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Rhizobium Nod Factors Reactivate the Cell Cycle during Infection and Nodule Primordium Formation, but the Cycle Is Only Completed in Primordium Formation

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Rhizobia induce the formation of root nodules on the roots of leguminous plants. In temperate legumes, nodule organogenesis starts with the induction of cell divisions in regions of the root inner cortex opposite protoxylem poles, resulting in the formation of nodule primordia. It has been postulated that the susceptibility of these inner cortical cells to *Rhizobium* nodulation (Nod) factors is conferred by an arrest at a specific stage of the cell cycle. Concomitantly with the formation of nodule primordia, cytoplasmic rearrangement occurs in the outer cortex. Radially aligned cytoplasmic strands form bridges, and these have been called preinfection threads. It has been proposed that the cytoplasmic bridges are related to phragmosomes. By studying the in situ expression of the cell cycle genes *cyc2*, *H4*, and *cdc2* in pea and alfalfa root cortical cells after inoculation with *Rhizobium* or purified Nod factors, we show that the susceptibility of inner cortical cells to *Rhizobium* is not conferred by an arrest at the G_2 phase and that the majority of the dividing cells are arrested at the G_0/G_1 phase. Furthermore, the outer cortical cells forming a preinfection thread enter the cell cycle although they do not divide.

INTRODUCTION

Rhizobia have the potential to induce the formation of a new organ, the root nodule, on the roots of leguminous plants. In these nodules, rhizobia are hosted by the plant and they reduce atmospheric nitrogen into ammonia (Newcomb, 1981; Hirsch, 1992). In temperate legumes, such as alfalfa and pea, nodule organogenesis starts with the induction of cell divisions in the inner cortical cell layers of the root (Libbenga and Harkes, 1973; Newcomb et al., 1979; Hirsch, 1992). These cells are fully differentiated and do not divide during normal plant development. Especially the inner cortical cells opposite protoxylem poles are susceptible to *Rhizobium* signals (Bond, 1948; Oinuma, 1948; Libbenga and Harkes, 1973). The dividing cortical cells form a nodule primordium; after penetration of the primordial cells by an infection thread, a meristem is formed at the distal part of the primordium, and this meristem produces cells that differentiate into the different nodule tissues (Libbenga and Bogers, 1974; Newcomb, 1981).

Concomitantly with the formation of a nodule primordium, *Rhizobium* induces the formation of infection threads by which the bacteria enter the plant. During the infection process, morphological changes are induced in the outer cortex of the root. Bakhuizen (1988) has shown that prior to infection thread penetration the nuclei move to the center of the outer cortical cells, and the cytoplasmic strands form bridges with a radial alignment. Because the infection thread will traverse the cortical cells by these radially aligned cytoplasmic bridges, they have been named preinfection threads (van Brussel et al., 1992). During the formation of the cytoplasmic bridges, microtubules rearrange in a manner similar to phragmosomes during mitosis. Therefore, it has been proposed that the cytoplasmic bridges are related to phragmosomes and that the outer cortical cells forming a preinfection thread enter the cell cycle, but because kinesis does not occur, they are supposed to become arrested in the G_2 phase (Bakhuizen, 1988; Kijne, 1992). Therefore, the processes induced by *Rhizobium* in both the inner and outer cortex of the root most likely require that fully differentiated root cells reenter the cell cycle, and the questions of how *Rhizobium* reactivates these cells and what determines the susceptibility of these cortical cells arise.

In recent years, a refined picture of eukaryotic cell cycle regulation has emerged. At the center of this regulatory network is the $p34^{cdc2}$ protein kinase, originally identified in fission and budding yeast. In yeast, a single kinase provides the functions required for both the G_1/S and G_2/M transitions (for reviews, see Nurse, 1990; Nasmyth, 1993), but in animals (for review, see Pines, 1993) and plants (for review, see Hirt and Heberle-

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Bors, 1994) several related kinases (including Cdc2 kinase), called cyclin-dependent protein kinases (CDKs), have evolved.

CDKs are not active as monomers (Poon et al., 1993), but only become active when associated with the cyclin regulatory subunit. The complex of CDK ($p34^{cdc2}$) and the cyclin that controls the G_2/M transition is named mitosis promoting factor (MPF) (Masui and Markert, 1971). Cyclins activate CDKs in a cell cycle, stage-specific manner. The stage specificity of cyclins is ensured mainly by their oscillating appearance in specific cell cycle stages. This is accomplished by regulation of the expression (Amon et al., 1993) and specific degradation of the respective cyclins (Glotzer et al., 1991; Tyers et al., 1992). There are, however, cyclins that do not change in abundance during the cell cycle, such as the *Saccharomyces cerevisiae* CLN3 (Tyers et al., 1993), the *Schizosaccharomyces pombe* Clg1 and Cdc13 (Bueno et al., 1991), and the animal C-, D-, and G-type cyclins (Leopold and O'Farrell, 1991; Lew et al., 1991; Xiong et al., 1991; Tamura et al., 1993).

In plants, *cdk* (*cdc2*) and cyclin genes have been isolated on the basis of sequence conservation (for review, see Hirt and Heberle-Bors, 1994). *cdk* genes have been identified from the dicotyledon species alfalfa (Hirt et al., 1991, 1993), Arabidopsis (Ferreira et al., 1991; Hirayama et al., 1991), pea (Feiler and Jacobs, 1990), petunia (Bergounioux et al., 1992), and soybean (Miao et al., 1993), as well as from the monocot species maize (Colasanti et al., 1991) and rice (Hashimoto et al., 1992). Several of these genes were shown to functionally substitute the yeast *cdc2* or *CDC28* genes. In radish and Arabidopsis, *cdc2* is expressed in dividing cells, such as the shoot and root meristem, but in contrast to the yeast and animal systems *cdc2* is also expressed in some nondividing cells such as the root pericycle cells. Because the root pericycle cells are mitotically reactivated when lateral root formation is initiated, it has been suggested that *cdc2* expression can also reflect the potential of a cell to divide (Martinez et al., 1992; Hemerly et al., 1993).

Cyclin clones have been isolated from alfalfa (Hirt et al., 1992), Antirrhinum (Fobert et al., 1994), Arabidopsis (Hemerly et al., 1992), and soybean (Hata et al., 1991). All of the sequences have homology to A- or B-type animal cyclins. In a functional assay for the G_2/M transition, the Arabidopsis and soybean cyclins were found to induce maturation after injection into Xenopus oocytes (Hata et al., 1991; Hemerly et al., 1992). Expression of the alfalfa and Antirrhinum cyclins was found to be restricted to cells in the G_2/M transition (Hirt et al., 1992; Fobert et al., 1994). Several other genes also have a strict cell cycle–specific expression in yeast and animal systems as well as in plants. For example, certain histone genes, such as *H2A* and *H3*, are specifically expressed at the end of the G_1 and S phases (Kapros et al., 1993; Tanimoto et al., 1993). Therefore, cyclin and histone genes are good molecular markers to study the induction of mitosis in cortical cells by *Rhizobium* and nodulation (Nod) factors.

The bacterial genes that play a key role in the induction of cell division and other early steps of nodulation are the so-called *nod* genes. The activity of the Nod proteins results in the synthesis and secretion of specific lipooligosaccharides named Nod factors. Purified Nod factors can mitotically reactivate cortical cells in a spatially controlled manner (Spaink et al., 1991; Truchet, 1991). The Nod factors of *R. meliloti* also stimulate cell divisions in an alfalfa suspension culture (Savouré et al., 1994). Furthermore, early nodulin genes (Nap and Bisseling, 1990) are expressed in such Nod factor–induced nodule primordia (Vijn et al., 1993), and preinfection threads are formed as well (van Brussel et al., 1992).

Why only specific cortical cells, namely inner cortical cells opposite a protoxylem pole, are mitotically activated even when roots are bathed in growth medium with Nod factor is not understood (Vijn et al., 1993). Several times it has been hypothesized that an arrest at a specific phase of the cell cycle determines the susceptibility of root cortical cells to *Rhizobium* and Nod factors. Wipf and Cooper (1938) first reported that a high percentage of disomatic (double number of chromosomes) mitosis occurs in the nodule meristems of several leguminous species, and they postulated that disomatic root cells in particular are mitotically activated by *Rhizobium*. This hypothesis has been supported by several other groups (see Libbenga and Bogers, 1974). More recently, Verma (1992) extended this hypothesis. He proposed that G_2-arrested cells in the root cortex maintain the phosphorylated inactive MPF. Nod factors are proposed to elicit *cdc25* expression, which leads to an activation of the MPF in the susceptible cells, whereas *cdc2* and cyclin gene expression is not required during the first divisions. Studies on *cdc2* expression have shown that certain cells intended with the potential to divide express *cdc2* (Hemerly et al., 1993). Therefore, it is possible that the susceptible cortical cells express *cdc2*. In this study, we used a cyclin B (*cyc2*), a histone (*H4*), and *cdc2* as probes to test these hypotheses. Furthermore, we studied whether outer cortical cells forming preinfection threads enter the cell cycle.

RESULTS

Cell Cycle Phase-Dependent Expression of *H4* and *cyc2*

To study the accumulation of *H4* and *cyc2* mRNA during the cell cycle, a synchronized alfalfa suspension culture was studied by RNA gel blot analysis, flow cytometry, and determination of the mitotic index (Figure 1). After aphidicolin treatment, 80% of the cells were arrested at the G_1/S phase transition point. Washing out the drug resulted in a rapid entry of the cells into S phase (80%, 3 hr) and high levels of *H4* mRNA accumulation. Entry of cells into G_2 (50%, 9 hr) resulted in low levels of *H4* mRNA but increased *cyc2* transcript levels. *cyc2* mRNA levels steadily increased through G_2 but declined sharply after onset of mitosis (15% mitotic index, 15 hr). In G_1 phase, low levels of *H4* and *cyc2* mRNA were detectable, but this is

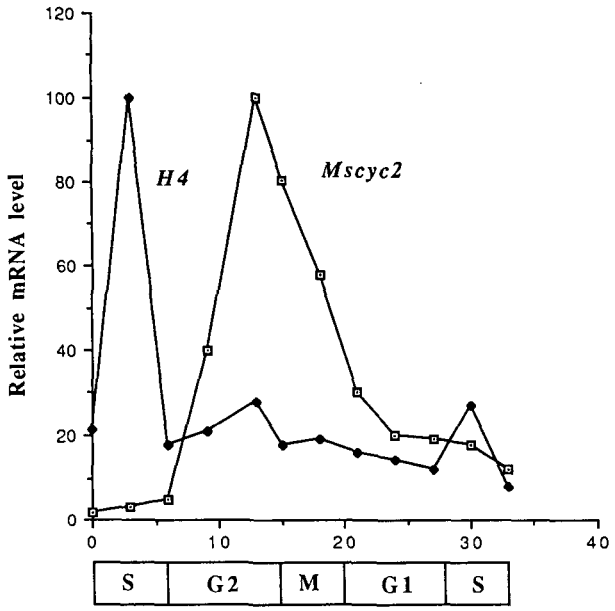

Figure 1. Cell Cycle–Dependent Expression of *H4* and *cyc2* in a Synchronized Suspension Culture of *M. varia.*

To determine the cell cycle stages, aphidicolin-arrested cells were released from the block and analyzed at different time points as indicated by flow cytometry as well as RNA gel blot analysis. Poly(A)$^+$ RNA was isolated from a synchronized *M. varia* suspension culture at different time points after aphidicolin treatment was completed. The same RNA gel blot was hybridized with radiolabeled *H4* and alfalfa *cyc2* (*Mscyc2*) fragments, respectively, and the hybridization signal was quantified by PhosphorImager analysis. The highest signal was arbitrarily designated 100%. The signal intensity representing relative mRNA level is plotted as a function of time. The corresponding cell cycle phases determined by flow cytometry are indicated by open bars.

probably a result of the cells that do not cycle synchronously. Upon entry of cells in the next S phase (50%, 30 hr), *H4* transcripts transiently accumulated. Our studies show that *H4* is a good marker for cells in S phase, whereas *cyc2* can be used to show that cells are at the transition of G_2 to M phase.

Cortical Cell Divisions

To investigate whether susceptibility of root cortical cells to *Rhizobium* mitogenic signals is determined by a G_2 phase arrest, we studied the expression of cell cycle genes in root cortical cells at different time points after inoculation with *Rhizobium* or purified Nod factors. Alfalfa seedlings were spot inoculated as described in Methods. To study the mechanism by which cell division is induced, spot-inoculated roots were harvested after 20 hr and before cell divisions occurred. Because the first divisions are only transversal (Libbenga and Harkes, 1973; Newcomb et al., 1979; Dudley et al., 1987), we analyzed longitudinal sections.

In Figures 2A, 3A, and 3C, examples of longitudinal sections of segments of alfalfa roots 20 hr postinoculation with rhizobia or Nod factors are shown. Roots inoculated with *Rhizobium* or purified Nod factors behaved similarly. In sections shown in Figures 2A, 3A, and 3C, several cortical cells contain a swollen nucleus that has migrated to the center of the cell at the site where the root was spot inoculated (the upper side of the sections shown in Figures 2A, 3A, and 3C), but none of the cortical cells has yet divided. The central position and the swollen appearance of the nuclei are the typical characteristics of root cortical cells that have been activated by *Rhizobium* but have not yet divided (Dudley et al., 1987; Bakhuizen, 1988). The sections shown in Figures 3A and 3C were hybridized with ^{35}S-labeled antisense *H4* mRNA probe, showing that *H4* is expressed in most cortical cells containing a swollen nucleus but that have not yet divided. Sections of seven different alfalfa roots were harvested 20 hr after spot inoculation with rhizobia (four roots) or purified Nod factors (three roots). They were analyzed for *H4* expression and ~60% of the cortical cells had a central nucleus containing *H4* mRNA. These *H4*-expressing cortical cells occurred only at the site that had been spot inoculated (Figures 3B and 3F). These results strongly suggest that G_0/G_1-arrested cells are reactivated by *Rhizobium* Nod factors.

Longitudinal sections of roots, 20 hr after inoculation with either *Rhizobium* or Nod factors, were hybridized with an antisense *cyc2* mRNA probe. An example is shown in Figures 2A and 2B. In general, the number of cells containing *H4* mRNA was found to be approximately five times higher than those containing *cyc2* mRNA. In the section shown in Figure 2B, one cortical cell containing a swollen nucleus expresses *cyc2*. *cdc2* expression was not studied in alfalfa roots 20 hr postinoculation, but studies with pea showed that this gene is induced during the first divisions in the cortical cells (see the following discussion).

In sections of uninoculated alfalfa roots, neither *H4* nor *cyc2* expression was detected in cortical cells. However, in several cambium cells of the vascular bundle of inoculated as well as control plants, both *cyc2* and *H4* are expressed (Figures 3D and 3E). Furthermore, hybridization with a sense *H4* or *cyc2* mRNA probe did not result in a signal above background level (data not shown).

To study the cell cycle events at a slightly later stage, sections were prepared from roots 2 days after inoculation with *Rhizobium*. At this stage, cells in the cortex have divided a few times, and a relatively broad primordium has been formed (Figures 2D and 2H). Hybridization with an antisense *cyc2* probe showed that the highest level of *cyc2* mRNA is present in cells at M phase (Figures 2D and 2E), which are indicated by the letters A, M, and P in the magnification shown in Figure 2C. In contrast, *cdc2* mRNA is present at a similar level in all

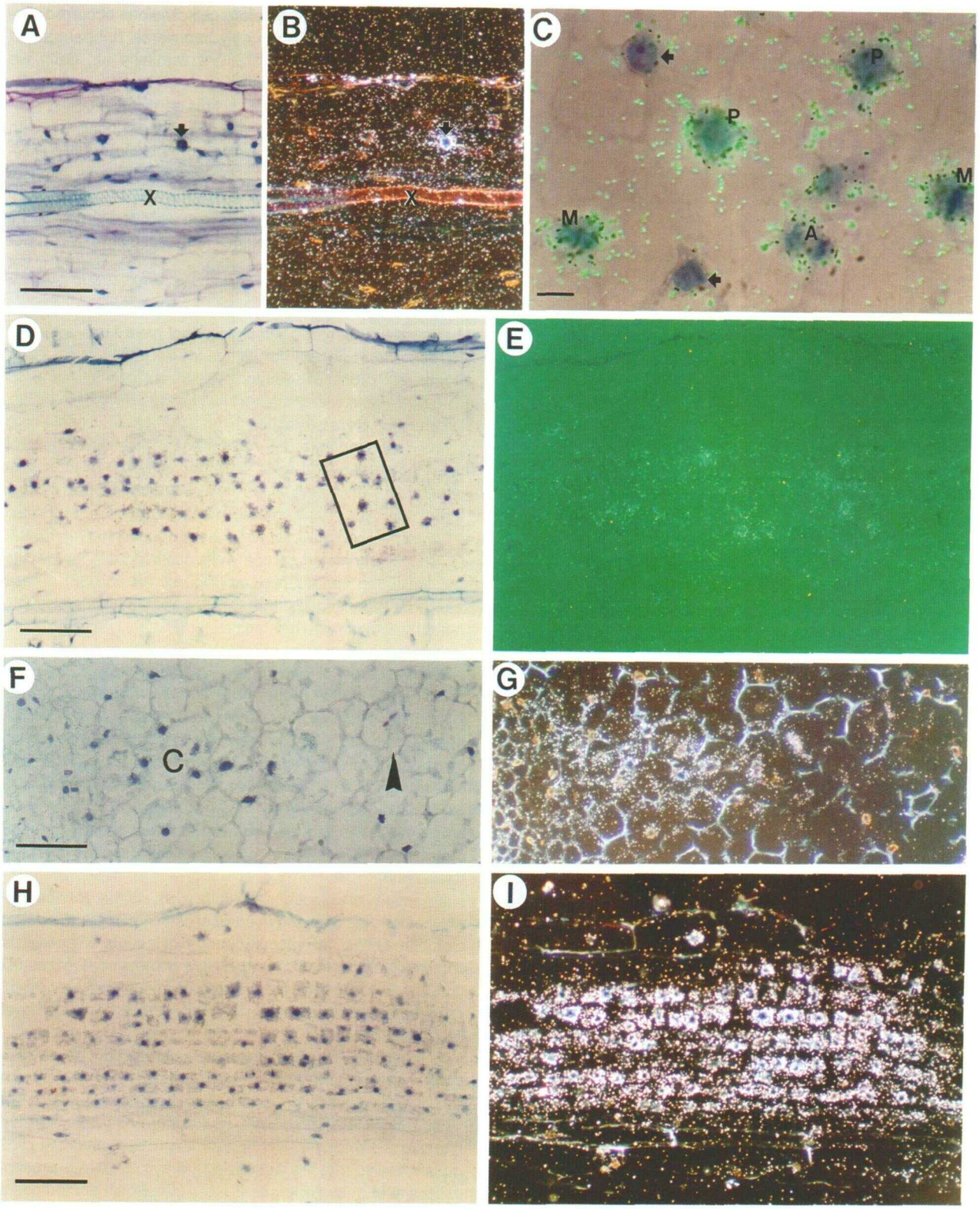

Figure 2. In Situ Localization of Cyc2 and Cdc2 Transcripts in Alfalfa and Pea Roots.

nodule primordium cells (Figures 2H and 2I). *H4* expression was studied in both alfalfa and pea nodule primordia. In both cases, a dispersed hybridization pattern was observed, and ~30% of the primordium cells contained H4 transcripts (Figures 3K and 3L). These results demonstrate that *cyc2* mRNA accumulates in a cell cycle phase–specific manner and the highest transcript level occurs during mitosis (M phase). *H4* is also expressed in a cell cycle–specific manner, whereas *cdc2* appears to be expressed at an equal level throughout the cell cycle.

cdc2 mRNA was detectable neither in root cortical cells at the time of spot inoculation (data not shown) nor in the uninfected root cortical cells that surround the nodule primordium (Figures 2H and 2I). However, *cdc2* is expressed in the root pericycle and cambium of both infected and uninoculated roots (data not shown).

Preinfection Threads

In pea roots, preinfection threads are formed in the outermost cell layers of the cortex, and these cells do not divide during nodulation (Figure 3G; Bakhuizen, 1988). In alfalfa, the formation of preinfection thread structures is less obvious. Cortical cells up to the hypodermal layer finally divide, and this might be the reason why it is hard to detect preinfection threadlike structures during alfalfa nodule formation. For this reason, we studied preinfection thread formation in pea only.

Serial cross-sections of pea roots, 1 day after inoculation, were made. An example is shown in Figure 3D. This part of the root is infected by *Rhizobium* at four different positions. These infection sites are characterized by the occurrence of a radial row of cells with prominent nuclei. It is noteworthy that three of these infection sites (I to III) are located exactly opposite a protoxylem point, while the infection site IV is positioned opposite a phloem and only involves a few cells, and therefore the infection might have been blocked. Magnifications of adjacent sections of the infection site I are shown in Figures 3G and 3I. By analyzing serial sections, the positions of the infection thread tips were identified at the four different infection sites. In one case, the infection thread has reached the outermost cortical cell layer (indicated by an arrowhead in Figure 3I), whereas in the other three cases the infection thread is still in an epidermal cell (data not shown).

The cross-sections were hybridized with an antisense *H4* mRNA probe, and as is shown in Figure 3E, *H4* mRNA is present in narrow radial rows of cortical cells in front of the infection thread tips. Even though several cells contain prominent nuclei, the organization of the cytoplasm is not clearly visible because tissues were paraffin embedded and the sections were pretreated with proteinase K before the in situ hybridization. For this reason, the cytoplasmic bridges are not readily apparent in these sections (Figures 3G and 3I). Cytological analysis of plastic-embedded, infected pea roots showed that the cytoplasmic rearrangements occur in three to four cells in front of the infection thread tip (Bakhuizen, 1988; W.-C Yang, unpublished results), indicating the expression of *H4* precedes the formation of cytoplasmic bridges. Because *H4* is expressed in the cells forming a preinfection thread (Figures 3H and 3J), they must have entered the cell cycle, although these cells do not progress through mitosis during nodulation.

By using a pea *cdc2* probe (pPscdc24RHa; see Methods), we showed that the level of *cdc2* mRNA is increased in cortical cells that form a preinfection thread as well as in dividing inner cortical cells (Figures 2F and 2G). In root cortical cells of uninoculated plants, *cdc2* expression is not detectable (data

Figure 2. (continued).

Alfalfa and pea seedlings were inoculated with *Rhizobium* as described in Methods. Longitudinal sections of alfalfa (**[A]** to **[E]**, **[H]**, and **[I]**) and cross-sections of pea (**[F]** and **[G]**) roots were analyzed for *cyc2* expression in **(A)** to **(E)** and *cdc2* expression in **(F)** to **(I)**. **(A)**, **(D)**, **(F)**, and **(H)** are bright-field micrographs in which dark dots represent hybridization signals. **(B)**, **(G)**, and **(I)** are dark-field micrographs in which bright grains represent hybridization signals. **(C)** is a combination of bright field and epipolarization; **(E)** is an epipolarization micrograph where shining dots are hybridization signals.

(A) A longitudinal section 20 hr after spot inoculation with *R. meliloti* showing an inner cortical cell having a swollen nucleus (arrow). The section was hybridized with the ^{35}S-labeled antisense *cyc2* mRNA probe. X, xylem. Bar = 50 μm.

(B) Dark-field micrograph of the section in **(A)** showing *cyc2* expression in the cell that is indicated by an arrow in **(A)**. X, xylem.

(C) An epipolarization/bright-field micrograph of the area boxed in **(D)** showing cell cycle–specific expression of *cyc2*. Note the two cells (arrows) do not contain a signal higher than background. A, anaphase; M, metaphase; P, prophase. Bar = 5 μm.

(D) A longitudinal section of an alfalfa root 48 hr after spot inoculation with *R. meliloti* showing a nodule primordium. This section was hybridized with the ^{35}S-labeled antisense *cyc2* mRNA probe. Bar = 50 μm.

(E) Epipolarization micrograph of the section in **(D)** showing a dispersed hybridization pattern.

(F) A cross-section of a pea root 1 day after inoculation with *R. l. viciae* hybridized with the pea ^{35}S-labeled antisense *cdc2* mRNA probe showing cortical cells (C) and cells forming a preinfection thread (arrowhead). Bar = 100 μm.

(G) Dark-field micrograph of the section in **(F)** showing *cdc2* expression.

(H) A serial section of the tissue in **(D)** was hybridized with the ^{35}S-labeled antisense *Mscdc2* mRNA probe. Bar = 100 μm.

(I) Dark-field micrograph of the section in **(H)** showing hybridization signals in all nodule primordial cells.

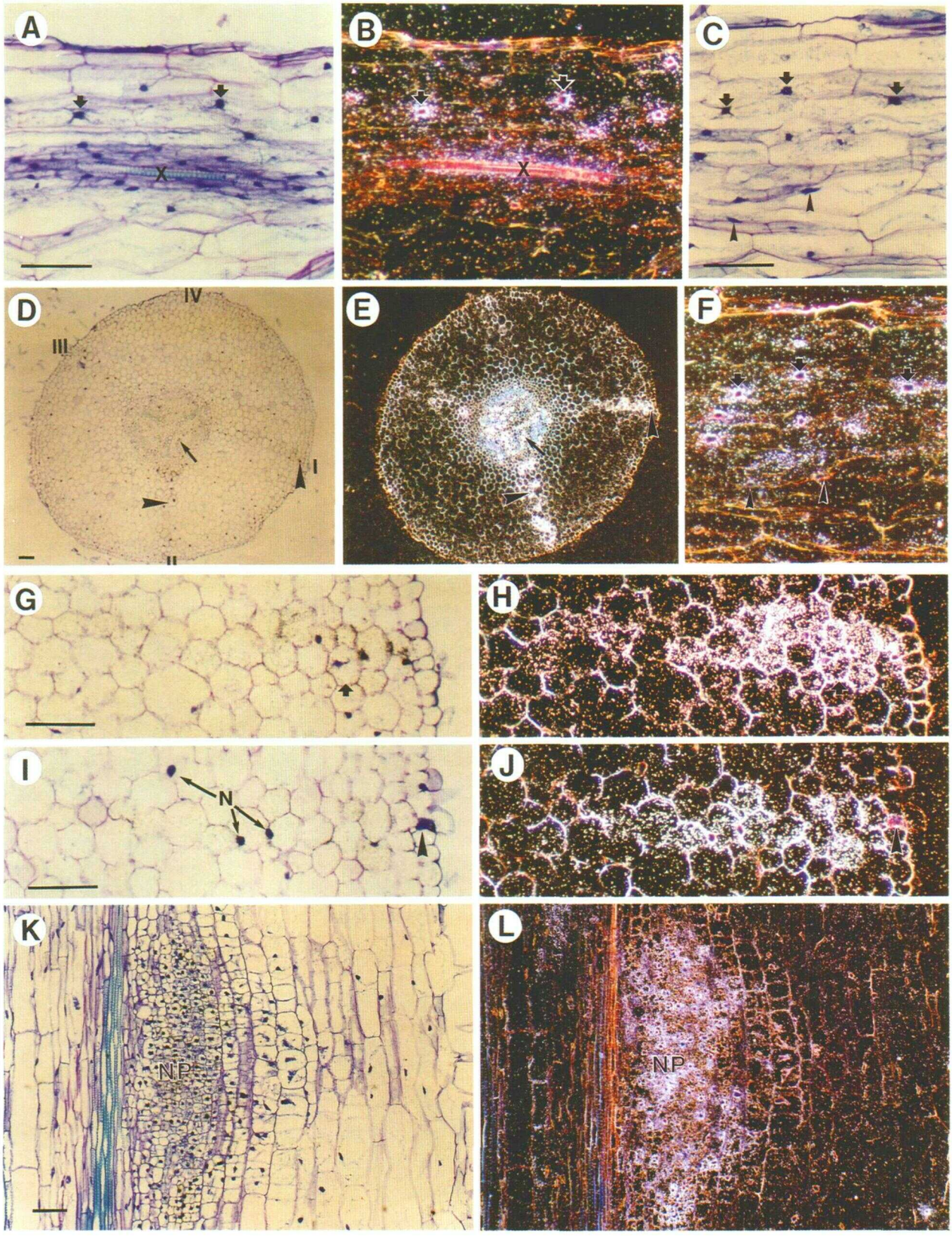

Figure 3. In Situ Localization of *H4* mRNA in Alfalfa and Pea Root Sections.

not shown). Therefore, it is unlikely that the susceptibility of inner cortical cells to *Rhizobium* is conferred by a constitutive *cdc2* expression.

Cross-sections of pea roots 1 day after inoculation were hybridized with a ^{35}S-labeled sense or antisense *PscycB* mRNA probe. Low hybridization signals were detected in the activated inner cortical cells, but no signal was detected in the preinfection thread structure (data not shown).

As is shown in Figures 3D and 3E, *H4* is also induced in the root inner cortical cells. Analysis of ~100 serial sections showed that most of these inner cortical cells had not yet divided (data not shown). This observation confirms the results with alfalfa showing that inner cortical cells express *H4* and thus had entered S phase before they divided for the first time.

To determine whether *H4* is transiently expressed in the outer cortex, we hybridized sections of pea roots 3 days after inoculation. As is shown in Figures 3K and 3L, *H4* mRNA is no longer detectable in the outer cortex, but a dispersed pattern of *H4*-expressing cells occurs in the now-formed nodule primordium (Figures 3K and 3L). At this stage, ~30% of the primordium cells contain *H4* mRNA, and this probably reflects the relative length of the S phase in the complete cell cycle. A similar percentage of cells expressing *H4* is found in Antirrhinum meristems (Fobert et al., 1994).

DISCUSSION

We studied the expression of the cell cycle–specific genes *H4*, *cdc2*, and *cyc2* at different stages of legume nodulation to address the following two questions. Is the susceptibility of root cortical cells to Nod factors controlled by an arrest at a specific stage of the cell cycle? Do root outer cortical cells that form a preinfection thread structure enter the cell cycle but become arrested at a stage before mitosis?

As a first step, we studied the expression of *cyc2*, *cdc2*, and *H4* during early stages of nodulation and in synchronized alfalfa suspension culture cells. These studies showed that *cyc2* is expressed at the highest level in cells that are in G_2 or mitosis and that the highest level of *H4* mRNA occurs during the S phase, whereas *cdc2* mRNA is present at an equal level throughout the cell cycle (L. Bögre, E. Heberle-Bors, and H. Hirt, unpublished results) and occurs in all nodule primordium cells. Our in situ hybridization data are consistent with the cell cycle–specific expression of these genes in Antirrhinum (Fobert et al., 1994), pea (Tanimoto et al., 1993), and alfalfa (Kapros et al., 1993). Thus, *H4* and *cyc2* show cell cycle–dependent expression in the plant and in in vitro suspension cultures. Because their expression is highly controlled during the cell cycle,

Figure 3. (continued).

Alfalfa and pea seedlings were inoculated with *Rhizobium* or Nod factor as described in Methods. Sections of alfalfa (**[A]**, **[B]**, **[C]**, and **[F]**) and pea (**[D]**, **[E]**, and **[G]** to **[L]**) roots were analyzed for *H4* expression. **(A)**, **(C)**, **(D)**, **(G)**, **(I)**, and **(K)** are bright-field micrographs in which dark dots represent hybridization signals. **(B)**, **(E)**, **(F)**, **(H)**, **(J)**, and **(L)** are dark-field micrographs in which hybridization signals are visible as white dots.

(A) A longitudinal section of an alfalfa root 20 hr after spot inoculation with *R. meliloti* 2011 showing inner cortical cells with a central swollen nucleus (arrows) at the site of inoculation (upper side of the section). This section was hybridized with the ^{35}S-labeled antisense *H4* mRNA probe. X, xylem. Bar = 50 μm.

(B) Dark-field micrograph of the section in **(A)** showing *H4* expression. Note that only a few cortical cells at the site of inoculation contain silver grains (arrows). X, xylem.

(C) An oblique longitudinal section of an alfalfa root 20 hr after spot inoculation with 10^{-5} M NodRm-IV(C16:2, Ac, S) hybridized with the ^{35}S-labeled antisense *H4* mRNA probe. Several activated cortical cells with a swollen nucleus (arrows) and inactivated cells (arrowheads) are indicated. Bar = 50 μm.

(D) A cross-section of a pea root 1 day after inoculation with *R. l. viciae*. Four infection sites (positions I to IV) are visible. This section was hybridized with the ^{35}S-labeled antisense *H4* mRNA probe. Arrowheads indicate cortical cells with a central nucleus. The arrow indicates cambium cells. Bar = 100 μm.

(E) Dark-field micrograph of the section in **(D)** showing localization of the *H4* transcript in cortical cells at and in front of the infection sites. Note positions I to III are opposite one of the protoxylem poles. Arrowheads show expression of *H4* in rhizobia-activated cortical cells. The arrow indicates *H4* expression in root cambium cells.

(F) Dark-field micrograph of the section in **(C)**. Hybridization signals are present in several activated cortical cells (arrows). Note lack of signals in inactivated cells (arrowheads).

(G) A serial section of the tissue in **(D)** was hybridized with the ^{35}S-labeled antisense *H4* mRNA probe. The arrow indicates a preinfection thread. Bar = 100 μm.

(H) Dark-field micrograph of the section in **(G)** showing localization of *H4* mRNA in the cells (arrow) forming a preinfection thread.

(I) A serial section of the tissue in **(D)** and **(G)** hybridized with the ^{35}S-labeled *H4* antisense mRNA probe. Arrowhead indicates the position of the infection thread. Two nuclei of cells forming a preinfection thread and one of a cortical cell are marked (N). Bar = 100 μm.

(J) Dark-field micrograph of the section in **(I)** showing *H4* expression in the cells forming a preinfection thread in front of the infection thread (arrowhead).

(K) A longitudinal section of a pea root 3 days after inoculation with *Rhizobium*. This section was hybridized with the ^{35}S-labeled antisense *H4* mRNA probe. NP, nodule primordium. Bar = 100 μm.

(L) Dark-field micrograph of the section in **(K)** showing a dispersed pattern of *H4* expression in nodule primordial cells (NP). Note lack of signals in the outer cortical cells.

they are very useful as markers to determine in situ the cell cycle phase of a root cortical cell, especially when they are combined with *cdc2*.

Cortical Cell Divisions

To study whether the susceptibility of root cortical cells to *Rhizobium* is determined by an arrest at a specific stage of the cell cycle, we used spot inoculation and in situ hybridization to follow the expression of cell cycle genes in root cortical cells. Plants were inoculated with *Rhizobium* or purified Nod factors, and expression of cell cycle genes was observed before the first cortical cell division. Our analyses showed that upon inoculation *H4* is induced in inner cortical cells before the first divisions take place. Approximately 60% of the cells with a centrally localized nucleus contain *H4* mRNA. Hence, we concluded that the majority of the root cortical cells that start to divide first pass the S phase before mitosis and thus were G_0/G_1 arrested at the time of inoculation. This proves that the susceptibility of root cortical cells is not determined by a G_2-arrested status.

All previous studies (see Libbenga and Bogers, 1974) that led to the hypothesis that a G_2 arrest confers susceptibility to *Rhizobium* involve measurements of DNA content or analyses of mitotic figures in cells of relatively old nodule primordia or of mature nodules. From these studies, the conclusion was drawn that the root cortical cells that become mitotically activated are disomatic cells, although root cortical cells were never studied during the first round of *Rhizobium*-induced cell divisions. Most likely the observed disomatic nodule cells result from endoreduplication that does not occur until a nodule meristem is formed (Libbenga and Bogers, 1974; Truchet, 1978).

Truchet (1978) measured the DNA content of cells of young pea nodule primordia 3 days after inoculation and found that these cells were monosomatic. A few days later, disomatic cells occur in the young nodules (Torrey and Barrios, 1969; Truchet, 1978), which are most likely formed by endoreduplication in monosomatic primordium cells (Truchet, 1978). Therefore, our studies on the induction of *H4* in root cortical cells in combination with the measurements of the DNA content in young primordium cells by Truchet (1978) strongly suggest that G_0/G_1–arrested monosomatic cells are reactivated by *Rhizobium* Nod factors.

cyc2 is induced by *Rhizobium* in cortical cells before they divide for the first time. Only ~10% of the cortical cells containing a central nucleus express *cyc2*, whereas ~60% of the cells contain *H4* mRNA. However, in nodule primordia (this study) as well as in Antirrhinum shoot and root meristems (Fobert et al., 1994), the number of cells containing *H4* mRNA is also approximately five times higher than those containing cyclin mRNA, and this probably reflects the relative time period during the cell cycle that these two genes are active. Therefore, our data on *cyc2* induction are consistent with the fact that *cyc2* is activated before a cortical cell divides. Furthermore, we showed that *cdc2* is activated in dividing cells; therefore, it is not likely that the susceptible cortical cells still contain sufficient MPF to support cell division as was proposed by Verma (1992).

Recently, it was postulated that *cdc2* expression in nondividing plant cells, similar to the root pericycle, contributes to the potential of these cells to divide (Hemerly et al., 1993). Here, we show that *cdc2* is induced in cortical cells that divide after inoculation with *Rhizobium*, whereas *cdc2* mRNA is not detectable in cortical cells of uninoculated roots. Hence, we concluded that the cortical cells that have the potential to divide after inoculation with *Rhizobium* do not have a higher *cdc2* expression level than the nonsusceptible cells.

Our studies show that susceptibility of cortical cells to *Rhizobium* Nod factors is not determined by an arrest at a specific stage of the cell cycle; other mechanisms have to determine this susceptibility. Cell divisions in the root cortex are induced by *Rhizobium* in a spatially controlled manner. In temperate legumes, the inner cortical cells located opposite protoxylem poles are most susceptible to *Rhizobium* (Libbenga and Bogers, 1974). Hence, it seems likely that positional cues from the stele control the susceptibility of the root cortical cells. The best candidate for a molecule that could provide this positional information is the so-called stele factor (Libbenga and Bogers, 1974). It has been shown that this compound can trigger cell divisions in the cortex of pea explants, and it is probably released from the protoxylem poles (Libbenga and Bogers, 1974; Smit et al., 1993). Hence, it seems probable that the mitotic reactivation of cortical cells involves the interplay of two oppositely oriented morphogen gradients. One morphogen is released by *Rhizobium* and is the Nod factor or a secondary signal generated by the Nod factor. The other morphogen is the stele factor that is released from the protoxylem poles. In Figures 3D and 3E, we showed that cortical cells in remarkably narrow radial rows reenter the cell cycle. The occurrence of such narrow rows of reactivated cells strongly supports the hypothesis that two oppositely oriented morphogen gradients control the reentry in the cell cycle.

Preinfection Thread

Previously, it has been shown that in a few cells in front of the infection thread tip the cytoplasm becomes radially aligned. Bakhuizen (1988) and Kijne (1992) postulated that when these cells enter the cell cycle a cell platelike structure is formed. Here, we show that in the pea outer cortical cells of the root that ultimately will form a preinfection thread *H4* expression is induced. Furthermore, the level of *cdc2* mRNA is increased but the pea *cyc2* homolog is not induced in these cells. Therefore, these outer cortical cells have entered the cell cycle, but most likely they become arrested in G_2 because the repression of the cyclin genes prevents the entry into mitosis. These observations suggest that part of the infection mechanism is derived from processes that control the cell cycle, and this makes it more easy to understand how Nod factors can trigger both infection-related processes and cell division.

Our observations show that cortical cells extending from the hypodermal layer to the innermost cortical cells reenter the cell cycle. Because the Nod factors secreted by *Rhizobium* induce preinfection thread formation as well as cortical cell divisions, these signal molecules have to induce the required cell cycle genes at an early stage. By a hitherto unresolved mechanism, the activated outer cortical cells become arrested in G_2, whereas the inner cortical cells continue the cell cycle.

METHODS

Plant Materials

Alfalfa (*Medicago sativa* cv AS13; Ferry Morse Seed Co., Mountain View, CA) seed were surface sterilized by incubation in 70% ethanol for 20 to 30 min, washed with sterile water and then in 0.1% (w/v) mercury chloride for 30 min, and finally washed five times with sterile water. The seed were germinated overnight at 21°C on an inverted Petri dish in the dark. Seedlings were transferred to 1% agar plate with BNM growth medium (Dudley et al., 1987; Cooper and Long, 1994) and grown at 21°C for 1 to 3 days with 16-hr-light and 8-hr-dark periods. The seedlings were then spot inoculated with *Rhizobium meliloti* 2011 (1×10^{10} cells per mL) according to Dudley et al. (1987) and Cooper and Long (1994) or with purified *R. meliloti* (nodulation) Nod factor NodRm-IV(C16:2, Ac, S) at a concentration of 1×10^{-5} M. *R. meliloti* 2011 was grown to late log phase in YEM medium (0.05% K_2HPO_4, 0.02% $MgSO_4$, 0.01% NaCl, 0.5% D-mannitol, 0.5% sodium-gluconate, 0.05% yeast extract, 0.16×10^{-7}% $CaCl_2$ [w/v], pH 6.9) in the presence of 10^{-9} M luteolin. NodRm-IV(C16:2, Ac, S) was purified from the culture medium of *R. meliloti* 2011 (a gift of J. Dénarié, CNRS-INRA, Toulouse, France) as described by Roche et al. (1991). Inoculation sites were marked with immersion oil containing charcoal.

Pea (*Pisum sativum* cv Rondo) seed were inoculated 3 days after sowing with *R. leguminosarum* bv *viciae* 248 as described by Scheres et al. (1990).

Isolation of Pea *H4*, *cdc2*, and a Partial Cyclin B cDNA Clones

During the process of screening a pea nodule cDNA library for clones representing genes that are expressed at elevated levels in nodules, we isolated an H4 cDNA clone (pPsH4) (W.-C. Yang and H. Franssen, unpublished data). For in situ hybridization, an ~200-bp XbaI fragment, representing the 5′ part of the insert of the pPsH4 cDNA clone, was subcloned into pBluescript KS+ yielding a plasmid pPsH4xs.

Several cDNA clones were isolated from a pea root hair cDNA library by using an alfalfa *cdc2*, *Mscdc2* clone (Hirt et al., 1991) as a probe. One of them was subcloned into pBluescript KS+ and designated pPscdc24, representing a full-length clone homologous to *Mscdc2* by sequence analysis. This cDNA clone was able to complement a yeast cell cycle–related *cdc2* mutant (data not shown). A 570-bp EcoRI-HindIII fragment containing the 5′ untranslated region and sequences coding for the first 140 amino acids of *cdc2* was then subcloned into a pBluescript KS+ vector yielding pPscdc24RHa.

A pea cDNA clone representing a cyclin B gene was isolated from a pea nodule cDNA library (Scheres et al., 1990) by polymerase chain reaction (PCR) using 5′-ATIC/TTIGTIGATTGGC/TTIGTIG/CAA/GGT as forward primer and 5′-TCTGGT/GGGA/GTAG/CATC/TTCC/TTCATATTT as the reverse primer representing conserved domains of amino acid sequences ILVDWLVE/QV and KYEEI/MYPPD/E, respectively, in cyclin B proteins (Hata et al., 1991).

Total phage DNA was isolated (Sambrook et al., 1989) and used to amplify cyclin B coding sequences in 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.2 mM deoxynucleotide triphosphates, 1 μM primers, and 0.2 units of Cetus (Perkin-Elmer) Taq polymerase. PCR was performed in a LEPREM PCR DNA thermal cycler (LEP Scientific) for 25 cycles of 94°C for 1 min, 45°C for 1.5 min, and 72°C for 1.5 min.

The reaction yielded DNA fragments of 200 bp, which were isolated and ligated into SmaI-digested pBluescript KS+ yielding pPscycB. The nucleotide sequence of the insert was determined (Sanger et al., 1977), and the insert displayed 89% identity to the comparable region in the soybean cyclin B gene (Hata et al., 1991).

In Situ Hybridization

Root segments were collected at different time points after inoculation and fixed in 4% paraformaldehyde supplemented with 0.25% glutaraldehyde in 10 mM sodium phosphate, 100 mM sodium chloride buffer, pH 7.2, for 4 hr (van de Wiel et al., 1990). Fixed material was dehydrated through routine ethanol series and embedded in paraffin.

Seven-micrometer-thick sections were hybridized with ^{35}S-UTP (1000 to 1500 Ci mmol^{-1}; Amersham International)–labeled antisense or sense mRNA probes according to a procedure derived from Cox and Goldberg (1988; and van de Wiel et al., 1990).

Preparation of mRNA Probes

To obtain the *H4* mRNA probe, the pPsH4xs plasmid was cut with SstI or BamHI and in vitro transcribed with T3 or T7 RNA polymerase to obtain antisense and sense mRNA probes, respectively.

pBluescript SK+ containing a 1.3-kb EcoRI-XbaI fragment of the alfalfa cDNA clone pMscyc2 (Hirt et al., 1992) was linearized with either XbaI or HindIII and in vitro transcribed with T3 or T7 RNA polymerase for sense and antisense cyclin mRNA probes, respectively. To obtain the *Mscdc2* mRNA probe, pMscdc2 containing a 500-bp SmaI fragment of the 5′ end of the insert (Hirt et al., 1991) was cut either with EcoRI and transcribed with T3 RNA polymerase for antisense mRNA synthesis or with BamHI and transcribed from the T7 promoter for sense mRNA synthesis.

For in situ hybridization, the pPscdc24RHa plasmid was linearized either with EcoRI and in vitro transcribed with T3 RNA polymerase for antisense mRNA production or with HindIII for sense mRNA synthesis with T7 polymerase. The pPscycB plasmid was linearized with either EcoRI or SacI and in vitro transcribed with T3 or T7 RNA polymerase, respectively, to obtain mRNA probes.

Plant Cell Culture Synchronization, Flow Cytometry, and ^{3}H-Thymidine Incorporation

A suspension culture of *M. varia* cv Rambler line A2 was used (Bögre et al., 1988). Subculturing was performed at 5-day intervals in 1:10 dilutions in Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid and 0.2 mg/L kinetin.

Synchronization of *M. varia* suspension culture cells was performed by treatment with aphidicolin (Sigma) for 24 hr. Aphidicolin was added after 0 and 12 hr to a final concentration of 10 μg/L at a cell density of 5 × 10^5 cells per mL. Cells were then washed five times with fresh medium and allowed to grow at the same density for the indicated times. The flow cytometric analysis was performed as described by Pfosser (1989) with slight modifications. Cells (1 × 10^5) were pelleted by centrifugation at 200*g* for 2 min, and 200 μL of enzyme solution (2% [w/v] cellulase Onozuka R10, 10% [w/v] pectinase [Sigma P5146], 0.6 M mannitol, 5 mM $CaCl_2$, 3 mM 2-[*N*-morpholino]ethanesulfonic acid, pH 5.7) was added to the cell pellet and incubated for 1 hr at 37°C to digest the cell wall. Released protoplasts were stained without being washed from the enzyme solution. Protoplasts were stained by mixing 100 μL of resuspended protoplasts with 400 μL of staining solution (10 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 4 μg/mL 4,6-diamino-2-phenylindole). Nuclei were released by passage through a needle, and the samples were subjected to flow cytometric analysis using a PAS2 flow cytometer (Partec, Münster, Germany).

The determination of the mitotic index was done by staining the intact cells in a solution containing 10 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 4 μg/mL 4,6-diamino-2-phenylindole and subsequent counting of the ratio of cells at M phase under the microscope.

RNA Extraction and Gel Blot Analyses

RNA isolation was performed according to Cathala et al. (1983). Poly(A)$^+$ RNA was isolated from 100 μg of total RNA with Dynabeeds according to the instructions of the manufacturer (Dynal, Oslo, Norway). Formamide agarose gel electrophoresis and RNA gel blot analyses were performed according to standard protocols (Sambrook et al., 1989). Radiolabeled probes were generated by random primed ^{32}P-labeling of inserts of the pMscyc2 (Hirt et al., 1992) or the pPsH4 (W.-C. Yang and H. Franssen, unpublished results). Hybridization signals were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

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