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DNA Replication Licensing Affects Cell Proliferation or Endoreplication in a Cell Type–Specific Manner

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In eukaryotic cells, the function of DNA replication licensing components (Cdc6 and Cdt1, among others) is crucial for cell proliferation and genome stability. However, little is known about their role in whole organisms and whether licensing control interfaces with differentiation and developmental programs. Here, we study *Arabidopsis thaliana* CDT1, its regulation, and the consequences of overriding licensing control. The availability of AtCDT1 is strictly regulated at two levels: (1) at the transcription level, by E2F and growth-arresting signals, and (2) posttranscriptionally, by CDK phosphorylation, a step that is required for its proteasome-mediated degradation. We also show that CDC6 and CDT1 are key targets for the coordination of cell proliferation, differentiation, and development. Indeed, altered CDT1 or CDC6 levels have cell type–specific effects in developing Arabidopsis plants: in leaf cells competent to divide, cell proliferation is stimulated, whereas in cells programmed to undergo differentiation-associated endoreplication rounds, extra endocycles are triggered. Thus, we propose that DNA replication licensing control is critical for the proper maintenance of proliferative potential, developmental programs, and morphogenetic patterns.

INTRODUCTION

The regulated function of prereplication complexes (pre-RC) is crucial to determine the ordered activation of DNA replication origins in every cell cycle and, as a consequence, to maintain genome integrity. Studies conducted in yeast have revealed that the assembly of pre-RC on origins starts at the end of mitosis and early G1 by the subsequent association of Cdc6 and Cdt1 proteins to the origin recognition complex (Cocker et al., 1996; Nishitani et al., 2000). In general terms, a similar series of events seem to occur also in Xenopus and mammalian cells (Coleman et al., 1996; Maiorano et al., 2000). The association of Cdc6 and Cdt1 to the origin recognition complex is a prerequisite for the loading of minichromosome maintenance (MCMs) proteins to chromatin, licensing the origins for a new replication round (Kelly and Brown, 2000; Bell and Dutta, 2002). The molecular components and the basic interactions of pre-RC components are strikingly conserved from yeast to metazoa (Kelly and Brown, 2000; Ritzi and Knippers, 2000; Bell and Dutta, 2002). However, the availability and dynamics of pre-RC proteins vary in different model systems. Such a fine control is achieved at different levels, including transcription, phosphorylation, binding of geminin to CDT1, subcellular localization, or proteolysis, depending on the protein and the organism (reviewed in Bell and Dutta, 2002; Blow and Hodgson, 2002; Diffley and Labib, 2002; Tanaka and Diffley, 2002). Studies on regulation of DNA replication in multicellular organisms are scarce and largely restricted to *Drosophila melanogaster* and *Caenorhabditis elegans* (Whittaker et al., 2000; Zhong et al., 2003). Thus, one of the major challenges ahead is to define whether regulation of DNA replication has cell type–specific properties and can affect key processes, such as developmental programs or morphogenetic patterns.

Very little is known about regulation of pre-RC components and DNA replication licensing in plants. Organogenesis is unique in plants because postembryonic development depends on a continuous balance between cell proliferation and differentiation. This together with the availability of the *Arabidopsis thaliana* genome sequence make it a powerful model for understanding DNA replication control and its possible implications on cell proliferation, differentiation, and development in the context of a whole organism.

Plants are intriguingly tolerant to changes in the level of cell cycle and DNA replication proteins, but the mechanisms are poorly understood (reviewed in Gutierrez et al., 2002; De Veylder et al., 2003; Dewitte and Murray, 2003). We have previously studied Arabidopsis CDC6 and shown that ectopic expression of *AtCDC6a* induces extra endoreplication cycles (Castellano et al., 2001). In the endoreplication cycle, repeated rounds of DNA replication take place without a succeeding mitosis leading to polyploid cells (Edgar and Orr-Weaver, 2001). Endoreplication is a physiological mode of full-genome rereplication, relatively frequent in many plant cell types, and associated with developmentally regulated processes such as hypocotyl elongation, trichome growth, or endosperm development (Hülskamp et al., 1999; Kondorosi et al., 2000; Larkins et al., 2001).

Thus, we wanted to study DNA replication licensing control in the context of a growing plant where both cell proliferation and endoreplication are tightly associated with particular

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differentiation programs. Here, we describe the identification of Arabidopsis CDT1a (AtCDT1a) and its regulation at the transcriptional and posttranslational level. We also describe the novel finding that altering the level of DNA replication licensing components has cell type–specific effects: in cells with proliferative potential, they stimulate cell renewal, whereas in cells that endoreplicate, extra endocycles are triggered.

RESULTS

AtCDT1 Proteins Contain Two Highly Conserved Motifs

To define the role of pre-RC components during plant growth and development, we focused on CDT1. A search in the Arabidopsis genome using amino acid motifs conserved among Cdt1 sequences led us to identify and clone two cDNAs, *AtCDT1a* and *AtCDT1b*, of the expected sizes based on RNA gel blot analysis (data not shown) and corresponding to the Munich Information Center for Protein Sequences codes At2g31270 and At3g54710, respectively. They encoded AtCDT1a (571 amino acids) and AtCDT1b (486 amino acids), but only AtCDT1a displays a basic pI, similar to animal CDT1 proteins (Maiorano et al., 2000; Arentson et al., 2002). Pairwise alignments revealed that AtCDT1a has 12.5, 18.9, 15.0, 14.2, 14.3, and 16.1% identity with its *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Xenopus laevis*, *D. melanogaster*, *C. elegans*, and *Homo sapiens* homologs, respectively (Maiorano et al., 2000; Nishitani et al., 2000; Whittaker et al., 2000; Wohlschlegel et al., 2000; Tanaka and Diffley, 2002; Zhong et al., 2003). A comparison among distantly related members allowed us to identify two highly conserved domains (Figure 1B): the central and the C-terminal domains that are similar to those required for interaction of mouse Cdt1 with MCM4 and geminin, respectively (Yanagi et al., 2002). In spite of this relatively low identity over the entire protein, that of these domains goes up to 30 to 40%. In addition, an Arg residue (R342) in the Drosophila Cdt1/DUP protein, critical for function (Whittaker et al., 2000), is conserved in both AtCDT1a (R137) and AtCDT1b (K70) (Figure 1B).

CDT1 cooperates with CDC6 in the loading of MCMs to the DNA replication origins, and the physical interaction between them has been described in different species (reviewed in Bell and Dutta, 2002). To know whether this interaction is conserved in plants, we performed pull-down assays. As expected, purified glutathione *S*-transferase (GST)-AtCDT1a was able to interact with in vitro translated AtCDC6a protein (Figure 1C). This interaction was also observed in the yeast two-hybrid system (data not shown). An in vitro DNA replication initiation assay, similar to that developed with Xenopus, is not available yet in Arabidopsis, but based on the properties of AtCDT1a cDNA and its binding to CDC6, it is reasonable to conclude that it encodes a putative Arabidopsis CDT1 homolog.

The Two *AtCDT1* Genes Are Expressed in Proliferating and Endoreplicating Cells

To establish whether both AtCDT1 could have similar functional relevance, we performed expression analysis of the two *AtCDT1* genes at different growth stages. To this end, transgenic plants expressing the β-glucuronidase (*GUS*) reporter gene under each of the *AtCDT1* promoters (*pCDT1a*:GUS and *pCDT1b*:GUS plants) were generated. The AtCDT1a promoter was stronger than that of AtCDT1b until 60 h after the seeds were transferred to light and 22°C, although both of them were active in similar locations. Afterwards, the two promoters showed identical expression patterns, both in location and intensity. Details are given below for *AtCDT1a* for simplicity. As mentioned above, the AtCDT1a promoter was active at very early stages (20 to 60 h after transferring to light; Figures 2A to 2C). At 40 h, a clear promoter activity already has been established at the shoot and root apical meristems. In 60-h-old cotyledons, a slight spotty pattern, over a background of positive GUS staining, was evident (Figure 2C). This corresponds, most likely, to epidermal cells of the stomatal lineage based on the staining pattern in cotyledons of 5-d-old seedlings (Figures 2D and 2E). A strong GUS activity can be also detected in meristematic locations, such as the shoot and the root apical meristems, and early leaf primordia (Figure 2D). Promoter activity is also detected in the emerging lateral roots (Figure 2F; 10-d-old seedlings). GUS activity was detected in guard cells of fully developed stomata (Figures 2G to 2I) as well as in progenitor cells of the stomatal lineage, such as primary and satellite meristemoids (Figure 2H). Later, it has disappeared in cotyledons (15 d old), and a decreasing gradient was apparent in leaves as they develop (Figure 2J). It should be noted that the AtCDT1a promoter is active in developing trichomes present in early leaf primordia (Figure 2K). The AtCDT1 promoters are also active in young flowers, particularly in developing anthers (Figure 2L) but not in mature flowers (Figure 2M) or in siliques (Figure 2N). This pattern of expression is consistent with a putative role of AtCDT1 proteins in DNA replication during both the cell cycle and the endocycle. Because of the similarity of the expression pattern of the two *AtCDT1* genes and the higher similarity of AtCDT1a with other counterparts, we focused our further study on *AtCDT1a*.

AtCDT1a Is an E2F Target Gene Downregulated by Antiproliferative Stimuli

Although the function of pre-RCs seems to be mechanistically conserved in eukaryotes (Bell and Dutta, 2002), the regulation of their components varies among species (Blow and Hodgson, 2002). Thus, we found important to study several aspects of AtCDT1a regulation in Arabidopsis. To begin to understand whether positive and negative regulators of cell proliferation affect *AtCDT1a* gene expression, we analyzed the putative promoter region. The in silico study revealed the presence of multiple potential binding sites for transcription factors, most notably it contained the sequences TTTCGCGG and TTTGGCGCG (reverse), consensus binding sites for the E2F/DP transcription factor, 186 and 113 bp upstream from the ATG (referred to the first T of the E2F/DP binding site).

To investigate whether *AtCDT1a* is a target of the E2F/DP transcription factor in vivo, we used real-time RT-PCR analysis of Arabidopsis plants expressing a dominant negative version of DP, the E2F heterodimeric partner (Ramirez-Parra et al., 2003). This revealed an approximately threefold decrease in *AtCDT1a* mRNA levels in the mutant compared with the control (Figure 3A),

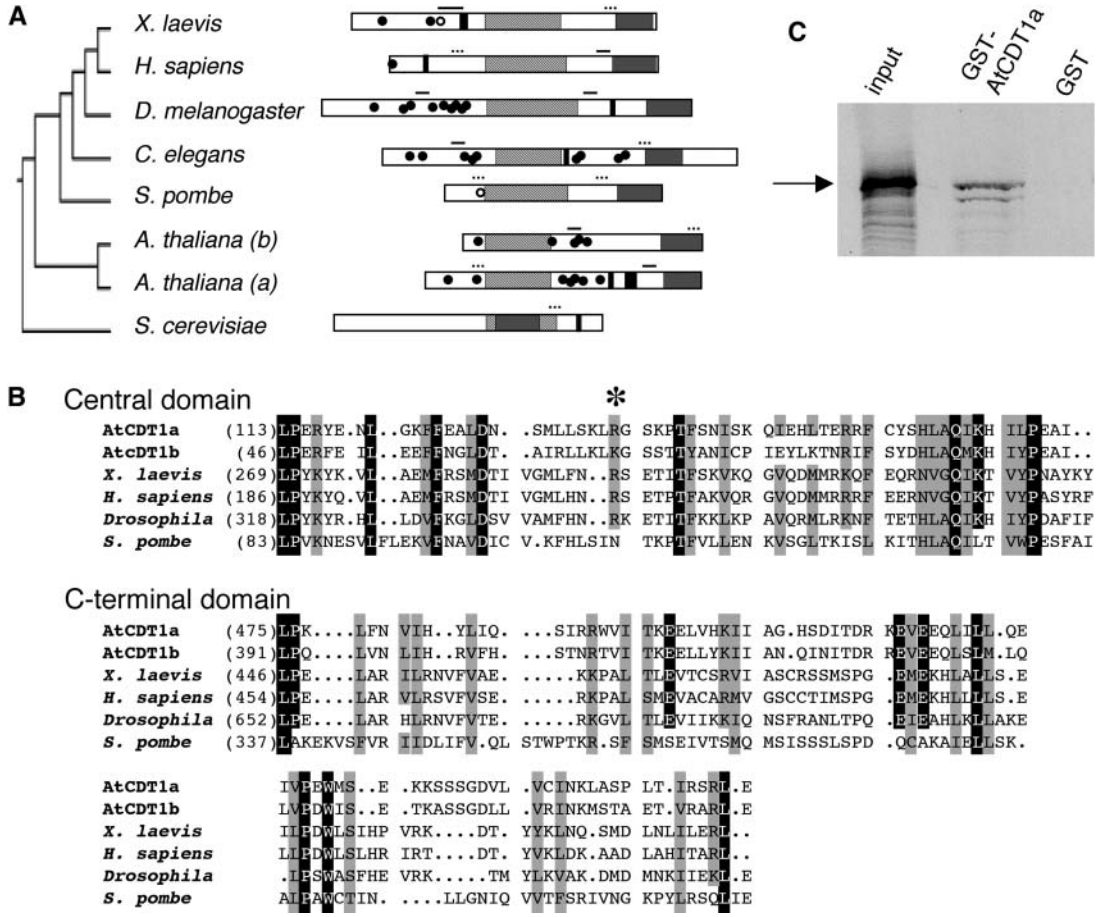


Figure 1. Arabidopsis CDT1a and CDT1b Proteins.

(A) Phylogenetic tree and domain organization of yeast, animal, and Arabidopsis CDT1 proteins. Two high homology regions define the central (hatched) and the C-terminal (gray) domains. Putative coiled-coil domains (lines and dots), PEST sequences (black), KEN sequences (open circles), and putative CDK phosphorylation sites (closed circles) are shown.

(B) Alignment of the highly conserved central and C-terminal domains of CDT1 proteins of various sources. Identical and conserved residues appear in black and gray boxes, respectively. The asterisk indicates the R/K residue conserved in CDT1 proteins.

(C) Interaction of in vitro transcribed and translated AtCDC6a protein (arrow) with purified protein GST-AtCDT1a. The bands below the full-length AtCDT1a protein correspond to partial translation products present in the bacterial lysate.

strongly supporting the idea that the E2F/DP binding site present in the AtCDT1a promoter is relevant for its transcriptional regulation. Two other E2F targets, *AtCDC6a* (Castellano et al., 2001; De Veylder et al., 2002; Ramirez-Parra et al., 2003) and *AtPCNA* (Egelkrout et al., 2001; Kosugi and Ohashi, 2002), used as controls, were also downregulated in the dominant negative DP plants (Figure 3A). Then, we tested whether treatments that negatively affect growth regulate *AtCDT1a* gene expression. Seedlings challenged with dehydration or abscisic acid (ABA), a plant hormone frequently associated with inhibition of growth (Finkelstein and Gibson, 2000) and DNA synthesis (Leung and Giraudat, 1998), showed an approximately threefold and sevenfold reduction of *AtCDT1a* gene expression, respectively (Figures 3B and 3C). As a control, we followed the response of the *Atrd29A* and the *AtCAB1* genes, which are known to be upregulated and downregulated, respectively, under these conditions (Capel et al., 1998; Oono et al., 2003). We further studied the effect of ABA using the *pCDT1a*:GUS transgenic plants. This analysis confirmed the results presented above because treatment of *pCDT1a*:*GUS* seedlings with ABA abolished GUS activity (Figure 3D). Interestingly, this effect was clearly observed in leaf primordia but not in the root meristems, suggesting an organ-specific regulation of the *AtCDT1a* promoter. Despite the fact that these experiments do not address whether *AtCDT1a* is a direct target of E2F/DP, a likely possibility based on our results and the presence of E2F binding sites in its putative promoter, we can conclude that *AtCDT1a* is downstream E2F and that antiproliferative stimuli impinge on its expression levels.

AtCDT1a Is Degraded by the Proteasome in a CDK-Dependent Manner

Little is known about the posttranscriptional regulation of CDT1, in particular in whole organisms. To begin to understand this in

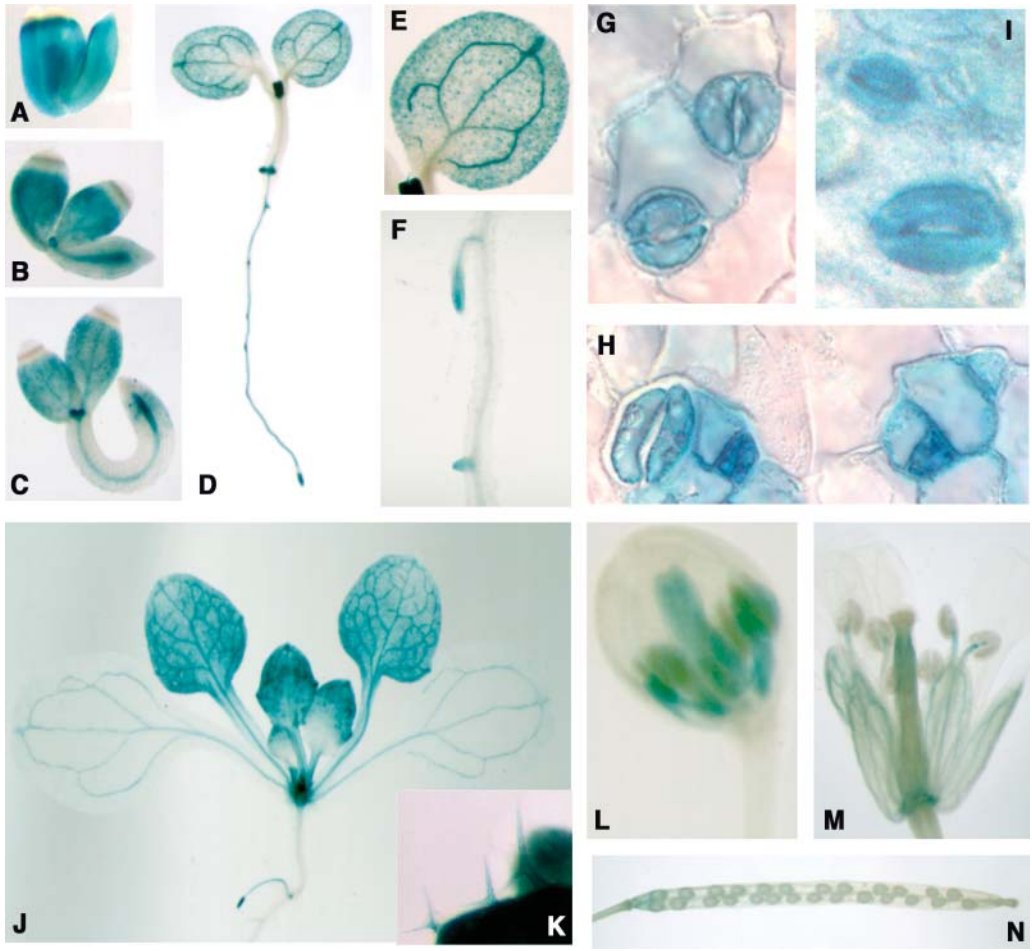


Figure 2. Expression Analysis of *AtCDT1* Genes.

(A) to **(C)** Arabidopsis *pAtCDT1a*:*GUS* seedlings at 20 **(A)**, 40 **(B)**, and 60 **(C)** h after transferring to light and 22°C.
(D) Four-day-old seedlings grown in the light.
(E) Detail of the cotyledon shown in **(D)**.
(F) Ten-day-old light-grown seedlings with developing lateral roots.
(G) to **(I)** Cells of the stomatal lineage in 5- to 10-d-old cotyledons. Fully developed stomata in 5- **(G)** and 10-d-old **(I)** cotyledons. Primary (left) and secondary (right) meristemoids in 5-d-old cotyledons **(H)**.
(J) Leaves at different stages of development (15-d-old seedlings).
(K) Detail of trichomes in leaf primordia.
(L) Young flowers.
(M) Mature flowers.
(N) Siliques.

Arabidopsis, we generated plants expressing constitutively under the control of the 35S promoter of *Cauliflower mosaic virus*, a C-terminally Myc-His–tagged *AtCDT1a* transgene (Figure 4A, top panel). Independent homozygous lines that expressed the transgene and had detectable levels of protein were selected for further analysis (Figure 4A, middle and bottom panels). We did not obtain plant expressing different amounts of AtCDT1a, suggesting a strong regulation at the protein level. Treatment of whole seedlings with the proteasome inhibitor MG132 stabilized AtCDT1a in planta (Figure 4B), demonstrating that it is degraded in vivo by the proteasome. Treatment with aphidicolin, which arrests cycling cells in early S-phase, did not allow detection of AtCDT1a unless MG132 was added (Figure 4B). Because this treatment was performed in whole seedlings, it is not easy to determine precisely the cell cycle phase where AtCDT1a is degraded, although it likely occurs in connection with the S-phase. If so, the situation would be similar to that of human (Nishitani et al., 2001; Li et al., 2003) and *C. elegans* (Zhong et al., 2003) Cdt1.

Proteins degraded through the proteasome frequently require prior phosphorylation. This has been shown for human Cdt1 in cultured human cells (Li et al., 2003), although the type of kinase(s) involved has not been identified. AtCDT1a, as well as the rest of the metazoan Cdt1, contains multiple CDK

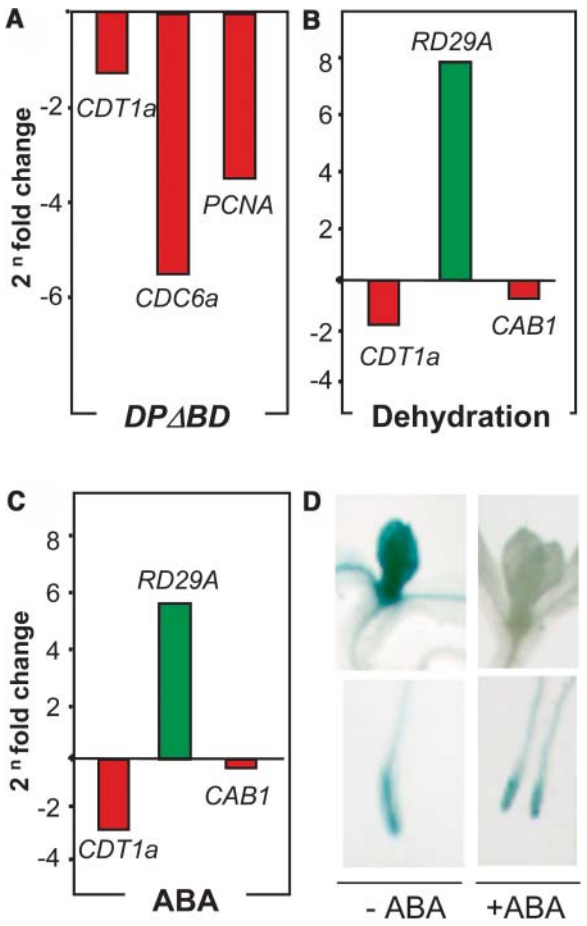


Figure 3. Regulation of *AtCDT1a* Gene Expression.

(A) Real-time RT-PCR of Arabidopsis transgenic seedlings expressing a dominant negative version of wheat (*Triticum aestivum*) DP (DPΔBD).
(B) and **(C)** Same as in **(A)** but with wild-type seedlings dehydrated for 6 h **(B)** or treated with 100 μM ABA **(C)**. In **(A)**, **(B)**, and **(C)**, values are referred to the fold change compared with untreated controls.
(D) Detection of GUS activity in ABA-treated *pAtCDT1a*:*GUS* plants. Note that promoter activity in leaf primordia (top panels), but not in the root meristems (bottom panels), is inhibited.

phosphorylation sites (Figure 1A), suggesting that CDK phosphorylation may affect AtCDT1a regulation. To determine whether a CDK-type activity could affect AtCDT1a stability, we incubated seedlings with roscovitine, a well-known CDK inhibitor. This treatment clearly led to accumulation of AtCDT1a (Figure 4C), as it occurred with MG132. Therefore, we conclude that AtCDT1a is subjected to proteasome-mediated proteolysis and hypothesize that degradation requires a CDK-dependent phosphorylation step.

AtCDT1a Interacts in Vitro and in Vivo with CDKA and Is an in Vitro Substrate of Cyclin D– and Cyclin A–Containing CDKA

To analyze whether CDK complexes phosphorylate AtCDT1a, we first asked whether it interacts in vivo with the major Arabidopsis CDKA-type kinase. To this end, extracts of AtCDT1-His-Myc transgenic seedlings were immunoprecipitated with anti-PSTAIRE antibodies, which recognize AtCDKA. Probing the immunoprecipitates with an anti-Myc antibody revealed the existence of a faint, but reproducible, band corresponding to AtCDT1a-His-Myc (Figure 5A). We confirmed the AtCDT1a/AtCDKA interaction in pull-down assays by incubating whole Arabidopsis cell extracts with GST-AtCDT1a. Kinase assays demonstrated that the AtCDT1a-bound products contained AtCDKA (Figure 5B, top panel) and phosphorylated AtCDT1a (Figure 5B, bottom panel). Thus, AtCDT1a interacts in vivo with a CDKA/cyclin complex that is able to phosphorylate AtCDT1a in vitro.

A diverse set of more than 30 A-, B-, and D-type cyclins are present in the Arabidopsis genome (Vandepoele et al., 2002). Because specific antibodies to identify individual Arabidopsis cyclins are not available, we analyzed the ability of AtCDT1a to interact in extracts with Arabidopsis A- and D-type cyclins. These cyclins participate in CDK/cyclin complexes that phosphorylate G1/S regulators in plants, for example, the retinoblastoma protein (Boniotti and Gutierrez, 2001) or AtE2Fc (del Pozo et al., 2002), and control plant cell cycle transitions (Gutierrez et al., 2002; De Veylder et al., 2003; Dewitte and Murray, 2003). Baculovirus-expressed AtCDKA, AtCYCD2;1, and AtCYCA2;2 bound to purified GST-AtCDT1a in pull-down assays (Figure 5C). Furthermore, insect cell extracts containing Arabidopsis CDK/cyclin combinations can phosphorylate AtCDT1a (Figure 5D) as well as histone H1 (data not shown). Altogether, these data show (1) that AtCDT1a can be phosphorylated, at least, by CDKA/CYCD2;1 and CYCA2;2 complexes in vitro, (2) that it interacts with CDK/cyclin complexes in vivo, and (3) that a CDK activity is required for AtCDT1a proteolysis. They also point to a role of a cyclin/CDK activity in regulating the availability of AtCDT1a by mediating its targeting to the proteasome.

Ectopic Expression of AtCDT1a Increases Nuclear Ploidy during Leaf Development

The availability of CDT1 and CDC6 is strictly controlled in eukaryotes (Blow and Hodgson, 2002). We have found that both transcriptional activation and proteolysis seem to regulate AtCDT1a. These results prompted us to study the consequences of overriding this control on (1) cell ploidy and (2) whether the licensing pathway is a target for coordinating cell proliferation and differentiation. To this end, we used AtCDT1a (Figure 4A) and AtCDC6a overexpressor plants (Castellano et al., 2001). Plants expressing ectopically AtCDT1a did not show obvious macroscopic phenotypes, indicating that plant growth is compatible with moderately, though not likely highly, increased levels of AtCDT1a. Based on previous reports (Galbraith et al., 1991), we analyzed the ploidy distribution of leaf nuclei by flow cytometry at different times after germination. We focused on leaves 1 and 2 that can be unequivocally identified. Such an analysis proved to be extremely useful because we found that leaf nuclear ploidy was significantly affected in the CDT1 and CDC6 overexpressors, but the effect was strongly dependent on the leaf developmental stage. Leaf primordia (9 d after germination) contained only 2C and 4C nuclei both in control and transgenic

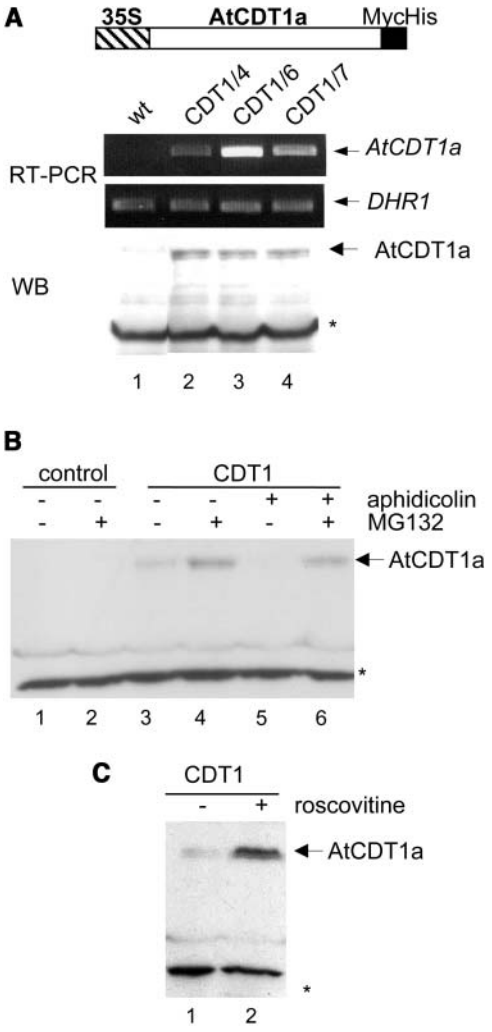


Figure 4. AtCDT1a Is Subjected to Proteasome-Mediated Degradation.

(A) Scheme of the construct used to generate transgenic plants ectopically expressing AtCDT1a-Myc-His (black) protein under the constitutive 35S promoter (hatched) of *Cauliflower mosaic virus* (top panel). Detection of *AtCDT1a-Myc-His* transgene expression by RT-PCR (middle panel) and protein gel blot (WB) with anti-Myc antibody (bottom panel) in whole trichloroacetic acid extracts of control (lane 1) and several transgenic lines (CDT1/4, 6, and 7; lanes 2 to 4) treated with the proteasome inhibitor MG132 (6-d-old seedlings treated with inhibitor for 8 h). The expression of *DHR1* was used as loading control for RT-PCR. The asterisk indicates an unspecific protein used as loading control in protein gel blot analysis.

(B) Levels of AtCDT1a-Myc-His detected by protein gel blot analysis with anti-Myc antibody in whole TCA extracts of 6-d-old Arabidopsis seedlings of control (lanes 1 and 2) or transgenic plants (CDT1; lanes 3 to 6) treated with MG132 or aphidicolin as indicated.

(C) Levels of AtCDT1a-Myc-His in whole TCA extracts of 6-d-old Arabidopsis seedlings of transgenic plants (CDT1) treated with the CDK inhibitor roscovitine.

plants (Figure 6A). Later, a switch to enter endocycles occurs, and 15 d after germination, CDT1 and CDC6 overexpressors start to show less 2C nuclei and more 8C nuclei than the controls (Figure 6A), indicating an increased endocycle potential in this organ. In fully developed leaves (28 d after germination), a further reduction in 2C and an increase in 8C and 16 C nuclei was evident in the transgenics relative to the controls. Thus, we conclude that CDT1 and CDC6 are able to induce, at least, one extra endocycle during leaf development. A summary of all these data are shown in Figure 6B, where the evolution of 2C through 16C nuclei during leaf development clearly reveals the increased endocycle potential in the CDT1 and CDC6 overexpressor plants. In the case of control plants, our observations are consistent with those of others (D. Inzé, personal communication). This overall effect is similar to express ectopically Cdc6 in human megakaryocytes (Bermejo et al., 2002), but is in clear contrast with the abnormal rereplication observed in other systems or cell types (Mihaylov et al., 2002; Vaziri et al., 2003; Zhong et al., 2003). We also analyzed the ploidy distribution of leaf nuclei in plants overexpressing both CDT1a and CDC6a obtained by crossing and found that they did not exhibit significant changes compared with either of the parent lines (data not shown). This indicates that although the two proteins may cooperate under normal conditions, an excess of either one is sufficient to trigger the effects observed.

Altering the Level of Licensing Components AtCDT1 and AtCDC6 Has Cell Type–Specific Consequences

It should be kept in mind that regulated DNA replication in whole organisms occurs in cells undergoing cell division cycles as well as endocycles, two processes whose balance in plants is particularly crucial for growth and development. To identify whether altering replication licensing in the context of a multicellular organism affects differentiation and morphogenesis, we focused on the leaf cell epidermis as a model tissue because it contains different cell types that follow two distinct developmental programs. In leaf primordia, some protoepidermal cells cease proliferation, undergo endoreplication cycles, and differentiate into trichomes, specialized leaf hairs located on the leaf surface (Hülskamp et al., 1999). Others, with a limited stem cell potential, proliferate to replenish the pool and eventually differentiate into cells of the stomata complexes, leaf pores involved in gaseous exchange (Nadeau and Sack, 2003).

Trichomes are polyploid cells with a well-established morphogenetic pattern where branching is genetically defined and associated to the occurrence of endocycles (Hülskamp et al., 1999). To assess whether increased levels of AtCDT1a and AtCDC6a could affect trichome morphogenesis, we analyzed the branching pattern in overexpressor plants. Compared with controls, the *AtCDT1a* and *AtCDC6a* overexpressor lines showed an approximately fivefold to sixfold increase in ≥4-branched trichomes (Figures 7A and 7B) as well as a low proportion of abnormal trichomes (Figure 7A, small panels). The increase in branch number, which occurred in plants overexpressing *AtCDT1* or *AtCDC6*, correlates with an increase in the nuclear size of trichomes, as observed by 4‘,6-diamidino-2-phenylindole (DAPI) staining (Figure 6B). As expected from the flow cytometry

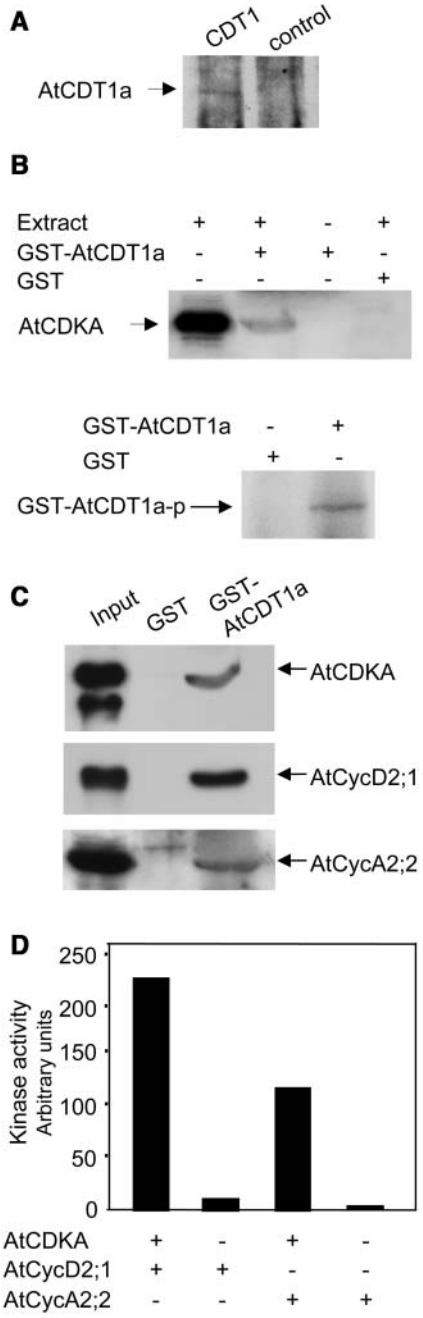


Figure 5. AtCDT1a Interacts with Arabidopsis CDKA, Cyclin D, and Cyclin A.

(A) Detection of AtCDT1a-Myc-His in immunoprecipitates of AtCDKA with anti-PSTAIRE antibody of extracts of control and transgenic plants (CDT1) expressing AtCDT1a.

(B) Detection of AtCDKA with anti-PSTAIRE antibodies in pull-down assays (top panel) of Arabidopsis cultured cell extracts incubated with GST-AtCDT1a or GST, as indicated, and kinase activity on GST-AtCDT1a of the bound material (bottom panel). GST-AtCDT1a-p indicates the phosphorylated protein.

(C) Interaction of GST and GST-AtCDT1a with AtCDKA (top panel), His-AtCycD2;1 (middle panel), and His-AtCycA2;2 (bottom panel) by pull-down assays of baculovirus-infected insect cell extracts expressing the indicated Arabidopsis proteins and subsequent detection by protein gel blot analysis with anti-PSTAIRE or anti-His antibodies.

(D) Kinase assays of baculovirus-infected insect cell extracts expressing the AtCDKA, AtCycD2;1, AtCycA2;2, or CDK/cyclin combinations, as indicated, using purified GST-AtCDT1a as substrate.

data, plants overexpressing both CDT1a and CDC6a did not show significant differences in the amount of ≥4-branched trichomes (data not shown). Quantification of the DNA content of individual trichome nuclei revealed that in AtCDT1a and AtCDC6a transgenics, a significant proportion of trichome nuclei (Figure 7B), not observed in the control, contain increased DNA content, strongly suggesting that they have undergone extra endocycles (Figure 7C, arrows). We did not find multicellular trichomes, as it has been observed in plants misexpressing *AtCYCB1;2* (Schnittger et al., 2002a) or *AtCYCD3;1* (Schnittger et al., 2002b). Therefore, we conclude that increasing the levels of AtCDT1a or AtCDC6a in cells that endoreplicate in association with a differentiation program promotes an increase in the ploidy level and, as a consequence, a change in their morphogenetic pattern, as observed here by a change in their branching pattern.

Stomata are epidermal structures made up of two guard cells surrounded by larger pavement cells (Serna and Fenoll, 2002; Nadeau and Sack, 2003). As depicted in Figure 8A, they originated from some protodermal cells (meristemoid mother cells [MMC]) that after an unequal division give rise to a meristemoid cell (M), a self-renewing cell that maintains its stem cell potential for up to three divisions. M cells can form the stomata guard mother cell at any time during these divisions and remain with a 2C DNA content. MMC identity also can be assumed by other epidermal cells of the stomatal complex (Nadeau and Sack, 2003). Compared with controls, transgenic AtCDT1a and AtCDC6a plants show significant changes in the stomata distribution (Figure 8B). Whereas the nonstomatal cell density (Figure 8C, nsd, number of nonstomatal cells/mm^2) was similar in all cases, AtCDC6a and AtCDT1a plants showed a twofold increase in stomatal density (Figure 8C, sd, number of stomata/mm^2). Consequently, a twofold decrease in the nonstomatal (ns) epidermal cell to stomatal (s) cell ratio occurred (Figure 8C, ns/s). These alterations in the ratio of leaf epidermal cell types lead to a significant increase in the stomatal index [Figure 8C, si, (sd/sd + epidermal cell density)*100]. These data are consistent with a higher production of satellite meristemoid cells that, eventually, develop into more stomata. They also suggest that, at least in leaves, stem cell initiation and/or maintenance as well as the final balance of differentiated cell types may depend on a correct function of the DNA replication licensing mechanism.

DISCUSSION

The function of DNA replication licensing components, such as Cdc6 and Cdt1, among others, is crucial for cell proliferation and genome stability, but little is known about this regulation occurring in whole organisms and in different cell types. We have approached these questions in Arabidopsis where a continuous balance between cell division and differentiation is crucial

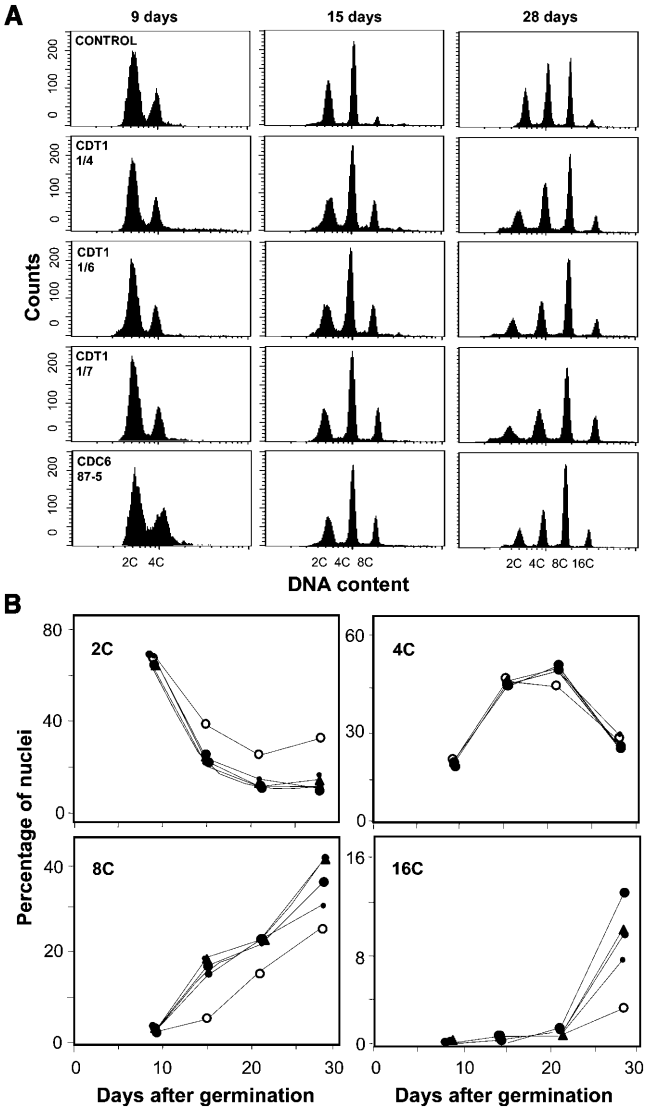


Figure 6. Ectopic Expression of AtCDT1a Increases Endoreplication Level.

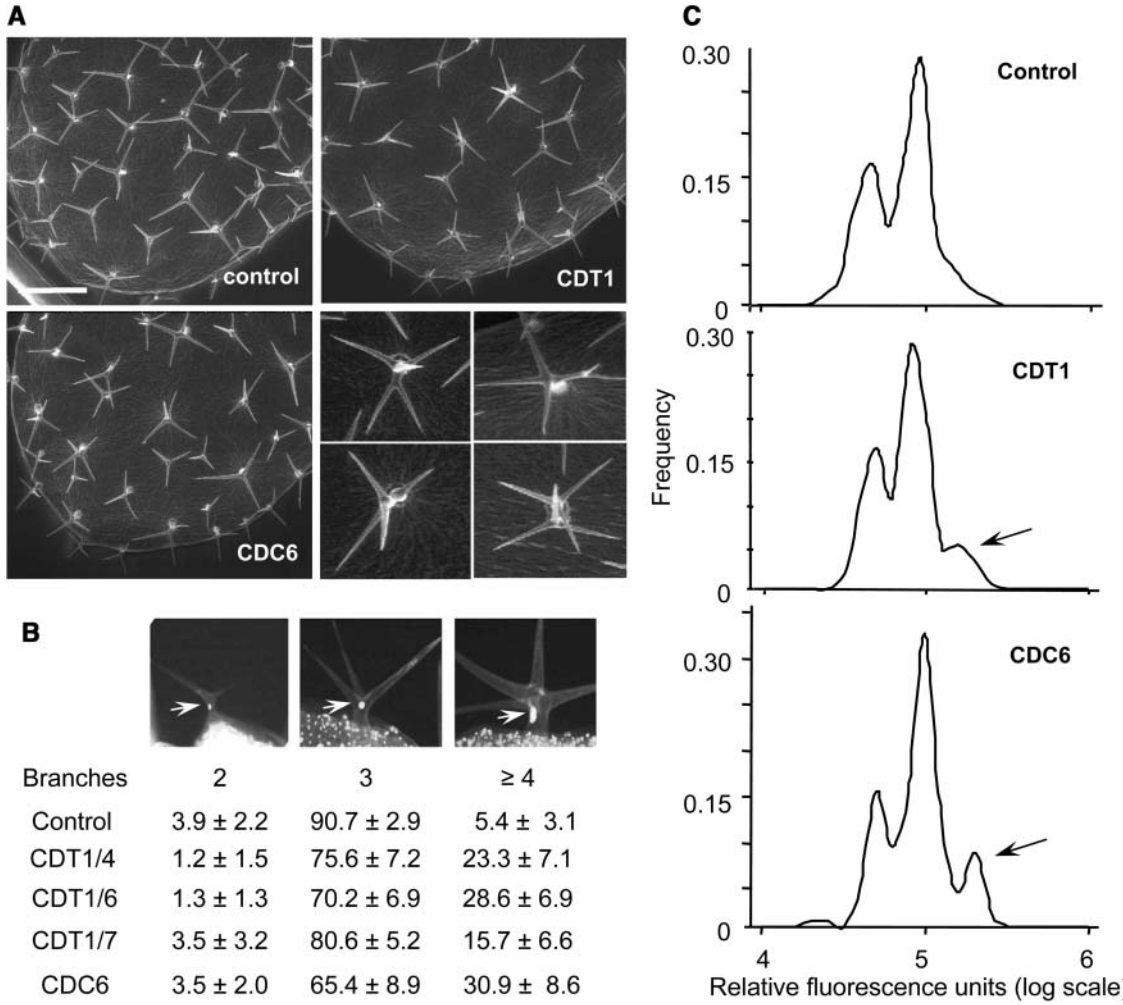
(A) Distribution of leaf nuclei with different DNA content in control, three independent CDT1 transgenic lines (CDT1/4, CDT1/6, and CDT1/7), and a CDC6 transgenic line (87-5; Castellano et al., 2001) at different times during leaf development. Flow cytometry profiles of leaf nuclei of 9-, 15-, and 28-d-old 1/2 leaves are shown. Note the increase in the 8C peak by 15 d and in the 16C peak by 28 d in CDT1 and CDC6 transgenic leaves.
(B) Summary of each ploidy peak distribution at different leaf developmental stages. Note that CDT1 and CDC6 decrease the amount of 2C nuclei concomitantly with an increase in 8C and 16C nuclei from day 15 onwards.

during postembryonic growth. Here, we show that availability of AtCDT1 is regulated both at the level of transcription and posttranscriptionally. Also, we show that altering the availability of DNA replication licensing components (e.g., AtCDT1 or AtCDC6) have cell type–specific consequences: in cells competent to divide, cell renewal is stimulated, whereas in cells competent to undergo differentiation-associated endoreplication rounds, extra endocycles are triggered.

Transcription is one level of *AtCDT1a* regulation. Arabidopsis plants expressing a dominant negative version of a DP protein that inhibits binding of E2F to DNA (Ramirez-Parra et al., 2003) show decreased *AtCDT1a* mRNA levels. This together with the presence of E2F binding sites in the putative AtCDT1a promoter strongly suggests that *AtCDT1a* is a downstream E2F target. A similar regulation has been reported for the *DUP* gene, the Drosophila Cdt1 homolog, based on the reduced *DUP* mRNA levels in Drosphila mutants in E2F genes (Whittaker et al., 2000). Our studies reinforce the role of E2F in the control of cell proliferation in animals and plants (De Veylder et al., 2002; Gutierrez et al., 2002; Shen, 2002; Stevaux and Dyson, 2002; Dewitte and Murray, 2003). Detailed promoter analysis should await the generation of appropriate tools to identify what E2F/DP combination regulates the *AtCDT1a* promoter.

Phosphorylation appears to be a second level of regulation of AtCDT1a availability. However, the CDK/cyclin complexes involved are not known yet. AtCDT1a contains seven potential CDK phosphorylation sites (S/TPxR/K), two of them in the N-terminal region are conserved in Cdt1 from other sources. We found that AtCDT1a interacts in vivo with AtCDKA as well as in extracts with A- and D-type cyclins. These data are strongly suggestive of AtCDT1a being a CDK/cyclin substrate in vivo. Based on the expression pattern of Arabidopsis cyclins (Dewitte and Murray, 2003), it is conceivable that, after initiation of S-phase, one or more CDK/cyclin complexes can phosphorylate AtCDT1a. The role of AtCDT1 phosphorylation is unknown, but it may be important for controlling protein stability. Degradation by the proteasome frequently requires prior phosphorylation of the target protein, and several examples exist both in animal cells (Carrano et al., 1999; Tedesco et al., 2002) and Arabidopsis (Castellano et al., 2001; del Pozo et al., 2002). We found that AtCDT1a, like other Cdt1 proteins, is degraded through a proteasome-mediated pathway (Li et al., 2003; Zhong et al., 2003; Liu et al., 2004) and that a CDK activity is required to reduce AtCDT1a levels, similar to results with human Cdt1 (Liu et al., 2004; Sugimoto et al., 2004). The possibility that CDK activity is an indirect requirement cannot presently be completely ruled out.

The retinoblastoma/E2F/DP pathway plays an important role in both proliferating and endoreplicating animal (Edgar and Orr-Weaver, 2000; Weng et al., 2003) and plant cells (Larkins et al., 2001). Because retinoblastoma and E2F/DP family members were identified in plants (reviewed in Gutierrez, 1998; Gutierrez et al., 2002; Vandepoele et al., 2002), accumulating evidence support their role in cell proliferation, endoreplication, and differentiation (Gutierrez et al., 2002; Shen, 2002). Arabidopsis E2Fa/DPa is one of the major regulators of these processes because its ectopic expression induces sustained cell proliferation in differentiated cotyledon and hypocotyl cells and extra rounds of endoreplication as measured in whole seedlings (De Veylder et al., 2002; Rossignol et al., 2002). Furthermore, when AtE2Fa is expressed in tobacco (*Nicotiana tabacum*), it promotes similar effects (Kosugi and Ohashi, 2003). Both *AtCDC6a* (Castellano et al., 2001; de Jager et al., 2001; De Veylder et al., 2002; Ramirez-Parra et al., 2003) and *AtCDT1a* (this work) are downstream targets of E2F in planta. This, together with the



Branches	2	3	≥ 4
Control	3.9 ± 2.2	90.7 ± 2.9	5.4 ± 3.1
CDT1/4	1.2 ± 1.5	75.6 ± 7.2	23.3 ± 7.1
CDT1/6	1.3 ± 1.3	70.2 ± 6.9	28.6 ± 6.9
CDT1/7	3.5 ± 3.2	80.6 ± 5.2	15.7 ± 6.6
CDC6	3.5 ± 2.0	65.4 ± 8.9	30.9 ± 8.6

Figure 7. Ectopic Expression of AtCDT1a Increases the Trichome Nuclear Ploidy and Branching Number.

(A) Scanning electron micrographs of the adaxial surface of the 3rd rosette leaf (14-d-old plants) in control, AtCDC6a (line 87-5; Castellano et al., 2001), and AtCDT1a (line CDT1/4) transgenic plants. Detail of abnormal trichomes with more than four branches and emerging from neighbor cells found in the AtCDT1a and AtCDC6a transgenic plants is shown in the smaller panels. Bar = 100 μm.

(B) Percentage of trichomes with two, three, and more than four branches of control and AtCDT1a (lines CDT1/4, CDT1/6, and CDT1/7) and AtCDC6a (line 87-5; Castellano et al., 2001) transgenic plants calculated on samples of 600 to 1000 trichomes in each case. DAPI-stained nuclei (arrows) of two-, three-, and four-branched trichomes are shown.

(C) DNA content distribution of individual trichome nuclei in the 3rd to 4th leaves of control and transgenic 14-d-old plants ectopically expressing AtCDT1a or AtCDC6a. Arrows point to trichome nuclei having undergone an extra endocycle.

results described here, is consistent with the idea that at least part of the phenotypes observed in plants with altered E2Fa/DPa activity may be related to altering DNA replication licensing in different cell types.

A strict regulation of licensing components is crucial for genome stability (Blow and Hodgson, 2002). Alterations of this control lead to deregulated DNA replication through mechanisms that operate differently depending on the organism and the cell type. Thus, overexpression of *S. pombe* Cdc18/Cdc6 induces origin refiring and in combination with Cdt1 produces uncontrolled DNA synthesis accumulating DNA contents of ≥64C (Gopalakrishnan et al., 2001; Yanow et al., 2001). In cultured animal cells, partial genome rereplication has been reported after overexpression of Cdt1 (Vaziri et al., 2003), whereas overexpression of Cdc6 in cultured megakaryocytes produces an extra endoreplication round (Bermejo et al., 2002). Differences also exist in licensing control between primary or transformed fibroblasts in culture (Shreeram et al., 2002). In Drosophila SD2 cells, partial genome rereplication occurs by silencing geminin, an inhibitor of Cdt1 function (Mihaylov et al., 2002). This issue has been less studied, so far, in whole organisms. In *C. elegans*, increased Cdt1 levels obtained by suppressing CUL-4, involved in Cdt1 proteolysis, leads to partial rereplication (Zhong et al., 2003). In *D. melanogaster*,

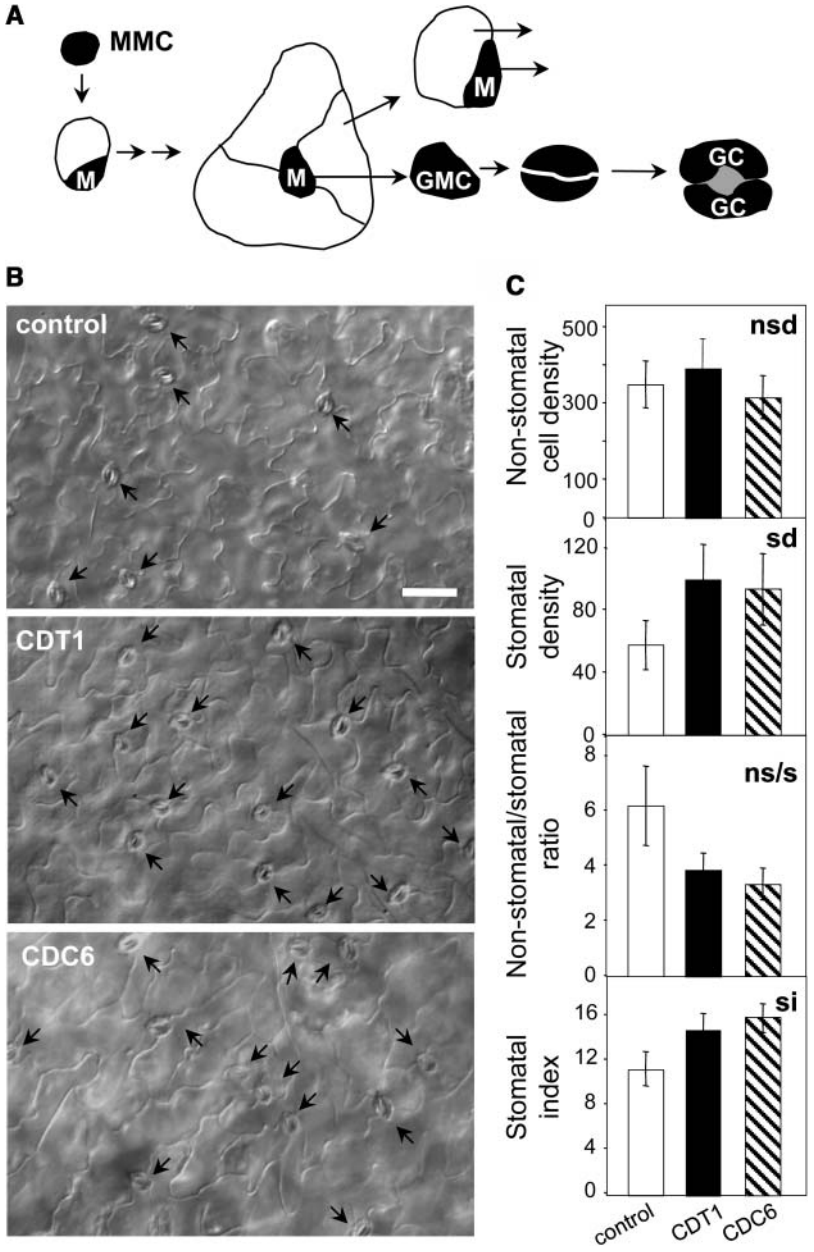


Figure 8. Ectopic Expression of AtCDT1a Increases the Amount of Stomata but Not the Nonstomatal Cell Number.

(A) Overview of cell proliferation and differentiation of different cell types during stomata development. GC, guard cell; GMC, guard mother cell.

(B) Adaxial epidermis (1st rosette leaf, 20-d-old plants) of control, AtCDT1a (line CDT1/4), and AtCDC6a (line 87-5; Castellano et al., 2001) ectopically expressing plants. Arrows point to stomata. Bar = 50 μm.

(C) Nonstomatal cell density (nsd; number of nonstomatal cells per mm^2), stomatal density (sd; number of stomata per mm^2), nonstomatal epidermal cells/stomata ratio (ns/s), and stomatal index [si; (sd/sd + epidermal cell density)*100] for control (white), AtCDT1a (black), and AtCDC6a (hatched) ectopically expressing plants. In each case, three different first leaves were analyzed and at least 1600 cells were scored. In all cases, the differences between control and transgenic plants were statistically significant ($P < 0.001$), except for the nonstomatal density.

overexpression of DUP/Cdt1 in a geminin mutant background leads to sustained ectopic gene amplification and to an increased proportion of cells that undergo endoreplication (Quinn et al., 2001). Interestingly, knockout mice lacking Skp2, the F-box responsible for targeting Cdt1 to the proteasome (Li et al., 2003), show increased ploidy levels (Nakayama et al., 2000), consistent with possible increased levels of Cdt1 and perhaps other licensing components.

Altogether, this variety of consequences of altering licensing control strongly suggests that different cell types respond

differently. The effects of altering CDT1 and CDC6 levels in cell types within an organ whose cells follow different developmental programs shed light on this topic. Our results are consistent with the model depicted in Figure 9 that highlights the proposal that altering replication licensing has cell type–specific effects. In cells whose developmental fate involves the occurrence of endoreplication cycles, increased levels of CDT1 or CDC6 proteins lead to an increase in the number of endocycles. In the case of trichomes, this increase led to a change in their morphogenetic pattern. In cells competent to divide and with a limited stem cell potential, alteration of licensing control seems to increase its proliferative potential. One possible mechanism to account for the increase in the amount of stomata in plants with increased levels of AtCDT1a or AtCDC6a would be that, at some stage in development, the differentiation potential of protoepidermal cells is altered, producing more cells that take the stomatal lineage. However, our results are more consistent with an alternative mechanism by which increased levels of licensing components stimulate the proliferation of subsidiary cells, precursor cells neighbor to meristemoids, to produce satellite meristemoids that eventually give raise to stomata. This is based on the fact that the amount of nonstomatal (pavement) cells is roughly maintained in the transgenic plants. This would not be expected if the division potential of early precursor cells (MMC) is stimulated. Because the *TMM* gene should be active in the CDT1a or CDC6a transgenics, stomatal clustering is not expected to occur (Nadeau and Sack, 2002), which is actually the case in our experiments. Therefore, our results support the idea that increased levels of licensing components increases the proliferative potential of the subsidiary cells next to meristemoids that divide asymetrically to generate new satellite meristemoids while maintaining the amount of nonstomatal cells (Figure 9). Interestingly, Cdt1 activity seems to be also a key target in animals for the coordination between proliferative potential and developmental control. Increasing Cdt1 levels in animals either by loss of geminin, the Cdt1 inhibitor, or by sequestering geminin with the homeobox-containing protein Six3 promotes retinal precursor cell proliferation (Del Bene et al., 2004).

The trichome phenotype (overendoreplication and overbranching) that we have observed in plants with altered AtCDT1a

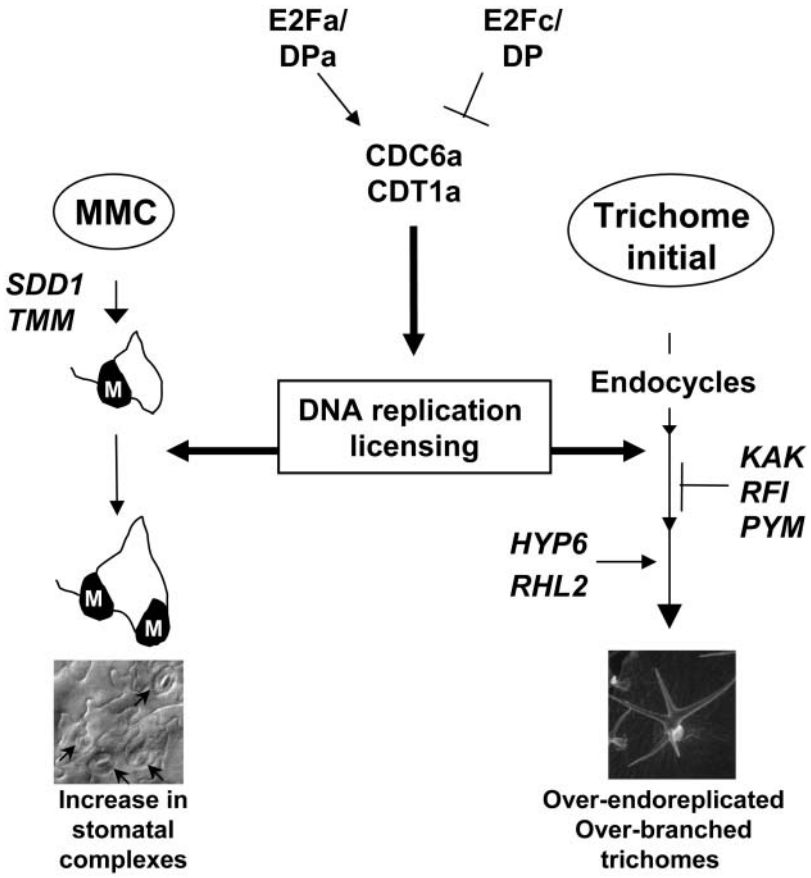


Figure 9. Model of DNA Replication Licensing Control Operating in Different Cell Types Based on Our Results (in Bold) in Leaves of Plants Ectopically Expressing AtCDT1 and AtCDC6.

Cells whose differentiation program involves the occurrence of endoreplication cycles (e.g., trichomes) are induced to undergo extra DNA replication rounds, and as a consequence, their morphogenesis is altered (overbranching) in cells competent to divide (e.g., secondary meristemoids). The sites where some genes relevant for this discussion can be putatively located are also indicated.

or AtCDC6a levels is opposite to that of plants lacking SPO11-1, the subunit A of topoisomerase VI (Hartung et al., 2002), and *hypocotyl6* and *root hairless2* mutants (Sugimoto-Shirazu et al., 2002) and similar to that of Arabidopsis *kaktus* (*kak*), *rastafari*, or *polychome* mutants (reviewed in Sugimoto-Shirazu and Roberts, 2003). Interestingly, the *KAK* gene, also named *UPL3*, encodes a HECT-domain E3 ligase required for proper control of the number of endocycles (Downes et al., 2003; El Refy et al., 2004). Likewise, the stomata phenotype of our plants with altered licensing components is reminiscent of the Arabidopsis *sdd1-1* mutant, except that stomatal clusters (groups of stomata that are not separated by intervening pavement cells) do not appear (Berger and Altmann, 2000). SDD1 is a subtilisin-like protease that generates an extracellular signal regulating the identity and fate of stem cells (i.e., meristemoids) (von Groll et al., 2002). Whether similar pathways are affected in CDT1a and CDC6a overexpressor plants and in the mutants with trichome and stomatal phenotypes is not known yet. However, experiments to alter replication licensing components in a cell type–specific manner at defined developmental stages and with different genetic backgrounds should help to further investigate in the future the coupling among DNA replication control, cell proliferation, and cell differentiation. Moreover, exploring whether the effects of altering CDT1/CDC6 levels displayed in leaf cells are maintained in other highly positionally regulated stem cell niches (e.g., the shoot or the root apical meristem) (Weigel and Jürgens, 2002) or if they are bypassed by other mechanisms opens a new avenue for future studies.

METHODS

Isolation of AtCDT1 cDNA Clones

AtCDT1a and AtCDT1b cDNA clones were obtained by PCR from a pACT2-cDNA library (Clontech, Palo Alto, CA), using primers derived from the *Arabidopsis thaliana* genome sequence, and subsequently cloned in the pCR2.1 vector (Invitrogen, Carlsbad, CA).

Transgenic Plants

For expression analysis, 1855- and 1860-bp fragments of the genomic region containing the putative *AtCDT1a* and *AtCDT1b* promoters, respectively, were isolated. These fragments included a piece of DNA encoding 11 N-terminal amino acids of each AtCDT1a and AtCDT1b and were fused in frame to the *GUS* coding sequence in a pBI101.1 vector. These constructs were used for transformation of Arabidopsis (*pCDT1a*:*GUS* and *pCDT1b*:*GUS* plants). Histochemical detection of GUS activity was done using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide with slight modifications (Castellano et al., 2001).

To generate plants expressing C-terminally Myc-His–tagged AtCDT1a protein, the *AtCDT1a* cDNA was cloned in frame with the Myc-His epitope into the pMHT-Gal vector (a gift of J. Diffley). The AtCDT1a-Myc-His fusion was cloned into the binary pRok vector (Baulcombe et al., 1986) under the control of the 35S promoter of *Cauliflower mosaic virus*. Arabidopsis plants transformed with an empty vector were used as controls. In all cases, Arabidopsis (Columbia-0 [Col-0] ecotype) plants were transformed with *Agrobacterium tumefaciens* by the floral dip method (Clough and Bent, 1998) and transgenic plants selected on MS agar plates containing 50 $\mu g\ mL^{-1}$ of kanamycin, growing under long-day conditions. AtCDT1a-Myc-His protein levels were determined by protein gel blot analysis of whole TCA extracts of 4-d-old seedlings and treated 8 to 12 h with the proteosome inhibitor MG132 (100 μM). Blots were probed with an anti-Myc monoclonal antibody raised against the 9E10 epitope (a gift of J. Diffley) at 1:1000 dilution. Transgenic Arabidopsis plants expressing a dominant negative version of DP (TmDPΔBD; Ramirez-Parra et al., 2003) or AtCDC6a (Castellano et al., 2001) have been described previously.

Real-Time RT-PCR

Total RNA was extracted using the Trizol reagent (Invitrogen). RT-PCR experiments were performed using the ThermoSript RT-PCR system (Invitrogen) using oligo(dT) and gene-specific primers. Estimation of relative mRNA abundance was made by real-time quantitative RT-PCR and was performed in a LightCycler FastStart DNA Master SYBR Green I (Roche, Indianapolis, IN) in a Lightcycler System (Roche) using the ubiquitin10 gene (*AtUBQ10*) expression as reference to normalize for differences of total RNA amount. After setting the amplification conditions, experiments were repeated twice. The difference between duplicates was within 10%.

Plant Extracts and Treatments

Protein extracts of Arabidopsis seedlings (grown under a long-day light regime) or cultured cells were prepared, except where TCA was used, in buffer A (del Pozo et al., 2002) in a final concentration of 500 mM NaCl. Extracts were adjusted to 150 mM of NaCl for immunoprecipitations or pull-down assays. MG132 treatment (100 μM) was performed by incubating 6-d-old seedlings for 8 h in 0.25× MSS medium. The RNA of transgenic plants expressing the dominant negative version of DP (Ramirez-Parra et al., 2003) was extracted from 7-d-old seedlings. For the dehydration treatment, wild-type Arabidopsis Col-0 seeds were germinated for 6 d in MSS agar plates, and then the plates were opened for 6 h in a laminar flow cabin. For the treatment with ABA, wild-type Arabidopsis Col-0 seeds were germinated and maintained for 7 d in liquid medium and then treated with 100 μM ABA for 3 h.

In Vitro Interaction and Phosphorylation Assays

The in vitro transcribed-translated AtCDC6a protein was obtained as described (Castellano et al., 2001). Full-length AtCDT1a was fused to GST in the PGEX vector (Pharmacia, Piscataway, NJ), expressed in *Escherichia coli* BL21 for 3 h at room temperature in the presence of 0.4 mM of isopropylthio-β-galactoside, and purified using glutathion-Sepharose beads (Pharmacia). Baculovirus-expressed proteins, in vitro phosphorylation, and in vitro pull-down assays using baculovirus extracts have been described elsewhere (del Pozo et al., 2002). Kinase assays were repeated twice, and the difference between duplicates was within 10%. The detection of AtCDKA was performed using the anti-PSTAIRE antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and the His-AtCYCD2;1 and His-AtCYCA2;2 was detected with anti-His antibody (Sigma, St. Louis, MO).

Optical and Scanning Electron Microscopy and Flow Cytometric Measurements

Epidermal leaf cells were observed by Nomarski microscopy using an Axiophot microscope (Carl Zeiss, Göttingen, Germany) after incubation of leaves in absolute ethanol overnight at room temperature and the same incubation with lactophenol (25% [w/v] phenol, 25% lactic acid, 25% glycerol, and 25% water). Images were captured with a digital Coolsnap FX camera (Roper Scientific, Trenton, NJ). Trichomes were observed by scanning electron microscopy using a FEI QUANTA 200 microscope (FEI-Philips, Eindhoven, The Netherlands) on unfixed material under low

vacuum conditions. The flow cytometric analysis of leaf nuclei was performed as previously described (Castellano et al., 2001). Quantification of DAPI-stained trichome nuclei was done with a Leica DM RA 2 epifluorescence microscope (Solms, Germany) equipped with a JVC digital camera KY-F70B (Friedberg, Germany). Fluorescence intensity of captured pictures was quantified with Diskus software version 4.30.19 (Technisches Büro Carl H. Hilgers, Königswinter, Germany). Software for data import and analysis was written by Maren Heese (Köln, Germany).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AJ421408 (AtCDT1a) and AJ421409 (AtCDT1b).

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