J Plant Growth Regul (1998) 17:221–230

Journal of
Plant
Growth
Regulation

Hormonal Control of Gene Expression During Reactivation of the Cell Cycle in Tobacco Mesophyll Protoplasts

S. A. Carle,*[,1,2] G. W. Bates,[1] and T. A. Shannon[2]

[1]Department of Biological Science, Florida State University, Tallahassee, FL 32306-3015, USA, [2]Biology Department, Hobart and William Smith Colleges, Geneva, NY 14456, USA

Received November 4, 1997; accepted October 7, 1998

Abstract. The roles of auxin and cytokinin in cell cycle reactivation were studied during the first 48 h of culture of mesophyll protoplasts of *Nicotiana tabacum.* Using hormone delay and withdrawal studies we found that auxin was required by 0–4 h of culture, whereas cytokinin was not required until hour 10–12, which is 6–10 h before S phase. Cycloheximide blocks division, indicating that protein synthesis is required. In an effort to detect a molecular response to either hormone, we examined the expression of the cell cycle marker, *cdc2. Cdc2* expression was detected by 12 h of culture, coincident with the timing of the cytokinin requirement and well before the entry into S. However, *cdc2* was partially induced by either auxin or cytokinin alone, suggesting that *cdc2* expression is not the primary target of either hormone. Our hormone delay experiments suggest that there are separate signal transduction pathways leading from auxin and from cytokinin to reactivation of the cell cycle and that these pathways converge before S. The underlying mechanisms for these distinct pathways remain to be elucidated.

Key Words. Auxin—Cytokinin—Tobacco—Protoplast—Development—*cdc2*

Auxin and cytokinin play integral roles in the regulation of plant development. One of the most fundamental effects of these hormones is their requirement for cell division in vitro (Das et al. 1956). They are also involved in the regulation of cell division in vivo (Ferreira et al. 1991, Klee and Estelle 1991). Despite the importance of these hormones, little is known about the molecular events involved in their responses. In particular, we have yet to identify the primary target of either hormone.

We have been studying the roles of auxin and cytokinin in the reinitiation of cell division in cultures of tobacco mesophyll protoplasts. During the maturation of tobacco leaves, mesophyll cells cease dividing when the leaf is 25% expanded (Dhillon and Miksche 1981). These cells are arrested predominantly in the G_0 phase of the cell cycle (Galbraith 1984). When cultured in the presence of auxin and cytokinin, tobacco mesophyll protoplasts reinitiate the cell cycle and divide 48 h later. During this period, tobacco mesophyll protoplasts proceed through a series of predictable changes, which include dedifferentiation, resynthesis of the cell wall, induction of the cell cycle, and activation of a wound response (Cooke and Meyer 1981, Grosset et al. 1990, Meyer and Cooke 1979, Nagata and Takebe 1970). Because large numbers of developmentally homogeneous mesophyll protoplasts can be isolated and because the reinitiation of cell division is dependent on auxin and cytokinin, tobacco mesophyll protoplasts provide a unique in vitro system for studying the hormonal control of reinitiation of the cell cycle.

Changes in gene expression are known to occur during protoplast development. Using in vitro translation and two-dimensional gel electrophoresis, it has been shown that the synthesis of many new proteins is activated (Fleck et al. 1980, Meyer et al. 1984b). However, leaf-specific proteins, such as Rubisco, are no longer synthesized (Fleck et al. 1980, Vernet et al. 1982). Auxin appears to play a role in regulating the expression of some of these genes, activating the expression of some (Meyer et al. 1984a) while inhibiting the expression of others (Grosset et al. 1990, Meyer et al. 1984a). Using differ-

Abbreviation: S phase, DNA synthesis phase of the cell cycle; BA, benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, naphthaleneacetic acid; TCA, trichloroacetic acid; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; kb, kilobases; bp, base pair(s); CDK, cyclin-dependent kinase; G_1, phase of cell cycle between M and S phases; G_2, phase of cell cycle between S and M phases; M phase, mitosis phase of the cell cycle.

*Author for correspondence.

ential screening of a cDNA library, Takahashi et al. (1993) have identified several genes (*parA, B, C*) whose transcription is auxin dependent and occurs early in protoplast development. The product of *parB* is a glutathione *S*-transferase, which conjugates glutathione to xenobiotics (Takahashi et al. 1993). *ParA* and *parC* are members of the same gene family and show homology to several genes identified in other plants, one of which shows glutathione *S*-transferase activity (Takahashi et al. 1995). However, the product of *parA* is localized in the nucleus and also shows homology to a 24-kDa *Escherichia coli* protein that binds to RNA polymerase (Takahashi et al. 1995). It is possible that the product *parA* is involved in transcriptional regulation.

Although auxin is known to stimulate expression of genes during protoplast culture, it has not yet been determined if protein synthesis is required for division or if the expression of genes identified so far correlates with the requirement for auxin or cytokinin. The current study seeks to address these issues. First, experiments were designed to determine the precise timing of the auxin and cytokinin requirements for reinitiation of cell division. Second, we determined the timing of expression of the cell cycle gene cyclin-dependent kinase p34^{cdc2}. Determination of the timing of the hormone requirement and expression of *cdc2* will facilitate determination of the primary action of cytokinin.

Materials and Methods

Tobacco Plants and in Vitro Culture

Tobacco plants (*Nicotiana tabacum* var. Xanthi) were grown in a growth chamber under a 16-h light/8-h dark cycle (light intensity 250 $\mu Em^{-1}\ s^{-1}$), 60% constant humidity, and at 25°C. Mesophyll protoplasts were isolated from leaves 8–10 cm long as follows. The leaves were sterilized by treatment with 10% commercial bleach for 6 min and rinsed three times with sterile water for 3 min each. Leaf peels were digested in CPW salts (Frearson et al. 1973) containing 0.1% Cellulysin (Calbiochem Corp., La Jolla, CA, USA) and 0.02% Pectolyase Y23 (Kanematsu Inc., Los Angeles, CA, USA). After a 10-h digestion the protoplasts were separated from cellular debris by flotation on 18% sucrose, washed to remove excess sucrose, and cultured in K_3G [K_3 salts and vitamins (Nagy and Maliga 1976) plus 0.42 M glucose] containing cytokinin [0.2 mg $liter^{-1}$ benzyladenine (BA)] and auxin [0.1 mg $liter^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) + 1 mg L^{-1} naphthaleneacetic acid (NAA)]. In some experiments, cytokinin, auxin, or both were omitted from the culture medium.

Determination of DNA Synthesis and Timing of Nuclear Division

DNA synthesis was measured by incorporation of [^{3}H]thymidine into trichloroacetic acid (TCA)-precipitable material as described by Zelcer and Galun (1976). Briefly, 5 μCi of [^{3}H]thymidine (50 Ci/mmol, ICN Radiochemicals, Costa Mesa, CA, USA) was added to samples of 2.5 × 10^5 protoplasts. After a 1-h incubation, the protoplasts were pelleted, washed twice with K_3G medium, resuspended in K_3G, and an equal volume of 10% TCA was added. An aliquot was removed to determine [^{3}H]thymidine uptake. As a carrier, salmon sperm DNA was added to the rest of the sample such that the final concentration was 0.18%. The rest of the sample was passed through a glass fiber filter (type GFA, Gelman Instrument Co., Ann Arbor, MI, USA), rinsed twice with 5% TCA and three times with 95% ethanol. The filters were dried, and the radioactivity was determined by liquid scintillation counting. All samples were done in duplicate.

Incorporation of [^{3}H]thymidine into TCA-precipitable material was corrected for [^{3}H]thymidine uptake in the following manner. In each experiment, the sample with the greatest uptake was arbitrarily set to 1.0. The counts taken up in each of the other samples were normalized to that for the sample with the greatest uptake. This gave a ratio of uptake for each sample. Then, for each sample the count of [^{3}H]thymidine incorporated was divided by the uptake ratio. This gave incorporation corrected for uptake.

The timing of nuclear division was determined by acetocarmine staining of protoplast samples at different times during culture and measurement of the percentage of cells in the sample which had undergone one or more nuclear divisions. Cells that had divided were identified by the presence of crosswalls and by the presence of a nucleus in each daughter cell. Cells with visible chromosomes, or anaphase or telophase mitotic figures, were also scored as having divided. Cells that had not divided contained no crosswalls and only a single nucleus. Finally, cells that lacked crosswalls but which had multiple nuclei were scored as having divided. The first divisions of tobacco protoplasts often result in the formation of some multinuceate cells because karyokinesis is not always followed by cytokinesis (Galbraith et al. 1981). Because some protoplasts undergo fusion during protoplast isolation, a fraction of freshly isolated protoplasts is observed to be multinucleate. In our protoplast isolates, no more than 5% (usually less than 2%) of the freshly isolated protoplasts were multinucleate.

Hormone Delay and Withdrawal Studies

Protoplasts were cultured in K_3G containing auxin (NAA + 2,4-D) or cytokinin (BA). At 13, 18, and 24 h after culture, the protoplasts were pelleted, washed twice with K_3G, and resuspended in medium containing auxin and cytokinin. At each time point, control samples (protoplasts cultured in both auxin and cytokinin) were also pelleted, washed twice with K_3G, and resuspended in medium containing auxin and cytokinin.

For cytokinin withdrawals, protoplasts were cultured in K_3G containing auxin and cytokinin. At 10, 19, and 24 h of culture the protoplasts were pelleted, washed twice, and resuspended in 10 mL of K_3G medium containing auxin only or auxin and cytokinin. The controls were cultured in auxin and cytokinin, and at each time point they were treated as described above except they were resuspended in auxin and cytokinin. The fraction of cells that had undergone nuclear division was assessed every 12 or 24 h. Nuclear divisions were visualized as described above.

RNA Isolation

Total RNA was isolated following the procedure of Wadsworth et al. (1988). Samples of 1–2 × 10^6 protoplasts were washed once in 10 mL of K_3G and taken up in 1 mL of extraction buffer (100 mM Tris-HCl,

20 mM aurintricarboxylic acid, 200 mM LiCl, 100 mM EDTA, and 100 mM 2-mercaptoethanol). Diethylpyrocarbonate was added at a concentration of 0.36 mL/mL of extraction buffer. The protoplasts were homogenized using a glass homogenizer set in ice. The mixture was extracted three times with 1 mL of phenol/chloroform/isoamyl alcohol (50:48:2) and then precipitated with an equal volume of cold (4°C) 6 M LiCl. After a 1-h incubation on ice, the RNA was pelleted and then washed three times with ice-cold 3 M LiCl, dissolved in 2% potassium acetate, and precipitated on ice with a final ethanol concentration of 71%. RNA concentrations were determined spectrophotometrically.

Probes for Northern Hybridizations

Clones of *Arabidopsis thaliana* histone H3 (clone A713) and H4 (clone A748) genes were obtained from Dr. C. Gigot (IMBMP-CNRS, France). A soybean 25 S ribosomal gene clone (pGmR1) was obtained from Dr. E. Zimmer (Department of Biochemistry, Louisiana State University, Baton Rouge, LA, USA). The histone and 25 S probes were labeled with either [^{32}P]dCTP or [^{32}P]dATP (NEN Life Sciences, Inc., Boston, MA), by the random primer method (Feinberg and Vogelstein 1983). The *cdc2* probe was prepared, in the presence of either [^{32}P]dCTP or [^{32}P]dATP (Schowalter and Somer 1989), by amplification of a subcloned PCR-generated tobacco *cdc2* fragment. The two oligonucleotides used for preparation of the probe were: 5′-GGTGAAGGAACTTACGGTGT-3′ and 5′-TTCTTTCAAGAGGGAGATTTC-3′.

Northern Blot Hybridization

Each 10-μg sample of total RNA was mixed with 1 μL of 0.4 μg/μL ethidium bromide, heated to 65°C for 10 min, separated on a 1.7% agarose/6% formaldehyde gel, and transferred to nitrocellulose as described by Sambrook et al. (1989). The filters were prehybridized overnight at 42°C in 50% formamide, 0.1% SDS, 6 × SSC, 5 × Denhardt's reagent, 50 mM sodium phosphate buffer, pH 6.5, 0.1 mg/mL heparin (Sigma Chemical Co., St. Louis, MO, USA), 0.1 mg/mL yeast tRNA (Sigma), and 0.01 mg/mL poly(A)RNA (Sigma). Hybridization was carried out at 42°C for 18–24 h. After hybridization the filters were washed. The final wash consisted of 0.1 × SSC and 0.1% SDS and was done at 42°C. Hybridizations were visualized and quantified using a Betascope 603 blot analyzer (Betagen Corp., Waltham, MA, USA). After visualization, the filters were washed in 0.05% SSC and 0.1% SDS for 1 h at 65°C to remove the probe. The filters were air dried and reprobed.

The amount of mRNA present was expressed as the percentage of hybridization to the 3-cm leaf (in the leaf studies) or the 24-h auxin + cytokinin sample (in the protoplast studies). The counts per minute hybridized to the 3-cm leaf or 24-h auxin + cytokinin sample was arbitrarily set at 100%.

Results

Identification of Tobacco Leaves in Which Cell Division Has Ceased

Because our aim is to study the hormonal regulation of cell cycle reactivation, it is important to determine the extent to which leaf cells used for protoplast isolation have ceased progressing through the cell cycle. Expression of the most abundant histone variants is replication dependent in plants (Kapros et al. 1992). Thus, we have used changes in the level of histone mRNAs to determine when cell division ceases during leaf development.

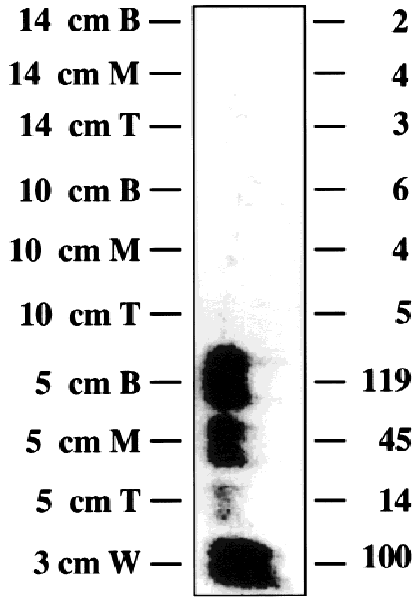

Fig. 1. Northern analysis of histone H4 expression in tobacco leaves of different sizes. Ten μg of total RNA was applied to each lane. A clone of the *A. thaliana* histone H4 gene was used as a probe. The *numbers* above each sample represent quantified histone H4 expression normalized to the value obtained for the 3-cm leaf sample. *T, M,* and *B* represent leaf tip, middle, and base, respectively. *W* represents whole leaf.

RNA was isolated from leaves 3–14 cm long, and expression of histones H3 and H4 was determined by Northern blotting. The results for histone H4 are shown in Fig. 1. Similar results were obtained for H3 (data not shown). The histone H4 probe hybridized to a single band on the blot. Based on the sizes of the 25 S (3.5 kb, Perry and Palukaitis 1990) and 18 S (1.8 kb, Schmidt-Puchta et al. 1989) ribosomal RNAs, the tobacco H4 transcripts is about 680 nucleotides. The *A. thalania* histone H4 transcript is known to be 700 nucleotides (Chaboute et al. 1988).

Histone H4 expression declined rapidly as the leaves grew beyond 5 cm in length, and it declined faster at the tip of the leaf than the base; this follows the known pattern for the cessation of cell division during tobacco leaf development (Digby and Firn 1985). Leaves 10 cm long had only 4–6% as much H4 mRNA as leaves 3 cm long. Leaves 14 cm long had slightly lower histone mRNA levels, but the yield of protoplasts was substantially lower than that from 10-cm leaves. Because the 10-cm leaves met our criterion of containing predominantly nondividing cells, we used 10-cm leaves for protoplast isolation for the remainder of the experiments.

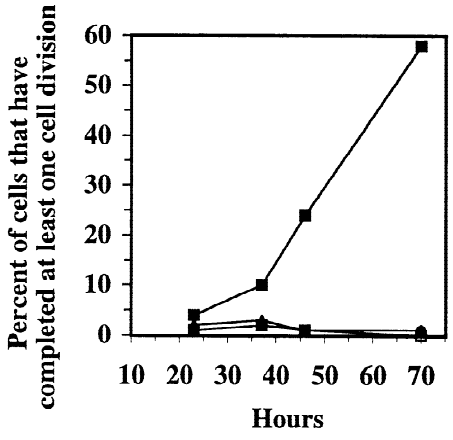

Fig. 2. Time course of nuclear division in tobacco mesophyll protoplasts cultured with and without hormones. Protoplasts were isolated from 10-cm leaves and cultured from time zero in K_3G medium supplemented with auxin and cytokinin (*solid squares*), auxin only (*triangles*), cytokinin only (*circles*), and no hormones (*open squares*). Protoplasts were sampled at the times indicated, fixed, stained with acetocarmine, and the percentage of protoplasts that had undergone nuclear division was determined visually.

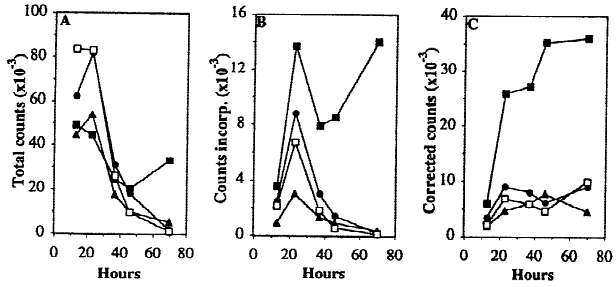

Fig. 3. Time course of DNA synthesis in tobacco mesophyll protoplasts and its dependence on hormones. Protoplasts were cultured as in Fig. 2 (all symbols are the same) and were labeled with [^{3}H]thymidine for 1 h just before sampling. All samples were done in duplicate. *Panel A*, uptake of [^{3}H]thymidine. After [^{3}H]thymidine labeling, the protoplasts were washed, resuspended in K_3G medium, and TCA was added. An aliquot of protoplasts was used to determine uptake. The data are expressed as the total counts taken up by 20,000 protoplasts. *Panel B*, incorporation of [^{3}H]thymidine. The rest of the sample in *panel A* was passed through a glass fiber filter and rinsed with TCA and ethanol. The data are expressed as the total number of counts incorporated into TCA-precipitable material/20,000 protoplasts. *Panel C*, incorporation of [^{3}H]thymidine after normalization of the data for uptake. The incorporation shown in *panel B* was corrected for uptake as described in the Materials and Methods section.

Nuclear Division and DNA Synthesis in Protoplasts Isolated from 10-Centimeter Leaves

In a typical batch of protoplasts cultured in complete medium (i.e. containing both auxin and cytokinin), nuclear division began at about 36 h of culture (Fig. 2). By 72 h, 58% of the protoplasts in complete medium had undergone at least one nuclear division; 11% had divided twice. If either hormone was omitted from the medium, the protoplasts did not divide.

The timing of DNA synthesis was assessed at 12-h intervals by pulse labeling with [^{3}H]thymidine. Both uptake and incorporation of [^{3}H]thymidine were measured. Uptake was high for the first 24 h of culture and then declined dramatically by 36 and 48 h (Fig. 3*A*). This trend was observed in all hormone combinations. However, uptake of [^{3}H]thymidine during the first 24 h was about 60% higher in protoplasts cultured in media lacking hormones or containing only cytokinin compared with media containing auxin alone or both auxin and cytokinin. By hours 36 and 48, the uptake of [^{3}H]thymidine was much less hormone dependent. Incorporation of [^{3}H]thymidine into TCA-precipitable material was low at 12 h and peaked at 24 h in all samples (Fig. 3*B*). The greatest incorporation was observed for protoplast samples cultured in media containing both auxin and cytokinin. These data suggest that DNA synthesis begins by 24 h in protoplasts cultured in complete medium. Protoplasts cultured in media containing only auxin had low levels of [^{3}H]thymidine incorporation (Fig. 3*B*). However, protoplasts cultured in the absence of hormones or in cytokinin alone showed 55–65% as much [^{3}H]thymidine incorporation at 24 h as did protoplasts cultured in complete medium (Fig. 3*B*). This incorporation dropped to low levels by 36 h in these protoplast samples.

The results described above show that [^{3}H]thymidine incorporation parallels uptake for protoplasts cultured in auxin alone, cytokinin alone, or in no hormones. However, incorporation was highest for protoplasts cultured in auxin plus cytokinin even though [^{3}H]thymidine uptake was significantly lower in these protoplasts than it was in those cultured in cytokinin alone or in no hormones. These data suggest that the incorporation of [^{3}H]thymidine is limited by uptake. Therefore, we normalized the incorporation data for the amount of [^{3}H]thymidine uptake in each sample compared with the sample having the highest uptake (i.e. the 12-h sample cultured in the absence of hormones). The results are shown in Fig. 3*C*. When plotted in this manner the data indicate only low levels of DNA synthesis in protoplasts cultured in the absence of auxin or cytokinin or both.

Effects of Delayed Auxin or Cytokinin Addition on Mitosis

Earlier work (Meyer and Cooke 1979) showed that cytokinin is required later during protoplast culture than auxin, but the precise timing of the hormone requirements is not known. To determine when each hormone acts, we cultured protoplasts in media lacking either

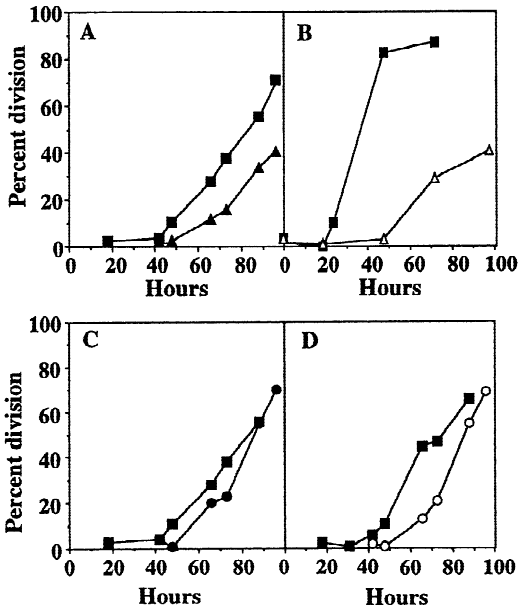

Fig. 4. Effect of a delay in auxin or cytokinin addition on the timing of nuclear division. Protoplasts were cultured in K_3G medium containing cytokinin (*panels A* and *B, triangles*) or auxin (*panels C* and *D, circles*), or both auxin and cytokinin (*solid squares* in all *panels*). At 18 h (*panels A* and *C*) or 24 h (*panels B* and *D*) of culture, the protoplasts were spun down, washed, and resuspended in K_3G containing both auxin and cytokinin. The fraction of protoplasts that had divided was determined every 12 h.

auxin or cytokinin for 13, 18, or 24 h; then the protoplasts were centrifuged, resuspended in complete medium, and the timing of mitosis was determined. The results of typical 18- and 24-h delay experiments are shown in Fig. 4. Statistical analysis of these results was complicated by the fact that the onset of mitosis could vary by as much as 12 h in different batches of protoplasts. To solve this problem, the time required for the initiation of mitosis in each sample of treated protoplasts was normalized by subtraction of the time required for the initiation of mitosis for a parallel sample of control protoplasts cultured in complete medium. Then, to calculate the timing of the hormone requirement, we subtracted the number of hours mitosis was delayed from the number of hours auxin or cytokinin addition was delayed. The results of these calculations are shown in Table 1.

We found that a delay in auxin addition had a greater effect on division than a delay in cytokinin addition. When protoplasts were cultured in cytokinin alone and auxin was added after 13 h, nuclear division was delayed by 9.4 h. Delaying auxin addition for 18 or 24 h delayed nuclear division for 17.8 and 36.8 h, respectively. By contrast, delaying cytokinin addition for 13, 18, or 24 h delayed mitosis for 4.3, 5.3, and 16.3 h, respectively. A two-way ANOVA, performed on the number of hours

Table 1. Timing of the auxin and cytokinin requirements during tobacco mesophyll protoplast culture as calculated from the hormone-delay experiments. Protoplasts were cultured in K_3G medium containing auxin only (cytokinin delay) or cytokinin only (auxin delay) for 13, 18, or 24 h. Then the protoplasts were washed, resuspended in complete medium containing both auxin and cytokinin, and the fraction of protoplasts that had divided was measured every 12 h. The number of hours that division was delayed was calculated by subtracting the number of hours it took the controls (protoplasts cultured continuously in complete medium) to reach 20% division from the number of hours it took the hormone-delayed samples to reach 20% division. The timing of the hormone requirement was calculated by subtracting the number of hours nuclear division was delayed from the number of hours auxin or cytokinin was withheld from the protoplasts. The data are presented as the averages ± the standard deviation for three to six replicates for each treatment.

Hormone and hours delayed	No. of hours division was delayed	Timing of hormone requirement (h)
Auxin		
13	9.4 ± 4.9	3.6
18	17.8 ± 10.0	0.3
24	36.8 ± 10.1	0.0
Cytokinin		
13	4.3 ± 6.7	9.0
18	5.3 ± 3.0	12.7
24	16.3 ± 4.6	7.7

division was delayed, showed that delay time had a significant effect ($F = 15.845$, $p < 0.0002$). Post hoc comparisons of 13- vs 24-h and 18- vs 24-h delays are statistically different for both auxin and cytokinin ($p < 0.0007$ and $p < 0.01$ for auxin, respectively; $p < 0.02$ and $p < 0.03$ for cytokinin, respectively). Post hoc comparisons of 13- vs 18-h delays were not significantly different for either hormone. The two-way ANOVA also showed that auxin and cytokinin affect the protoplasts differently ($F = 18.883$, $p < 0.0005$).

These hormone delay studies indicate that auxin is required at least by the 4th hour of culture and may be required from time zero. Cytokinin is not needed until later, sometime between 7.7 and 12.7 h of culture. Cytokinin withdrawal experiments were used to examine more carefully the timing of cytokinin action. Protoplasts were cultured in complete medium and then switched to medium containing auxin as the only hormone after 10, 19, or 24 h. Fig. 5*A* shows that the protoplasts did not divide if they were switched into auxin-only medium at hour 10. This result refines the conclusions of the hormone delay experiments; it appears that cytokinin is not required until after 10–12 h of culture. As Fig. 5*B* shows, when cytokinin was removed from the medium at hour 19 of culture, there was a 50% decrease in the number of protoplasts that divided compared with the controls. Removal of cytokinin at 24 h resulted in a smaller reduction in the number of cells that divided. When these cultures were observed for 120 h, only 3–6% of the protoplasts

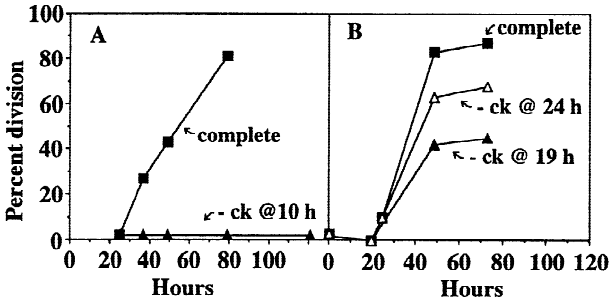

Fig. 5. Effect of removing cytokinin from the medium on the timing of nuclear division. At 10, 19, or 24 h of culture, protoplasts cultured in complete medium were spun down, washed twice, and resuspended in medium lacking cytokinin. The fraction of protoplasts that had divided was determined every 12 h. *Panel A,* cytokinin (*ck*) withdrawal at 10 h of culture (*solid triangles*). Control, protoplasts cultured continuously in complete medium (*solid squares*). *Panel B,* cytokinin withdrawal at 19 h (*solid triangles*) and 24 h (*open triangles*) of culture. Control protoplasts cultured as in *panel A* (*solid squares*).

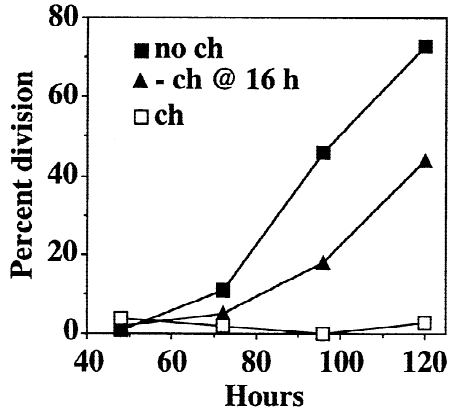

Fig. 6. Effect of cycloheximide on the timing of nuclear division. Protoplasts were cultured K_3G medium containing auxin and cytokinin plus cycloheximide (*ch*) from time zero. *Open squares,* cycloheximide left in the medium continuously. *Solid triangles,* cycloheximide was washed out of the medium after 16 h. *Solid squares,* control protoplasts cultured without cycloheximide. The data plotted for the cycloheximide removal are the average of two replicate samples. The fraction of protoplasts that had divided was determined every 24 h.

divided a second time whereas protoplasts cultured in complete media had formed colonies by 120 h of culture.

Requirement for Protein Synthesis

Previous studies have shown major changes in the pattern of gene expression during protoplast development (Fleck et al. 1980, Meyer et al. 1984a, 1984b, Vernet et al. 1982). Although changes in gene expression occur during protoplast development, it is not clear if protoplast development requires new gene expression. As shown in Fig. 6, we found that protoplasts cultured in the presence of cycloheximide did not divide, and when cultured in cycloheximide for just the first 16 h their division was delayed by 15–20 h. These data suggest that protein synthesis is required for protoplast development and that activation of new gene expression is required early in protoplast development. At this time, it is not known what genes may be activated or what roles they may play.

Timing of Cyclin-dependent Kinase p34^{cdc2} Expression

It has been difficult to determine the primary targets of auxin and cytokinin in protoplasts because of batch to batch variations, which can shift the timing of division by several hours. A molecular marker whose expression is activated coincident with the timing of the hormone requirement would facilitate the determination of the targets of auxin and cytokinin. Hemerly et al. (1993) showed that GUS is not expressed in freshly isolated mesophyll protoplasts of transgenic tobacco plants carrying the GUS gene driven by the *Arabidopsis cdc2* promoter. However, after 72 h in culture GUS is expressed by protoplasts cultured in media containing auxin plus cytokinin. Setiady et al. (1996) found that *cdc2* is expressed constitutively in synchronized tobacco BY-2 cells; however, expression of *cdc2* declines when these cells stop dividing. These data suggest that *cdc2* expression may be critical for cell cycle reactivation.

Probes prepared from *Arabidopsis cdc2* clones were unable to detect *cdc2* transcripts on Northern blots of RNA from young leaves or shoot tips of tobacco. We therefore used PCR to clone a tobacco *cdc2* homolog for use in Northern blotting. Primers were designed to match the conserved ends of the ATP binding site and the PSTAIR region of the *Arabidopsis cdc2a* gene (Ferreira et al. 1991). These regions correspond to the conserved amino acids GEGTYG and REISLLKE, respectively (Miao et al. 1993). The primers were used to amplify a portion of the *cdc2* gene from tobacco genomic DNA. The resulting 130-bp fragment was subcloned and sequenced (data not shown). The deduced amino acid sequences of our tobacco fragment and a previously isolated tobacco *cdc2* gene (Setiady et al. 1996) differ by one amino acid. The amplified tobacco *cdc2* gene fragment contains the conserved PSTAIR motif found in all *cdc2* genes.

Northern blots of RNA isolated from tobacco leaves and protoplasts were probed with the tobacco *cdc2* fragment. The *cdc2* probe hybridized to two bands on the blot. The strongest hybridization was to a message about 1.6 kb in length. The *cdc2* messages from tobacco and other plants are approximately the same length (alfalfa, 1.4 kb, Hirt et al. 1991; *Arabidopsis,* 1.4 kb, Ferreira et

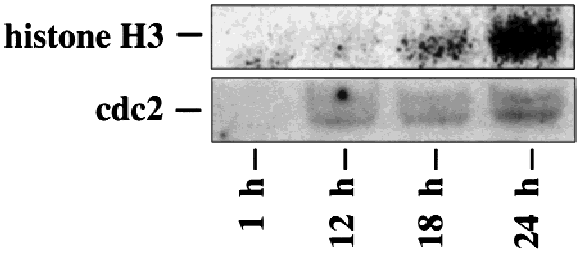

Fig. 7. Time course of *cdc2* and histone H3 expression in tobacco mesophyll cells cultured in K_3G medium containing both auxin and cytokinin. Ten μg of total RNA was applied to each lane. A PCR-amplified *cdc2* tobacco fragment and a clone of the *A. thaliana* histone H3 gene were used as probes.

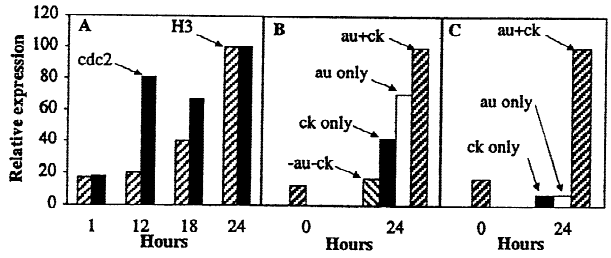

Fig. 8. *Panel A,* Quantified data for expression of *cdc2* (*solid bars*) and histone H3 (*striped bars*) in tobacco mesophyll protoplasts cultured in auxin (*au*) and cytokinin (*ck*). The data represent quantified expression of the Northern blots shown in Fig. 7. *Panel B,* effect of hormones on *cdc2* expression. Protoplasts were cultured for 24 h in K_3G medium containing both auxin and cytokinin (*thick striped bars*), auxin only (*white bar*), cytokinin only (*black bar*), or no hormones (*thin striped bar*). *Panel C,* effect of hormones on histone H3 expression. Symbols are the same as in *panel B.* Samples were normalized to the 24-h sample in complete medium.

al. 1991; rice, 1.5 kb, Hashimoto et al. 1992; petunia, 1.4 kb, Bergounioux et al. 1992; tobacco, 1.4 kb, Setiady et al. 1996). Expression of *cdc2* in 10-cm leaves was two-fold lower than in 3-cm leaves (data not shown). In freshly isolated protoplasts, *cdc2* expression was four-fold lower than in 10-cm leaves. As shown in Figs. 7 and 8*A*, *cdc2* was activated in protoplasts by 12 h, and an increase in histone expression was not detected until 18 h. No increase in *cdc2* expression was detected in protoplasts cultured without hormones. However, protoplasts cultured in either auxin alone or cytokinin alone showed a partial activation of *cdc2* expression (Fig. 8*B*). This result contrasts with histone expression, which requires the presence of both auxin and cytokinin (Fig. 8*C*).

Discussion

When protoplasts were cultured in auxin and cytokinin, we found that [^{3}H]thymidine incorporation began between 12 and 24 h. These data confirm earlier observations (Cooke and Meyer 1981, Takahashi et al. 1993) and indicate that DNA synthesis begins before 24 h of culture. Galbraith et al. (1981) criticized the use of [^{3}H]thymidine incorporation for the measurement of DNA synthesis in protoplasts because of possible changes in internal thymidine pools during protoplast culture. For the following reasons we believe our [^{3}H]thymidine incorporation data are reliable, particularly when incorporation is corrected for uptake. Like Cooke and Meyer (1981), we observed that the uptake of [^{3}H]thymidine changes during culture and that uptake was highest for protoplasts cultured in the absence of auxin. However, the incorporation of [^{3}H]thymidine paralleled uptake for protoplasts cultured in hormone-deficient media but not for protoplasts cultured in media containing both auxin and cytokinin. For protoplast samples cultured in hormone-deficient media, when [^{3}H]thymidine uptake was low incorporation was proportionately low, and when uptake was high so was incorporation. These data suggest that [^{3}H]thymidine incorporation is driven by uptake in these samples. By contrast, the incorporation of [^{3}H]thymidine into DNA was higher at all time points in protoplasts cultured in complete medium than in those cultured in hormone-deficient media even though [^{3}H]thymidine uptake was lower in complete medium. We conclude that the increase in [^{3}H]thymidine incorporation between 12 and 24 h in complete medium reflects the onset of the S phase. When the incorporation data were normalized to take into account differences in uptake, the timing of DNA synthesis was not changed, but its hormone dependence was. The normalized data indicate that DNA synthesis was low and did not change over time for protoplasts cultured in the absence of auxin or cytokinin or both.

Our histone H3 expression data provide independent evidence supporting this view. Histone expression has been shown to increase during S phase in vegetative tissues, suspension cultures, and protoplasts (Kapros et al. 1992, Lepetit et al. 1992). We found that the first detectable increase in histone expression was at 18 h when protoplasts were cultured in the presence of auxin plus cytokinin, and no increase in histone expression was observed when protoplasts were cultured in the absence of either hormone. Thus, the timing and hormone dependence of histone expression parallel our normalized [^{3}H]thymidine incorporation data. Taken together, these data indicate that the protoplasts first enter S phase at about 18–20 h of culture and that entry into S requires both auxin and cytokinin. These data support recent observations that *Petunia* protoplasts also require both auxin and cytokinin for reactivation of the cell cycle and entry into S phase (Trehin et al. 1998).

The [^{3}H]thymidine incorporation data do indicate that some DNA is synthesized by protoplasts cultured in the

absence of either auxin or cytokinin. We found that the TCA-precipitable [^{3}H]thymidine recovered from protoplasts cultured in cytokinin alone was solubilized by treatment with DNase (data not shown). Thus, this [^{3}H]thymidine incorporation reflects real DNA synthesis. However, the relative magnitudes of DNA synthesis which occur in protoplasts cultured in hormone-deficient vs complete media cannot be known without measurement of the internal thymidine pools. If the thymidine pool expands during S phase then our [^{3}H]thymidine incorporation data may underestimate the differences in DNA synthesis. The DNA synthesized by protoplasts cultured in the absence of auxin or cytokinin could reflect entry into S phase by a subpopulation of protoplasts, or it could be caused by DNA repair or replication of organelle DNA.

The pattern of histone expression suggests that the majority of cells in tobacco leaves cease dividing by the time the leaves are 10 cm long. This confirms earlier cytological observations (Avery 1933, Dhillon and Miksche 1981). Galbraith (1984) found that 80% of the cells in mature tobacco leaves and freshly isolated protoplasts contain a 2C level of DNA, thus the majority of cells are arrested in G_1. Based on our [^{3}H]thymidine incorporation and histone expression data, tobacco mesophyll protoplasts take about 18–20 h to reinitiate the cell cycle, pass through G_1, and enter S phase. Entry into S requires both auxin and cytokinin, but our hormone delay and withdrawal studies suggest that these hormones act at different steps. Auxin is required very early; it is needed somewhere between 0 and 4 h of protoplast culture. Cytokinin acts later; the initial cytokinin-requiring step occurs somewhere between 10 and 12 h of culture, which is 6–10 h before S phase. These data confirm and extend earlier observations in tobacco protoplasts (Cooke and Meyer 1981, Meyer and Cooke 1979) and more recent observations in *Petunia* protoplasts (Trehin et al. 1998). In contrast to these results, Zhang et al. (1996) and Laureys et al. (1998) report that cytokinin is required for the completion of G_2 in *Nicotiana plumbaginifolia* and tobacco BY-2 suspension culture cells, but it is not needed during G_1. Our cytokinin delay experiments suggest that mesophyll protoplasts do not require cytokinin for the first 13 h of culture. When cytokinin was withdrawn from the cultures at 10 h none of the protoplasts divided, but if cytokinin was withdrawn at 19 h or 24 h 50% or 65% divided. Because 19 h is approximately at about the G_1–S border in the fastest developing protoplasts, our interpretation is that cytokinin is needed for completion of G_1 and that protoplasts that had already entered S phase when cytokinin was withdrawn complete G_2 and divide. It is also possible that by 19–24 h some of the protoplasts had accumulated a sufficient store of cytokinin to get them through a cytokinin-requiring step in G_2. However, if that were the case some of the protoplasts in the 19- and 24-h cytokinin withdrawal samples would be expected to divide a second time, and none did. Regardless of the situation in G_2, our results and those of Trehin et al. (1998) point to a requirement for cytokinin before the onset of S phase in mesophyll protoplasts, which is not found in suspension culture cells. This difference in cytokinin requirement may reflect the fact that mesophyll protoplasts are nondividing when placed in culture and have to reinitiate the cell cycle, whereas suspension culture cells are actively proliferating. The stress of protoplast preparation could also lead to an additional cytokinin requiring step that is not seen in the other systems.

Our data support and extend the suggestions of Hemerly et al. (1993) and Trehin et al. (1998) that *cdc2* expression is activated before S phase; in fact, we detected increasing levels of *cdc2* by 12 h of culture, which is 6 h before S phase. It is unclear whether the *cdc2* expression assayed here, or that studied by Hemerly et al. or Trehin et al., represents a cyclin-dependent kinase (CDK) involved in the G_1 to S or the G_2 to M transition. Tobacco contains at least two *cdc2*-like genes (Carle and Bates unpublished results, Setiady et al. 1996), whereas *Petunia* may contain as many as four *cdc2*-like genes (Bergounioux et al. 1992). Because we used a region of high homology as a *cdc2* probe, it is unlikely that the expression we observe is gene specific. However, the timing of *cdc2* expression and the lack of expression in hormone-free media are consistent with this CDK having a role early in reinitiation of the cell cycle. Although the timing of *cdc2* expression is coincident with the timing of the cytokinin requirement, the fact that it is partially activated by either auxin or cytokinin alone suggests that *cdc2* expression is not the primary target of either hormone. We found that auxin was slightly more effective than cytokinin in activating *cdc2* expression. Hemerly et al. (1993) observed the opposite relationship, whereas Trehin et al. (1998) found that auxin alone could not activate *cdc2* expression in *Petunia* protoplasts. These difference may reflect the fact that Hemerly et al. were studying the expression of an *Arabidopsis cdc2* promoter in tobacco rather than a homologous tobacco *cdc2* promoter, or it may be because they assayed *cdc2* expression late during protoplast culture (72 h vs 24 h in our study). Although Trehin et al. examined expression of *cdc2* at the same time we did, they used a nearly full-length *cdc2* probe instead of a region of high homology to all *cdc2* genes. It is possible that different *cdc2* genes display differential sensitivity to the hormones. Both our results and those of Trehin et al. (1998) show that both auxin and cytokinin were required for full expression of *cdc2* in mesophyll protoplasts. These studies suggest that either auxin or cytokinin alone may be sufficient to activate the expression of *cdc2;* however, both hormones are needed for full expression.

Most of the work on cell cycle reinitiation in mesophyll protoplasts has focused on the role of auxin because auxin acts rapidly in this system and induces gene

expression (Meyer et al. 1984a, Takahashi et al. 1993). However, understanding the role of plant hormones during reactivation of cell division will require elucidation of the role of cytokinin as well auxin. Our hormone delay experiments suggest that there are separate signal transduction pathways leading from auxin and from cytokinin to reactivation of the cell cycle machinery and that these pathways converge in mid G_1 of the first cell cycle. The observation that both auxin and cytokinin partially induce *cdc2* expression may indicate that multiple signal transduction pathways are involved in cell cycle reactivation. In a study of *Nicotiana* suspension culture cells, Dominov et al. (1992) have proposed that cytokinin reinforces the effect of auxin in cell division by blocking feedback inhibition of the auxin response pathway. This observation could explain why cytokinin plus auxin supports a higher level of *cdc2* expression in protoplasts than auxin alone, but it fails to explain the cytokinin-induced expression of *cdc2*. What remains to be detected in protoplasts is a distinct molecular response to cytokinin alone before S phase. Recently, Ehness and Roitsch (1997) demonstrated cytokinin-induced expression of an extracellular invertase and a glucose transporter in *Chenopodium* suspension culture cells. They suggest that these genes may underlie the role of cytokinin in cell division. Direct evidence for this proposal has not yet been presented, and whether cytokinin has the same role in cell cycle reinitiation in mesophyll protoplasts and cell cycle maintenance in suspension culture cells remains unclear.

Acknowledgments. We thank Dr. David Droney for help with the statistical analysis. This work was supported in part by BARD Project IS-1631-89, USDA NRICGP Grant 93370193-37301-9569, a Florida State University COFRS grant (to G. W. B.), a Sigma Xi grant-in-aid of research (to S. A. C.), and a Howard Hughes medical institute grant (to T. A. S.).

References

Avery GS (1933) Structure and development of the tobacco leaf. Am J Bot 20:565–592

Bergounioux C, Perennes C, Hemerly A, Quin L, Sarda C, Inze D, Gadal P (1992) A *cdc2* gene of *Petunia hybrida* is differentially expressed in leaves, protoplasts and during various cell cycle phases. Plant Mol Biol 20:1121–1130

Chaboute M, Chaubet N, Clement B, Gigot C, Philipps G (1988) Polyadenylation of histone H3 and H4 mRNAs in dicotyledonous plants. Gene (Amst) 71:217–223

Cooke R, Meyer Y (1981) Hormonal control of tobacco protoplast nucleic acid metabolism during in vitro culture. Planta 152:1–7

Das NK, Patau K, Skoog F (1956) Initiation of mitosis and cell division by kinetin and indoleacetic acid in excised tobacco pith tissue. Physiol Plant 9:640–651

Dhillon S, Miksche J (1981) DNA changes during sequential leaf senescence of tobacco (*Nicotiana tabacum*). Physiol Plant 51: 291–298

Digby J, Firn R (1985) Growth substances and leaf growth. In: Baker N, Davies W, Ong C (eds) Control of leaf growth. Cambridge University Press, Cambridge, pp 57–76

Dominov JA, Stenzler L, Lee S, Schwarz JJ, Leisner S, Howell SH (1992) Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. Plant Cell 4:451–461

Ehness R, Roitsch T (1997) Coordinated induction of mRNAs for extracellular invertase and a glucose transporter in *Chenopodium ruburm* by cytokinins. Plant J 11:539–548

Feinburg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Ferreira P, Hemerly A, Villarroel R, Van Motagu M, Inzé D (1991) The *Arabidopsis* functional homolog of the $p34^{cdc2}$ protein kinase. Plant Cell 3:531–540

Fleck J, Durr A, Fritsch C, Lett MC, Hirth L (1980) Comparison of proteins synthesized in vivo and in vitro by mRNA from isolated protoplasts. Planta 148:453–454

Frearson EM, Power JB, Cocking EC (1973) The isolation, culture and regeneration of *Petunia* leaf protoplasts. Dev Biol 33:130–137

Galbraith DW (1984) Flow cytometric analysis of the cell cycle. In: Vasil IK (ed) Cell culture and somatic cell genetics of plants. Vol 1. Academic Press, New York, pp 765–777

Galbraith DW, Mauch TJ, Shields BA (1981) Analysis of the initial stages of plant protoplast development using 33258 Hoechst: Reactivation of the cell cycle. Physiol Plant 51:380–386

Grosset J, Marty I, Chartier Y, Meyer Y (1990) mRNAs newly synthesized by tobacco mesophyll protoplasts are wound inducible. Plant Mol Biol 15:485–496

Hashimoto J, Hirabayashi T, Hayano Y, Hata S, Ohashi Y, Suzuka I, Utsugi T, Toh EA, Kikuchi Y (1992) Isolation and characterization of cDNA clones encoding *cdc2* homologues from *Oryza sativa:* A functional homologue and cognate variants. Mol Gen Genet 233:10–16

Hemerly AS, Ferreira P, Engler JDA, Montagu MV, Engler G, Inzé D (1993) *cdc2A* expression in *Arabidopsis* is linked with competence for cell division. Plant Cell 5:1711–1723

Hirt H, Pay A, Gyorgyey J, BaKo L, Nemeth K, Bogre L, Schweyen RJ, Heberle-Bors E, Dudits D (1991) Complementation of a yeast cell cycle mutant by an alfalfa cDNA encoding a protein kinase homologous to $p34^{cdc2}$. Proc Natl Acad Sci USA 88: 1636–1640

Kapros T, Bögre L, Németh K, Bako L, Györgyey J, Wu S-C, Dudits D (1992) Differential expression of histone H3 gene variants during cell cycle and somatic embryogenesis in alfalfa. Plant Physiol 98:621–625

Klee H, Estelle M (1991) Molecular genetic approaches to plant hormone biology. Annu Rev Plant Physiol Plant Mol Biol 42:529–551

Laureys F, Dewitte W, Witters E, Van Montagu M, Inzé D, Van Onckelen H (1998) Zeatin is indispensable for the G_2–M transition in tobacco BY-2 cells. FEBS Lett 426:29–32

Lepetit M, Ehling M, Chaubet N, Gigot C (1992) A plant histone gene promoter can direct both replication-dependent and -independent gene expression in transgenic plants. Mol Gen Genet 231: 276–285

Meyer Y, Aspart L, Chartier Y (1984a) Auxin-induced regulation of protein synthesis in tobacco mesophyll protoplasts cultivated in vitro. I. Characteristics of auxin-sensitive proteins. Plant Physiol 75:1027–1033

Meyer Y, Aspart L, Chartier Y (1984b) Auxin-induced regulation of protein synthesis in tobacco mesophyll protoplasts cultivated in

vitro. II. Time course and level of auxin control. Plant Physiol 75:1034–1039

Meyer Y, Cooke R (1979) Time course of hormonal control of the first mitosis in tobacco mesophyll protoplasts cultured in vitro. Plant 147:181–185

Miao G, Hong Z, Verma DPS (1993) Two functional soybean genes encoding $p34^{cdc2}$ protein kinase are regulated by different plant developmental pathways. Proc Natl Acad Sci USA 90:943–947

Nagata T, Takebe I (1970) Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta 92:301–308

Nagy JI, Maliga P (1976) Callus induction and plant regeneration from protoplasts of *Nicotiana sylvestris.* Z Pflanzenphysiol 78:453–455

Perry K, Palukaitis P (1990) Transcription of tomato ribosomal DNA and the organization of the intergenic spacer. Mol Gen Genet 221:102–112

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Harbor Laboratories, Cold Spring Harbor, NY

Schmidt-Puchta W, Kutemeier G, Gunther I, Haas B, Sanger H (1989) Cloning and sequence analysis of the 18 S ribosomal RNA gene of tomato and a secondary structure model for the 18 S rRNA of angiosperms. Mol Gen Genet 219:17–25

Schowalter DB, Sommer SS (1989) The generation of radiolabeled DNA and RNA probes with polymerase chain reaction. Anal Biochem 177:90–94

Setiady YY, Sekine M, Hariguchi N, Kouchi H, Shinmyo A (1996) Molecular cloning and characterization of a cDNA clone that encodes a cdc2 homolog from *Nicotiana tabacum.* Plant Cell Physiol 37:369–376

Takahashi Y, Hasezawa S, Kusaba M, Nagata T (1995) Expression of the auxin-regulated *parA* gene in transgenic tobacco and nuclear localization of its gene products. Planta 196:111–117

Takahashi Y, Ishida S, Nagata T (1993) Functions and modulations of auxin-regulated genes. J Plant Res 106:357–367

Trehin C, Planchais S, Glab N, Perennes C, Tregear J, Bergounioux C (1998) Cell cycle regulation by plant growth regulators: Involvement of auxin and cytokinin in the re-entry of *Petunia* protoplasts into the cell cycle. Planta 206:215–224

Vernet T, Fleck J, Durr A, Fritsch C, Pinck M, Hirth L (1982) Expression of the gene coding for the small subunit for ribulose biphosphate carboxylase during differentiation of tobacco plant protoplasts. Eur J Biochem 126:489–494

Wadsworth G, Redinbough M, Scandalois J (1988) A procedure for the small-scale isolation of plant RNA suitable for RNA blot analysis. Anal Biochem 172:279–283

Zelcher A, Galun E (1976) Culture of newly isolated tobacco protoplasts: Precursor incorporation into protein, RNA, and DNA. Plant Sci Lett 7:331–336

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