The Plant Cell, Vol. 13, 2671–2686, December 2001, www.plantcell.org © 2001 American Society of Plant Biologists

Expression and Stability of Arabidopsis CDC6 Are Associated with Endoreplication

M. Mar Castellano,[a] J. Carlos del Pozo,[a] Elena Ramirez-Parra,[a] Spencer Brown,[b] and Crisanto Gutierrez[a,1]

[a] Centro de Biologia Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

[b] Institut des Sciences du Végétal, Unité Propre de Recherche 2355, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette, France

Studies on the CDC6 protein, which is crucial to the control of DNA replication in yeast and animal cells, are lacking in plants. We have isolated an Arabidopsis cDNA encoding the AtCDC6 protein and studied its possible connection to the occurrence of developmentally regulated endoreplication cycles. The *AtCDC6* gene is expressed maximally in early S-phase, and its promoter contains an E2F consensus site that mediates the binding of a plant E2F/DP complex. Transgenic plants carrying an *AtCDC6* promoter–β-glucuronidase fusion revealed that it is active in proliferating cells and, interestingly, in endoreplicating cells. In particular, the extra endoreplication cycle that occurs in dark-grown hypocotyl cells is associated with upregulation of the *AtCDC6* gene. This was corroborated using *ctr1* Arabidopsis mutants altered in their endoreplication pattern. The ectopic expression of AtCDC6 in transgenic plants induced endoreplication and produced a change in the somatic ploidy level. AtCDC6 was degraded in a ubiquitin- and proteosome-dependent manner by extracts from proliferating cells, but it was degraded poorly by extracts from dark-grown hypocotyl endoreplicating cells. Our results indicate that endoreplication is associated with expression of the *AtCDC6* gene and, most likely, the stability of its product; it also apparently requires activation of the retinoblastoma/E2F/DP pathway. These conclusions may apply to endoreplicating cells in other tissues of the plant and to endoreplicating cells in other eukaryotes.

INTRODUCTION

The growth of eukaryotic organisms, particularly plants, depends on the balance between cell division and cell elongation. DNA replication is a tightly regulated process that is confined to the S-phase of the cell cycle. The initiation of DNA replication, which has been studied extensively in *Saccharomyces cerevisiae*, depends on the activation of prereplicative complexes by the sequential association and/or release of cellular DNA replication factors at origins (Donaldson and Blow, 1999; Kelly and Brown, 2000; Ritzi and Knippers, 2000). The yeast origin recognition complex (ORC) binds to replicator DNA sequences (Bell and Stillman, 1992) and remains bound to these elements throughout the cell cycle (Diffley et al., 1994). The CDC6 protein plays an essential role because it associates in G1 with ORC, conferring on cells the ability to initiate DNA replication (Kelly et al., 1993; Liang et al., 1995; Piatti et al., 1995). CDC6 is a member of a large family of nucleotide triphosphatases (NTPases) that includes several ORC proteins, minichromosome maintenance (MCM) proteins, and replication factor C (RF-C) (Perkins and Diffley, 1998). The nucleotide triphosphate (NTP) binding domain of CDC6 is necessary for a functional interaction with ORC (Wang et al., 1999), allowing the loading of MCM proteins (Kelly and Brown, 2000; Mizushima et al., 2000).

The function of CDC6 in ensuring the activation of DNA replication origins once per cell cycle is finely regulated at different levels. CDC6 assembles into preinitiation complexes, but once origins are activated, CDC6 function is no longer necessary for origin function. It is in this context that significant differences occur among different eukaryotes. In animal cells, CDC6 expression is regulated at the G1/S transition by E2F transcription factors (Hateboer et al., 1998; Ohtani et al., 1998; Yan et al., 1998). In human cells, the CDC6 protein is targeted for proteolysis by the anaphase-promoting complex (APC) in early G1 (Mendez and Stillman, 2000; Petersen et al., 2000). In contrast, in yeast, the E3 Skp-Cullin-F-box (SCF) complex targets CDC6 for degradation during S-phase (Drury et al., 1997; Sanchez et al.,

[1] To whom correspondence should be addressed. E-mail cgutierrez@cbm.uam.es; fax 34-91-397-4799.

Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.010329.

1999). Furthermore, overexpression of the Cdc18 protein, the *Schizosaccharomyces pombe* CDC6 homolog, causes multiple rounds of DNA replication in the absence of mitosis (Nishitani and Nurse, 1995; Jallepalli and Kelly, 1996); interestingly, however, expression of the CDC6 protein in *S. cerevisiae* and human cells fails to do so (Liang and Stillman, 1997; Petersen et al., 2000). These differences suggest that although the mechanism by which CDC6 protein activates DNA replication origins might be conserved through evolution (Kelly and Brown, 2000), distinct regulatory pathways contribute to its activity in eukaryotic organisms with distinct growth characteristics.

Knowledge of proteins that regulate DNA replication in plants is lacking. Recent studies indicate that the molecular components that control plant cell cycle events are more related to those present in higher eukaryotes than to those present in yeast (reviewed by Gutierrez, 1998, 2000; Mironov et al., 1999; Meijer and Murray, 2001). This factor and the unique growth and developmental features of plants make the study of CDC6 and its regulation very attractive. Furthermore, DNA replication events other than those that take place during the cell cycle occur during endoreplication, the short circuit in which repeated rounds of DNA replication take place in the absence of intervening mitosis (reviewed by Edgar and Orr-Weaver, 2001). This physiological mode of genome rereplication is a frequent event in plants, associated in many cases with developmentally regulated processes such as hypocotyl elongation, trichome growth, and endosperm development (reviewed by Traas et al., 1998; Kondorosi et al., 2000; Larkins et al., 2001).

Given the crucial role of CDC6 in the initiation of DNA replication, understanding its function during endoreplication may help to determine the mechanisms that allow S-phase to be either coordinated with or dissociated from mitosis. Yet, studies on CDC6 in relation to the endoreplication process are lacking in eukaryotes. Thus, we have chosen Arabidopsis as a model system to examine, first, the structure of the *AtCDC6* gene in plants, and second, CDC6 dynamics in relation to developmentally regulated DNA replication and endoreplication events.

Here, we report the isolation of an Arabidopsis cDNA clone encoding a protein whose domain structure is conserved in yeast and animal CDC6. The *AtCDC6* gene is expressed maximally in early S-phase, consistent with the presence in its promoter of an E2F consensus site. Using wild-type and mutant plants with altered endoreplication patterns, we show that *AtCDC6* gene expression is upregulated in cells undergoing developmentally regulated endocycles. Furthermore, ectopic expression of AtCDC6 induces endoreplication. AtCDC6 is degraded in a proteosome-dependent manner in suspension cell extracts but is degraded poorly in extracts from hypocotyl cells. Our results show that both AtCDC6 expression and stabilization are associated with the occurrence and maintenance of endoreplication cycles during Arabidopsis growth.

RESULTS

Isolation of Arabidopsis CDC6 cDNA

With the aim of identifying the Arabidopsis *CDC6* gene, we initially used as a query the sequence of the human CDC6 protein against the available databases. We identified a sequence from chromosome 2, which when used as a query retrieved all of the reported yeast and animal CDC6 protein sequences. To assess whether the putative open reading frame (ORF) identified was actually expressed and to determine its amino acid sequence, we used polymerase chain reaction (PCR) to isolate the AtCDC6 cDNA from an Arabidopsis cDNA library prepared from proliferating cultured cells, as described in Methods. Two specific overlapping fragments were obtained that originated a full-length cDNA (1755 bp) containing a 1527-bp ORF encoding AtCDC6 (508 amino acids; predicted molecular mass, 56.3 kD). This cDNA clone has 3′ and 5′ untranslated regions 191 and 37 bp long, respectively, and the 5′ untranslated region contains two in-frame stop codons, indicating that it might be a full-length clone. Comparison of the predicted translation of the AtCDC6 cDNA isolated here with the genomic sequence indicates that the *AtCDC6* gene contains 16 exons and 15 introns. One of these, 81 bp long, located near the C-terminal end (data not shown) had not been recognized before in the putative ORF deduced from the published genomic sequence of Arabidopsis chromosome 2 (Lin et al., 1999; accession number AC005496).

Alignment of the AtCDC6 amino acid sequence with those of CDC6 proteins from other sources (Figure 1) using the ClustalW package revealed an overall similarity score of 19 and 22% with *S. cerevisiae* CDC6 and Cdc18, its homolog in *S. pombe*, respectively, of 29% with *Xenopus laevis* Cdc6, and of 26% with human CDC6 proteins. We also confirmed the presence of amino acid motifs typically conserved in all members, such as the Walker A (**G**XX**G**X**GK**S; box 1) and B (**DE**X**D**; box 3) domains of the NTP binding motif (Walker et al., 1982) and other motifs conserved with Orc1p (Gavin et al., 1995) and the RFC clamp loader (Perkins and Diffley, 1998; boxes 1 through 6). Interestingly, AtCDC6 contains three putative CDK phosphorylation sites (**S**/**TP**X**K**/**R**) near its N terminus (positions 10, 60, and 66), which might be functionally equivalent to those present in yeast and human CDC6 proteins (Jiang et al., 1999; Petersen et al., 1999; Calzada et al., 2000). A putative nuclear localization signal (**KRKL**I**SD**; starting at residue 36 in AtCDC6), similar to that of wheat E2F (Ramirez-Parra et al., 1999), is present in its N terminus. AtCDC6 also contains six **R**XX**L** sequences (positions 259, 275, 351, 358, 483, and 504) that might be involved in proteolysis. Interestingly, AtCDC6, as in *X. laevis* CDC6p (Coleman et al., 1996), lacks a **KEN** amino acid motif, which was shown recently to mediate, in cooperation with the RXXL motifs, the APC-dependent degradation of human CDC6 (Petersen et al., 2000).

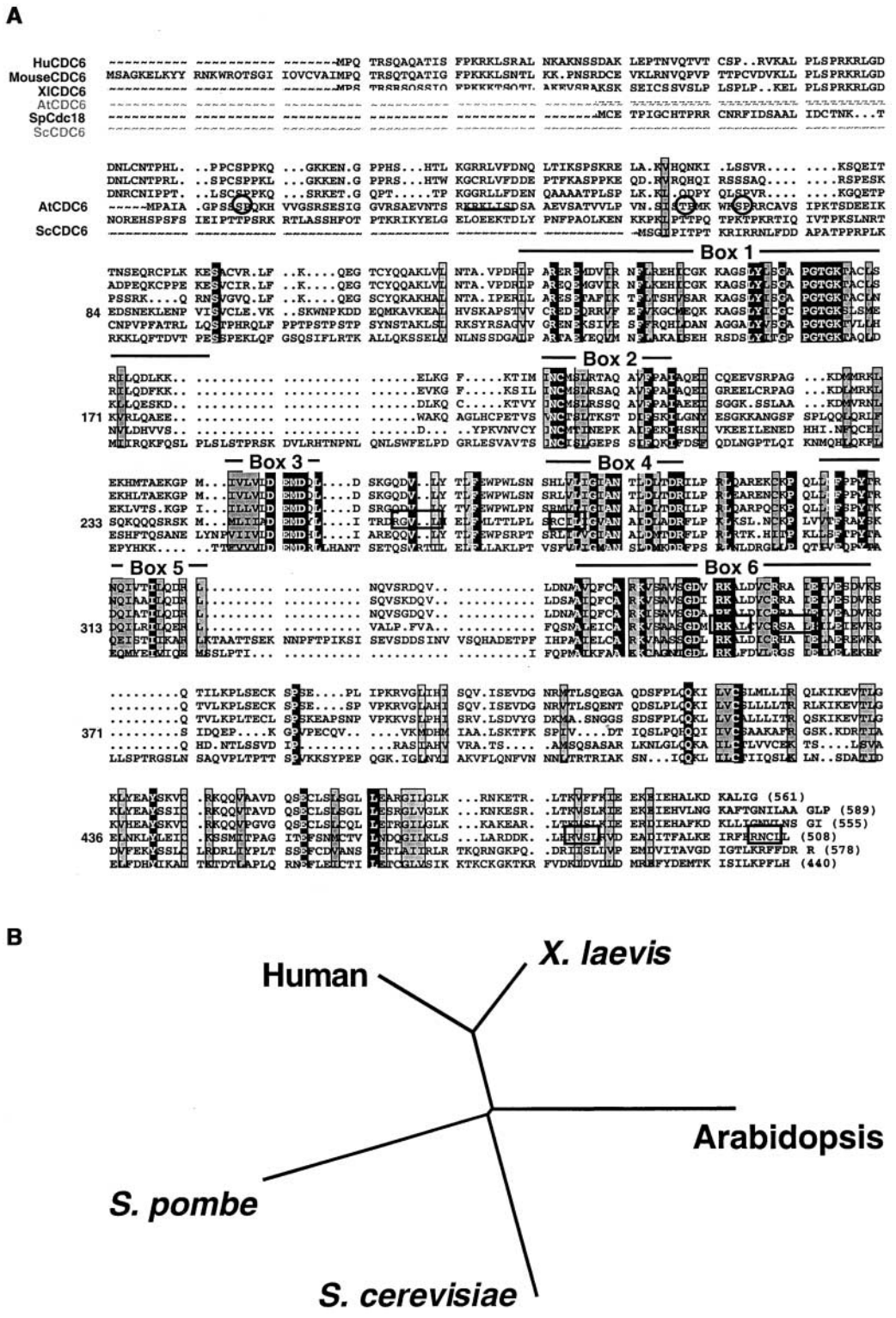


Figure 1. AtCDC6 Protein.

(A) Alignment of AtCDC6 with human (accession number AF022109), mouse (AJ009559), *X. laevis* (U66558), *S. cerevisiae* (X13118), and *S. pombe* (L16793) CDC6 proteins. Highly conserved motifs (boxes 1 through 6), including invariant (black background) and conserved (gray background) residues, are shown. Relevant features of the AtCDC6 protein are shown, including amino acid positions (left), the putative nuclear localization signal (underlined), the three potential CDK phosphorylation sites (circles), and the RXXL motifs (small boxes).
(B) Evolutionary tree of yeast, animal, and Arabidopsis CDC6 proteins.

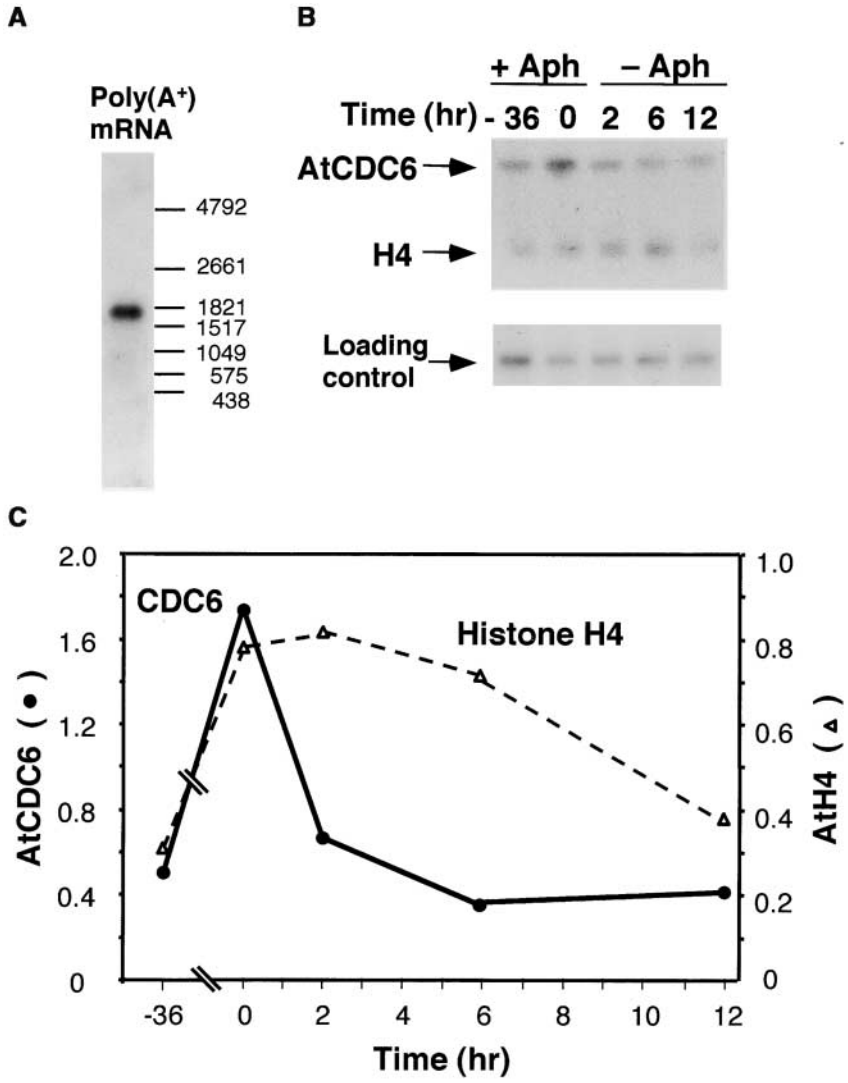


Figure 2. Expression of the *AtCDC6* Gene in Suspension Cultured Cells.

(A) Detection of the *AtCDC6* transcript by RNA gel blot hybridization of poly(A$^+$) mRNA.
(B) Cell cycle–dependent expression of the *AtCDC6* gene. RNA gel blot analysis of poly(A$^+$) mRNA prepared from cells treated with aphidicolin (Aph) for 36 hr and then released from the aphidicolin block for 2, 6, and 12 hr. In the same filter, we detected the levels of histone H4 as a marker of S-phase cells and of AtL3 as a loading control.
(C) Quantitation of the blot shown in **(B)**. Values are arbitrary units that refer to the intensity of the loading control.

AtCDC6 mRNA Accumulates in Early S-Phase Cycling Cells

RNA gel blot analysis showed that a single band of ~1.8 kb, consistent with the size of the AtCDC6 cDNA isolated here, could be detected in poly(A$^+$) mRNA preparations of asynchronously growing cultured cells (Figure 2A). This finding corroborated further that it is a full-length cDNA clone.

To gain insight into the cell cycle regulation of *AtCDC6* gene expression, we determined the *AtCDC6* mRNA levels in Arabidopsis cultures in which the fraction of actively dividing cells respond to an aphidicolin treatment. RNA gel blot analysis (Figure 2B), whose quantification is shown in Figure 2C, was performed in cells arrested at G1/S with an aphidicolin treatment and released from the block. Aphidicolin-blocked cells showed a significantly increased level

of *AtCDC6* mRNA, which decreased rapidly to very low levels by 2 to 6 hr after aphidicolin removal (Figure 2C). We used a histone H4 probe to monitor progression through the S-phase (Reichheld et al., 1995); we found that the histone *H4* mRNA level remained high up to 6 hr after drug removal, when the *AtCDC6* mRNA level was already very low. This finding indicates that the *AtCDC6* mRNA level peaked early in S-phase and decreased rapidly as cells progressed into S-phase. Similar conclusions were obtained with an oryzalin treatment, which arrests cells in mitosis (data not shown). Together, these data indicate that *AtCDC6* gene expression is upregulated at the G1/S transition in cycling cells.

E2F Binds to the *AtCDC6* Promoter Sequences in Vitro

Upregulation of gene expression at the G1/S transition in mammalian cells is dependent on E2F/DP transcription factors. Because a retinoblastoma-related/E2F pathway also operates in plants (Gutierrez, 1998; de Jager and Murray, 1999), we were interested in studying whether the regulation of *AtCDC6* gene expression might be mediated by E2F. The availability of the genomic sequence upstream from the 5′ end of the isolated cDNA clone allowed us to search for putative transcription factor binding sites in a region upstream from the AtCDC6 coding sequence. Analysis of this sequence (http://transfac.gbf-braunschweig.de/TRANSFAC/) revealed the existence of numerous putative binding sites for transcription factors. Among them, 182 bp upstream from the putative initiator methionine, we found the sequence TTTCCCGC (Figure 3A), which matches the human E2F-1 consensus DNA binding sequence (Helin et al., 1992). To determine whether it was actually able to direct E2F binding, we performed electrophoretic mobility shift assays (EMSA) using plant E2F (Ramirez-Parra et al., 1999) and DP (Ramirez-Parra and Gutierrez, 2000) proteins expressed in *Escherichia coli* and purified as glutathione *S*-transferase and maltose-binding protein fusion proteins, respectively. EMSA performed with a double-stranded probe (E2CDC6wt; Figure 3A) containing the core sequence TTTCCCGC within its genomic context revealed the formation of a specific complex (Figure 3B). Complex formation was barely detectable when a small amount (50 ng) of E2F alone was used (detectable only in overexposed films; data not shown). Using this amount of E2F, we found that adding increasing amounts of purified DP protein significantly increased the amount of complex formed. This complex was specific because it did not appear (1) when another probe (E2CDC6mut; Figure 3A) containing two point mutations in the core sequence that abolish E2F binding (Helin et al., 1992) was used, and (2) with DP protein alone (Figure 3B), as reported previously (Ramirez-Parra and Gutierrez, 2000). This finding indicated that the *AtCDC6* gene may be an E2F/DP target.

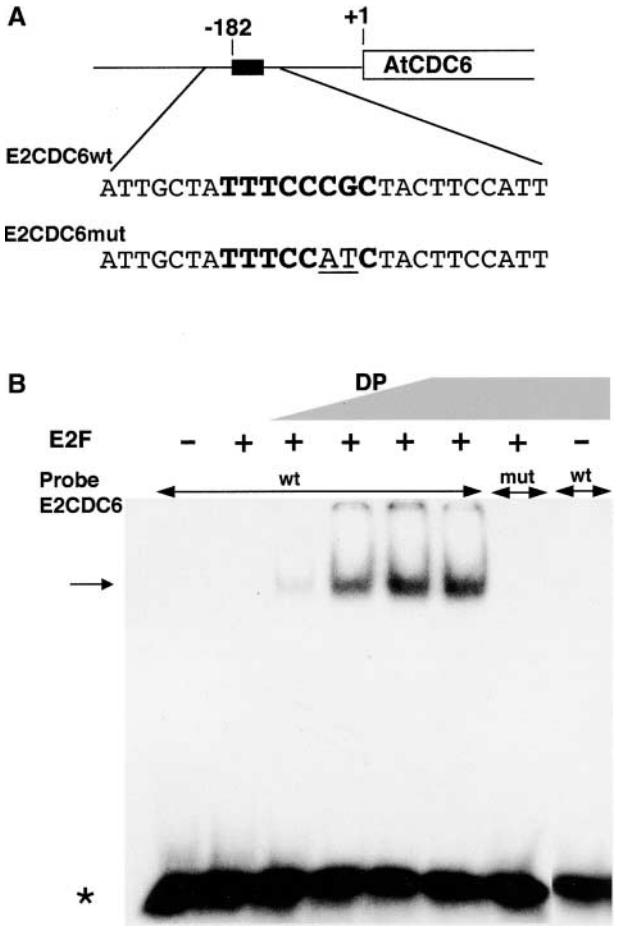


Figure 3. An E2F Binding Site in the *AtCDC6* Gene Promoter Mediates E2F/DP-DNA Complex Formation.

(A) Scheme of the AtCDC6 promoter region containing an E2F consensus sequence (black box). Numbering refers to the A residue of the initiator methionine (+1) and to the first T residue of the E2F site (−182). Below is the nucleotide sequence (top strand) of the indicated promoter region from which synthetic oligonucleotide probes were prepared. Probe E2CDC6wt consists of the sequence present in the promoter that contains an E2F site (boldface). Probe E2CDC6mut consists of the same sequence except for the two point mutations (underlined) that destroy the E2F binding site.

(B) EMSA using the indicated ^{32}P-labeled probe and purified MBP-DP alone (1 μg), and GST-E2F (50 ng) alone or with increasing amounts of purified MBP-DP (0 to 200 ng). Free DNA probes (asterisk) and DNA–protein complexes (arrow) were fractionated on native polyacrylamide gels.

AtCDC6 Promoter Is Active in Dividing and Endoreplicating Cells

To analyze the organ- and tissue-specific expression pattern of the *AtCDC6* gene during Arabidopsis seedling development,

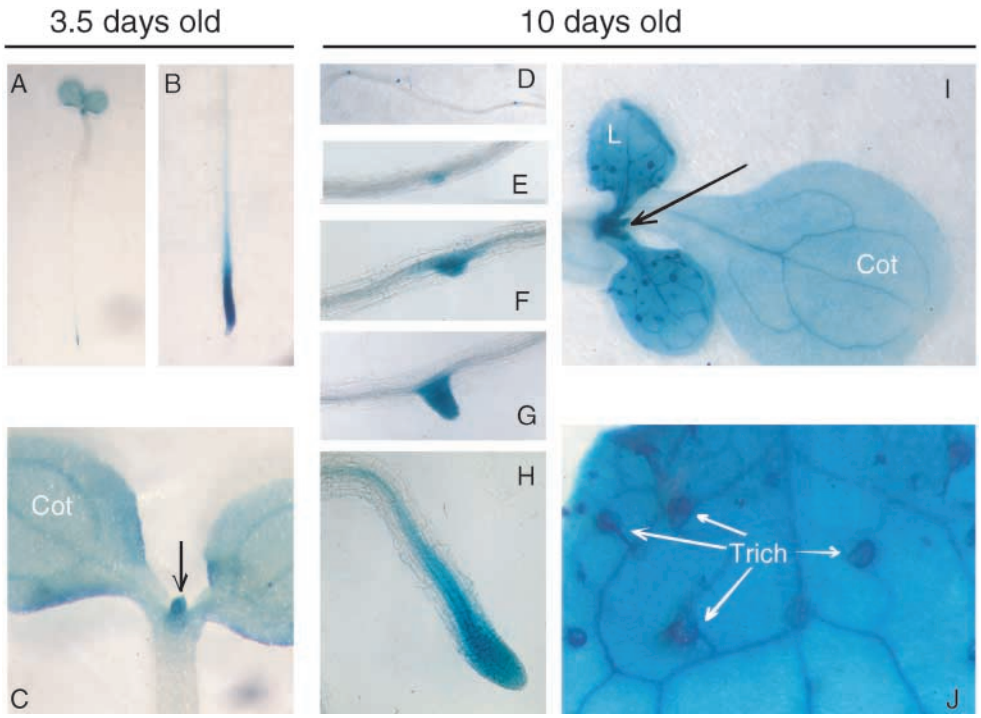


Figure 4. Tissue- and Cell-Specific Activity of the *AtCDC6* Gene Promoter.

Histochemical localization of GUS activity in transgenic Arabidopsis seedlings, grown for 3.5 **[A]** to **[C]**) or 10 (**[D]** to **[J]**) days in the light, carrying the *AtCDC6* promoter–GUS translational fusion.

(A) A representative 3.5-day-old seedling.

(B) Detail of the strong GUS activity restricted to the root meristem.

(C) The first pair of leaf primordia also are stained. The cotyledons (Cot) show some background GUS activity as well.

(D) to **(H)** Lateral roots at different growth stages in 10-day-old seedlings.

(I) This plant contains the second pair of leaf primordia (arrow) together with the expanding first pair of leaves, which are GUS positive. In addition, these new leaves show strong GUS activity, scattered on their surface, corresponding to the presence of developing trichomes.

(J) Basal part of the growing leaf showing GUS staining in the trichomes (Trich).

we isolated a genomic fragment containing the *AtCDC6* promoter and generated transgenic plants expressing the β-glucuronidase (GUS) gene under its control. Histochemical localization of GUS activity in Arabidopsis seedlings (Figure 4A) indicated that the *AtCDC6* promoter was very active, as expected, in proliferating cells such as root meristems (Figures 4B and 4D to 4H), leaf primordia (Figures 4C and 4I), and young growing leaves (Figure 4I). A strong GUS signal also appeared associated with the development of lateral roots from the very early stages of lateral root primordia (Figures 4D to 4H) until a full lateral root was formed (Figure 4H).

In addition, GUS activity also was detected in other locations. Thus, a strong signal was present in the basal part of trichomes (Figures 4I and 4J), which are highly modified single cells that undergo several endoreplication cycles associated with their morphogenetic development (Oppenheimer, 1998; Traas et al., 1998). A clear gradient was observed in the GUS signal in trichomes, the strongest signal occurring in trichomes located toward the basal part of the growing leaf, whereas it was undetectable in those located toward the leaf tip (Figures 4I and 4J), consistent with the spatiotemporal developmental pattern of trichomes in the growing leaf (Hülskamp et al., 1994). In addition, the young expanded cotyledons (Figures 4C and 4I), composed largely of cells that had endoreplicated during seed maturation (Lemontey et al., 2000), gave weak GUS staining. This study led us to conclude that *AtCDC6* expression is associated with cell division and endoreplication. In addition, our results, together with those obtained for animal CDC6 proteins, suggest that the activity of the *AtCDC6* gene promoter may be a useful marker of cell proliferation.

High Expression of *AtCDC6* during Dark-Regulated Endoreplication Cycles

To analyze *AtCDC6* expression during endoreplication, we chose the growing hypocotyl, in which the occurrence of developmentally regulated endoreplication cycles is well characterized (reviewed by Traas et al., 1998). Typically, during photomorphogenic development, 3.5-day-old light-grown seedlings have expanded green cotyledons and short hypocotyls (Figure 5A). Histochemical detection of GUS activity in *AtCDC6* promoter–GUS transgenic plants indicated that in light-grown seedlings, the hypocotyl cells appeared negative (Figure 5B). In situ hybridization analysis of light-grown Arabidopsis seedlings revealed undetectable levels of *AtCDC6* transcripts in hypocotyl cells with both the antisense probe (Figure 5C) and the control sense probe (Figure 5D). It should be mentioned that GUS activity in the cotyledons varies in different transgenic lines and is dependent within lines on growth time and conditions. These observations may be attributable, at least in part, to differences in the stability of the GUS protein in relation to the physiological state of cotyledon cells. Therefore, a full understanding of the molecular basis of these differences awaits further research in the future.

In contrast to those grown in the light, dark-grown seedlings are characterized by a skotomorphogenic pathway that leads to the formation of closed cotyledons and enlarged hypocotyls (Figure 5A). This is the result of a massive cell enlargement concomitant with the occurrence of one extra endoreplication round in a large proportion of hypocotyl cells, which become 16C, 3 to 5 days after germination (Gendreau et al., 1998). GUS activity was very high in 4.5-day-old dark-grown hypocotyl cells (Figures 5E and 5H). This expression pattern, which occurred in all transgenic lines analyzed, was in agreement with the in situ hybridization analysis of dark-grown hypocotyl cells, in which the *AtCDC6* gene was highly expressed (Figures 5F and 5I), compared with the negative light-grown tissue (Figure 5C) and sense controls (Figures 5G and 5J). Furthermore, an acropetal increasing gradient of *AtCDC6* expression was observed (Figure 5F), coincident with the known pattern of enlargement that takes place concomitant with the development of the extra endoreplication round, the ploidy level describing an acropetal gradient. This result strongly suggests that increased *AtCDC6* expression is associated with the occurrence of an extra endocycle in hypocotyl cells of dark-grown seedlings.

AtCDC6 Expression in Arabidopsis Mutants with Altered Endoreplication Pattern and Ploidy Level

To corroborate the putative association between *AtCDC6* gene expression and the endoreplication process, we took advantage of an Arabidopsis mutant with an altered pattern of endoreplication. Hypocotyl cells of seedlings carrying a

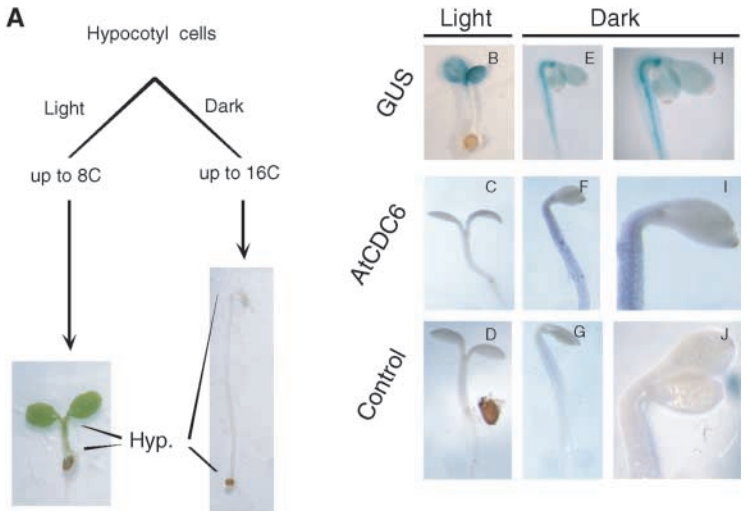


Figure 5. *AtCDC6* Gene Is Highly Expressed in Endoreplicating Dark-Grown Hypocotyl Cells.

(A) shows the effect of light on growing Arabidopsis seedlings in relation to the ploidy level of the hypocotyl (Hyp.) cells. The DNA contents reach up to 16C in the dark because of an extra endoreplication cycle that is absent in the light-grown seedlings. Arabidopsis seedlings were grown either in the light (**[B]** through **[D]**) or in the dark (**[E]** through **[J]**). GUS activity was monitored in transgenic plants carrying the *AtCDC6* promoter–GUS fusion (**[B]**, **[E]**, and **[H]**), and the AtCDC6 transcripts were detected in wild-type 3.5-day-old plants by in situ hybridization using an antisense probe (AtCDC6; **[C]**, **[F]**, and **[I]**). A sense AtCDC6 probe was used as a control (**[D]**, **[G]**, and **[J]**).

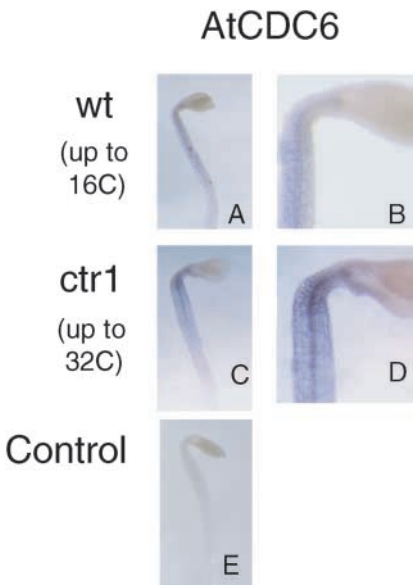


Figure 6. *AtCDC6* Gene Expression in Arabidopsis Mutants with Altered Endoreplication Patterns in Hypocotyl Cells.

Wild-type (wt; **[A]** and **[B]**) and *ctr1* (**[C]** and **[D]**) Arabidopsis plants were grown in the dark for 3.5 days. The maximum DNA content reached by hypocotyl cells is shown in parenthesis (wild type, 16C; *ctr1*, 32C). *AtCDC6* transcripts were detected by in situ hybridization using an antisense probe, and seedlings are shown under low (**[A]** and **[C]**) and high (**[B]** and **[D]**) magnification. A control corresponding to a wild-type plant processed with a sense AtCDC6 probe is shown in **(E)**. For simplicity, controls for the mutant plants, which also were negative, are not shown.

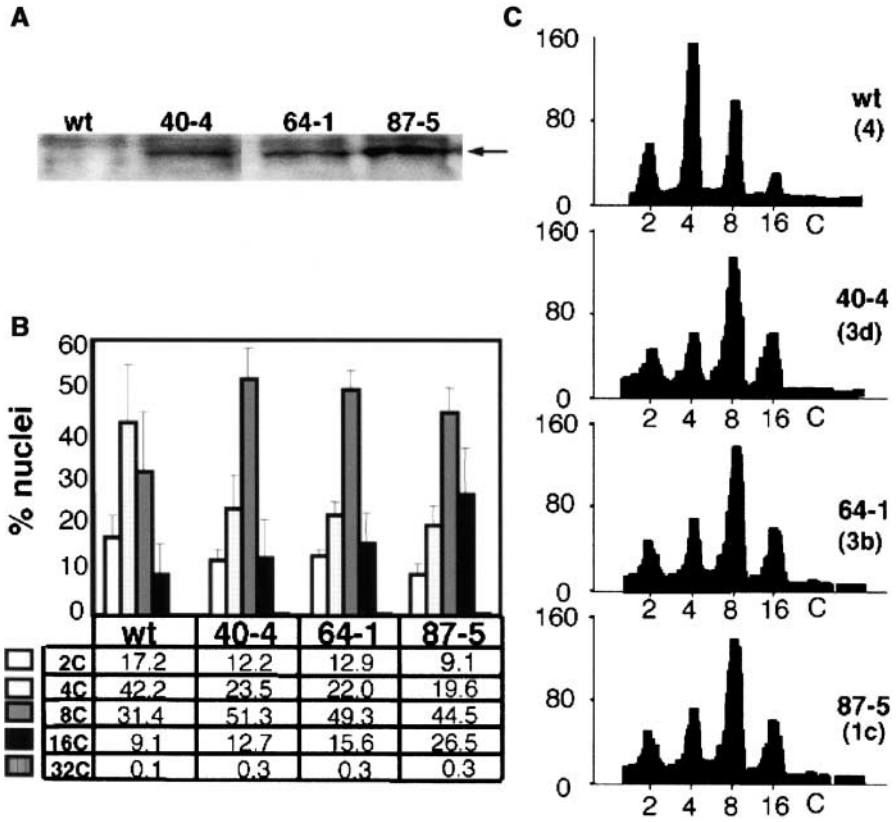
mutation in the *CTR1* (for *CONSTITUTIVE TRIPLE RESPONSE1*) gene, which is involved in ethylene signaling (Chang and Shockey, 1999), undergo one extra endoreplication round in a large proportion of cells that become 32C in the absence of light (Gendreau et al., 1999). In situ hybridization experiments revealed that *AtCDC6* gene expression was much higher in hypocotyl cells of dark-grown *ctr1* mutant seedlings (Figures 6C and 6D) than in wild-type seedlings (Figures 6A and 6B), whereas the sense controls were negative (Figure 6E). Therefore, these results reinforce the idea that the occurrence of extra endocycles during hypocotyl growth is associated with and requires the maintenance of increased levels of *AtCDC6* gene expression.

Ectopic Expression of *AtCDC6* Affects Endopolyploidy Levels

Unscheduled expression of the CDC6 protein in *S. pombe*, but not in *S. cerevisiae* or human cells, leads to changes in the ploidy level. The pattern of *AtCDC6* gene expression in endoreplicating cells of wild-type and *ctr1* mutant plants strongly suggested that AtCDC6 may be one of the factors required for the maintenance of endoreplication cycles. To determine whether AtCDC6 affects the ploidy level, we generated transgenic Arabidopsis plants expressing constitutively a hemagglutinin (HA)-tagged AtCDC6 protein. Three different transgenic lines that expressed detectable levels of the HA-AtCDC6 protein (Figure 7A) were chosen for further analysis. Wild-type Arabidopsis leaves undergo endoreplication cycles to yield a mean ploidy level distribution of the kind shown in Figure 7B, with a major peak of 4C cells. A clear difference was observed in the HA-AtCDC6–expressing plants, in which the mean distribution was, in all three lines, significantly shifted from lower levels of ploidy to a higher ploidy level, with a major peak of 8C cells and an increase of the 16C peak. In one of the lines (87-5), the frequency of the 16C nuclei was almost triple that of the wild type (Figure 7B). These mean distributions were generated by averaging the results from 12 to 17 different plants of each line to account for experimental variability (bars in Figure 7B indicate the standard deviation in each case). Student's *t* test demonstrated that the decreases in the frequency of 2C and 4C nuclei and the increases in the frequency of 8C and 16C nuclei of the HA-AtCDC6–expressing plants compared with wild-type plants were statistically significant (P values ranged between 0.025 and 0.005). Representative histograms belonging to one plant of each line are shown in Figure 7C. These results indicate that the ectopic expression of *AtCDC6* is sufficient to induce endoreplication and most likely is one of the factors that contributes to maintain endoreplication competence.

AtCDC6 Is Degraded Efficiently in Dividing Cell Extracts but Poorly in Endoreplicating Cell Extracts

The balance between expression and proteolytic degradation has been described in other systems as a key regulatory step of CDC6 activity (Piatti et al., 1996; Drury et al., 1997, 2000; Kominami and Toda, 1997; Sanchez et al., 1999; Calzada et al., 2000). We have generated polyclonal sera that recognized AtCDC6 protein expressed in bacteria or yeast, but the very low amounts of AtCDC6 protein in cell extracts has precluded, thus far, the analysis of protein levels under different settings (M.M. Castellano and C. Gutierrez, unpublished data). Therefore, to investigate the stability of the AtCDC6 protein, we used AtCDC6 protein in a cell-free assay (Naidoo et al., 1999; Osterlund et al., 2000). We found that in vitro–transcribed and –translated, ^{35}S-labeled AtCDC6 was degraded efficiently within minutes in extracts prepared from suspension-cultured cells (Figure 8A). As a control, we used in vitro–translated luciferase that was not degraded under similar conditions (Figure 8A), indicating that the AtCDC6 proteolysis observed in this assay was not caused by the presence of unspecific proteases in the plant cell extract. To determine whether the destruction of AtCDC6 was proteosome dependent, we used the specific and well-char-



	wt	40-4	64-1	87-5
2C	17.2	12.2	12.9	9.1
4C	42.2	23.5	22.0	19.6
8C	31.4	51.3	49.3	44.5
16C	9.1	12.7	15.6	26.5
32C	0.1	0.3	0.3	0.3

Figure 7. Expression of *AtCDC6* Affects the Somatic Ploidy Level.

(A) Protein gel blot analysis of the expression of HA-tagged AtCDC6 in wild-type (wt) and transgenic (lines 40-4, 64-1, and 87-5) Arabidopsis tissue extracts. The band corresponding to HA-AtCDC6 is marked with an arrow.

(B) Graphic representation of the mean percentage of nuclei with DNA content of 2C (white), 4C (pale gray), 8C (gray), 16C (black), and 32C (vertical lines) of the wild-type and HA-AtCDC6–expressing transgenic plant lines. Error bars indicate the standard deviation for 12 to 17 independent plants of each line analyzed. The mean values of each frequency class are shown in the table below.

(C) Representative histograms (abscissa with log scale) belonging to one plant of each line analyzed. Plant numbers are shown in parentheses.

acterized proteosome inhibitor MG132, which effectively prevented AtCDC6 degradation when added to the cell-free assay (Figure 8A). In this cell-free system, AtCDC6 remained stable when the reaction was performed in the presence of a mutated ubiquitin protein (not shown) carrying a K-to-A substitution at position 48, which prevents polyubiquitination of target proteins, thus blocking their degradation (Haas and Siepmann, 1997). Together, these results indicate that AtCDC6 is degraded in a ubiquitin- and proteosome-dependent manner by suspension-cultured cell extracts.

To investigate whether this AtCDC6 proteolytic activity could be detected in whole-plant extracts, different regions of dark-grown seedlings were processed. Extracts from cotyledons degraded AtCDC6 in vitro (Figure 8B), although less efficiently than cultured cell extracts. Interestingly, AtCDC6 was more stable in extracts prepared from the upper (Hyp. 1) than from the lower (Hyp. 2) part of hypocotyls of dark-grown seedlings (Figure 8B). This result suggests that the occurrence of extra endoreplication cycles in dark-grown hypocotyls is associated with increased stability of AtCDC6.

DISCUSSION

In this work, we have isolated an Arabidopsis cDNA encoding a key regulatory protein of cellular DNA replication, and we have shown that the promoter contains a consensus site for the E2F transcription factor and binds E2F/DP in vitro. We also analyzed *AtCDC6* gene expression during seedling growth and addressed the possible participation of the CDC6 protein in the endoreplication process associated with Arabidopsis growth and development. To this end, we have studied *AtCDC6* gene expression in wild-type, *AtCDC6*

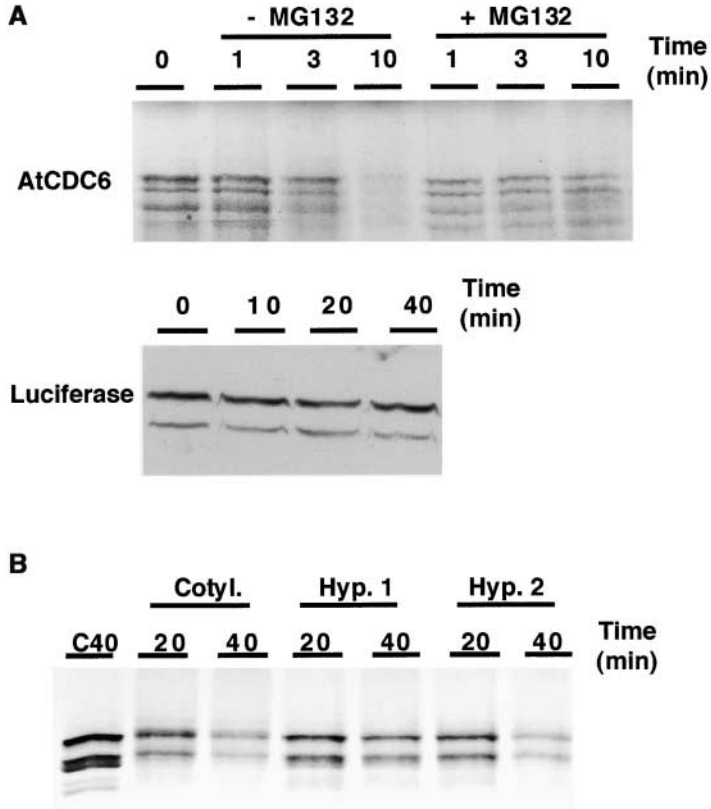


Figure 8. AtCDC6 Protein Is Degraded in Cycling Cells through a Ubiquitin-Dependent Pathway.

(A) Time course of cell-free degradation of AtCDC6. In vitro transcribed–translated AtCDC6 was incubated for the times indicated in the absence or presence of the proteosome inhibitor MG132, with extracts prepared from proliferating cells. In the absence of extract, AtCDC6 was not degraded (data not shown). An unrelated protein (luciferase) was used as a control for unspecific degradation under similar assay conditions.
(B) Proteolytic degradation of AtCDC6 by extracts from different regions of the seedling in the absence (C40) or in the presence of extracts from three different regions of Arabidopsis seedlings grown in the dark for 3 days. Cotyl., cotyledons; Hyp. 1, upper part of hypocotyls; Hyp. 2, lower part of hypocotyls.

promoter–GUS transgenic, and mutant Arabidopsis plants with altered endoreplication patterns and ploidy levels. We propose that the occurrence of endoreplication cycles is associated with increased levels of expression and stability of AtCDC6.

Domain Organization of CDC6 in Metazoa and Metaphyta

CDC6 is essential for DNA replication in archaea, yeast, and metazoa, but information is not available in plants. The isolation of an AtCDC6 cDNA extends the family to metaphyta in which, based on its domain organization, CDC6 also may regulate DNA replication origin activity. AtCDC6, like some members of the AtORC complex (M.M. Castellano and C. Gutierrez, unpublished data), contains conserved amino acid motifs, including those present in ORC1 and the RF-C clamp loader (Perkins and Diffley, 1998), the NTP binding domain, the nuclear localization signal motif, and three potential cyclin-dependent kinase (CDK) phosphorylation sites. Because various CDK and A-, B-, and D-type cyclins have been identified in Arabidopsis (Mironov et al., 1999), it will be interesting to determine whether AtCDC6 is actually phosphorylated, by which CDK/cyc complexes, and the functional consequences of its modification. AtCDC6 contains six RXXL motifs, and interestingly, AtCDC6 shares with the *X. laevis* Cdc6 protein (Coleman et al., 1996) the lack of a **KEN** box, which is crucial for human Cdc6 (Petersen et al., 2000). The unique plant body architecture suggests that regulation of its activity in relation to growth and development may have distinct features.

While this work was in progress, we identified another genomic sequence and isolated a cDNA, which we have named AtCDC6b (accession number AJ310357), encoding another CDC6 protein (M.M. Castellano and C. Gutierrez, unpublished

data). The AtCDC6b protein shares 74% identity in primary sequence with the AtCDC6 protein (79% identity at the DNA level). Although both AtCDC6 proteins share the six characteristic motifs described, interestingly, the AtCDC6b protein contains only one consensus (**S/TPXK/R)** CDK phosphorylation site (position 54; M.M. Castellano and C. Gutierrez, unpublished data). Thus, it seems that the Arabidopsis genome contains two *AtCDC6* genes, a rare situation in eukaryotes, although it is frequent in the Arabidopsis genome (Arabidopsis Genome Initiative, 2000). The use of the *AtCDC6* promoter in the expression experiments and the AtCDC6 protein in the degradation assays led us to ensure that our main conclusions refer, at least, to this gene. The high similarity between the two AtCDC6 proteins suggests that they may play similar roles, but potential differences in the roles of the two *AtCDC6* genes, if any, remain to be determined.

AtCDC6 Expression and Stability in Relation to Endoreplication

The availability of CDC6 depends on a strict balance of cell cycle–regulated expression, localization, and proteolytic activity. *S. cerevisiae* Cdc6 and *S. pombe* Cdc18 transcripts are high in late mitotic cells, and a second peak has been detected in *S. cerevisiae* when G1 is prolonged (Kelly et al., 1993; Piatti et al., 1995; Zwerschke et al., 1995). In contrast, in human cells, transcription of the *CDC6* gene is upregulated at the G1/S transition by E2F transcription factors (Hateboer et al., 1998; Ohtani et al., 1998; Yan et al., 1998). We have found that in proliferating cultured cells, *AtCDC6* gene expression peaks in early S-phase. We also found that the *AtCDC6* gene is expressed in proliferating cells of different plant organs (e.g., root meristems, lateral roots, and leaf primordia) but is undetectable in differentiated cell types, consistent with a role of AtCDC6 in DNA replication. This, together with the presence of an E2F consensus site in the *AtCDC6* promoter that directs binding of purified plant E2F/DP, strongly points to an E2F-dependent regulation of *AtCDC6* gene expression. Future work will seek to identify which of the complexes formed by the six different E2F and the two DP Arabidopsis proteins (Magyar et al., 2000; Ramirez-Parra and Gutierrez, 2000) regulate *AtCDC6* gene expression during the cell cycle and/or in different cell types.

Unscheduled expression of *S. cerevisiae* CDC6 in G2 does not result in extensive genome rereplication (Piatti et al., 1996; Drury et al., 1997). Interestingly, overexpression of the *S. pombe* Cdc18p (Nishitani and Nurse, 1995; Jallepalli and Kelly, 1996) results in repeated rounds of DNA replication in the absence of mitosis. In contrast, the expression of human CDC6 in cultured cells does not (Petersen et al., 2000), although S-phase entry is accelerated (Stoeber et al., 1998; Petersen et al., 2000). These data indicate that different strategies have evolved in eukaryotes and suggest that the regulation in different cell types or in whole organisms may differ from what is being observed in cultured cells.

One controlled mode of rereplication occurs when cells undergo endoreplication cycles, a common event in plants, frequently occurring in association with or before cell differentiation (Traas et al., 1998; Kondorosi et al., 2000; Larkins et al., 2001). We have found that the *AtCDC6* gene is highly expressed in cells undergoing the endoreplication cycles (e.g., in trichomes or in dark-grown hypocotyl cells). This observation was corroborated using *ctr1* seedlings. The *CTR1* gene, which encodes a Raf-related serine/threonine kinase, acts as a negative regulator of the ethylene signaling pathway (Chang and Shockey, 1999). Mutation of the *CTR1* gene leads to altered endoreplication patterns and ploidy levels of hypocotyl cells (Gendreau et al., 1998, 1999). The hypocotyl cells of the *ctr1* mutant undergo one extra endoreplication cycle and have increased *AtCDC6* gene expression in these cells. On the basis of our observations in two different cell types of wild-type and mutant plants, it is conceivable that the initiation and maintenance of developmentally induced endoreplication cycles are associated with increased levels of *AtCDC6* gene expression and perhaps of other E2F-responsive genes. This idea is reinforced by our finding that ectopic expression of AtCDC6 is sufficient to induce a change in the somatic ploidy level in the leaves of transgenic Arabidopsis plants. Whether this applies to endoreplication events occurring in other cell types remains to be confirmed.

In addition to regulation at the level of gene expression, CDC6 availability depends on cell cycle–regulated, proteosome-dependent proteolysis. In yeast, CDC6 is targeted for ubiquitin-dependent proteolysis by the SCF complex soon after the G1/S transition (Piatti et al., 1996; Drury et al., 1997, 2000; Kominami and Toda, 1997; Sanchez et al., 1999; Calzada et al., 2000). In multicellular eukaryotes, the situation seems to be different, because human Cdc6 is targeted for proteolysis by the APC complex in early G1 (Mendez and Stillman, 2000; Petersen et al., 2000). We found that AtCDC6 is degraded by the proteosome in dividing cell extracts and plant tissue extracts but is degraded only poorly in endoreplicating cell extracts. Whether the pathway involved is SCF or APC remains to be determined. Thus, endoreplicating cells, characterized by the absence of typical G2, M, and G1 phases, also have an apparent defect in AtCDC6 proteolysis. One or more of the cellular factors required may be missing in endoreplicating hypocotyl cells. The AtCDC2b kinase does not seem to be a limiting factor because plants underexpressing this gene have a normal endoreplication pattern in hypocotyl cells (Yoshizumi et al., 1999).

A variety of pathways seems to contribute to triggering and maintaining endoreplication competence during development in association with the regulated expression of specific sets of genes. Leaving the mitotic cycle to initiate endoreplication requires the *fzr* gene in Drosophila embryonic epidermal cells (Sigrist and Lehner, 1997) or its alfalfa homolog, the *ccs52* gene, during legume nodule formation (Cebolla et al., 1999). Several *fzr* genes have been identified in the Arabidopsis genome. The CCS52/FZR protein may function as an activator of the ubiquitin-dependent APC

pathway (Fang et al., 1998; Lorca et al., 1998; Zachariae et al., 1998; Cebolla et al., 1999) to induce proteolysis of mitotic cyclins (Sigrist and Lehner, 1997; Yamaguchi et al., 1997). In support of this, alfalfa CCS52 induces the proteolysis of mitotic cyclins when it is expressed in *S. pombe* and affects endopolyploidy (Cebolla et al., 1999). In addition to the requirements for specific gene products to trigger endoreplication, other cellular functions seem to be required for the maintenance of endoreplication cycles (reviewed by Larkins et al., 2001). Extensive endoreplication rounds occur during endosperm (Grafi and Larkins, 1995) and fruit (Joubés et al., 1999) development, during which the activity of an S-phase–related kinase is increased. This activity could inactivate transcriptional repression by retinoblastoma-related proteins (Grafi et al., 1996; Gutierrez, 1998) and, as a consequence, could lead to the upregulation of E2F-responsive genes (e.g., *AtCDC6*) and perhaps other G1/S-regulated genes that are required for the occurrence of endoreplication. Our data showing that ectopic expression of AtCDC6 enhances the endopolyploidy level are fully supportive of this idea.

In conclusion, the basic molecular mechanism for the activation of DNA replication origins by CDC6 might be conserved in metazoa and metaphyta. Our results indicate that studying the roles of DNA replication proteins during growth and development in different systems is a challenging endeavor that should help in delineating CDC6 function and regulation in whole organisms. In addition, the different settings in which endoreplication occurs in plant cells offer the possibility to define the roles of cellular factors that regulate the activation of DNA replication origins and to determine the mechanisms by which S-phase is coordinated with or dissociated from mitosis.

METHODS

Plant Material

The *Arabidopsis thaliana* suspension culture (obtained from J. Murray, Institute of Biotechnology, Cambridge, UK) was maintained in liquid Murashige and Skoog (MS) (1962) salts supplemented with vitamins, naphthylacetic acid, and kinetin with continuous shaking at 26°C. The culture medium was changed every 7 days after a 1:10 dilution of cells. Arabidopsis seedlings (Columbia [Col-0] ecotype) were grown in MS salts supplemented with 1% sucrose and 1% agar either in the dark or in the light (16 hr of light and 8 hr of dark), as indicated in Results, at 22°C. The *ctr1* mutant seed were a gift of R. Solano (Nacional de Biotecnologia, Madrid, Spain).

Isolation of AtCDC6 cDNA by Polymerase Chain Reaction Amplification

A pACT2-based cDNA library (a kind gift of C. Konzc, Max-Planck Institut für Zuchtungsforschung, Cologne, Germany), prepared from Arabidopsis suspension cultured cells, was subjected to polymerase chain reaction (PCR) (using Taq polymerase) to amplify overlapping DNA fragments containing the 5′ and 3′ cDNA regions encoding AtCDC6. To amplify the 5′ region, we used the primers pACT2 (5′-ATGATGAAGATACCCCACCAAAC-3′) and GCME (5′-GCCTTCTTC-TGCTCCATACAAC-3′); for the 3′ region, we used primers VSKAP (5′-ATGTATCTAAGGCACCGTCAA-3′) and GAD10rev (5′-GCACGA-TGCACAGTTGAAGT-3′). After cloning, we confirmed by sequencing that the two fragments corresponded to the genomic sequence identified. To isolate the coding region, we used the 5′ and 3′ fragments as templates with the oligonucleotides MET (5′-AAATGGATCCTA-CAATGCCTGCAATCG-3′) and GCME and oligonucleotides VSKAP and STOP (5′-TCATAGAAGACAGTTGCGGAAGA-3′), respectively, in separate PCR reactions (using Pfu polymerase). The two products were mixed in the absence of exogenously added primers, annealed, and extended (94°C for 2 min, followed by 10 cycles at 94°C for 2 min, 50°C for 10 min, and 72°C for 2 min). Then, the MET and STOP oligonucleotides were added and the reaction was continued (94°C for 2 min, followed by 25 cycles at 94°C for 30 sec, 65°C for 30 sec, 72°C for 4 min, and 72°C for 7 min). The final product was purified, cloned and sequenced, and found to match fully with the coding sequence of AtCDC6.

Electrophoretic Mobility Shift Assays of E2F/DP Complexes with DNA

The binding mixture contained 20 mM Hepes, pH 7.9, 12% glycerol, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM $MgCl_2$, 1 μg of salmon sperm DNA, and bacterially purified GST-TmE2F (Ramirez-Parra et al., 1999) and MBP-DP (Ramirez-Parra and Gutierrez, 2000) proteins, as indicated in Results. Binding reactions were incubated for 20 min at 4°C, and the DNA–protein complexes were fractionated by electrophoresis though 4% polyacrylamide gels at 4°C in 0.5 × Tris-borate/EDTA (TBE) buffer. Synthetic oligonucleotides (Isogen Bioscience, Maarssen, The Netherlands) spanning the AtCDC6 gene promoter region containing a consensus E2F binding site (E2CDC6wt, 5′-ATT-GCTATTTCCCGCTACTTCCATT-3′, top strand) was end labeled with γ-^{32}P-ATP, annealed with an excess of the cold complementary bottom strand, and used as a binding substrate. The oligonucleotide mutated in the E2F binding site (E2CDC6mut, 5′-ATTGCTATTTCC-ATCTACTTCCATT-3′, top strand) was labeled and annealed as described for the wild-type probe.

RNA Extraction and RNA Gel Blot Analysis

Total RNA from Arabidopsis suspension-cultured cells was prepared, at the indicated times in Figure 2, after grinding the cell pellet in liquid nitrogen with the Trizol reagent (GIBCO-Invitrogen-Life Technologies, Groningen, The Netherlands) according to the manufacturer's instructions. Poly(A^+) mRNA was isolated from the total RNA preparation using PolyATtract mRNA isolation system IV (Promega). Partial synchronization was achieved by treatment with aphidicolin (4 μg/mL) for 36 hr and then releasing the culture into fresh medium lacking the drug. Aliquots of the mRNA samples were denatured, fractionated on a 1.2% agarose gel supplemented with 2.2 M formaldehyde, and transferred to a Zeta-Probe membrane (Bio-Rad). The filters were hybridized with random-primed ^{32}P-labeled probes (Boehringer Mannheim) consisting of a 5′ terminal region (the first 460 nucleotides) of the *AtCDC6* cDNA, a wheat histone H4 probe (Q. Xie

and C. Gutierrez, unpublished data), or a ribosomal protein AtL3 probe (Kim et al., 1990) as a loading control. RNA molecular mass markers were purchased from Boehringer Mannheim.

In Situ Hybridization

The sense (control) and antisense RNA probes were prepared with the digoxigenin RNA labeling kit (Boehringer Mannheim), as described by the manufacturer, after in vitro transcription from the T7 and T3 promoters of a full-length *AtCDC6* cDNA cloned into the pBluescript KS+ vector. The samples were treated with alkali in 0.1 M carbonate buffer, pH 10.2, at 60°C for 1 hr. Sterilized Arabidopsis seed, kept at 4°C for 2 days, were grown vertically in MS modified medium supplemented with 1% sucrose and 1% agar either in the dark or in the light (16 hr of light and 8 hr of dark), as indicated in Results, at 22°C.

Preparation of Whole Cell Extracts

Protein extracts from Arabidopsis cell cultures or seedlings were prepared in buffer A containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 1 mM phenylmethylsulfonyl fluoride, and 1 × protease inhibitor cocktail tablet (Complete Mini EDTA-free; Boehringer Mannheim). Arabidopsis cultured cells (~5 g fresh weight) were grounded in buffer A at 4°C and kept on ice for 20 min. The samples were centrifuged at 10,000*g* for 15 min at 4°C, and the supernatant was divided into aliquots and stored at −80°C. Etiolated seedlings were grown in the dark for 3 to 4 days, and protein extracts from excised cotyledons and hypocotyls were prepared as described above for cultured cells.

In Vitro Assay of AtCDC6 Stability

AtCDC6 was obtained by in vitro transcription-translation using the TNT-T7 kit (Promega) in the presence of both ^{35}S-labeled methionine and cysteine. Incubations of ^{35}S-AtCDC6 with protein extracts were performed at 32°C in a buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 7.5 mM $MgCl_2$, 0.1% Tween 20, 0.2 mM DTT, 5 mM ATP, 0.2 mg/mL ubiquitin, 5 mM creatine phosphate, and 0.1 unit/mL creatine kinase. Protein extracts from cultured cells or dark-grown seedling tissues were added to a final concentration of 2 mg/mL. The proteosome inhibitor MG132 was used at 100 μM. The incubations were stopped by adding loading buffer, the samples were boiled for 4 min, and the ^{35}S-AtCDC6 product was analyzed by gel electrophoresis on 10% polyacrylamide gels.

Transgenic Plants

To generate Arabidopsis plants expressing the translational *AtCDC6* promoter–β-glucuronidase (GUS) fusion, an ~2.3-kb genomic fragment spanning from positions −2250 to +18 (+1 being the A residue of the initiator methionine) was amplified from genomic DNA by PCR using primers C6D (5′-TTTTGGATCCTGTGAGATCCGCTTAGTT-3′) and C6inv60 (5′-TTTTCCCGGGTCCGGCGATTGCAGGCATT-3′). The resulting PCR fragment was gel purified and cloned into the pCR2.1 vector, digested with BamHI and SmaI, and transferred to the pBI101.3 binary vector in frame with the GUS coding sequences.

To generate plants expressing a hemagglutinin (HA)-tagged AtCDC6 protein, the AtCDC6 cDNA was cloned in frame with the HA epitope into the pPily plasmid (Ferrando et al., 2000). The HA-AtCDC6 fusion under the control of a double 35S promoter of *Cauliflower mosaic virus* was cloned into the binary pBIN19 vector. This HA-AtCDC6 clone was used to transform Arabidopsis plants.

Agrobacterium tumefaciens–mediated transformation of Arabidopsis plants was performed by means of an infiltration procedure (Clough and Bent, 1998). Transformed seedlings (T0 generation) were selected on MS agar plates containing 50 μg/mL kanamycin and transferred to soil. T2 homozygous plants were selected for further analysis. HA-AtCDC6 protein levels were determined by protein gel blot analysis of extracts in whole protein extracts prepared by grinding 4-day-old seedlings in TBS buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40) in the presence of 1 × protease inhibitor cocktail (Boehringer Mannheim), 1 mM phenylmethylsulfonyl fluoride, and 20 μM MG132 at 4°C for 30 min. Proteins (20 μg) were fractionated on 10% polyacrylamide gels and electroblotted to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA) according to the manufacturer's instructions. Blots were probed with a monoclonal antibody against the HA epitope (Eurogentec, Seraing, Belgium) at a dilution of 1:2000. A goat anti-mouse horseradish peroxidase–conjugated IgG was used as a secondary antibody.

Histochemical Detection of GUS Activity

Detection of GUS activity was performed on primary and/or secondary transformants using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide as described (Jefferson et al. 1987), with slight modifications.

Flow Cytometric Analysis of Leaf Nuclei

Plants were grown in a glasshouse at 23°C with 18 hr of light. Initially, all major tissues were dissected and analyzed by cytometry, but only results concerning the second to the fourth rosette leaves are presented. Two leaves were cut to eliminate the petiole and midrib. The remaining lamina was chopped in 600 μL of cold nuclear isolation buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM [4-morpholino]propanesulfonate, pH 7.0, 0.1% [w/v] Triton X-100) (Galbraith et al., 1983). This crude preparation of isolated nuclei was filtered through 48-μm nylon mesh and stained with 5 μg/mL 4′,6-diamidino-2-phenylindole (Sigma). DNA histograms corresponding to 5000 isolated nuclei were made with an ELITE ESP cytometer (Beckman-Coulter, Roissy, France) using UV excitation and gates to eliminate debris or doublets (as described in Coba de la Peña and Brown, 2001). To account for physiological or experimental variability, this histogram was generated from a mixture of these two leaves and repeated independently using 12 to 17 different plants from each line to be compared. The frequency of nuclei at each somatic ploidy level was determined. For different transgenic lines and control plants (wild type Col-0), means were compared by Student's *t* test.

Accession Number

The accession number for the AtCDC6 protein described in this article is AJ271606.

ACKNOWLEDGMENTS

We are indebted to J.A.H. Murray for the Arabidopsis cell suspension culture, to C. Koncz for the Arabidopsis cDNA library, to A. Ferrando for plasmids, to R. Solano for the *ctr1* seed, to G. Morata, M. Calleja, and F.J. Diaz-Benjumea for help with microscope facilities, and to J. Diffley, J.A. Tercero, J. Mendez, and E. Martinez-Salas for suggestions. M.M.C was the recipient of a predoctoral fellowship from the Ministerio de Educacion y Cultura and Comunidad de Madrid (CAM), and E.R.-P. and J.C.d.P. were recipients of postdoctoral fellowships from CAM. This work was partially supported by Grants PB96-0919 (Direccion General de Enseñanza Superior), BMC2000-1004 (Ministerio de Ciencia y Tecnologia), and 07B/0020/98 and 07G/0033/00 (CAM) and by an institutional grant from Fundación Ramón Areces.

Received August 6, 2001; accepted September 19, 2001.

REFERENCES

Arabidopsis Genome Initiative. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408,** 796–815.

Bell, S.P., and Stillman, B. (1992). ATP-dependent recognition of eukaryotic origins of replication by a multiprotein complex. Nature **357,** 128–134.

Calzada, A., Sanchez, M., Sanchez, E., and Bueno, A. (2000). The stability of the Cdc6 protein is regulated by cyclin-dependent kinase/cyclin B complexes in *Saccharomyces cerevisiae*. J. Biol. Chem. **275,** 9734–9741.

Cebolla, A., Vinardell, J.M., Kiss, E., Oláh, B., Roudier, F., Kondorosi, A., and Kondorosi, E. (1999). The mitotic inhibitor ccs52 is required for endoreduplication and ploidy-dependent cell enlargement in plants. EMBO J. **18,** 4476–4484.

Chang, C., and Shockey, J.A. (1999). The ethylene-response pathway: Signal perception to gene regulation. Curr. Opin. Plant Biol. **2,** 352–358.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis. Plant J. **16,** 735–743.

Coba de la Peña, C.T., and Brown, S.C. (2001). Flow cytometry. In Plant Cell Biology: Practical Approach, 2nd ed, C. Hawes and B. Satiat-Jeunemaître, eds (Oxford, UK: Oxford University Press), pp. 85–106.

Coleman, T.R., Carpenter, P.B., and Dunphy, W.G. (1996). The Xenopus Cdc6 protein is essential for the initiation of a single round of DNA replication in cell-free extracts. Cell **87,** 53–63.

de Jager, S.M., and Murray, J.A.H. (1999). Retinoblastoma protein in plants. Plant Mol. Biol. **41,** 295–299.

Diffley, J.F.X., Cocker, J.H., Dowell, S.J., and Rowley, A. (1994). Two steps in the assembly of complexes at yeast replication origins in vivo. Cell **78,** 303–316.

Donaldson, A.D., and Blow, J.J. (1999). The regulation of replication origin activation. Curr. Opin. Genet. Dev. **9,** 62–68.

Drury, L.S., Perkins, G., and Diffley, J.F.X. (1997). The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. EMBO J. **16,** 5966–5976.

Drury, L.S., Perkins, G., and Diffley, J.F.X. (2000). The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle. Curr. Biol. **10,** 231–240.

Edgar, B.A., and Orr-Weaver, T.L. (2001). Endoreplication cell cycles: More for less. Cell **105,** 297–306.

Fang, G., Hongtao, Y., and Kirschner, M.W. (1998). Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G_1. Mol. Cell **2,** 163–171.

Ferrando, A., Farras, R., Jasik, J., Schell, J., and Koncz, C. (2000). Intron-tagged epitope: A tool for facile detection and purification of proteins expressed in Agrobacterium-transformed plants. Plant J. **22,** 553–560.

Galbraith, D.W., Harkins, K.R., Maddox, J.M., Ayres, N.M., Sharma, D.P., and Firoozabady, E. (1983). Rapid flow cytometry analysis of the cell cycle in intact plant tissues. Science **220,** 1049.

Gavin, KA., Hidaka, M., and Stillman, B. (1995). Conserved initiation proteins in eukaryotes. Science **270,** 1667–1671.

Gendreau, E., Höfte, H., Grandjean, O., Brown, S., and Traas, J. (1998). Phytochrome controls the number of endoreduplication cycles in the *Arabidopsis thaliana* hypocotyl. Plant J. **13,** 221–230.

Gendreau, E., Orbovic, V., Höfte, H., and Traas, J. (1999). Gibberellin and ethylene control endoreduplication levels in the *Arabidopsis thaliana* hypocotyl. Planta **209,** 513–516.

Grafi, G., and Larkins, B.A. (1995). Endoreduplication in maize endosperm: Involvement of M phase-promoting factor inhibition and induction of S phase-related kinases. Science **269,** 1262–1264.

Grafi, G., Burnett, R.J., Helentjaris, T., Larkins, B.A., DeCaprio, J.A., Sellers, W.R., and Kaelin, W.G., Jr. (1996). A maize cDNA encoding a member of the retinoblastoma protein family: Involvement in endoreduplication. Proc. Natl. Acad. Sci. USA **93,** 8962–8967.

Gutierrez, C. (1998). The retinoblastoma pathway in plant cell cycle and development. Curr. Opin. Plant Biol. **1,** 492–497.

Gutierrez, C. (2000). DNA replication and cell cycle in plants: Learning from geminiviruses. EMBO J. **19,** 792–799.

Haas, A.L., and Siepmann, T.J. (1997). Pathways of ubiquitin conjugation. FASEB J. **11,** 1257–1268.

Hateboer, G., Wobst, A., Petersen, B.O., Le Camp, L., Vigo, E., Sardet, C., and Helin, K. (1998). Cell cycle–regulated expression of mammalian CDC6 is dependent on E2F. Mol. Cell. Biol. **18,** 6679–6697.

Helin, K., Lees, J.A., Vidal, M., Dyson, N., Harlow, E., and Fattaey, A. (1992). A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell **70,** 337–350.

Hülskamp, M., Miséra, S., and Jürgens, G. (1994). Genetic dissection of trichome cell development in Arabidopsis. Cell **76,** 555–566.

Jallepalli, P.V., and Kelly, T.J. (1996). Rum1 and Cdc18 link inhibition of cyclin-dependent kinase to the initiation of DNA replication in *Schizosaccharomyces pombe*. Genes Dev. **10,** 541–552.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS

fusions: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6,** 3901–3907.

Jiang, W., Wells, N.J., and Hunter, T. (1999). Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. Proc. Natl. Acad. Sci. USA **96,** 6193–6198.

Joubés, J., Phan, T.-H., Just, D., Rothan, C., Bergounioux, C., Raymond, P., and Chevalier, C. (1999). Molecular and biochemical characterization of the involvement of cyclin-dependent kinase A during the early development of tomato fruit. Plant Physiol. **121,** 857–869.

Kelly, T.J., and Brown, G.W. (2000). Regulation of chromosome replication. Annu. Rev. Biochem. **69,** 829–880.

Kelly, T.J., Martin, G.S., Forsburg, S.L., Stephen, R.J., Russo, A., and Nurse, P. (1993). The fission yeast cdc18+ gene product couples S phase to START and mitosis. Cell **74,** 371–382.

Kim, Y., Zhang, H., and Scholl, R.L. (1990). Two evolutionarily divergent genes encode a cytoplasmic ribosomal protein in *Arabidopsis thaliana*. Gene **93,** 177–182.

Kominami, K., and Toda, T. (1997). Fission yeast WD-repeat protein pop1 regulates genome ploidy through ubiquitin-proteosome-mediated degradation of the CDK inhibitor rum1 and the S-phase initiator cdc18. Genes Dev. **11,** 1548–1560.

Kondorosi, E., Roudier, F., and Gendreau, E. (2000). Plant cell–size control: Growing by ploidy? Curr. Opin. Plant Biol. **3,** 488–492.

Larkins, B.A., Dilkes, B.P., Dante, R.A., Coelho, C.M., Woo, Y., and Liu, Y. (2001). Investigating the hows and whys of DNA endoreduplication. J. Exp. Bot. **52,** 183–192.

Lemontey, C., Mousset-Déclas, C., Munier-Jolain, N., and Boutin, J.-P. (2000). Maternal genotype influences pea seed size by controlling both mitotic activity during early embryogenesis and final endoreduplication level/cotyledon cell size in mature seed. J. Exp. Bot. **51,** 167–175.

Liang, C., and Stillman, B. (1997). Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. Genes Dev. **11,** 3375–3386.

Liang, C., Weinreich, M., and Stillman, B. (1995). ORC and Cdc6p interact and determine the frequency of initiation in the genome. Cell **81,** 667–676.

Lin, X., et al. (1999). Sequence and analysis of chromosome 2 of the plant *Arabidopsis thaliana*. Nature **402,** 761–768.

Lorca, T., Castro, A., Martinez, A.-M., Vigneron, S., Morin, N., Sigrist, S., Lehner, C., Doree, M., and Labbe, J.-C. (1998). Fizzy is required for activation of the APC/cyclosome in Xenopus extracts. EMBO J. **17,** 3565–3575.

Magyar, Z., Atanassova, A., de Veylder, L., Rombauts, S., and Inzé, D. (2000). Characterization of two distinct DP-related genes from *Arabidopsis thaliana*. FEBS Lett. **486,** 79–87.

Meijer, M., and Murray, J.A.H. (2001). Cell cycle controls and the development of plant form. Curr. Opin. Plant Biol. **4,** 44–49.

Mendez, J., and Stillman, B. (2000). Chromatin association of human origin recognition complex, Cdc6, and minichromosome maintenance proteins during the cell cycle: Assembly of prereplication complexes in late mitosis. Mol. Cell. Biol. **20,** 8602–8612.

Mironov, V., de Veylder, L., van Montagu, M., and Inzé, D. (1999). Cyclin-dependent kinases and cell division in higher plants—The nexus. Plant Cell **11,** 509–521.

Mizushima, T., Takahashi, N., and Stillman, B. (2000). Cdc6p modulates the structure and DNA binding activity of the origin recognition complex in vitro. Genes Dev. **14,** 1631–1641.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 473–497.

Naidoo, N., Song, W., Hunter-Ensor, M., and Sehgal, A. (1999). A role for the proteosome in the light response of the timeless clock protein. Science **285,** 1737–1741.

Nishitani, H., and Nurse, P. (1995). $p65^{cdc18}$ plays a major role controlling the initiation of DNA replication in fission yeast. Cell **83,** 397–405.

Ohtani, K., Tsujimoto, A., Ikeda, M., and Nakamura, M. (1998). Regulation of cell growth–dependent expression of mammalian CDC6 gene by the cell cycle transcription factor E2F. Oncogene **17,** 1777–1785.

Oppenheimer, D.G. (1998). Genetics of plant cell shape. Curr. Opin. Plant Biol. **1,** 520–524.

Osterlund, M.T., Hardtke, C.S., Wei, N., and Deng, X.W. (2000). Targeted destabilization of HY5 during light-regulated development of Arabidopsis. Nature **405,** 462–466.

Perkins, G., and Diffley, J.F.X. (1998). Nucleotide-dependent prereplicative complex assembly by Cdc6p, a homolog of eukaryotic and prokaryotic clamp-loaders. Mol. Cell **2,** 23–32.

Petersen, B.O., Lukas, J., Sorensen, C.S., Bartek, J., and Helin, K. (1999). Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. EMBO J. **18,** 396–410.

Petersen, B.O., Wagener, C., Marinoni, F., Kramer, E.R., Melixetian, M., Denchi, E.L., Gieffers, C., Matteucci, C., Peters, J.-M., and Helin, K. (2000). Cell cycle– and cell growth–regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1. Genes Dev. **14,** 2330–2343.

Piatti, S., Lengauer, C., and Nasmyth, K. (1995). Cdc6 is an unstable protein whose de novo synthesis in G_1 is important for the onset of S-phase and for preventing a "reductional" anaphase in the budding yeast *Saccharomyces cerevisiae*. EMBO J. **14,** 3788–3799.

Piatti, S., Böhm, T., Cocker, J.H., Diffley, J.F.X., and Nasmyth, K. (1996). Activation of S-phase-promoting CDKs in late G_1 defines a "point of no return" after which Cdc6 synthesis cannot promote DNA replication in yeast. Genes Dev. **10,** 1516–1531.

Ramirez-Parra, E., and Gutierrez, C. (2000). Characterization of wheat DP, a heterodimerization partner of the plant E2F transcription factor which stimulates E2F-DNA binding. FEBS Lett. **486,** 73–78.

Ramirez-Parra, E., Xie, Q., Boniotti, M.B., and Gutierrez, C. (1999). The cloning of plant E2F, a retinoblastoma-binding protein, reveals unique and conserved features with animal G_1/S regulators. Nucleic Acids Res. **27,** 3527–3533.

Reichheld, J.P., Gigot, C., and Chaubet-Gigot, N. (1995). Multilevel regulation of histone gene expression during the cell cycle in tobacco. Nucleic Acids Res. **26,** 3255–3262.

Ritzi, M., and Knippers, R. (2000). Initiation of genome replication: Assembly and disassembly of replication-competent chromatin. Gene **245,** 13–20.

Sanchez, M., Calzada, A., and Bueno, A. (1999). The Cdc6 protein is ubiquitinated in vivo for proteolysis in *Saccharomyces cerevisiae*. J. Biol. Chem. **274,** 9092–9097.

Sigrist, S.J., and Lehner, C.F. (1997). Drosophila fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. Cell **90,** 671–681.

Stoeber, K., Mills, A.D., Kubota, Y., Krude, T., Romanowski, P., Marheineke, K., Laskey, R.A., and Williams, G.H. (1998). Cdc6 protein causes premature entry into S phase in a mammalian cell-free system. EMBO J. **17,** 7219–7229.

Traas, J., Hülskamp, M., Gendreau, E., and Höfte, H. (1998). Endoreduplication and development: Rule without dividing? Curr. Opin. Plant Biol. **1,** 498–503.

Walker, J.E., Saraste, M., Runswick, M.J., and Gay, N.J. (1982). Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1,** 945–951.

Wang, B., Feng, L., Hu, Y., Huang, S.H., Reynolds, C.P., Wu, L., and Jong, A.Y. (1999). The essential role of *Saccharomyces cerevisiae* CDC6 nucleotide-binding site in cell growth, DNA synthesis and Orc1 association. J. Biol. Chem. **274,** 8291–8298.

Yamaguchi, S., Murakami, H., and Okayama, H. (1997). A WD-repeat protein controls the cell cycle and differentiation by negatively regulating Cdc2/B-type cyclin complex. Mol. Biol. Cell **8,** 2475–2486.

Yan, Z., DeGregori, J., Shohet, R., Leone, B., Stillman, B., Nevins, J.R., and Williams, R.S. (1998). Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells. Proc. Natl. Acad. Sci. USA **95,** 3603–3608.

Yoshizumi, T., Nagata, N., Shimada, H., and Matsui, M. (1999). An Arabidopsis cell cycle–dependent kinase-related gene, *CDC2b*, plays a role in regulating seedling growth in darkness. Plant Cell **11,** 1883–1895.

Zachariae, W., Schwab, M., Nasmyth, K., and Seufert, W. (1998). Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. Science **282,** 1721–1724.

Zwerschke, W., Rottjakob, H.-W., and Küntzel, H. (1995). The *Saccharomyces cerevisiae* CDC6 gene is transcribed at late mitosis and encodes a ATP/GTPase controlling S phase initiation. J. Biol. Chem. **269,** 23351–23356.