The Cytokinin Requirement for Cell Division in Cultured *Nicotiana plumbaginifolia* Cells Can Be Satisfied by Yeast Cdc25 Protein Tyrosine Phosphatase. Implications for Mechanisms of Cytokinin Response and Plant Development

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Cultured cells of *Nicotiana plumbaginifolia*, when deprived of exogenous cytokinin, arrest in G2 phase prior to mitosis and then contain cyclin-dependent protein kinase (CDK) that is inactive because phosphorylated on tyrosine (Tyr). The action of cytokinin in stimulating the activation of CDK by removal of inhibitory phosphorylation from Tyr is not a secondary downstream consequence of other hormone actions but is the key primary effect of the hormone in its stimulation of cell proliferation, since cytokinin could be replaced by expression of *cdc25*, which encodes the main Cdc2 (CDK)-Tyr dephosphorylating enzyme of yeast (*Saccharomyces cerevisiae*). The *cdc25* gene, under control of a steroid-inducible promoter, induced a rise in cdc25 mRNA, accumulation of $p67^{Cdc25}$ protein, and increase in Cdc25 phosphatase activity that was measured in vitro with Tyr-phosphorylated Cdc2 as substrate. Cdc25 phosphatase activity peaked during mitotic prophase at the time CDK activation was most rapid. Mitosis that was induced by cytokinin also involved increase in endogenous plant CDK Tyr phosphatase activity during prophase, therefore indicating that this is a normal part of plant mitosis. These results suggest a biochemical mechanism for several previously described transgene phenotypes in whole plants and suggest that a primary signal from cytokinin leading to progression through mitosis is the activation of CDK by dephosphorylation of Tyr.

Cell cycle control is essential for the initiation and maintenance of meristems and for the regulation of organogenesis. Auxin and cytokinin hormones are implicated in cell cycle control since they strongly influence the division of cells that are in culture and are means by which Agrobacterium infection causes cell proliferation (e.g. for review, see Srivastava, 2002). Cytokinin influences cell division activity in embryos and mature plants through altering the size and activity of meristems as seen when cytokinin levels are altered, by transgenes such as *IPT* or *CKX* (Medford et al., 1989; Werner et al., 2001), and by mutations such as *amp1* (Chaudhury et al., 1993). Furthermore, defects in cytokinin signal transduction that have been caused by mutation of the receptor gene *AHK4* (*wol* mutant allele) or knockouts in the *AHK* gene family can result in insufficient division of vascular precursors in the root (Mähönen et al., 2000) or general suboptimal cell division in meristems (Nishimura et al., 2004). It has therefore been suggested that morphogenic effects of cytokinin may primarily occur through influence on cell cycle regulation (e.g. Werner et al., 2001).

There is evidence that cytokinin regulates the cell cycle at both the G1/S phase and G2/M phase progressions. Entry into S phase requires hormone dependent accumulation of D-cyclins in cultured cells from both plant and animal kingdoms. D-cyclin accumulation increases activity of cyclin-dependent protein kinase (CDK) enzymes that release transcription factors for genes of DNA replication (for review, see Gutierrez et al., 2002). One mode of action of cytokinin is clearly by induction of D-cyclins (Riou-Khamlichi et al., 1999; for review, see Murray et al., 2001). A second cell cycle control point, at the G2/M progression, is also a potential point of regulation by cytokinins. Initiation of mitosis is universally regulated as a checkpoint that blocks mitosis if nuclear DNA is incompletely replicated or is damaged (Rhind and Russell, 2001; Preuss and Britt, 2003), but in plants entry to mitosis is also responsive to developmental and physiological status and to hormonal signals, and it is a more frequent point of arrest in plants than it is in metazoa (Van't Hof, 1974; Zetterberg and Larsson, 1985). The G2/M progression in plants can be accelerated, as by hormone stimulus in excised stems of rice (*Oryza sativa*; Sauter and Kende, 1992), or can be slowed as in seedling leaves of water stressed wheat (*Triticum aestivum*; Schuppler et al., 1998) or in oxidatively stressed tobacco (*Nicotiana tabacum*) seedlings (Reichheld et al., 1999). Arrest in G2 phase prior to mitosis also participates in developmental control since pericycle cells that arrest in G2 phase are the source of lateral root initials (Beeckman et al., 2001).

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A contribution of cytokinins to regulating the G2/M phase progression is indicated by several lines of evidence, including the requirement for cytokinin and the synthesis of cytokinin at mitotic initiation. In the cell line of *Nicotiana plumbaginifolia* studied here, when cytokinin is limiting, arrest occurs in G2 phase indicated by a 2C nuclear-DNA content (John et al., 1993), and freshly isolated tobacco cells without cytokinin also can traverse S phase (and may repeat it) but cannot progress to mitosis (Valente et al., 1998). Consistent with this the BY2 cell line of tobacco, which is autonomous for cytokinin, abruptly accumulates zeatin by several orders of magnitude at mitotic initiation (Redig et al., 1996) and is unable to enter mitosis if this accumulation is inhibited but is able to resume mitotic progress if zeatin is added (Laureys et al., 1998). It seems therefore that cytokinin is involved in mitotic initiation and in some cell types exogenous cytokinin may be stringently required at this point. However, the requirement for exogenous cytokinin at G1/S progression is sometimes more apparent (e.g. Riou-Khamlichi et al., 1999). It has therefore been suggested that dependence upon cytokinin may be more stringent at either G1/S or G2/M phase progressions depending on the different endogenous levels of cyclin-D or activators of mitotic cyclin/CDK complexes in particular cell types (John and Zhang, 2001).

An eventual impact of cytokinin on CDK enzymes can be anticipated from the central role of these enzymes in division, which has been indicated by slower division when CDK is mutated (Hemerly et al., 1995) or is inhibited (e.g. Cleary et al., 2002) and by faster division when active CDK is microinjected or CDK activators are locally induced (Hush et al., 1996; Wyrzykowska et al., 2002). Hormones that stimulate division also induce CDK and cyclin proteins (Gorst et al., 1991; Hata et al., 1991; Riou-Khamlichi et al., 1999), and additional posttranslational controls are also suspected to operate since lateral root meristems begin with division in cells that are not initially expressing more division proteins than their neighbors (Hemerly et al., 1993). The posttranslational CDK activator Cdc25 can induce lateral root and shoot initiation (McKibbin et al., 1998; Suchomelova et al., 2004) and, when locally expressed, induces zones of cell division in leaves (Wyrzykowska et al., 2002).

We now report that the key effect of cytokinin on G2/M progression in tobacco cells is exerted on CDK through posttranslational modification of phosphorylation. In yeast (*Saccharomyces cerevisiae*) and animal cells phosphorylation of CDK at Tyr-15, and to a lesser extent at Thr-14, restrains entry into nuclear division. Mitosis is initiated when inhibiting phosphate is removed by the activator phosphatase Cdc25 (Millar et al., 1991; Norbury and Nurse, 1992). In plants, the presence of inhibitory phosphorylation in CDK has been detected by the in vitro activation of plant CDK by Cdc25 phosphatase from yeast or Drosophila (Zhang et al., 1996; Mészáros et al., 2000). Furthermore, significance of such phosphorylation has been indicated by effects on development from the posttranslational CDK activator *cdc25* and from modified CDK that does not accommodate inhibitory phosphorylation (see "Discussion").

We now report evidence that posttranslational activation of CDK is a primary mechanism of cytokinin action at mitosis and that the activation is not a secondary consequence of the many actions of the hormone in other aspects of growth. Extensive actions of cytokinin have been detected in developmental, nutritional, and organelle metabolism and are underlined by numerous effects on transcript levels (for reviews, see Schmülling et al., 1997; Hoth et al., 2003). Surprisingly, we have found that cytokinin could be replaced in initiating and supporting mitosis by the induced expression of a single transgene; the CDK-Tyr phosphatase Cdc25. This evidence, together with our observation of the pattern of endogenous CDK Tyr phosphatase activity seen in the cell cycle and supported by assessment of reported transgenic phenotypes, leads us to suggest that posttranslational activation of CDK is a normal part of plant mitosis and is a primary mode of cytokinin action that underlies several morphogenic effects of cytokinin in the whole plant.

RESULTS

Cell Lines Inducibly Expressing *cdc25*

To test whether an essential action of cytokinin in the cell cycle is the activation of protein Tyr phosphatase activity directed to CDK, we inducibly expressed the fission yeast CDK regulator Cdc25 phosphatase by joining the *cdc25* gene to a modified plant promoter described by Schena et al. (1991) that contained rat glucocorticoid response elements. This promoter is responsive to rat glucocorticoid receptor protein in the presence of dexamethasone and provides inducible gene expression without any observed side effects in Nicotiana. The glucocorticoid response element-*cdc25*, together with the constitutively expressed nopaline synthase promoter-glucocorticoid receptor-protein construct of Schena et al., was inserted into the vector pBin19, which contains *pnos:nptII* for kanamycin resistance, and was introduced into cells of *N. plumbaginifolia* by electroporation into protoplasts (Taylor and Larkin, 1988). Microcolonies that formed on solid CS V (Zhang et al., 1996) medium containing 50 μg mL^{-1} kanamycin were returned to liquid culture and allowed to resume division in suspension, then cell lines were tested for their ability to divide in medium containing dexamethasone and auxin (2,4-dichlorophenoxyacetic acid [2,4-D]) without cytokinin. Initial tests made at 10 μM dexamethasone revealed little increase in cell number, but we noticed that many cells arrested with condensed chromosomes without progression to metaphase, consistent with some form of mitotic aberration. On testing with lower concentrations of dexametha-

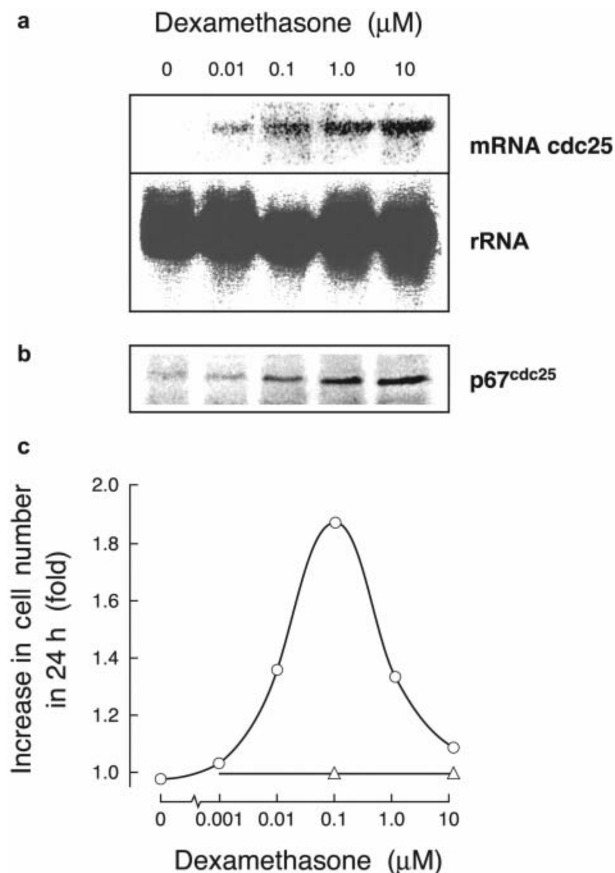

Figure 1. Cdc25 expression could induce cell division without added cytokinin in cells containing dexamethasone-inducible *cdc25.* A, cdc25 mRNA was detected by northern blot in 60-μg aliquots of total RNA extracted after 12-h induction with dexamethasone when equal RNA loading was indicated by stripping and reprobing for 18S rRNA. B, Cdc25 protein (p67^{cdc25}) was detected by western blotting of 50-μg aliquots of total soluble protein extracted after 12-h induction, probed with polyclonal rabbit antibody against Cdc25 catalytic core; equal loading and transfer was tested by staining protein on the membrane using iron chelate (Patton et al., 1994) that was removed before blocking and probing. C, Increase in cell number by 24 h after dexamethasone treatment of transgenic cells (○), and nontransgenic cells treated identically (△). Cells had been arrested in G2 phase, by culture without hormone and then with auxin only, prior to dexamethasone treatment.

sone, cell proliferation was observed in several lines. These were further tested for induced expression of Cdc25 protein in medium lacking added kinetin but containing 0.1 μM dexamethasone. Cdc25 protein was detected in western blots by antibody that was raised against glutathione-*S*-transferase (GST)-Cdc25 fusion protein expressed in *Escherichia coli*, exactly as described by Millar et al. (1991).

Three independent cell lines were analyzed biochemically and had similar properties. All contained yeast *cdc25* DNA (detected in Southern blots; data not shown) and in 0.01 to 10 μM dexamethasone all accumulated 2 kb cdc25 mRNA and 67 kD Cd25 protein in an inducer dependent manner (Fig. 1, A and B). Effects of inducer on division were tested in cells that had been arrested at the G2 phase hormonal control point by depletion of auxin and cytokinin followed by provision of auxin only. Effect on division was tested by induction with 0.01 to 1.0 μM dexamethasone (Fig. 1C), and a sharp optimum concentration of 0.1 μM inducer was observed in independent clones, consistent with a requirement for a critical optimum Cdc25 activity. No cell division was observed without inducer or in untransformed cells treated with dexamethasone (Fig. 1C). Results from one representative transgenic cell line are shown.

Synchronous Induction of Cdc25 Phosphatase, CDK Activation, and Mitosis

In subsequent experiments, cells were brought to arrest at the cytokinin control point in late G2 phase through incubation without added hormone, followed by incubation with auxin (2,4-D) alone, to bring cells to arrest with 2C DNA content (Zhang et al., 1996). Potential to synchronously enter mitosis could then be measured following the addition of cytokinin or transgene induction. Prophase progression under these conditions was slower than in cells that have not been interrupted by lack of cytokinin, which facilitated study of the timing of increase in enzyme activities.

To investigate how well *cdc25* supported progression to mitosis, division was followed in a culture that was divided and treated with either the transgene inducer dexamethasone or the cytokinin kinetin, or remained untreated. Mitosis was dependent upon stimulation but was completed as rapidly when initiated by *cdc25* expression as by hormone (Fig. 2). The daughter cells resulting from Cdc25 induction were fully viable, indicating that mitosis initiated by mitotic phosphatase is functionally normal and suggesting that CDK activation by phosphatase could be a natural part of cytokinin action. The daughter cells could

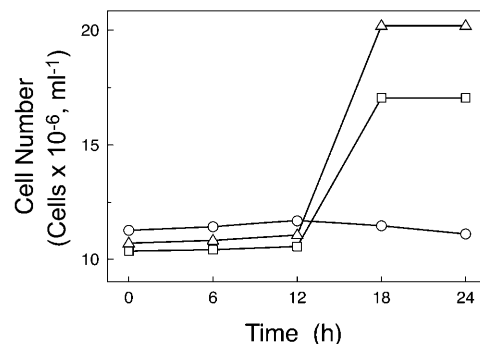

Figure 2. Cell division induced by *cdc25* expression (△) or by cytokinin stimulation (□) proceeded with similar rapidity. Prior to the sampling period, cells were brought to arrest in G2 phase by culture without hormone and then with auxin only, and at zero time received either cytokinin at normal medium concentration or dexamethasone at 0.1 μM; control received no addition (○).

proliferate indefinitely with dexamethasone replacing exogenous cytokinin, and they yielded a 9-fold increase in cell number in 7 d as did control cultures with cytokinin.

To test whether induced mitosis involved appearance of Cdc25 enzyme activity, we developed an assay for Cdc25 Tyr phosphatase activity that was based upon the in vitro activation of CDK1 (Cdc2) protein kinase enzyme of fission yeast. Yeast CDK1 was prepared for use as an assay reagent in a form that is responsive to activation by Cdc25 by extraction from yeast cells containing a temperature conditional mutation in Cdc25 phosphatase. At the restrictive temperature of 35°C, this mutation caused accumulation of phosphate on Tyr-15 of CDK1, which inhibited its H1 histone kinase activity (Millar et al., 1991). This Tyr-phosphorylated CDK1 was used as substrate in the first phase of a Cdc25 phosphatase activity assay that involved in vitro removal of the inhibitory phosphate from the CDK1 by Cdc25. Then, in a second phase of the Cdc25 activity assay, antibody-bound phosphatase enzyme was removed and the extent of its action was measured by the resulting CDK1 H1 histone kinase activity.

Induced Cdc25 enzyme activity was detected after 6-h induction with dexamethasone (Fig. 3, left) in enzyme purified by anti-GST-Cdc25 antibody. Specificity of Cdc25 enzyme recovery by the antibody was indicated by low activity recovered by preimmune serum and serum precompeted with freeze/thaw-inactivated GST-Cdc25.

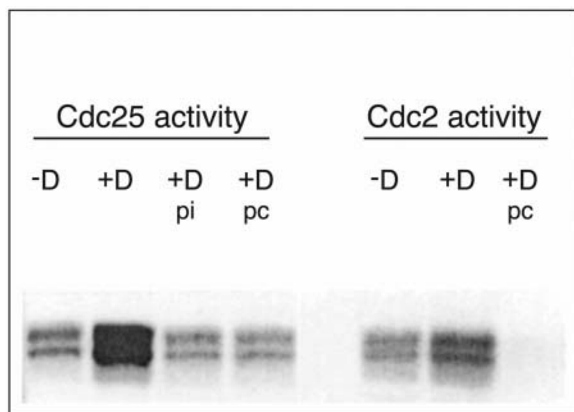

Figure 3. Cdc25 Tyr phosphatase and CDK activity measured in transgenic cells progressing from the late G2 phase hormonal control point through mitosis. At left, Cdc25 activity was induced by 0.1 μM dexamethasone (+D) and was recovered from cells 6 h after induction by immune serum but not preimmune serum (pi) or precompeted serum (pc). Cdc25 activity was measured in vitro by its activation of yeast cdc2 kinase activity, which was then assayed by [^{32}P] transferred to H1 histone. At right, plant CDKA was activated in vivo by 0.1 μM dexamethasone induction (+D) of Cdc25 activity; CDKA was recovered by CDKA-specific serum but not by serum precompeted with peptide antigen (pc). The CDK sample shown was taken at 6 h after induction but activation continued beyond that time (Fig. 5).

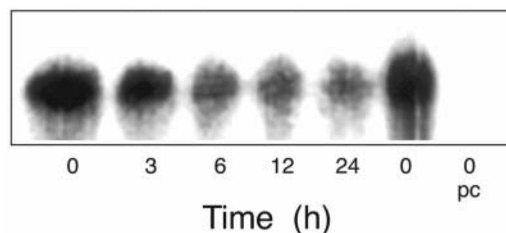

Figure 4. Phosphotyrosine declined in CDKA after Cdc25 was induced, as cells progressed through prophase to mitosis. Times of sampling after treatment with 0.1 μM dexamethasone are indicated. CDKA was recovered immunologically as in Figure 3 and probed for phosphotyrosine in western blots. Specific recovery of CDKA was indicated by elimination of CDKA from the immunoprecipitate fraction by precompetition with CDKA peptide (pc), and consistent loading and transfer was indicated by the repeat loading of the 0-h sample.

To test the hypothesis that induced Cdc25 activity in Nicotiana stimulated mitosis through activation of cell cycle kinase, CDKA kinase was recovered with antibody specific for its carboxyterminal peptide, and its H1 histone kinase activity was assayed. CDKA was selected for study as the class of plant CDK that has most extensive involvement in the cell cycle, being required for both DNA replication and mitosis (Mironov et al., 1999). Members of this class of CDK are recognized by their close sequence similarity to the CDK1 of other eukaryotes (Joubès et al., 2000) and by their capacity to complement mutated CDK1 function in yeast, as has been shown for Nicotiana CDKA (Setiady et al., 1996). CDKA activity was found to increase markedly, after induction of Cdc25 with dexamethasone, in G2 phase-arrested cells (Fig. 3, right). Specific recovery of the CDKA kinase was indicated by elimination of recovery after precompetition of the antibody with free CDKA carboxyterminal peptide.

These indications that Cdc25 acted by affecting CDKA were further tested by direct measurement of Tyr phosphate in the enzyme, which revealed a progressive decline in phosphate through the first 12 h after induction with dexamethasone (Fig. 4). To more rigorously test whether the decline in phospho-Tyr in CDK followed induction of Cdc25 activity and correlated with the rise in CDKA protein kinase activity, the timing of the increase in these enzyme activities was measured. Following induction with dexamethasone, Cdc25 activity rose rapidly (Fig. 5) and remained elevated until 12 h, which correlated with the period during which phosphotyrosine in CDKA declined to a basal level (Fig. 4) and CDKA activity increased to its peak (Fig. 5), consistent with the hypothesis that the decline in phosphotyrosine in CDKA was caused by Cdc25 activity and resulted in rising CDKA activity.

A Normal Mechanism in Plant Mitosis

To explore the possibility that Cdc25 induced division because Tyr phosphatase activity directed to

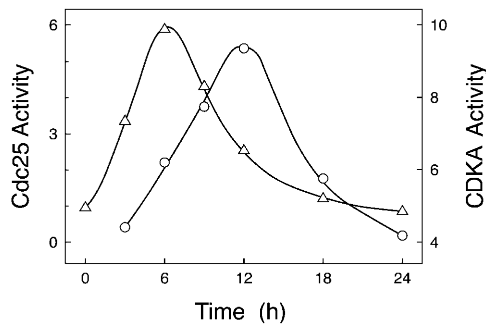

Figure 5. Cdc25 activity induced by dexamethasone (△) increased before CDK activity rose (○), and Cdc25 activity remained high during the time CDK activity rose rapidly; both activities measured as in Figure 3. The decline in activity after 12 h correlates with entry into anaphase that preceded cell number increase as seen in Figure 2.

CDKA is a normal part of plant mitosis, a control portion of culture was examined after treatment with cytokinin instead of dexamethasone (Fig. 6). The induction of mitosis by hormone involved a very similar increase in Cdc25-like phosphatase activity recoverable by anti-GST-Cdc25 that showed a slightly slower initial increase than was observed when the transgene was induced (Fig. 5) but again occurred in the period up to 12 h during which CDKA activity increased. The absence of a lower total Cdc25-like catalytic activity than when yeast enzyme was expressed (Fig. 5) is consistent with the evidence from other kingdoms that different amounts of the mitotic inducer phosphatase are accommodated and activity is stabilized by homeostatic controls that act on Cdc25 enzyme (Kovelman and Russell, 1996) through complex phosphorylations (Bulavin et al., 2003) and ubiquitin-directed proteolysis (Nefsky and Beach, 1996; Mailand et al., 2000).

The hypothesis was further investigated by testing the potential for activation by Cdc25. Through the prophase period of 3 to 12 h extracted CDK activity increased, but total activity after in vitro activation was relatively constant (Fig. 7), therefore showing that in vivo-activated enzyme could not be additionally activated by phosphatase and indicating that the previous in vivo activation had been by phosphatase. Since CDKA amount is essentially constant through the division cycle of cultured Nicotiana cells (Setiady et al., 1996; Zhang et al., 1996) this indicates that the rising CDK activity in plant prophase is due to progressive removal of inhibitory Tyr phosphate from CDK. The persistence of some capacity for activation by Cdc25 up to 12 h correlates with persistence of a basal level of phosphotyrosine in the enzyme (Fig. 4). Anaphase was shown to involve a different activity control since the sharp decline in CDK activity after 12 h could not be reversed by incubation with GST-Cd25 (Fig. 7), consistent with activity in anaphase being limited by another cause such as the proteolytic elimination of B-cyclins (King et al., 1995), which has been observed in maize and Nicotiana (Mews et al., 1997; Genschik et al., 1998). The same relative timing of phosphatase and CDK activities and mitosis was consistently observed in different experiments and in all three transgenic lines that were investigated. Furthermore, when phosphatase and CDK activity peaks were a few minutes earlier or later in different cultures, mitosis was proportionately earlier or later (data not shown), consistent with phosphatase activation of CDK again being the determinant of mitotic timing.

A further indication that cytokinin is required as inducer of Cdc25 phosphatase activity was the consistently low level of Cdc25-like activity in cells limited by cytokinin, as at 0 h in Figures 3, 5, 6, and 8 and the rise in Cdc25-like activity when stimulated by cytokinin. To test more rigorously whether such rise is normally involved at mitosis, nontransgenic cells were compared with transgenic cells as a source of CDK-activating phosphatase recoverable by anti-GST-Cdc25, and plant CDK was used as the substrate enzyme to test activation capacity. For this purpose, plant CDK enzyme that was phosphorylated on Tyr (as shown in Fig. 4) was taken from cells arrested at the G2 control point (Fig. 8, lanes 1–3) or after 3-h stimulation with cytokinin (Fig. 8, lanes 4–6) when activation of CDK was in progress (as shown in Fig. 6). A CDK Tyr phosphatase activity, unambiguously of plant origin because from nontransgenic cells (lane 5), was found to be slightly more active than Cdc25 enzyme recoverable from transgenic cells that had also been induced for 6 h (lane 6), indicating that a plant Cdc25-like Tyr phosphatase participates in plant mitosis.

DISCUSSION

A Key Early Event in the Stimulation of Mitosis

Cytokinin is necessary in cultured cells for both the initiation of mitosis and the dephosphorylation of Tyr in CDK (Zhang et al., 1996). But only if such Tyr dephosphorylation is directly stimulated by cytokinin when promoting cell division, not a downstream consequence of the many other effects of the hormone, will presence of the hormone be substituted by

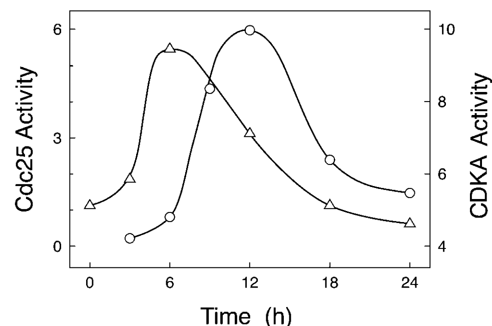

Figure 6. Stimulation with the hormone cytokinin, not transgene inducer, also caused increase in Cdc25 activity (△) that coincided with the time of activation of CDK activity (○).

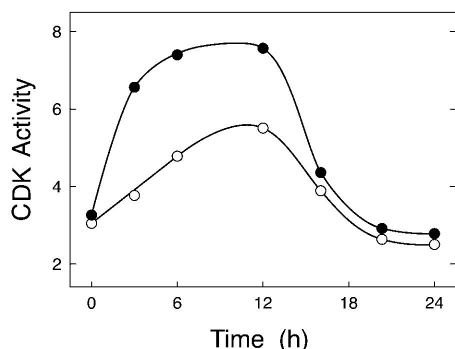

Figure 7. Activity in CDK (○) that had been extracted from cells that were induced by cytokinin to enter prophase could be further activated in vitro by GST-Cdc25, and the total activity after reaction with Cdc25 (●) was relatively constant through prophase (3–12 h), indicating that activation in vivo reduced the capacity for further activation by Tyr phosphatase. Low CDK activity after 12 h, while cells progressed through anaphase, could not be increased by phosphatase in vitro and was probably not due to inhibitory phosphorylation.

increase in protein phosphatase. Unexpectedly we observed that the yeast Cdc25 phosphatase, which is specific for Tyr dephosphorylation of CDK (Millar et al., 1991), was able to substitute for cytokinin in supporting completion of the cell cycle and recurrent further divisions. Therefore, one major function of cytokinin in the cell cycle is to cause the activation of phosphatase that activates CDK. In the cell type studied here no other action of cytokinin was necessary. In other cell types, the induction of D-cyclins by cytokinin can be a more critically limiting factor that arrests the cell cycle in G1 phase when cytokinin is limiting (see introduction).

We suggest that cytokinin stimulation of Tyr dephosphorylation in CDK is a normal part of prophase progression in plants. Consistent with this, cells that were synchronously progressing through prophase without induction of a transgene contained CDKA that declined in capacity to be activated by Cdc25 as the enzyme became progressively activated in vivo (Fig. 7) and as its phosphotyrosine content declined (Fig. 4). Furthermore, a plant Tyr phosphatase activity rose following cytokinin stimulation coincident with mitotic activation of CDKA, and the phosphatase was able to activate yeast and plant CDK (Figs. 3, 6, and 8). Identity of the plant mitotic-Tyr phosphatase remains uncertain although it was recovered in immunoprecipitates with antibody against the catalytic region of yeast Cdc25. Close structural similarity of the plant Tyr phosphatase with Cdc25 cannot be assumed since genome sequencing has not yet shown a plant protein phosphatase with extensive similarity to Cdc25. Furthermore, it is not certain that the plant mitotic inducer must be very similar since Cdc25 is not the only mitosis inducing phosphatase in yeast but rather works in tandem with the protein phosphatase Pyp3 (Millar et al., 1992). Both phosphatases are essential for normal mitotic timing in yeast, and Pyp3 can replace Cdc25 when pyp3 is expressed at high level (Rhind and Russell, 2001). Pyp3 shares some structural features with Cd25 in the catalytic region; therefore, the immunoprecipitated plant mitotic phosphatase that was measured here may in part resemble either of these phosphatases and may also have unique features. Our observations underline the importance of further study of plant CDK phosphatases in the intact plant. In addition, the signal transduction pathway from cytokinin receptor to cell cycle catalysts is not yet resolved, but it might be significant that cytokinin signal transduction proceeds by phosphate transfer (for reviews, see Haberer and Kieber, 2002; Kakimoto, 2003) and that CDK-regulating phosphatases are under phosphorylation control (Kovelman and Russell, 1996; Bulavin et al., 2003).

Our findings indicate that cytokinin, in addition to stimulating G1/S progression through induction of D-cyclins (for review, see Murray et al., 2001), also has an important action at G2/M through activation of CDK Tyr phosphatase. We cannot eliminate the possibility that CDK phosphatase also stimulates progress through S phase by maintaining activity of CDK/cyclin complexes that are active in mid-cell cycle, as occurs in mammalian cells (Mailand et al., 2000). However, in the cells that we have studied, a clear mitotic role for cytokinin-induced CDK activation is indicated by the fact that cells without cytokinin arrest in G2 phase and can be stimulated to initiate mitosis with either cytokinin or with transgenic expression of CDK-directed Tyr phosphatase activity, and furthermore, this phosphatase activity is low in the absence of cytokinin and rises as a normal part of mitosis that is dependent on cytokinin.

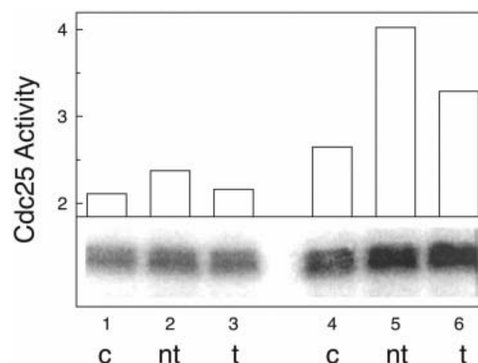

Figure 8. Cdc25-like CDK activating activity was detectable in a plant enzyme recovered by anti- Cdc25 from nontransformed cells (nt) as well as from transformed cells (t), after 6-h induction with cytokinin or dexamethasone, respectively. Cdc25-like activity was assayed using as substrate plant CDK of high phosphotyrosine content (Fig. 4) and low kinase activity (Fig. 3) because isolated from cells, either before stimulation with cytokinin (lanes 1–3), or after brief (6-h) stimulation with cytokinin (lanes 4–6). The activating Tyr phosphatase was immunoprecipitated with anti-Cdc25 either from nontransgenic cells stimulated with cytokinin (lanes 2 and 5) or from transgenic cells induced with 0.1 μM dexamethasone (lanes 3 and 6). Control incubations received no activating enzyme (c). Both nontransgenic and transgenic cells contained Cdc25-like plant-CDK-activating enzyme activity when progressing to mitosis (lanes 5 and 6).

Biochemical Basis of Cytokinin and Cytokinin-Like Phenotypes in Planta

In plant tissues there are also indications from transgene effects that cytokinin regulation of meristem activity involves modulation of CDK phosphorylation. Because *cdc25* expression, while substituting for cytokinin, increased the proportion of CDK that was dephosphorylated and active, we hypothesize that high cytokinin is signaled by Tyr-dephosphorylated CDK. Several transgenic and hormone treatment studies are consistent with this. Increased cell division and initiation of growth in lateral shoots follows both raised cytokinin level (e.g. Pillay and Railton, 1983; Klee and Romano, 1994; Werner et al., 2001) and also induced *cdc25* expression in Nicotiana (Suchomelova et al., 2004). Furthermore, localized induction of *cdc25* expression caused localized cell division in the developing leaf (Wyrzykowska et al., 2002), all consistent with the cytokinin stimulation of cell proliferation in shoot tissue (Werner et al., 2001). A further indication that Tyr-dephosphorylated CDK is a signal of high cytokinin has come from the effect of modified Arabidopsis (*Arabidopsis thaliana*) CDKA (CDKA Ala-4, Phe-15) that lacks sites for inhibitory phosphorylation, which was observed to increase lateral branch formation (Hemerly et al., 1995). We now interpret this as another example of high cytokinin being signaled by Tyr-dephosphorylated CDK.

In the root also, in spite of high exogenous cytokinin being inhibitory to formation of lateral organs (for review, see Srivastava, 2002), cytokinin at low concentration induces lateral roots in many plants such as pea (Wightman et al., 1980) and lettuce (Biddington and Dearman, 1982). Again, the transgenic expression of *cdc25* has the same stimulatory effect as cytokinin on induction of lateral roots (McKibbin et al., 1998). We therefore suggest that the phenotypes of transgenes that encode CDK or its modifiers can be explained by active Tyr-dephosphorylated CDK acting as a positive biochemical signal of cytokinin presence, stimulating cell proliferation and plant development.

MATERIALS AND METHODS

Cell Culture

Suspension cultures of *Nicotiana plumbaginifolia* were grown in CS V medium with 9 μM synthetic auxin (2,4-D) and 0.23 μM kinetin and were brought to arrest at the cytokinin control point by omission of kinetin, and mitotic activity and cell numbers were monitored after fixing protoplasting and DNA staining, as described by Zhang et al. (1992, 1996).

Antibodies

Polyclonal antibodies were raised in rabbits. The carboxyterminal peptide KRITARNALEHEYFKDIGYVP of the tobacco (*Nicotiana tabacum*) CDKA, which has been shown by complementation to be a functional homolog of Cdc2 (Setiady et al., 1996), was synthesized chemically, purified by HPLC, and conjugated to keyhole limpet hemocyanin carrier at a ratio of 3 μmol peptide/12 mg keyhole limpet hemocyanin for use as antigen. Antibody was also raised against a GST-cdc25 catalytic core fusion protein, which was synthesized in *Escherichia coli* DH5α, as described (Millar et al., 1991), and used as antigen without coupling to carrier. Antigens were introduced, in 1-mg amounts, at multiple subcutaneous points in rabbits at 5-week intervals, and the appearance of antibodies was determined by western blotting. For testing anti-CDKA, soluble proteins from proliferating tobacco tissue were loaded at 25 μg/lane, and for testing anti-cdc25, soluble proteins from *E. coli* cells containing pGEX25-BD that had been induced by 0.1 mM isopropylthio-β-galactoside for 3 h were loaded at 0.5 μg/lane.

Assay of CDK and Cdc25 Activities

CDK and Cdc25 enzymes were extracted from cells that were frozen and ground in liquid nitrogen. CDK was extracted at 0°C in NDE buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 15 mM dithiothreitol (DTT), 3 μg mL^{-1} leupeptin, 20 mM EGTA, pH 7.4, 80 mM β-glycerophosphate, pH 7.4, 1 mM ortho vanadate, 30 mM NaF, 0.2 mM ammonium molybdate, p-nitrophenylphosphate with phenylmethylsulfonyl fluoride freshly added to 0.5 mM, and a soluble protein fraction was obtained as the supernatant after centrifugation at 14,000*g*. For immunoprecipitation of CDKA 10 μL of antibody was added to volumes of extract that contained soluble protein from 5 mL of culture (5–10 mg protein) and, after reaction for 3 h at 4°C, was sedimented with 35 μL protein A beads, then washed for 10-min intervals three times with HDW buffer that contained 2 mM EDTA, pH 7.4, 150 mM NaCl, 1% NP40, 5 μg mL^{-1} leupeptin, 0.2 mM orthovanadate, 50 mM NaF, pH 7.4, 10 mM $NaP0_4$, pH 7.0, then washed once with HBK buffer that contained 25 mM HEPES, pH 7.4, 1 mM EGTA, pH 7.4, 5 mM $MgCl_2$ 160 mM KCl to which 1 mM DTT was added just before use. Beads with CDKA were washed twice with cold-assay buffer lacking isotope and H1 histone and then suspended in this buffer before assay of H1 kinase activity by addition of aliquots of enzyme to 50 μL final volumes of assay that contained 25 mM HEPES, pH 7.3, 25 mM β-glycerophosphate, 10 mM $MgCl_2$, 10 mM EGTA, pH 7.3, 1 mM DTT, 0.1 mg mL^{-1} H1 histone, 2 μM (10 μCi) [γ-^{32}P]ATP, incubated at 30°C for 10 min and the reaction stopped by addition of 10 μL of 1% acetic acid. Phosphate transferred to H1 histone was measured by transfer of 24-μL aliquots to strips of Whatman (Clifton, NJ) p81 paper, then scintillation counting radioactivity in histone after extensive washing in 75 mM phosphoric acid, and was also visualized by neutralizing the reaction mix with NaOH and adjusting to pH 7.8 with 0.5 M Tris-HCl before SDS PAGE on 12% gel and exposure of the fixed, washed, and dried gel in a PhosphorImager.

Cdc25 was extracted from cell grindate in PDE buffer, containing 25 mM MOPS, pH 7.2, 100 mM NaCl, 10 mM DTT, 5 mM EDTA, 1 mM EGTA, 1% NP40, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin 3 μg mL^{-1}, aprotinin 20 μg mL^{-1}. Immunoprecipitates of Cdc25 were obtained with 10 μL of antibody added to soluble protein from 5 mL of culture (5–10 mg protein) for 3 h at 4°C, sedimented with 35 μL protein A beads, then washed for 10-min intervals three times with HDW buffer supplemented with 2 μM spermidine and then washed once with HBK buffer supplemented with 2 μM spermidine. Cdc25 assays were conducted in two stages; first, immunoprecipitates of Cdc25-like protein from 500 μg plant protein were incubated for 30 min at 30°C in Cdc25 assay buffer (Millar et al., 1991) with substrate 0.25 μg Tyr-phosphorylated Cdc2 that had been purified with p13^{suc1}-beads exactly as described by Zhang et al. (1996) from 500 μg protein of either temperature-arrested *cdc25-22* mutant fission yeast or from plant cells. The Cdc25 phosphatase reaction was stopped by sedimenting the beads that carried antibody-bound Cdc25 and, in the second stage of the assay, the extent of activation of the substrate yeast Cdc2 kinase was determined by its catalytic activity with [γ-^{32}P]ATP and H1 histone as substrates. Histone was separated by SDS PAGE and assays to be compared were run and exposed together in a PhosphorImager.

CDKA Phosphotyrosine

Phosphotyrosine was detected in CDKA enzyme that was recovered by antibody, essentially as for CDK activity assay, from 10 mg soluble plant protein with NDE buffer modified by increase of sodium vanadate to 2.5 mM and addition of 1 mM phosphotyrosine, and the immune complex was washed twice with HDW buffer supplemented to 1 mM with sodium vanadate. After separation by SDS PAGE on 12% gel a western blot was probed with anti-phosphotyrosine mouse monoclonal (PY99, Santa Cruz Biotechnology, Santa Cruz, CA) and [^{125}I]-second antibody then detected by PhosphorImager, as described (Zhang et al., 1996).

Northern Blots

RNA was extracted from cells ground in liquid nitrogen into 2 volumes of 10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% SDS, and 2 volumes of phenol:chloroform:iso-amylalcohol (25:24:1, v/v) at 4°C and fractionated. Northern blots were probed with the 650-bp *Bgl*II-*Xba*I fragment of *cdc25* made radioactive by nick translation (Sambrook et al., 1989) and equal loading was tested by reprobing the membrane for 18S rRNA using a probe from wheat (*Triticum aestivum*) 18 S rRNA (kindly provided by R. Appels, CSIRO, Canberra, Australia).

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to requisite permission from any third party owners of all or parts of the material. Obtaining any permission will be the responsibility of the requestor.

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LITERATURE CITED

Beeckman T, Burssens S, Inzé D (2001) The peri-cell-cycle in Arabidopsis. J Exp Bot **52:** 403–411

Biddington NL, Dearman AS (1982) The involvement of the root apex and cytokinins in the control of lateral root emergence in lettuce seedlings. Plant Growth Regul **1:** 183–193

Bulavin DV, Higashimoto Y, Demidenko ZN, Meek S, Graves P, Phillips C, Zhao H, Moody SA, Appella E, Piwnica-Worms H, et al (2003) Dual phosphorylation controls Cdc25 phosphatases and mitotic entry. Nat Cell Biol **5:** 545–551

Chaudhury AM, Letham DS, Craig S, Dennis ES (1993) amp1: a mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. Plant J **4:** 907–916

Cleary AL, Fowke LC, Wang H, John PCL (2002) The effect of ICK1, a plant cyclin-dependent kinase inhibitor, on mitosis in living plant cells. Plant Cell Rep **20:** 814–820

Genschik P, Criqui MC, Parmentier Y, Derevier A, Fleck J (1998) Cell cycle-dependent proteolysis in plants: identification of the destruction box pathway and metaphase arrest produced by the proteasome inhibitor MG132. Plant Cell **10:** 2063–2075

Gorst JR, Sek FJ, John PCL (1991) Level of $p34^{cdc2}$-like protein in dividing, differentiating and dedifferentiating cells of carrot. Planta **185:** 304–310

Gutierrez C, Ramirez-Parra E, Castellano M, Carlos del Pozo JC (2002) G_1 to S transition: more than a cell cycle engine switch. Curr Opin Plant Biol **5:** 480–486

Haberer G, Kieber JJ (2002) Cytokinins: new insights into a classic phytohormone. Plant Physiol **128:** 354–362

Hata S, Kouchi H, Suzuka I, Isii T (1991) Isolation and characterisation of cDNA clones for plant cyclins. EMBO J **10:** 2681–2688

Hemerly A, de Almeida Engler J, Bergounioux C, Van Montagu M, Engler G, Inzé D, Ferriera P (1995) Dominant negative mutants of Cdc2 kinase uncouple cell division from iterative plant development. EMBO J **14:** 3925–3936

Hemerly AS, Ferriera PCG, de Almeida Engler J, Van Montagu M, Engler G, Inzé D (1993) cdc2a expression in Arabidopsis is linked with competence for cell division. Plant Cell **5:** 1711–1723

Hoth S, Ikeda Y, Morgante M, Wang X, Zuo J, Hanafey MK, Gaasterland T, Tingey SV, Chua NH (2003) Monitoring genome-wide changes in gene expression in response to endogenous cytokinin reveals targets in *Arabidopsis thaliana.* FEBS Lett **554:** 373–380

Hush JM, Wu L, John PCL, Hepler LH, Hepler PK (1996) Plant mitosis promoting factor disassembles the microtubule preprophase band and accelerates prophase progression in Tradescantia. Cell Biol Int **20:** 275–287

John PCL, Zhang K (2001) Cytokinin control of cell proliferation in plant development. *In* D Francis, ed, The Plant Cell Cycle and Its Interfaces, CRC Press, Boca Raton, FL, pp 190–211

John PCL, Zhang K, Dong C, Diederich L, Wightman F (1993) $p34^{cdc2}$ related proteins in control of cell cycle progression, the switch between division and differentiation in tissue development and stimulation of division by auxin and cytokinin. Aust J Plant Physiol **20:** 503–526

Joubès J, Chevalier C, Dudits D, Heberle-Bors E, Inzé D, Umeda M, Renaudin JP (2000) CDK-related protein kinases in plants. Plant Mol Biol **43:** 607–620

Kakimoto T (2003) Perception and signal transduction of cytokinins. Annu Rev Plant Biol **54:** 605–627

King RW, Peters JM, Tugendreich S, Rolfe M, Hieter P, Kirschner MW (1995) A 20S complex containing CDC27 and CDC16 catalyses the mitosis-specific conjugation of ubiquitin to cyclin B. Cell **81:** 279–288

Klee H, Romano C (1994) The roles of phytohormones in development as studied in transgenic plants. CRC Crit Rev Plant Sci **13:** 311–324

Kovelman R, Russell P (1996) Stockpiling of Cdc25 during a DNA replication checkpoint arrest in *Schizosaccharomyces pombe.* Mol Cell Biol **16:** 86–93

Laureys F, Dewitte W, Van Montagu M, Inzé D, Van Onckelen H (1998) Zeatin is indispensable for the G2-M transition in tobacco BY-2 cells. FEBS Lett **426:** 29–32

Mähönen AP, Bonke M, Kauppinen L, Riikonen M, Benfey PN, Helariutta Y (2000) A novel two-component hybrid molecule regulates vascular morphogenesis of the Arabidopsis root. Genes Dev **14:** 2938–2943

Mailand N, Falck J, Lukas C, Syljuasen RG, Welcker M, Bartek J, Lucas J (2000) Rapid destruction of human Cdc25A in response to DNA damage. Science **288:** 1425–1429

McKibbin RS, Halford NG, Francis D (1998) Expression of fission yeast *cdc25* alters the frequency of lateral root formation in transgenic tobacco. Plant Mol Biol **36:** 601–612

Medford JI, Horgan R, El-Sawi Z, Klee HJ (1989) Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentyl transferase gene. Plant Cell **1:** 403–413

Mészáros T, Miskolczi P, Ayaydin F, Pettkó-Szandtner A, Peres A, Magyar Z, Horváth GV, Bakó L, Fehér A, Dudits D (2000) Multiple cyclin-dependent kinase complexes and phosphatases control G(2)/M progression in alfalfa cells. Plant Mol Biol **43:** 595–605

Mews M, Sek FJ, Moore R, Volkmann D, Gunning BES, John PCL (1997) Mitotic cyclin distribution during maize cell division: implications for the sequence diversity and function of cyclins in plants. Protoplasma **200:** 128–145

Millar JBA, Lenaers G, Russell P (1992) *Pyp3* PTPase acts as a mitotic inducer in fission yeast. EMBO J **11:** 4933–4941

Millar JBA, McGowan CH, Lenaers G, Jones R, Russell P (1991) $p80^{cdc25}$ mitotic inducer is the tyrosine phosphatase that activates $p34^{cdc2}$ kinase in fission yeast. EMBO J **10:** 4301–4309

Mironov VV, De Veylder L, Van Montagu M, Inzé D (1999) Cyclin-dependent kinases and cell division in plants—the nexus. Plant Cell **11:** 509–522

Murray JAH, Doonan J, Riou-Khamlichi C, Meijer M, Oakenfull EA (2001) G1 cyclins, cytokinins and the regulation of the G1/S transition. *In* D Francis, ed, The Plant Cell Cycle and Its Interfaces. CRC Press, Boca Raton, FL, pp 19–41

Nefsky B, Beach D (1996) Pub1 acts as an E6-AP-like protein ubiquitin ligase in the degradation of cdc25. EMBO J **15:** 1301–1312

Nishimura C, Ohashi Y, Sato S, Kato T, Tabata S, Ueguchi C (2004) Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. Plant Cell **16:** 1365–1377

Norbury C, Nurse P (1992) Animal cell cycles and their control. Annu Rev Biochem **61:** 441–470

Patton WF, Lam L, Su Q, Lui M, Erdjumentbromage H, Tempst P (1994) Metal chelates as reversible stains for detection of electroblotted proteins: application to protein microsequencing and immunoblotting. Anal Biochem **220:** 324–335

Pillay I, Railton ID (1983) Release of axillary buds from apical dominance in intact, light grown seedlings of *Pisum sativum* L. following application of cytokinin. Plant Physiol **71:** 972–974

Preuss SB, Britt AB (2003) A DNA-damage-induced cell cycle checkpoint in Arabidopsis. Genetics **164:** 323–334

Redig P, Shaul O, Inzé D, Van Montagu M, Van Onckelen H (1996) Levels of endogenous cytokinins, indole-3-acetic acid and abscisic acid during the cell cycle of synchronized tobacco BY-2 cells. FEBS Lett **391:** 175–180

Reichheld J-P, Vernoux T, Lardon F, Van Montagu M, Inzé D (1999) Specific checkpoints regulate plant cell cycle progression in response to oxidative stress. Plant J **17:** 647–656

Rhind N, Russell P (2001) Roles of the mitotic inhibitors Wee1 and Mik1 in the G2 DNA damage and replication checkpoints. Mol Cell Biol **21:** 1499–1508

Riou-Khamlichi C, Huntley R, Jacqmard A, Murray JAH (1999) Cytokinin activation of Arabidopsis cell division through a D-type cyclin. Science **283:** 1541–1544

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sauter M, Kende H (1992) Gibberellin induced growth and regulation of the cell division cycle in deepwater rice. Planta **188:** 362–368

Schena M, Lloyd AM, Davies RW (1991) A steroid-inducible gene expression system for plant cells. Proc Natl Acad Sci USA **88:** 10421–10425

Schmülling T, Schäffer S, Romanov G (1997) Cytokinins as regulators of gene expression. Physiol Plant **100:** 505–519

Schuppler U, He P-H, John PCL, Munns R (1998) Effect of water stress on cell division and Cdc2-like cell cycle kinase activity in wheat leaves. Plant Physiol **117:** 667–678

Setiady YY, Sekine M, Hariguchi N, Kouchi H, Shinmyo A (1996) Molecular cloning and characterisation of a cDNA clone that encodes a Cdc2 homolog from *Nicotiana tabacum.* Plant Cell Physiol **37:** 369–376

Srivastava LM (2002) Plant Growth and Development. Academic Press, San Diego, pp 303–379

Suchomelova P, Velgova D, Masek T, Francis D, Rogers HJ, Marchbank AM, Lipavska H (2004) Expression of the fission yeast cell cycle regulator cdc25 induces de novo shoot formation in tobacco: evidence of a cytokinin-like effect by this mitotic activator. Plant Physiol Biochem **42:** 49–55

Taylor BH, Larkin PJ (1988) Analysis of electroporation efficiency in plant protoplasts. Aust J Biotechnol **1:** 52–57

Valente P, Tao W, Verbelen J-P (1998) Auxins and cytokinins control DNA replication and deduplication in single cells of tobacco. Plant Sci **134:** 207–215

Van't Hof J (1974) Control of the cell cycle in higher plants. *In* GM Padilla, IL Cameron, A Zimmerman, eds, Cell Cycle Controls. Academic Press, New York, pp 77–85

Werner T, Motyka V, Strnad M, Schmülling T (2001) Regulation of plant growth by cytokinin. Proc Natl Acad Sci USA **98:** 10478–10492

Wightman F, Schneider EA, Thimann KV (1980) Hormonal factors controlling the initiation and development of lateral roots. II. Effects of exogenous growth factors on lateral root formation in pea roots. Physiol Plant **49:** 304–314

Wyrzykowska J, Pien S, Shen WH, Fleming AJ (2002) Manipulation of leaf shape by modulation of cell division. Development **129:** 957–964

Zetterberg A, Larsson O (1985) Kinetic analysis of regulatory events in G1 leading to proliferation or quiescence of Swiss 3T3 cells. Proc Natl Acad Sci USA **82:** 5365–5369

Zhang K, Yasumasa T, John PCL (1992) Mitotic arrest in tobacco caused by the phosphoprotein phosphatase inhibitor okadaic acid. Plant Cell Physiol **33:** 677–688

Zhang K, Letham DS, John PCL (1996) Cytokinin controls the cell cycle at mitosis by stimulating the tyrosine dephosphorylation and activation of $p34^{cdc2}$ -like H1 histone kinase. Planta **200:** 2–12