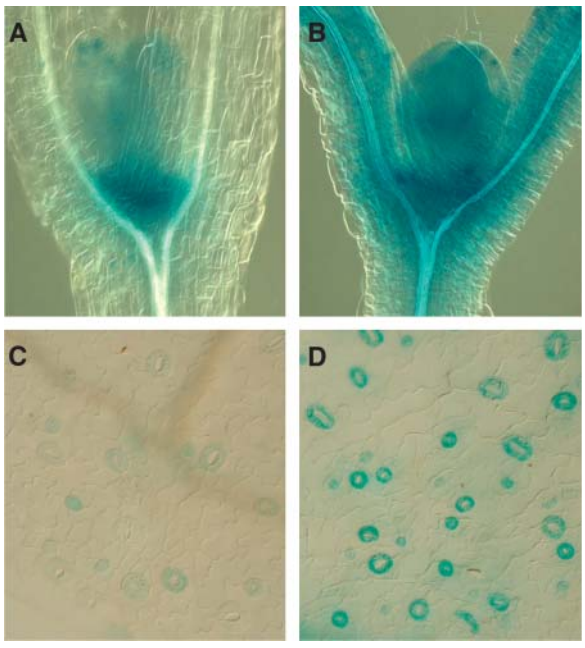

Figure 7. Activity of the *CDKB1;1* Promoter Fused to the GUS Reporter (pCDKB;1:GUS) in Wild-Type (Col-0) and *E2Fa-DPa*–Overexpressing Plants Visualized by Histochemical Staining.

(A) GUS activity in the shoot apex of untransformed plants.
(B) GUS activity in the shoot apex of *E2Fa-DPa* transgenic plants.
(C) GUS activity in stomata of untransformed plants.
(D) GUS activity in stomata of *E2Fa-DPa* transgenic plants.

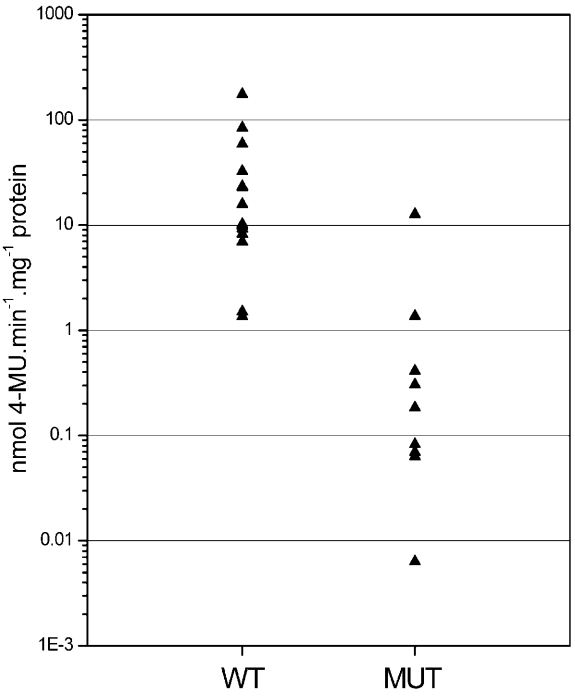

Figure 8. Quantification of Wild-Type and Mutant *CDKB1;1* Promoter Activity.

GUS activity was measured fluorometrically in protein extracts prepared from transgenic BY-2 cells transformed with the wild type ($n = 14$) or mutant (MUT; $n = 14$) *CDKB1;1* promoter fused to the *GUS* reporter gene. 4-MU, 4-methylumbelliferone.

This untimely exit of the mitotic cell cycle results in leaves with fewer but larger cells than those in wild-type leaves, which have a higher ploidy level. An increase in cell size in response to an inhibition of cell division has been observed previously and can be attributed to an uncoupling of cell division and cell expansion (Hemerly et al., 1995; De Veylder et al., 2001). Because of the observed correlation between ploidy level and cell dimension, an increase in ploidy level in response to a perturbation in the cell cycle is often seen as a mechanism to accommodate the increase in cell size. As such, the extra endoreduplication observed in the *CDKB1;1.N161* plants might be triggered by the need for cell growth. However, the amplified ploidy level is more readily a primary phenotype triggered by CDKB1;1.N161 rather than a secondary effect induced by cell expansion because an increase in the endoreduplication level could already be observed when cell number and size were indistinguishable between wild-type and transgenic lines. Because both wild-type and *CDKB1;1.N161*-overexpressing plants exit the endocycle at the same stage during leaf development (leaf 1 at 21 d after sowing), the higher ploidy levels observed in the *CDKB1;1.N161* plants can be largely attributed to the earlier start of the endocycle program.

Whereas overproduction of the dominant negative CDKB1;1.N161 stimulates endoreduplication, overexpression of the wild-type *CDKB1;1* gene had no effect on the ploidy distribution of wild-type plants. This discrepancy can be explained by the lack of a regulatory factor (such as a cyclin) for CDKB1;1 activity. Indeed, overexpression of *CDKB1;1* in a wild-type background does not significantly alter the amount of extractable CDK activity (Boudolf et al., 2004). A good candidate to act in concert with CDKB1;1 is the CYCA3;2 cyclin, whose overproduction has been demonstrated to strongly inhibit the endocycle (Yu et al., 2003).

CDKB1;1 transcription is regulated through an *E2F cis*-acting element in its promoter, which is surprising because E2F-DP transcription factors are presumed to operate at the G1-S transition, whereas CDKB1;1 kinase activity peaks at G2-M. However, in synchronized cultures, *CDKB1;1* transcript levels increase from the G1-S transition onwards, followed by an increase in CDKB1;1 protein and associated activity during S-phase (Porceddu et al., 2001; Sorrell et al., 2001; Breyne et al., 2002; Menges et al., 2002). In all eukaryotes, DNA replication is initiated by the stepwise binding of proteins to the origins of replication (Larkins et al., 2001; Nishitani and Lygerou, 2002). After binding of the DNA replication-licensing proteins ORC, CDC6, CDC18, and CDT1 to these origins, which form the prereplication complex, replication is initiated by the recruitment of MCM proteins. As soon as replication starts, the MCM proteins dissemble from the replication complex, and reformation of the complex is inhibited until sister chromatids are separated in mitosis. In fission yeast, the mitotic cyclin B/cdc2 complex has been demonstrated to prevent the relicensing of replicated DNA by association with the replication complex during chromosome duplication, preventing the MCM proteins to reinitiate DNA synthesis (Wuarin et al., 2002). Similarly, CDKB1;1 activity might repress the endocycle in plants by association with the prereplication complex. This hypothesis suggests that besides its anticipated role at the G2-M transition, CDKB1;1 also plays a role earlier in the cell cycle.

Previously, we have demonstrated that E2Fa-DPa regulates both the mitotic and endoreduplication cycles of Arabidopsis. Cells in which this heterodimeric transcription factor are overexpressed were shown to either undergo extra mitotic divisions or to endoreduplicate. The decision to divide mitotically or to endoreduplicate was postulated to depend on the presence of an MIF (De Veylder et al., 2002). Upon the coexpression of *CDKB1;1.N161*, the ectopic cell division phenotype of the E2Fa-DPa transgenic plants was repressed, whereas the endoreduplication phenotype is enhanced. These data illustrate that factors important for mitosis, which are stimulated by *E2Fa-DPa* overexpression are titrated out by CDKB1;1.N161. Therefore, CDKB1;1 is the most probable candidate to be part of

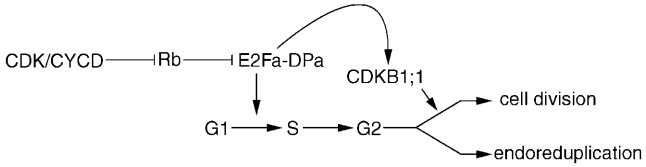

Figure 9. Model Illustrating the Interaction between E2Fa-DPa and CDKB1;1 Activity to Drive the Mitotic Cell Cycle.

For details, see text. Rb, retinoblastoma.

the MIF. Cells that possess CDKB1;1 activity undergo mitotic division, whereas those that lack it are stimulated by E2Fa-DPa to enter the endocycle (Figure 9).

Although the switch between mitosis and endocycle almost certainly occurs at the protein level by the specific destruction of M-phase–specific regulators by the CCS52 complex, the absence of *CDKB1;1* transcripts in polyploid tissues suggests a mechanism that is operational to suppress the transcriptional activation of *CDKB1;1* in endoreduplicating cells. The Arabidopsis E2F factors can be functionally subdivided into activators (E2Fa and E2Fb) and a repressor (E2Fc) (De Veylder et al., 2003). It is possible that the *CDKB1;1* promoter is associated sequentially with different E2F proteins, with repressing E2Fs replacing an activating E2F complex when cells shift from the mitotic cell cycle into the endocycle. However, in contrast with the proliferating cell nuclear antigen promoter of tobacco, in which the *E2F* binding sites function as negative regulatory elements that repress transcription in mature leaves (Egelkrout et al., 2001, 2002), deletion of the *E2F* binding site in the *CDKB1;1* promoter does not result in increased transcriptional activity in maturing leaves (data not shown), strongly suggesting that *CDKB1;1* transcription is solely regulated by activating E2Fs. Therefore, the difference in *CDKB1;1* promoter activity in mitotically dividing versus endoreduplicating cells might rather be regulated through the interaction of E2F with other transcriptional activators and repressors bound to the promoter. The combinatorial nature of E2F transcription factors has been demonstrated in mammals, in which activating E2Fs are recruited to a promoter via synergistic interactions with adjacent transcription factors (Schlisio et al., 2002; Giangrande et al., 2003, 2004). At the moment, the nature of the transcription factor that would distinguish an endoreduplicating cell from a mitotically dividing cell remains unknown.

Although our understanding of how cell cycle transitions are regulated has gradually improved, we hardly comprehend how the different transitions communicate with each other. The transcriptional induction of *CDKB1;1* by E2F-DP transcription factors suggests a mechanism by which the G1-S and G2-M cell cycle checkpoints communicate. The existence of such linkage has already been hinted by the observation that ectopic expression of the D-type cyclin CYCD3;1 not only induces DNA replication but also cell division in Arabidopsis trichomes (Schnittger et al., 2002). In budding yeast, the combination of chromatin immunoprecipitation assays with DNA microarray analysis has revealed that proteins that operate as activators during one stage of the cell cycle can contribute to the transcriptional activation of proteins that function during the next stage, forming a fully connected regulatory circuit (Simon et al., 2001). The scarce data available suggest that a similar circuit might be operational in plants. However, it is clear that to fully understand cell cycle functioning, experiments aimed at unraveling connections between different checkpoints will be crucial.

METHODS

Generation of Transgenic Plants

Plants harboring the wild-type *CDKB1;1* or mutant *CDKB1;1.N161* gene under the control of *CaMV35S* (Boudolf et al., 2004) and plants coexpressing the *DPa* and *E2Fa* genes (De Veylder et al., 2002) were constructed as described previously. Triple *CaMV35S:CDKB1;1.N161-E2Fa-DPa* plants were obtained by crossing double homozygous *CaMV35S:E2Fa-DPa* plants with homozygous *CaMV35S:CDKB1;1.N161* plants.

Kinematic Analysis of Leaf Growth

Leaf growth was analyzed kinematically as described (De Veylder et al., 2001). Briefly, wild-type and CDKB1;1.N161-overproducing plants were germinated and grown in round 12-cm Petri dishes filled with 100 mL of 1× MS medium (Duchefa, Haarlem, The Netherlands) and 0.6% plant tissue culture agar (LabM, Bury, UK) at 22°C and 65 $\mu E\ m^{-2}\ s^{-1}$ radiation in a 16-h-light/8-h-dark photoperiod. From day 5 until day 28 after sowing, plants were harvested, cleared overnight in methanol, and subsequently stored in lactic acid for microscopy. The youngest plants were mounted on a slide and covered. The leaf primordia were observed under a microscope fitted with differential interference contrast optics (DMLB; Leica, Wetzlar, Germany). The total (blade) area of leaves 1 and 2 of each seedling were first determined from drawing-tube images with the public domain image analysis program ImageJ (version 1.30; http://rsb.info.nih.gov/ij/). At older stages, the primordia were digitized directly with a charge-coupled device camera mounted on a binocular (Stemi SV11; Zeiss, Jena, Germany). Cell density was determined from scanned drawing-tube images of outlines of at least 20 cells of the abaxial epidermis located 25 and 75% from the distance between the tip and the base of the leaf primordium (or blade once the petiole was present), halfway between the midrib and the leaf margin. In the youngest primordia (up to day 6), a single group of cells was drawn. The following parameters were determined: total area of all cells in the drawing, total number of cells, and number of guard cells. From these data, we calculated the average cell area and estimated the total number of cells per leaf by dividing the leaf area by the average cell area (averaged between the apical and basal positions). Finally, average cell division rates for the whole leaf were determined as the slope of the $\log_2$-transformed number of cells per leaf, which was done using five-point differentiation formulas (Erickson, 1976).

Flow Cytometric Analysis of Leaves

Leaves were chopped with a razor blade in 300 μL of buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 3-[*N*-morpholino]propanesulfonic acid, pH 7, and 1% Triton X-100) (Galbraith et al., 1991). To the supernatants, 1 μL of 4',6-diamidino-2-phenylindole from a stock of 1 mg/mL was added, which was filtered over a 30-μm mesh. The nuclei were analyzed with the BRYTE HS or CyFlow flow cytometer, using Win-Bryte software (Bio-Rad, Hercules, CA) or FloMax software (Partec, Münster, Germany), respectively.

Epidermal Peels and Measurements of Nuclear DNA Quantity

The protocol for making epidermal peels was adapted from Melaragno et al. (1993). Three-week-old plants were fixed in a solution of three-quarters 95% ethanol and one-quarter glacial acetic acid for 2 h at room temperature and stored in 70% ethanol at 4°C. Fixed tissue was soaked first in water and then in 0.5 M EDTA, pH 8. Leaves were placed with the abaxial epidermis side down on a glass slide and held in place with forceps, while tissue was removed from the abaxial epidermis with another pair of fine forceps. A drop of 4′,6-diamidino-2-phenylindole at a concentration of 0.005 mg/mL in McIlvaines's buffer, pH 4.1 (60 mL 0.1 mol/L citric acid + 40 mL 0.2 mol/L Na_2HPO_4), was placed on the epidermal peel. Peels were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and observed under a 20× objective on a Zeiss Axioskop equipped with an Axiocam CCD camera (Zeiss).

Images were obtained with the Axiovision software and were analyzed in gray scale with the public domain image analysis program ImageJ (version 1.28; http://rsb.info.nih.gov/ij/). Relative fluorescence units were measured as integrated density, which is the product of the area and the average fluorescence of the selected nucleus.

Promoter Analysis

The intergenic region between the CDKB1;1 (At3g54180) open reading frame and the upstream open reading frame (486 bp) was isolated from Col-0 genomic DNA by PCR using the 5′-AAAAAGCAGGCTGAAGATG-CAAAAGAAGAAATTGCTGGTGG-3′ and 5′-AGAAAGCTGGGTTTCTGA-GAGGTTTCGTAAAATTGAACTGTG-3′ primers, and the resulting PCR fragment was cloned into the GATEWAY pDONR 207 vector (Invitrogen, Carlsbad, CA) by *att*B × *att*P recombination reaction. The resulting plasmid was used to mutate the E2F binding site by PCR with the outward-oriented 5′-AAGGAAAAACGAAGTGACGTGGAAG-3′ and 5′-CTCCTTTGGATTTTGAATTCGTTCG-3′ primers. The PCR fragment was phosphorylated with the T4 polynucleotide kinase and self-ligated. The introduced mutations were confirmed by sequencing. The wild-type and mutant promoter fragments were subsequently recombined into the pKGWFS7 vector by *att*L × *att*R recombination reaction at the GATEWAY recombination site located in front of the gene coding for the enhanced green fluorescent protein-GUS (Karimi et al., 2002), resulting in the pCDKBWT and pCDKBmut vectors, respectively. The vectors were used to transform tobacco (*Nicotiana tabacum*) BY-2 cells as described (Joubès et al., 2004). For both constructs, 14 calli were selected whose GUS activity was measured as described (Zambre et al., 2003).

RT-PCR Analysis

RNA was extracted from leaves of *Arabidopsis thaliana* Heynh. (ecotype Col-0) with TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed on 3 μg of total RNA with the Superscript RT II kit (Invitrogen) and oligo(dT)$_{18}$ according to the manufacturer's instructions. A 1-μL aliquot of the total reverse transcription reaction volume (20 μL) was used as template in the semiquantitative RT-PCR amplification, ensuring that the amount of amplified product remained in linear proportion to the initial template present in the reaction. Ten microliters from the PCR reaction was separated on a 0.8% agarose gel and transferred onto Hybond N$^+$ membranes (Amersham Biosciences, Little Chalfont, UK). The membranes were hybridized at 65°C with fluorescein-labeled probes (Gene Images random prime module; Amersham Biosciences). The hybridized bands were detected with the CDP Star detection module (Amersham Biosciences). Primers used were as follows: 5′-GGTGGTGACATGT-GGTCTGTTGG-3′ and 5′-CGCAGTGTGGAAACACCCGG-3′ for *CDKB1;1* and 5′-GGCTCCTCTTAACCCAAAGGC-3′ and 5′-CACACCATCACCA-GAATCCAGC-3′ for *ACT2* (At3g18780).

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