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 G2 phase and that the majority of the dividing cells are arrested at the Go/G1 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). 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 G2 phase and that the majority of the dividing cells are arrested at the Go/G1 phase. Furthermore, the outer cortical cells forming a preinfection thread enter the cell cycle although they do not divide.
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<sup>cdc2</sup>) and the cyclin that controls the G<sub>2</sub>/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* Cyc2 (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 cy-
Cell Cycle Genes and Root Nodule Initiation

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 (MscycZ) fragments, respectively, and the hybridization signal was quantified by PhosphorImager analysis. The highest signal was arbitrarily designated 1000/0. 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 G2 to M phase.

Cortical Cell Divisions

To investigate whether susceptibility of root cortical cells to Rhizobium mitogenic signals is determined by a G2 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 35S-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 G0/G1-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
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–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 protoxylom 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 (pPsdc24RhA; 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 not shown).

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 35S-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 35S-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 35S-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 35S-labeled antisense Msdc2 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 *PsycB* 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 G2 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. Bøgre, 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 vitro suspension cultures. Because their expression is highly controlled during the cell cycle,

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**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
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 $\sim$10% of the cortical cells containing a central nucleus express cyc2, whereas $\sim$80% 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 cyc2 mRNA and the $H4$ and cyc2 expression is oppositely oriented. 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 cyc2 gene prevents the entry into mitosis. 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 cyc2 gene prevents the entry into mitosis. These
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 G2, 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) mercuric chloride for 30 min, and finally washed five times with sterile water. The seed were germinated overnight at 21°C. The seedlings were then spot inoculated with *Rhizobium meliloti* 2011 was grown to late log phase in YEM medium (0.05% K2HP04, 0.05% yeast extract, 0.16 x 10⁻⁷ M CaCl₂ [w/v], pH 6.9) and grown at 21°C for 1 to 3 days with 16-hr-light and 8-hr-dark periods.

**Preparation of mRNA Probes**

To obtain the h4 mRNA probe, the pPsH4xs plasmid was cut with SstI and BamHI and in vitro transcribed with T3 or T7 RNA polymerase, respectively. The pPscycB plasmid was linearized with either EcoRI or SalI and in vitro transcribed with T3 or T7 RNA polymerase for sense and antisense cyclin mRNA probes, respectively.

**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 35S-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).

**Plant Cell Culture Synchronization, Flow Cytometry, and 3H-Thymidine Incorporation**

A suspension culture of *M. varia* cv Rambler line A2 was used (Bögere 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.


