The Arabidopsis D-type Cyclins CycD2 and CycD3 Both Interact in Vivo with the PSTAIRE Cyclin-dependent Kinase Cdc2a but Are Differentially Controlled*

J. M. Sandra Healy‡, Margit Menges, John H. Doonan§, and James A. H. Murray¶

From the Institute of Biotechnology, University of Cambridge, Cambridge CB2 1QT, United Kingdom and the §Department of Cell Biology, John Innes Centre, Colney Lane, Norwich NR4 7UH, United Kingdom

Received for publication, October 4, 2000, and in revised form, November 20, 2000
Published, JBC Papers in Press, November 28, 2000, DOI 10.1074/jbc.M009074200

D-type cyclins (CycD) play key roles in linking the Arabidopsis cell cycle to extracellular and developmental signals, but little is known of their regulation at the post-transcriptional level or of their cyclin-dependent kinase (CDK) partners. Using new antisera to CycD2 and CycD3, we demonstrate that the CDK partner of these Arabidopsis cyclins is the PSTAIRE-containing CDK Cdc2a. Previous analysis has shown that transcript levels of CycD2 and CycD3 are regulated in response to sucrose levels and that both their mRNA levels and kinase activity are induced with different kinetics during quiescence (1). Here we analyze the protein levels and kinase activity of CycD2 and CycD3. We show that CycD3 protein and kinase activity parallel the abundance of its mRNA and that CycD3 protein is rapidly lost from cells in stationary phase or following sucrose removal. In contrast to both CycD2 and the regulation of its own mRNA levels, CycD2 protein is present at constant levels. CycD2 kinase activity is regulated by sequestration of CycD2 protein in a form inaccessible to immunoprecipitation and probably not complexed to Cdc2a.

The cell cycle in eukaryotic organisms is primarily controlled by cyclin-dependent kinases (CDKs) in complexes with their activating and substrate-specifying partners, cyclins. The action of these protein complexes is perhaps best understood in yeast, in which a single CDK interacts with different cyclins during the G1 and G2/M phases of the cell cycle (2). In comparison with yeast, relatively little is known about the control of the plant cell cycle, but complementation of yeast strains lacking G1 cyclins has been used to identify the functionally equivalent proteins in plants (3, 4). These approaches identified plant D-type (CycD) cyclins as less related to the yeast G1 cyclins than to mammalian and Drosophila D-type cyclins that function during G1 phase to control cell cycle commitment in response to growth and mitogenic signals (5, 6). CycD cyclins have low overall similarity with animal D-type cyclins (7) but share key features, including an LXXLX motif at the N terminus, which in both animal and plant D-type cyclins is the binding site for the retinoblastoma (Rb) protein (8–10). The interaction between animal D-type cyclins and Rb results in phosphorylation of Rb by the cyclin D-CDK complexes, and the resultant phosphorylated Rb is unable to bind (and therefore inhibit) E2F transcription factors involved in the G1/S transition and progression through S phase (reviewed in Refs. 11 and 12). The transcription of the mammalian D-type cyclins is dependent on stimulation by serum growth factors, and thus D-type cyclins act as mediators of external signals in the progression of the cell cycle (5, 6).

Higher organisms express multiple CDKs, which bind distinct cyclins, and different CDKs are therefore characterized by sequence differences in their cyclin interaction domain. In animals, mitotic cyclins (A- and B-type) bind CDKs that contain the conserved cyclin-binding sequence PSTAIRE, whereas D-type cyclins bind CDK4 and -6, which have the sequence P(I/T)XV(L/I)RE (reviewed in Ref. 13). Plants express two main types of cell cycle regulating CDKs: those containing the PSTAIRE sequence (CDK-a or Cdc2a proteins) and a novel CDK class with the variant sequence PTT(a/T)XRE, which shows cell cycle regulation (reviewed in Refs. 14 and 15). Little is known of cyclin-CDK partnerships in plants, although an alfalfa A-type cyclin CypA2 has recently been shown to associate with the PSTAIRE-containing Cdc2a (16), as has a tobacco cycD3 (17).

The isolation of plant Rb and E2F-like proteins and their interaction in vitro therefore suggests overall similarities between G1/S control in animals and plants (17–21). Evidence that an in vitro assembled complex of tobacco CycD3 and the PSTAIRE CDK Cdc2a can phosphorylate a tobacco Rb-related protein also substantiates the broad parallels between plant and mammalian systems (17). In addition, mRNA levels of the Arabidopsis cyclin D2 (CycD2) and cyclin D3 (CycD3) genes are controlled by external growth signals. In an Arabidopsis cell culture, CycD2 and CycD3 mRNA levels are induced by sucrose and CycD3 mRNA levels increase in response to the presence of the plant hormone cytokinin (1, 22). CycD1 expression is not detectable in this culture. Overexpression of the Arabidopsis Cyclin D2 gene in tobacco has also been shown to increase overall plant growth rate (23), and high level expression of CycD3 results in extopic cell divisions and altered growth (22). Taken together, these results suggest that plant CycD levels may serve to integrate such signals with commitment of cells to the cell cycle (5, 6).

This paper is available on line at http://www.jbc.org

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

* This work was supported by Biotechnology and Biological Sciences Research Council (BBSRC) Grants G02552 and P09509, a BBSRC studentship (to M. M.), and Aventis CropScience. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dipartimento di Genetica, IV Piano, Torre A, Università di Milano, 20133 Milan, Italy.

¶ To whom correspondence should be addressed: Institute of Biotechnology, University of Cambridge, Tennis Court Rd., Cambridge CB2 1QT, United Kingdom. Tel.: 44-1223-334166; Fax: 44-1223-334162; E-mail: j.murray@biotech.cam.ac.uk.

§ The abbreviations used are: CDK, cyclin-dependent kinase; WCE, whole cell extract; IVT, in vitro translated.

1 The abbreviations used are: CDK, cyclin-dependent kinase; WCE, whole cell extract; IVT, in vitro translated.

2 S. de Jager, M. Menges, U.-M. Bauer, and J. A. H. Murray, submitted for publication.
division, paralleling the role of D-type cyclins of animals.

Despite the significance of CycD activity for cell cycle control, development, and growth, almost nothing is known of the control of protein levels or kinase activity of CycD kinases in plants. This is particularly significant, since post-transcriptional and post-translational controls are of considerable importance in determining CDK-cyclin activity (24). Moreover, the CDK partner of the Arabidopsis CycDs is unknown. Here we utilize antibodies that specifically detect CycD2 and CycD3 proteins of Arabidopsis to analyze CycD protein levels in an Arabidopsis cell culture and during plant growth, to identify the CDK partner of these cyclins, and to analyze the kinase activity and associations of the cyclin-CDK complexes in response to external growth signals.

**EXPERIMENTAL PROCEDURES**

**Arabidopsis Cell Suspension and Plant Growth**—Suspension cultures of Arabidopsis thaliana ecotype Landsberg erecta (25, 26) were maintained as previously described (1). For cell cycle reentry experiments, quiescent cells were prepared from cultures 7 days after the previous subculture; the cells were washed and subcultured into MS medium with hormones and all supplements except sucrose. After 24 h, sucrose was added to the culture, and time point samples were taken as the cells reentered the cell cycle (1, 22). Similarly, for cell cycle exit experiments, 3-day (exponential cell growth phase) cells were harvested and replaced in MS medium containing hormones and all supplements except sucrose, and samples were taken at specified times. Arabidopsis seedlings (Landsberg erecta) were grown in liquid medium as previously described (1). Plants were grown to flowering at 22°C in 16 h of light in a growth room.

**Protein Methods**—Polyclonal rabbit antisera were raised against full-length Arabidopsis CycD2 expressed with a 6-histidine tag in Escherichia coli and against Arabidopsis CycD3 C-terminal peptide MVGAENSEKKKPIHLHPWAIVATP by the antibody facility at the Babraham Institute. Antiserum against a common C-terminal peptide found in Arabidopsis Cdc2b and in its tobacco homologue CdkB1 has been described (data not shown) and antiserum against Arabidopsis Cdc2a was produced using the C-terminal peptide ARAALEHEYFKDLGGMP at the Babraham Institute. Procedures for protein extraction, SDS-polyacrylamide gel electrophoresis, Western blot analysis, immunoprecipitations, and histone H1 protein kinase assays have been described (1, 23). Antiserum was used at 1:1000 dilution and incubated with Western blots overnight at room temperature. For competition of the antisera with CycD3 peptide or CycD2 protein, the appropriate antiserum was incubated with CycD3 peptide (at a concentration of 1 μM) or CycD2 protein (40 μg of E. coli-expressed CycD2) for 1 h at 37°C before probing the Western blot.

**In Vitro Translation**—CycD2 and CycD3 cDNAs were cloned into pET23 (Novagen, Madison, WI), and proteins were produced using a T7 coupled transcription-translation rabbit reticulocyte lysate expression system with incorporation of [35S]methionine (Promega, Madison, WI).

**RESULTS**

**Specific Antiseras That Immunodetect and Immunoprecipitate Arabidopsis CycD2 and CycD3 in Active Kinase Complexes**—For analysis of Arabidopsis cycD2 and cycD3 cyclin proteins, rabbit antibodies were generated against full-length CycD2 protein and against a CycD3 C-terminal peptide. To verify that these antisera specifically detect the intended proteins, they were used to probe Western blots of Arabidopsis whole cell extract (WCE) and in vitro translated (IVT) radiolabeled CycD2 and CycD3; the antibody probes were used both directly and in competition with the relevant purified CycD2 protein or CycD3 peptide (Fig. 1A). The CycD2 antibody detected IVT CycD2 protein of ~46 kDa and a protein of the same molecular weight in whole cell extracts (Fig. 1B). The CycD3 antibody detected a ~46 kDa protein in IVT extracts but not in whole cell extracts (Fig. 1B). In competition experiments using whole cell extracts, the CycD2 antibody specifically competed away the ~46 kDa band, while the CycD3 antibody was unable to compete with the ~46 kDa band (Fig. 1C). These results indicate that the antibodies specifically detect the intended proteins.

**CycD2 and CycD3 protein levels during vegetative development and in mature tissues**—A, levels of CycD2, CycD3, and Cdc2a in seedlings/plants of 4, 5, 7, 9, 14, and 17 days grown in vitro. B, levels of CycD2 and CycD3 in shoots and roots of 9-day seedlings. C, presence of CycD2 and CycD3 in leaf, stem, root, and flower of mature soil-grown Arabidopsis.
size in WCE. Neither were detected when the antibody was preincubated with purified CycD2 protein. Similarly, the CycD3 antibody detected IVT CycD3 and a protein of the same size (60 kDa) from the WCE, and these bands were not detected after preincubation of the antibody with purified CycD3 peptide. No cross-reactivity was observed between the CycD2 and CycD3 antisera (not shown). These results were confirmed by immunoblots of plants overexpressing CycD2 or CycD3, in which a stronger signal was observed (not shown).

The quantitative immunoprecipitation of CycD2 and CycD3 by these antibodies was demonstrated by the recovery of IVT CycD2 and CycD3 from the lag phase (days 1 and 2) through exponential growth (days 3–6) to stationary phase (day 7) (1). The upper band visible in some CycD2 blots is nonspecific and not competed by preincubation of the CycD2 antiserum with purified CycD2 protein (data not shown). B, protein gel blots showing levels of CycD2, CycD3, Cdc2a, and Cdc2b in response to removal of sucrose from medium of 3-day (exponential phase) suspension-cultured cells. 3d, WCE before sucrose removal; further lanes are extracts at times (in hours) after replacing cells in fresh medium lacking sucrose. Short (above) and longer (below) exposures of CycD3 gel blots are shown to demonstrate the continued presence of low levels of CycD3 for at least 7 h. C, protein kinase activity (measured in arbitrary units) of CycD2 and CycD3 immunoprecipitates against histone H1 at the times indicated. Background phosphorylation observed using preimmune serum for immunoprecipitation has been subtracted from the data. Corresponding protein gel (Western) blots for the extracts used for kinase assays are shown above the graphs.
presence of CycD2 and CycD3 was examined during Arabidopsis vegetative development (Fig. 3A). CycD2 levels are lower in 4-day seedlings but are subsequently present at a constant level in whole seedling/plant extracts (5–17 days), whereas CycD3 is present at similar levels from 4 to 14 days but subsequently declines at 17 days. In contrast, the kinase partner Cdc2a is present at a constant level in these protein extracts. The distribution of the protein between roots and shoots was examined in 9-day seedlings. CycD2 is primarily expressed in the shoots, although a weak signal was detected in root extract (Fig. 3B), while CycD3 was detected less readily than D2 and primarily in the root. In flowering Arabidopsis plants, CycD2 was more abundant in leaves and stem and detectable at a lower level in flowers but appeared to be absent from root extracts. In contrast, CycD3 was present only in the root, where it was detected weakly.

CycD3 but Not CycD2 Protein Level Is Higher in Actively Dividing Cells—Levels of CycD2 and CycD3 were examined throughout the growth cycle from samples taken on consecutive days after subculture. This culture reaches maximum cell density after 6 days (1). The level of CycD2 remained fairly constant throughout the growth cycle, compared with CycD3 which decreased dramatically after 6 days, corresponding to the onset of stationary phase (Fig. 4A). CycD3 is therefore present only in cells from actively dividing cultures.

Differential Abundance and Kinase Activity of CycD2 and CycD3 Proteins after Removal of Growth Stimulation Signals—Previous analysis has shown the dependence of CycD2 and CycD3 gene expression on sucrose availability (1). To measure the response of CycD2 and CycD3 protein levels and kinase activity to the removal of such growth stimulation signals, sucrose was removed from an exponentially growing (3-day) cell culture, and samples were taken at specific times (Fig. 4B). CycD2 protein levels remained relatively constant, but CycD3 protein levels decreased rapidly with ~90% of CycD3 being degraded within 2 h of sucrose removal. Longer exposures of this blot show that low levels of CycD3 persist for at least 7 h, but by 24 h CycD3 is almost undetectable (Fig. 4B). In contrast to CycD2 mRNA, CycD2 protein levels are constant following sucrose removal, whereas CycD3 protein rapidly declines in abundance. The abundance of Cdc2a and Cdc2b proteins after removal of sucrose remained constant for at least 24 h (Fig. 4B).

When the kinase activity of immunoprecipitations was examined in a similar experiment, a somewhat different pattern was observed. In contrast to the constant abundance of CycD2 protein, CycD2-associated kinase activity started to decline within 4 h of sucrose removal and by 24 h was reduced by more than 50%. CycD3 kinase activity decreased to 50% of its original level within 4 h and then declined more gradually up to 24 h. However, although CycD3 protein was almost undetectable after 24 h, there was still one-third of the kinase activity observed in the exponentially growing cells. This may suggest that much of the early decline in CycD3 levels may be due to a reduction of cyclin not associated with active kinase complexes.

Differential Accumulation and Activity of CycD2 and CycD3 in Cells Reentering the Division Cycle—Arabidopsis cells deprived of sucrose for 24 h resume division and show a relatively synchronous entry into S phase when sucrose is readded (1, 22). CycD2 and CycD2 mRNA levels decline after sucrose removal and increase after readdition. CycD2 mRNA increases within 1 h of sucrose addition, whereas CycD3 mRNA accumulates only after 4 h, corresponding to late G1 phase. To examine the
Characterization of Arabidopsis CycD2 and CycD3 Proteins

Summary model of CycD2 and CycD3 mRNA levels (1), protein abundance, association with Cdc2a, and kinase activity levels in an Arabidopsis cell culture during the transition from exponential growth to stationary phase and during reentry into the cell cycle.

|                 | Active division | Quiescent cells | Sucrose removal | Cell cycle reentry | Late G1 |
|-----------------|-----------------|-----------------|-----------------|-------------------|--------|
| CycD2 mRNA      | ++              | ++              | ++              | ++/++             | ++     |
| Protein         | ++              | ++              | ++              | ++/++             | ++     |
| Association     | CycD2-Cdc2a     | X-CycD2-Xb      | X-CycD2-Xb      | ++/++             | ++     |
| Kinase activity | ++              | ++              | ++              | ++/++             | ++     |
| CycD3 mRNA      | ++              | ++              | ++              | ++/++             | ++     |
| Protein         | ++              | ++              | ++              | ++/++             | ++     |
| Association     | CycD3-Cdc2a     | Cdc2a           | Cdc2a           | ++/++             | ++     |
| Kinase activity | ++              | ++              | ++              | ++/++             | ++     |

See "Discussion" for details.

* Increases within 1 h.

† CycD2 is shown complexed in nondividing cells with unknown protein X. Rising CycD2-associated kinase activity in early G1 may result from liberation of CycD2 from these complexes or from de novo synthesized CycD2.

‡ Increases within 2 h of sucrose addition.

§ Note that CycD3 not complexed to Cdc2a may be present in exponentially growing cells, suggesting possible regulation at the level of complex assembly.

abundance of CycD2 and CycD3 proteins and their associated kinase activity as cells reenter the cell cycle, early stationary phase (7-day) cells were prepared by removing sucrose from the growth medium for 24 h, and then the levels of D-type cyclins (Fig. 5A) and kinase activity of their immunoprecipitations (Fig. 5B) were observed after sucrose was readded. CycD2 was present at relatively high levels in stationary phase cells before (data not shown) and after sucrose removal. CycD2 was present at a low level before (data not shown) sucrose removal and absent after 24 h without sucrose, consistent with results observed after sucrose removal from an exponentially growing cell culture (Fig. 4B). Fig. 5A shows that in samples taken every hour after sucrose readdition, CycD2 remains at a constant level. During this time, cells progress through G1, reaching S phase after 6 h (1, 22). CycD3 protein was absent in early G1 cells but started to accumulate rapidly at 4–5 h in late G1, coincident with the increase in CycD3 mRNA (1, 22). The abundance of Cdc2a, the CDK partner of CycD2 and CycD3, was constant during this experiment.

The kinase activities of CycD2 and CycD3 were examined in a separate experiment (Fig. 5B) and showed that despite the constant protein abundance of CycD2, its associated kinase activity is strongly regulated, being very low after sucrose removal and increasing within 2 h of sucrose readdition, as previously reported (1). CycD2 kinase activity continues to increase up to 24 h after sucrose readdition, despite no change in protein abundance (Fig. 5B). In contrast, CycD3 kinase activity starts to increase only after 4 h and largely reflects mRNA levels and protein abundance during G1/S phase, although a further increase in kinase activity is seen in cells 24 h after sucrose addition (Fig. 5B). We conclude that CycD2 is subject to strong post-translational regulation.

**CycD2 Does Not Interact with Cdc2a in Quiescent Cells**—The experiments described above show that CycD2 protein abundance remains constant in actively dividing and nondividing Arabidopsis suspension cells, although CycD2-associated kinase activity is only found in actively dividing cells. To analyze the nature of this post-translational regulation, we first examined whether the CDK partner is present. Both CycD2 and Cde2a were found in quiescent cells (prepared as for the cell cycle reentry experiment) as well as exponentially growing cells, although Cde2a levels were lower than in exponentially growing cells (Fig. 6A). However, the differences in the kinase activity of the CycD2-Cdc2a complex were marked between the two growth phases, and almost no activity was present in quiescent cells (Fig. 6A). To investigate whether the lack of kinase activity in quiescent cells was due to the inability of CycD2 and Cdc2a to interact, CycD2 was immunoprecipitated from both quiescent and exponentially growing cells to identify if similar amounts of Cdc2a were coimmunoprecipitated. The amount of Cdc2a coimmunoprecipitated in quiescent cell extract was much lower (Fig. 6B, c), suggesting that the interaction between Cdc2a and CycD2 was inhibited in the quiescent state. However, replotting these blots with CycD2 antibody (Fig. 6B, g) showed that the quantity of CycD2 protein immunoprecipitated from quiescent cell extract was much lower than from exponential cell extract although CycD2 was equally abundant in quiescent cells (Fig. 6A). Thus, anti-CycD2 antiserum efficiently immunoprecipitates CycD2 from exponential cell extract but not from quiescent cells, a result that was observed in several repeated experiments. From this experiment, it is therefore impossible to determine whether CycD2 and Cdc2a are associated in quiescent cells, since CycD2 is not immunoprecipitated.

To determine whether this inefficient immunoprecipitation is a feature of quiescent cell extract or particular to CycD2, Cdc2a was immunoprecipitated directly from quiescent and exponentially growing cells, and the eluates were immunoblotted with the same antiserum (Fig. 6C, c and d). Cdc2a was immunoprecipitated in proportion to its abundance (Fig. 6A) from cells in both phases of growth, suggesting that the inefficient immunoprecipitation of CycD2 in quiescent cells was a behavior specific to CycD2. The eluted Cdc2a immunoprecipitates were immunoblotted with anti-CycD2 antiserum (Fig. 6C, a and b). CycD2 was efficiently coimmunoprecipitated by the anti-Cdc2a antiserum from exponential cells (Fig. 6C, a) but not from quiescent cells (Fig. 6C, b). This demonstrates that the majority of CycD2 present in quiescent cells is not associated with Cdc2a; nor, as we show in Fig. 6B, is it accessible for immunoprecipitation by the CycD2 antiserum.

We also noted that the small amount of CycD2 immunoprecipitated from quiescent cells by the CycD2 antiserum (Fig. 6B, g) and the amount of CycD2 coimmunoprecipitated by the Cdc2a antiserum (Fig. 6C, b) is a similar proportion of the equivalent immunoprecipitations from exponential cells (compare Fig. 6B (g) with Fig. 6B (f), and compare Fig. 6C (b) with...
Characterization of Arabidopsis CycD2 and CycD3 Proteins

DISCUSSION

Plant D-type (CycD) cyclins play important roles in controlling the cell cycle in development and in response to external signals (1, 3, 4, 22, 23, 29). However, despite the importance of post-transcriptional mechanisms in regulating cyclin-CDK activity (24, 30), previous studies have examined either the consequences of CycD misexpression (22, 23), the developmental regulation of their gene expression, or the response of mRNA levels to external signals (1, 3, 4, 22, 29).

Here we show that the development of specific antisera to CycD2 and CycD3 of Arabidopsis allows the identification of the CDK partner of these cyclins and analysis of their protein abundance and kinase activity during development and during the response to sucrose removal and addition. We show that both CycD2 and CycD3 associate with the Arabidopsis PSTAIRE CDK Cdc2a and that CycD2 and CycD3 show strikingly different modes of regulation at the protein level.

Tobacco CycD3 has previously been shown to associate with a CDK of the PSTAIRE type in vitro and in BY-2 cell extract (17), and Arabidopsis CycD1 was identified in a yeast two-hybrid screen using Cdc2a as a bait (31). Moreover, Arabidopsis CycD2 expressed in tobacco forms functional kinase complexes with the PSTAIRE tobacco CDK Cdc2a (23). Here we present the first evidence that endogenous Arabidopsis CycD2 and CycD3 interact with and form functional kinase complexes with Cdc2a in vivo, whereas these cyclins do not interact with the non-PSTAIRE CDK Cdc2b. These results are in contrast to mammalian and Drosophila D-type cyclins, which do not form functional complexes with PSTAIRE CDKs but rather associate with the non-PSTAIRE-containing CKD4 and CKD6 (2, 32).

Immunoprecipitates of CycD2 and CycD3 were found to exhibit in vitro protein kinase activity against added histone H1 as previously reported (1, 23) and against coimmunoprecipitated proteins from the Arabidopsis cell extract. The identity of these proteins is currently unknown, but since CycD2 and CycD3 interact with maize Rb in vitro and in yeast two-hybrid assays (10), one candidate is the Arabidopsis homologue of the Rb protein. The suitability of histone H1 as a substrate for plant CycD kinases (1, 23) is further confirmed, highlighting a further difference from animal cyclin D-CDK4 complexes for which histone H1 is a poor substrate. Since Arabidopsis Rb was unpublished at the time of this work (33), we have been unable to confirm its suitability as an in vitro substrate of Arabidopsis CycD kinases, although this would be predicted from previous analysis in vitro (10, 17).

Mammalian D-type cyclins are highly unstable proteins whose synthesis is linked to the presence of external growth signals (5, 6, 24–36). Previous analysis has shown that mRNA levels of plant CycD respond to external signals of hormones and sucrose levels (1, 3, 22, 29, 37). Arabidopsis CycD2 and CycD3 mRNA levels decline on sucrose removal and are induced on its readdition, although the magnitude of the CycD3 response is twice that of CycD2. Here we examined the protein levels and kinase activity of CycD2 and CycD3 and found strikingly different results.

When sucrose-starved cells were induced to reenter the cell cycle, CycD3 protein was detectable 4–5 h after the readdition of sucrose, at the same time as CycD3 mRNA and kinase activity are induced (1). Since we found that Cdc2a is present throughout the sucrose starvation, this suggests that CycD3 kinase activity is regulated by CycD3 mRNA abundance during this experiment. The importance of transcriptional regulation of CycD3 activity is supported by the strong phenotypes produced in vivo from its overexpression (22).

When sucrose was removed from cells in a midexponential phase culture, the majority of CycD3 was no longer present after 1–2 h, suggesting that in this situation it is rapidly turned over. A residual level of CycD3 persists for several hours, and the reduction in kinase activity is less abrupt than the loss of CycD3 protein, suggesting that CycD3 not present in active kinase complexes is turned over more rapidly. In this regard, it is interesting to note the low amount of Cdc2a coimmunoprecipitated with CycD3 (Fig. 2). We note that the response of CycD3 to sucrose removal appears to be an immediate and specific response to loss of this signal, since the levels of other cell cycle proteins remain constant for 24 h after sucrose removal, for example the kinases Cdc2a and Cdc2b (Fig. 4B), although Cdc2b abundance is cell cycle-regulated and the protein is only present from S to M phases (14). We conclude that changes in CycD3 level are an immediate response to sucrose removal and not an indirect consequence of a cessation of cell division caused by declining intracellular carbohydrate levels.

CycD2 regulation is strikingly different from CycD3 and D-type cyclins in other organisms. Despite the regulation of its mRNA during sucrose removal and reappl y(1), CycD2 protein remains almost constant in abundance throughout the sucrose starvation and reentry into the cell cycle, although its kinase activity is strongly regulated, being activated more rapidly than that of CycD3. Further investigation showed that CycD2 present in quiescent or sucrose-starved cells is not associated with Cdc2a and is not accessible for immunoprecipitation. This suggests regulation of CycD2-associated kinase activity by a novel

![Fig. 7. Summary model of CycD2 and CycD3 protein associations with Cdc2a in an Arabidopsis cell culture during the transition from quiescent cells, through cell cycle reentry to active division. See “Discussion” and Table I for details. CycD2 is shown complexed with an unknown protein X in quiescent (nondividing) cells; the rising CycD2-associated kinase activity in early G1, may result from the liberation of CycD2 from these complexes; alternatively, CycD2 may be synthesized de novo (not illustrated).](image308x478 to 555x720)
post-translational mechanism involving its sequestration in an inactive form. It is unknown whether the reappearance of CycD2-Cdc2a kinase activity within 2 h of sucrose readdition is a result of release and activation of existing CycD2 or the rising levels of CycD2 activity result from de novo synthesis. The increase in CycD2 mRNA shortly after sucrose readdition may be consistent with the latter explanation. We conclude that the kinase activity of CycD2-containing complexes is not dependent on the level of CycD2 protein, in contrast to the situation observed with CycD3.

Based on the data presented here, we present a model for activation of cell division in Arabidopsis cells (Table I and Fig. 7). In quiescent cells, CycD2 protein is sequestered and presumably inactive, since it is not associated with its CDK partner. CycD3 protein is absent. After stimulation of division, CycD2 mRNA accumulates, CycD2 protein associates in an active form with Cdc2a, and kinase activity starts to accumulate within 2 h in early G1 phase. The amount of CycD2 present in this active form is unknown. After 4 h, in late G1, CycD3 transcript levels increase sharply, accompanied by an increase in CycD3 protein levels and kinase activity. This is followed by entry into S phase after ~6 h.

The work presented here and elsewhere (reviewed in Ref. 14) suggests that the overall similarities between plant and mammalian controls of the cell cycle are overlaid by complex and important differences in the control, interactions, and targets of cell cycle regulators in plants.

Acknowledgments—We thank Bart den Boer, Marc de Block, and Masami Sekine for useful discussions.

REFERENCES
1. Riou-Khamlichi, C., Menges, M., Healy, J. M., and Murray, J. A. H. (2000) Mol. Cell. Biol. 20, 4519–4521
2. Pines, J. (1995) Adv. Cancer Res. 66, 181–212
3. Soni, R., Carmichael, J. P., Shah, Z. H., and Murray, J. A. H. (1995) Plant Cell 7, 85–103
4. Dahl, M., Meskenie, I., Bugre, L., Ha, D. T. C., Swohoda, I., Hubmann, R., Hirt, H., and Heberle-Bors, E. (1995) Plant Cell 7, 1847–1857
5. Sherr, C. J. (1993) Cell 73, 1059–1065
6. Sherr, C. J. (1994) Cell 79, 551–555
7. Renaudin, J.-P., Doonan, J. H., Freeman, D., Hashimoto, J., Hirt, H., Inze, D., Jacobs, T., Kouchi, H., Rouze, P., Sauter, M., Savoure, A., Sorrell, D. A., Sundaresan, V., and Murray, J. A. H. (1996) Plant Mol. Biol. 32, 1003–1018
8. Ewen, M. E., Sluss, H. K., Sherr, C. J., Matsushime, H., Kato, J., and Livingston, D. M. (1993) Cell 73, 487–497
9. Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E., and Sherr, C. J. (1993) Genes Dev. 7, 331–342
10. Huntley, R., Healy, S., Freeman, D., Lavender, P., de Jager, S., Greenwood, J., Makker, J., Walker, E., Jackman, M., Xie, Q., Bannister, A. J., Kouzarides, T., Guttierrez, C., Donnan, J. H., and Murray, J. A. H. (1998) Plant Mol. Biol. 37, 155–169
11. Sherr, C. J. (1996) Science 274, 1672–1677
12. Dryson, N. (1998) Genes Dev. 12, 2245–2262
13. Morgan, D. O. (1997) Annu. Rev. Cell Dev. Biol. 13, 261–291
14. Mironov, V., De Leyder, L., Van Montagu, M., and Inze, D. (1999) Plant Cell 11, 509–521
15. Huntley, R. P., and Murray, J. A. H. (1999) Curr. Opin. Plant Biol. 2, 440–446
16. Roudier, F., Fedorova, E., Gyorgyey, J., Feher, A., Brown, S., Kondorosi, A., and Kondorosi, E. (2000) Plant J. 23, 73–83
17. Nakagami, H., Sekine, M., Marama, H., and Shinmyo, A. (1999) Plant J. 18, 243–252
18. Xie, Q., Sanz Burgos, A. P., Hannon, G. J., and Gutierrez, C. (1996) EMBO J. 15, 4900–4908
19. Grafi, G., Burnett, R. J., Helenjari, T., Larkins, B. A., Decaprio, J. A., Sellers, W. R., and Kaelin, W. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8962–8967
20. Ramirez Parra, E., Xie, Q., Boniotti, M. B., and Gutierrez, C. (1999) Nucleic Acids Res. 27, 3527–3533
21. Sekine, M., Ito, M., Uemukai, K., Maeda, Y., Nakagami, H., and Shinmyo, A. (1999) FEBS Lett. 460, 117–122
22. Riou-Khamlichi, C., Huntley, R., Jacqmard, A., and Murray, J. A. H. (1999) Science 283, 1541–1544
23. Cockcroft, C. E., den Boer, B. G., Healy, J. M., and Murray, J. A. H. (2000) Nature 405, 575–579
24. Lew, D. J., and Kornbluth, S. (1996) Curr. Opin. Cell Biol. 8, 795–804
25. May, M., and Leaver, C. (1993) Plant Physiol. 103, 621–627
26. Fuerst, R. A. U., Soni, R., Murray, J. A. H., and Lindsey, K. (1996) Plant Physiol. 112, 1025–1033
27. Diehl, J. A., Zindy, F., and Sherr, C. J. (1997) Genes Dev. 11, 957–972
28. Diehl, J. A., and Sherr, C. J. (1997) Mol. Cell. Biol. 17, 7362–7374
29. Gaudin, Y., Lunnness, P. A., Fobert, P. R., Towers, M., Riou-Khamlichi, C., Murray, J. A. H., Coen, E., and Doonan, J. H. (2000) Plant Physiol. 122, 1137–1148
30. Sherr, C. J., and Roberts, J. M. (1995) Genes Dev. 9, 1149–1163
31. Hieleboid, S., Hendriko, T., Bant, G., Inze, D., Yasseur, J., and Hilbert, J. L. (2000) J. Exp. Bot. 51, 1189–1200
32. Xiong, Y., Zhang, H., and Beach, D. (1992) Cell 71, 505–514
33. Kong, L. J., Orozco, B. M., Roe, L. J., Nagar, S., Ou, S., Peifer, H. S., Dufresne, T., Miller, A. B., Grusswe, W., Robertson, D., and Hanley-Bowdson, L. (2000) EMBO J. 19, 3485–3495
34. Matsushime, H., Ewen, M. E., Strom, D. K., Kato, J.-Y., Hanks, S. K., Roussel, M. F., and Sherr, C. J. (1992) Cell 71, 323–334
35. Sewing, A., Burger, C., Brusselbach, S., Schalk, C., Lucibello, F. C., and Muller, R. (1993) J. Cell Sci. 104, 545–555
36. Baldwin, V., Lukas, J., Marote, M. J., Pagano, M., and Draetta, G. (1993) Genes Dev. 7, 812–821
37. De Leyder, L., de Almeida Engler, J., Burssens, S., Manevski, A., Leserc, B., Van Montagu, M., Engler, G., and Inze, D. (1999) Plant Cell 10, 453–462