The EMBO Journal vol.13 no.5 pp.1093–1102, 1994

Activation of the cell cycle machinery and the isoflavonoid biosynthesis pathway by active *Rhizobium meliloti* Nod signal molecules in *Medicago* microcallus suspensions

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Communicated by A.Kondorosi

We have shown that treatment of *Medicago* microcallus suspensions with the cognate *Rhizobium meliloti* Nod signal molecule NodRm-IV(C16:2,S) can modify gene expression both qualitatively and quantitatively. At concentrations of 10^{-6}–10^{-9} M, this host specific plant morphogen but not the inactive non-sulfated molecule stimulated cell cycle progression as indicated by the significantly enhanced thymidine incorporation, elevated number of S phase cells, increase in kinase activity of the p34^{cdc2}-related complexes and enhancement of the level of expression of several cell cycle marker genes, the histone *H3*-1, the *cdc2Ms* and the cyclin *cycMs2*. The presented data suggest that at least part of the physiological role of the Nod factor may be linked to molecular events involved in the control of the plant cell division cycle. *In situ* hybridization experiments with antisense *H3*-1 RNA probe indicated that only certain cells of the calli were able to respond to the Nod factor. High (10^{-6} M) but not low (10^{-9} M) concentrations of the active Nod factors induced the expression of the isoflavone reductase gene (*IFR*), a marker gene of the isoflavonoid biosynthesis pathway in most callus cells. Our results indicate that *Medicago* cell responses to the Nod signal molecules can be investigated in suspension cultures.

Key words: cell cycle/early nodulin/*Medicago*/Nod factor/phytoalexin biosynthesis

Introduction

The development of nitrogen-fixing root nodules involves the co-ordinated expression of both plant and bacterial genes (for review, see Fisher and Long, 1992). The bacterial common and host specific *nod* gene products determine the production of lipo-oligosaccharide Nod signal molecules. A sulfated and acylated β-1,4-tetrasaccharide of D-glucosamine, designated as NodRm-IV(C16:2,S), has been shown to be the major Nod factor in *Rhizobium meliloti* (Lerouge *et al.*, 1990). Recently, a number of related lipo-oligosaccharide Nod signal molecules, produced by *R.meliloti* (Roche *et al.*, 1991b; Schultze *et al.*, 1992), *R.leguminosarum* (Spaink *et al.*, 1991, 1992), *Rhizobium* sp. strain NGR234 (Price *et al.*, 1992) and *Bradyrhizobium japonicum* (Sanjuan *et al.*, 1992), have been identified. Specific modifications of the chito-oligosaccharide backbone appear to determine host specificity as well as biological activity of these Nod signal molecules (Roche *et al.*, 1991a; Spaink *et al.*, 1991; Schultze *et al.*, 1992). For example, in the case of the *R.meliloti* Nod factors, the presence of a sulfate group at the reducing end of the chito-oligosaccharide backbone is essential for activity on the host plant *Medicago*. In contrast, modification of the acyl chain by hydrogenation does not inactivate the Nod factor but renders it ~100-fold less active.

The Nod signals evoke various responses on their legume hosts. At pico- to nanomolar concentrations, purified *R.meliloti* NodRm and *R.leguminosarum* NodRlv factors induced, within a few hours or a day, deformation and branching of root hairs (Lerouge *et al.*, 1990; Roche *et al.*, 1991b; Spaink *et al.*, 1991; Schultze *et al.*, 1992) and expression of the early nodulin *Enod12* gene (Kondorosi *et al.*, 1992; Pichon *et al.*, 1993), identified from pea (Scheres *et al.*, 1990) and from diploid and tetraploid *Medicago* species (Pichon *et al.*, 1992; Allison *et al.*, 1993). The NodRlv factor elicits the formation of cytoplasmic bridges proposed to be pre-infection threads (van Brussel *et al.*, 1992). Both NodRm and NodRlv factors induce cell division in the inner cortex and formation of nodule primordia (Spaink *et al.*, 1991; Truchet *et al.*, 1991) which, in the case of alfalfa (*Medicago sativa*), give rise to the development of empty nodules (Truchet *et al.*, 1991). Though the perception and transduction mechanisms of the Nod signals are still unknown, these observations and studies of Schmidt *et al.* (1988) suggest that this signalling pathway may be related to a more direct effect on cell division.

Plant cells have the unique property of totipotency, and even highly differentiated cells can be stimulated to undergo cell division and organogenesis. The major control points of the cell cycle are located at the G_1–S and G_2–M transitions (Jacobs, 1992; Dudits *et al.*, 1993). One of the genes involved in the regulation of the cell cycle and traverse of these transition points is the *cdc2/CDC28* gene encoding the protein kinase p34^{cdc2}. The G_2–M transition is regulated by maturation promoting factor (MPF) which is composed of the p34^{cdc2} and the B type cyclin. Phosphorylation or dephosphorylation of p34 and the synthesis/degradation of cyclins provide at least two potential levels of regulation of the cell cycle progression (Jacobs, 1992).

In recent years, significant progress has been achieved in the isolation and characterization of cell cycle genes in higher plants (for review see Jacobs, 1992; Dudits *et al.*, 1993). In agreement with the concept of a unified eukaryotic cell cycle control, homologues of both *cdc2/CDC28* and cyclin genes have been cloned from a variety of plant species, including *Medicago* (Hirt *et al.*, 1991, 1992). Furthermore, phase specific *cdc2*-related kinase complexes were detected in cultured *Medicago* cells (Magyar *et al.*, 1993). Several studies have demonstrated that specific histone gene variants

are expressed in a cell cycle dependent manner and, therefore, can serve as markers in studies in the control of the cell cycle (Kapros *et al.*, 1992, 1993; Lepetit *et al.*, 1992).

In this paper, we describe a comprehensive analysis of the alteration of gene expression in the Nod factor-treated *Medicago* microcallus suspensions which provided more homogeneous and substantially higher amounts of the Nod factor responsive material than intact roots containing only few reacting cells. In addition to the detection of overall changes in transcription pattern, the flow cytometric analysis of nuclei and the demonstrated increases in thymidine incorporation, $p34^{cdc2}$ kinase activity and expression of selected cell cycle marker genes from *M.sativa* suggest that the cognate Nod factor stimulates cell cycle progression. Moreover, expression of the early nodulin *MsEnod12* gene and genes of the phenylpropanoid pathway were also investigated. The significance of Nod factor activated cell divisions in the reprogramming of root cells for nodule organogenesis, the possible mode of action of Nod factors in nodule initiation and the advantages of the use of cell cultures for these studies are discussed.

Results

NodRm-IV(C16:2,S) influences gene expression in both embryogenic and non-differentiated Medicago cell cultures

Rhizobia induce the formation of nodule primordia in the emerging root hair zone of the root at a few foci. Molecular analysis of Nod factor-induced cell division in the intact roots is very difficult due to the low number of Nod factor-responsive cells. Therefore, we tested whether *Medicago* suspension cultures could be used for this purpose. The suspension cultures were derived from callus tissues of RA3, an embryogenic line of *M.sativa* that consisted of dedifferentiated microcalli (MCS) grown in the presence of hormones or differentiated embryos that developed from MCS after a short auxin shock in hormone-free medium (Dudits *et al.*, 1991). Both the MCS and the embryogenic cultures contained not only tetraploid cells but also a high percentage of octoploid cells. Moreover, the MCS consisted of dividing and quiescent cells (data not shown).

In order to test the responsiveness of these cultures to Nod factor treatment, alterations of gene expression of cultured *Medicago* cells upon treatment with Nod factor was studied by two dimensional gel electrophoresis of the *in vitro* translation products of total RNA from the various samples. Embryogenic and MCS cultures were grown for 2, 5, 8, 24 and 48 h in the absence or presence of low concentrations (10^{-9} and 10^{-10} M) of NodRm-IV(C16:2,S), which were biologically active in the root hair deformation assay, and with a high concentration (10^{-6} M) of Nod factor which had an inhibitory effect on root hair deformation (Schultze *et al.*, 1992). The control cultures were supplied with water in the same volume as the Nod factor.

Total RNA isolated from the cells was translated *in vitro* in the presence of [^{35}S]methionine. Autoradiograms of the labelled proteins on two dimensional gels showed ~850 reproducible spots in all samples. In all cases, both in the non-differentiated and in the embryogenic cultures, the Nod factor treatment caused substantial quantitative as well as qualitative changes. By comparing spot intensities using the Bio Image analysis package, most frequently only quantitative changes were detected. Figure 1 shows an example for differential gene expression in the embryogenic culture after a 5 h incubation with water (a) or with 10^{-9} M Nod factor (b). The majority of changes affected only the level of gene expression. However, new protein spots as well as disappearance of those detected in the control cells were also found.

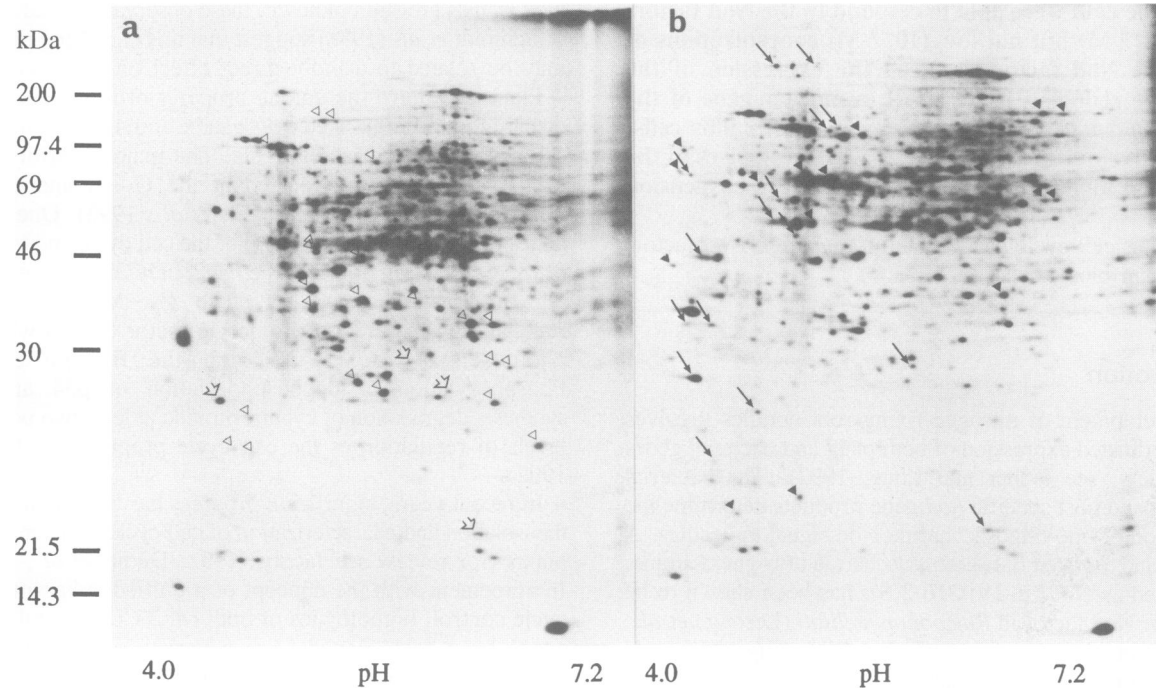

Fig. 1. 2D-gel analysis of the *in vitro* translation products of embryogenic *Medicago* cell cultures treated for 5 h either with water (**a**) or with 10^{-9} M NodRm-IV(C16:2,S) (**b**). Changes in the polypeptide patterns caused by the Nod factor are indicated as follows: →, new induced spots; enhanced spots; ⇨, repressed spots; ▷, diminishing spots.

A summary of changes in gene expression is given in Figure 2 for the embryogenic cultures treated with 10^{-6} M, 10^{-9} M or 10^{-10} M Nod factor. The spots altered in the Nod factor-treated samples could be divided into four classes, representing (i) induced new spots (I), (ii) repressed undetectable spots (R), (iii) enhanced spots (E) and (iv) spots with decreased intensity (D). The most significant changes relative to the control were observed at the shortest (5 h) incubation period where >30% of the spots differed from the control sample. In samples taken after longer periods of Nod factor treatment (8–24 h), only 15–20% of the protein spots differed from the control. At 24 h, both low and high Nod factor concentrations were assayed for gene expression. Gene expression was affected most strongly (17%) at 10^{-9} M, less (15%) at 10^{-6} M and least (8%) at 10^{-10} M Nod factor concentrations. Treatment for 48 h resulted again in more significant differences in gene expression and in the appearance of the highest number of new spots, indicating that the Nod factor had not only a transient but also a long term effect on plant gene expression.

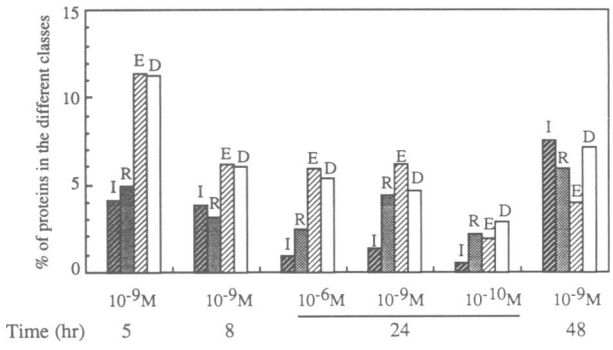

Fig. 2. Changes in the *in vitro* translation products elicited by Nod factor in the embryogenic culture. The 2D-gel patterns of protein spots after the different treatments were compared with the appropriate control supplied with water for the same incubation period. As described in Materials and methods the spots were grouped into four classes representing induced (new) (I, ■), repressed (undetectable) (R, ▩), enhanced (E, ▨) and decreased (D, □) spot intensities.

Parameters of cell cycle progression are modified by NodRm-IV(C16:2,S) in cultured Medicago cells

The finding that the purified Nod factor can induce the development of dividing foci in the inner cortex of the root (Truchet *et al.*, 1991) suggested that the Nod factor interacts (either directly or indirectly) with the cell cycle control elements. To analyse the possible involvement of the Nod factor in controlling the cell cycle, nuclei of MCS and embryogenic cultures treated with 10^{-6} M (F6) or 10^{-9} M (F9) concentrations of the cognate Nod factor NodRm-IV(C16:2,S) or with a 10^{-9} M concentration of the non-sulfated Nod factor (NS9) NodRm-IV(C16:2) were analysed by flow cytometry for the distribution of cells at different phases of the cell cycle. The frequencies of nuclei in G_1, S, and G_2–M phases of the tetraploid cycle in cells treated with Nod factors were compared with those of water-treated cells. Namely, for each time point, the frequencies observed in the treated cells were related to those of the control samples (=100). Figure 3 presents these relative frequencies of nuclei in cells treated with the sulfated and the non-sulfated Nod factors. Nuclei in both MCS and embryogenic cultures treated with the cognate Nod factor differed significantly from the control. After treatment for 5 h (Figure 3A), a significant increase of nuclei in the S phase was observed. In MCS, treatment with 10^{-6} M Nod factor (F6) was more effective, resulting in a 146% relative increase of nuclei in S phase (in other words, 2.46-fold higher than the control values), in contrast to the 46% relative increase at 10^{-9} M concentration (F9). In the embryogenic cultures, the 10^{-9} M concentration (F9) produced a higher relative increase (80%) of nuclei in S phase than did the control. In this latter culture treated with either low or high concentrations of Nod factor, the number of nuclei in G_2–M phase was significantly reduced which might reflect G_2–M transition and initiation of the G_1 phase of the tetraploid cycle or passage into the octoploid cycle. The difference in the distribution of nuclei between the control and the cognate Nod factor-treated cells was maintained even after a 3 day growth period (Figure 3B). In contrast, the number of nuclei in the different phases was very similar in cells treated with

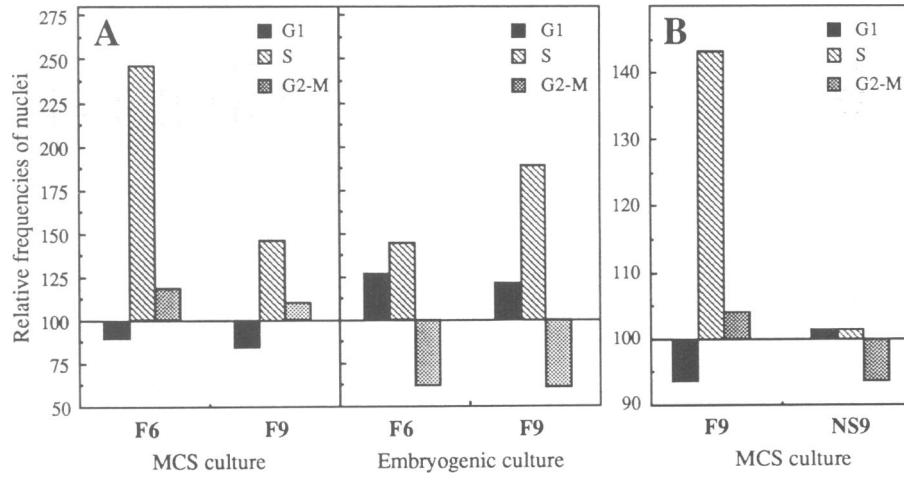

Fig. 3. Frequencies of nuclei in G_1, S and G_2–M compartments of the tetraploid cell cycle after treatment with NodRm-IV(C16:2,S) (F) or with NodRm-IV(C16:2) (NS) relative to water-treated controls (=100). Percentages of nuclei in the different phases were analysed by flow cytometry. The maximum standard deviation in the number of nuclei at the different phases was 8%. (**A**) MCS and embryogenic cultures treated for 5 h with 10^{-6} M (F6) or 10^{-9} M (F9) NodRm-IV(C16:2,S). (**B**) MCS treated with F9 and a 10^{-9} M (NS9) concentration of the non-sulfated Nod factor NodRm-IV(C16:2) for 72 h.

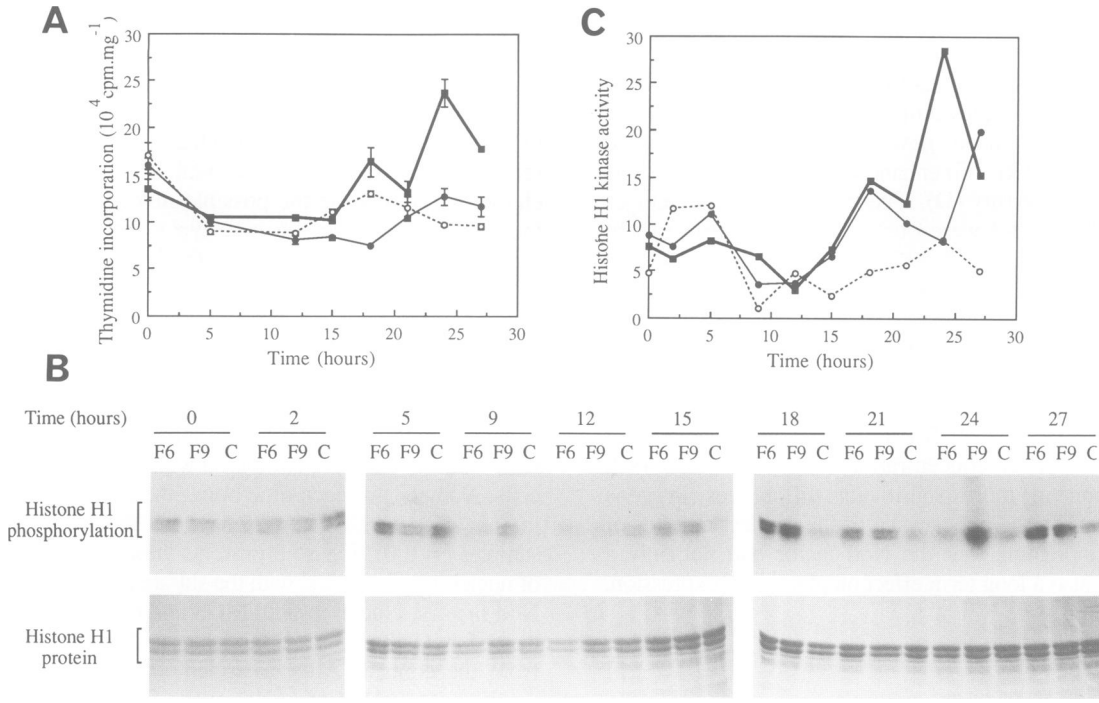

Fig. 4. Effects of NodRm-IV(C16:2,S) on thymidine incorporation and kinase activity of p34^{cdc2}-related complexes in MCS. (**A**) Thymidine incorporation of MCS treated with water (C) (---○---) or with 10^{-6} M (F6) (—●—) and with 10^{-9} M (F9) (—■—) NodRm-IV(C16:2,S) for different time periods. (**B**) Upper panel: autoradiograms showing histone H1 kinase activity of the p34^{cdc2} complexes in the same MCS used in panel A. Lower panel: detection of the amount of histone H1 protein used for the assay by staining the polyacrylamide gels with Coomassie blue. (**C**) Densitometric evaluation of the histone H1 phosphorylation autoradiograms shown in B, same nomenclature as in A.

the non-sulfated NodRm-IV(C16:2) or with water (Figure 3B). This indicated that modifications in the cell cycle parameters of the cultured cells depended on the presence of active Nod factor.

The influence of Nod factor on cell cycle was also followed by measurement of [^{3}H]thymidine incorporation, which reflects cells undergoing DNA synthesis. In these experiments, the dedifferentiated MCS cultures were used usually at 72 h after subculturing. As shown in Figure 4A, at zero time elevated [^{3}H]thymidine incorporation was measured both in the control (water-treated) and in the Nod factor-treated cells. In the control cells, a moderate peak appeared 18 h after of the start of the experiment, possibly due to partial synchrony of the culture. In cells supplemented with NodRm-IV(C16:2,S) at 10^{-9} M concentration (F9), a significant increase of [^{3}H]thymidine incorporation was detected after 24 h of Nod factor treatment. In contrast, *Medicago* cells in the presence of 10^{-6} M NodRm-IV(C16:2,S) (F6) exhibited only a low [^{3}H]thymidine peak at 24 h. [^{3}H]thymidine incorporation of MCS treated with 10^{-9} M NodRm-IV(C16:2) (NS9) for 24 h was identical to that of the control cells (data not shown).

Based on the recent demonstration of the cell division dependent activity of p34^{cdc2} kinases in higher plants (Colasanti *et al.*, 1991; Magyar *et al.*, 1993), we tested the histone H1 kinase activity of p34^{cdc2} complexes purified by p13^{suc1}–Sepharose affinity chromatography from protein extracts of MCS, used also for the [^{3}H]thymidine incorporation experiment (Figure 4B and C). For the water-treated control culture (C), autoradiograms of the phosphorylated histone H1 indicated a modest increase in the p34^{cdc2}-related kinase activity at two time periods (2–5 and 24 h), shortly after the peaks of [^{3}H]thymidine incorporation (Figure 4A). MCS cultures treated for 15–27 h with the Nod factor had a higher phosphorylation activity than the control. At 10^{-9} M concentration (F9), stimulation of p34^{cdc2}-related kinase activity was more pronounced and the phosphorylation pattern followed closely the pattern of [^{3}H]thymidine uptake, peaking at 24 h after addition of the Nod factor to the culture.

The Nod factor alters transcription of cell cycle marker genes in Medicago microcallus suspension

Expression of the genes *cdc2Ms*, *cycMs2* (pivotal cell cycle control genes) and histone *H3*-1 (a marker of the DNA synthesis phase) was studied in Nod factor-treated cells. Using *cdc2Ms* DNA as a hybridization probe, Northern analysis of RNA samples isolated from MCS grown in the absence or presence of Nod factor revealed two transcripts (of 1.4 kb and 1.2 kb; Figure 5A). In the control cultures (C), the accumulation of *cdc2*-related transcripts showed a definite expression pattern during the studied culture period suggesting partial synchrony of the cells. Accumulation of both *cdc2* mRNAs reached the highest level at 8 h. In the Nod factor-treated cultures, a prompt reaction to the Nod factor at both high and low concentrations was observed 2 h after treatment: the level of the 1.4 kb *cdc2* transcript was increased. In contrast, the amount of the 1.2 kb mRNA was reduced shortly after the treatment. After 5 h, in the control samples and in cells treated with 10^{-6} M Nod factor (F6) both transcripts were present in similar amounts. After 8 h, while the transcripts accumulated in the control, the level of transcripts was diminished in the Nod factor-treated cells. At 24 and 48 h, the *cdc2* transcripts were present in low amounts in the control cells and only cells treated with 10^{-9} M NodRm-IV(C16:2,S) (F9) for 24 h exhibited increased levels. These results indicate that addition of Nod factor to

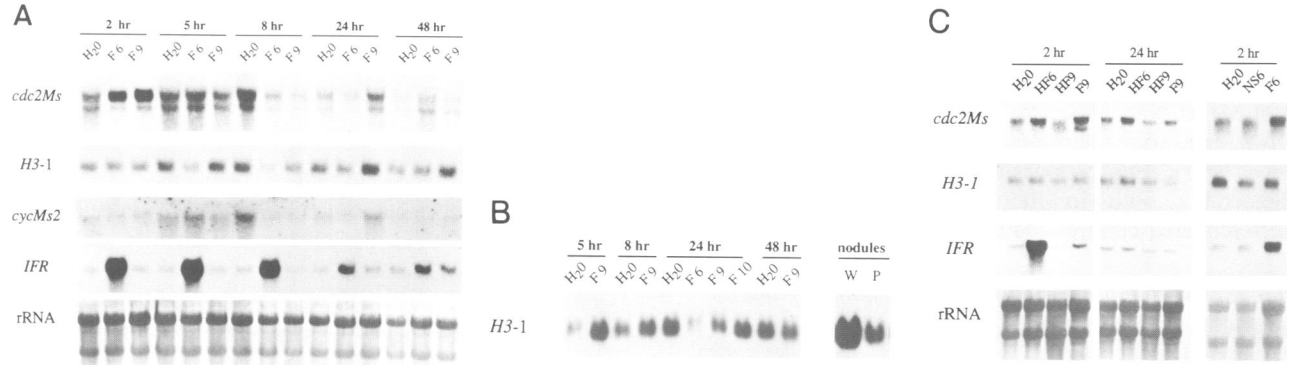

Fig. 5. Expression of cell cycle marker genes (*cdc2Ms*, histone *H3*-1 and *cycMs2*) and the isoflavonoid pathway marker gene *IFR* in Nod factor-treated *Medicago* cell cultures. (**A**) MCS treated for 2, 5, 8, 24 and 48 h with water (C) or 10^{-6} M (F6) or 10^{-9} M (F9) NodRm-IV(C16:2,S). The same blot was hybridized consecutively with the different ^{32}P-labelled DNA probes. (**B**) Expression of the *H3c*-1 gene in embryogenic suspension cultures as well as in non-fixing, white (W) and nitrogen-fixing, pink (P) nodules. 10^{-10} M NodRm-IV(C16:2,S) for 24 h (F10), F6 and F9 as in panel A. (**C**) Expression of *cdc2Ms*, *H3*-1 and *IFR* in MCS treated with 10^{-6} M (HF6) or 10^{-9} M (HF9) NodRm-IV(C16:0,S), 10^{-6} M (NS6) NodRm-IV(C16:2) as well as with water, F6 and F9. The amount of RNA samples used for the Northern analysis was quantified by staining the same filter with methylene blue which allowed the visualization of the rRNA.

MCS cell culture affected the level and timing of the expression of the cell cycle gene *cdc2Ms*.

Expression of the histone *H3*-1 gene, an S phase specific marker gene (Kapros *et al.*, 1992), was studied both in MCS (Figure 5A) and in embryogenic cell suspensions (Figure 5B). In the control MCS, considerable accumulation of histone *H3*-1 transcript was observed in cells at 5 and 8 h, again indicating a cell population cycling in partial synchrony. However, the Northern blot analysis indicated a continuous basic level of expression of this gene during the culture period, probably due to the lack of complete synchrony of the cell cultures. Comparison of the histone *H3*-1 transcript levels in the control and cells treated with 10^{-9} M Nod factor (F9) revealed a stimulatory effect of the Nod factor after long incubation periods (24 and 48 h). At 10^{-6} M concentration (F6), the NodRm-IV(C16:2,S) repressed histone *H3*-1 expression within 2–5 h after application of the factor. In the embryogenic culture (Figure 5B), similarly to MCS, a low concentration of Nod factor (F9) induced expression of the *H3*-1 gene which was especially pronounced after 5 and 8 h treatments. At 24 h, a repressive effect of the high concentration of Nod factor (F6) on the *H3*-1 gene expression was detected.

To test whether the observed marked increase in *H3*-1 transcript level in Nod factor-treated differentiated tissue may have relevance to the role of Nod factor in nodule induction, total RNAs isolated from nitrogen-fixing and from immature non-fixing nodules were blotted with the same *H3*-1 probe. As shown in Figure 5B, a high level of the *H3*-1 transcripts was observed in the young nodules, supporting the involvement of *H3*-1 gene expression during nodule development.

Expression of *cycMs2*, classified as a B type cyclin gene (G_2–M phase specific) (Hirt *et al.*, 1992), was low in MCS (Figure 5A). In the control, the highest expression was found at 8 h which was shifted to 5 h in cells treated with the high concentration of Nod factor (F6). A low concentration of Nod factor (F9) resulted in a significant increase in the *cycMs2* expression at 24 h, in comparison with other samples.

Our overall conclusion from the studies on the expression of the three cell cycle marker genes was that the Nod factor resulted in elevated accumulation of transcripts in the cells. It has to be mentioned that the increase in mRNA levels in cells treated with 10^{-9} M Nod factor for 24 h coincided with the peak of [^{3}H]thymidine incorporation in the experiment shown in Figure 4A.

In order to test whether all these changes depended on the presence of active Nod factor, MCS cultures were treated with the cognate Nod factor as well as with modified derivatives that were either inactive on *Medicago*, such as the non-sulfated Nod factor NodRm-IV(C16:2) (NS), or had reduced activity, such as the hydrogenated NodRm-IV(C16:0,S) (HF), which was ~100-fold less active in root hair deformation assay than the cognate Nod factor (Figure 5C). The hydrogenated Nod factor was used at 10^{-6} M (HF6) and 10^{-9} M (HF9) concentrations and the effects were compared with those of 10^{-9} M NodRm-IV(C16:2,S) (F9). The non-sulfated Nod factor was used at 10^{-6} M (NS6) and its effect was compared with that of treatment with a 10^{-6} M (F6) concentration of the cognate Nod factor. Expression of *cdc2Ms* was not altered in samples treated with either the non-sulfated Nod factor or with the hydrogenated Nod factor at 10^{-9} M concentration (HF9) and was similar to the expression in the water-treated samples. The hydrogenated Nod factor, however, stimulated expression of *cdc2Ms* at 10^{-6} M concentration (HF6) after both 2 h and 24 h treatments. In this experiment, probably due to shifted timing in cycling of the cells, enhancement of *cdc2Ms* expression at 24 h was not detectable in samples treated with 10^{-9} M concentration of the cognate Nod factor. Expression of *H3*-1 in the Nod factor-treated cells at these time points was similar to the control, except for HF6 which stimulated expression of *H3*-1 at 24 h.

The early nodulin gene MsEnod12 is not induced by Nod factor in cell cultures

Analysis of two dimensional gels of the *in vitro* translation products as well as expression of cell cycle marker genes indicated an effect of Nod factor on gene expression in suspensions of cultured *Medicago* cells. However, these experiments did not reveal whether early nodulin genes responding to the Nod factor are expressed in cell cultures. *MsEnod12* had been shown to be inducible by the Nod factor in *Medicago* roots (Kondorosi *et al.*, 1992; Pichon *et al.*, 1993) and was detected in young nodules (Pichon *et al.*,

1992; Allison *et al.*, 1993). To investigate its expression in *Medicago* cell cultures, MCS and embryogenic cultures were treated with different concentrations (10^{-6} M, 10^{-9} M or 10^{-10} M) of Nod factor for 5, 8, 24 and 48 h and total RNA samples were hybridized to a *MsEnod12* probe (Allison *et al.*, 1993). Expression of *MsEnod12*, however, was undetectable both in the embryogenic and in the dedifferentiated cultures (data not shown).

Induction of the isoflavone reductase gene by high Nod factor concentration

The lipo-oligosaccharide Nod factors have structural similarity to the chito-oligosaccharide elicitors, suggesting that Nod factors might mimic these elicitors and induce similar plant reactions (Barber *et al.*, 1989; Ren and West, 1992; Yamada *et al.*, 1993). This hypothesis was tested by studying the expression of the isoflavone reductase (*IFR*) gene, a marker gene of the isoflavonoid pathway (Paiva *et al.*, 1991), in MCS incubated with low and high concentrations of NodRm-IV(C16:2,S), the reduced factor NodRm-IV(C16:0,S) and the non-sulfated factor NodRm-IV(C16:2). As shown in Figure 5A, expression of the *IFR* gene was detectable at a low level in MCS treated with water (C) or with the low (10^{-9} M) concentration of the Nod factor (F9). In cells treated with 10^{-6} M NodRm-IV(C16:2,S) (F6), however, the expression of the *IFR* gene was rapidly induced, reached a maximum level between 2 and 8 h and then declined (24 and 48 h). Similarly, a 10^{-6} M (HF6) but not 10^{-9} M (HF9) concentration of the hydrogenated NodRm-IV(C16:0,S) also induced the expression of *IFR* (Figure 5C). In contrast, a 10^{-6} M concentration of the non-sulfated factor (NS6) did not stimulate expression of the *IFR* (Figure 5C). These results suggest that only high concentration of the active Nod factors but not that of the inactive Nod factor can elicit the activation of the isoflavonoid pathway.

In situ localization of histone H3-1 and IFR transcripts in microcallus cultures

Microcallus cell suspensions were incubated with 10^{-6} M or 10^{-9} M NodRm-IV(C16:2,S) or with the same volume of water for 5 or 24 h. The microcalli were then fixed and embedded and 8 μm sections were prepared from them and used for *in situ* hybridizations using antisense ^{35}S-labelled RNA probes for the histone *H3*-1 and *IFR* genes, respectively. Figure 6 shows the *in situ* localization of the *H3*-1 mRNA in microcalli samples fixed 24 h after the treatment. While the *H3*-1 transcript was hardly detectable in microcalli incubated with water or 10^{-6} M NodRm-IV(C16:2,S) (Figure 6A and B), its accumulation was observed in certain cells of microcalli treated with 10^{-9} M NodRm-IV(C16:2,S) (Figure 6C and D).

In situ localization of *IFR* transcripts in microcalli was carried out on samples taken after 5 h of the treatment (Figure 7). In microcalli treated with 10^{-9} M NodRm-IV(C16:2,S) (Figure 7C) or water (Figure 7A), no hybridization signal was detected. In contrast, high and homogeneously distributed transcript levels of *IFR* were found in microcalli treated with 10^{-6} M NodRm-IV(C16:2,S) (Figure 7B and D). Control hybridizations using the *H3*-1 or *IFR* DNA probes did not give signals above the background (data not shown). In both cases, the *in situ* hybridizations coincided with the transcript levels detected by Northern analysis after the same incubation periods (Figure 5A).

Discussion

We have reported here on molecular changes induced by the *R.meliloti* Nod factors in cultured *Medicago* cells. For the first time, it is demonstrated that not only the differentiated root hair cells or root cortical cells but also *Medicago* cells grown in microcallus suspension are able to respond to the major *R.meliloti* Nod signal molecule NodRm-IV(C16:2,S). Evidence is provided that the cognate Nod factor but not its inactive non-sulfated derivative (i) affected gene expression both quantitatively and qualitatively, (ii) stimulated cell cycle progression and (iii) at high concentration, activated a marker gene of the isoflavonoid pathway.

Molecular and biochemical analyses of the Nod signal transduction pathway necessitate substantial amounts of responsive material. The host legume roots, however, contain only few reactive cells in a limited region (in the emerging root hair zone) that are masked by the mass of non-responsive cells. Therefore, we explored the potential use of alfalfa cell suspensions to study the early events in *Medicago* cells in response to the *R.meliloti* Nod signal molecules. These cultures represent either dedifferentiated callus cells (MCS) grown in the presence of plant hormones or embryogenic cells differentiated after auxin shock (Dudits *et al.*, 1991). The dedifferentiated MCS cells, however, still exhibit root related traits as shown by expression of root specific transcripts homologous to pTUO4 from soybean (Hong *et al.*, 1987) and a gene coding for another proline-rich protein in alfalfa (unpublished results). These cells have the potential for root differentiation under hormone-free conditions. Analysis of the *in vitro* translation products demonstrated that the Nod factors may influence gene expression in *Medicago* cells. The kinetics of the changes in the 2D-gel protein pattern exhibited two peaks in the alteration of gene expression. The first, transient one was observed in cells treated for 5 h, the second one after 48 h of treatment.

One particular property of plants is that cell division and organogenesis are initiated at distinct sites throughout their life cycle. Little is known about the components and sequential events of plant cell proliferation, although some basic elements conserved in all eukaryotic cells have been detected (Norbury and Nurse, 1989; Jacobs, 1992; Magyar *et al.*, 1993). It has been suggested that the regular cell division processes of plants must differ partly from those of animals or yeast (Dudits *et al.*, 1993). It is likely that plant hormones are involved in mitotic regulation. However, the signal transduction pathway for none of the hormonal growth factors has been functionally identified. Nod factors produced by rhizobia belong to a new class of plant morphogens. They are host specific and required for the development of a new organ, the root nodule, in their legume hosts under nitrogen limitation. Nodule organogenesis involves new cell division in the root cortex suggesting that the Nod factor might interact at certain points with the cell cycle machinery, either directly or indirectly, most probably via internal plant hormone production or their signal transduction pathways (Verma, 1992).

Our results show that in *Medicago* microcallus suspension

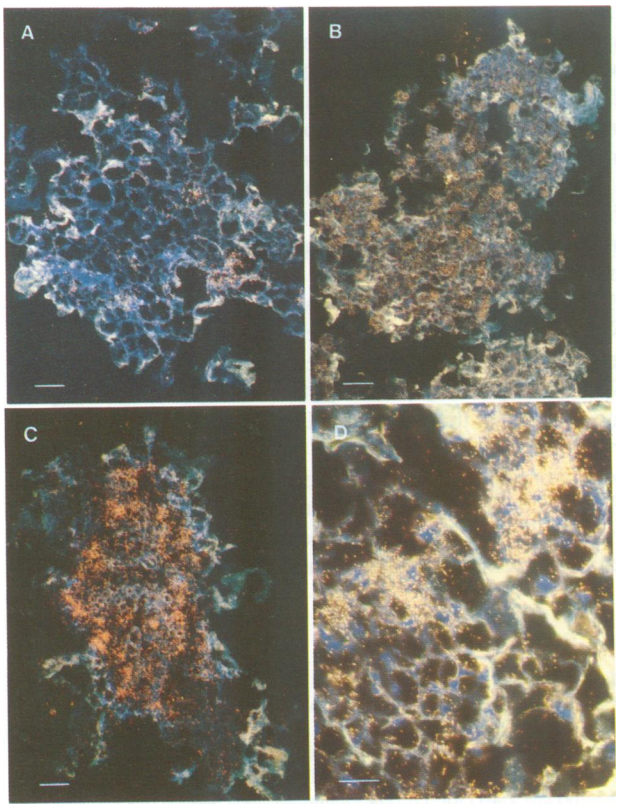

Fig. 6. *In situ* localization of *H3*-1 mRNA in *M.sativa* microcalli. 8 μm transverse sections of microcalli treated for 24 h with water (**A**) or 10^{-6} M (**B**) or 10^{-9} M (**C** and **D**) NodRm-IV(C16:2,S) were hybridized with ^{35}S-labelled antisense *H3*-1 RNA probe. Orange spots observed in the sections with dark-field microscopy represent hybridization signals. Exposure time was constant for all the treatments. In panels A–C, bars = 10 μm; in panel D, bar = 3 μm.

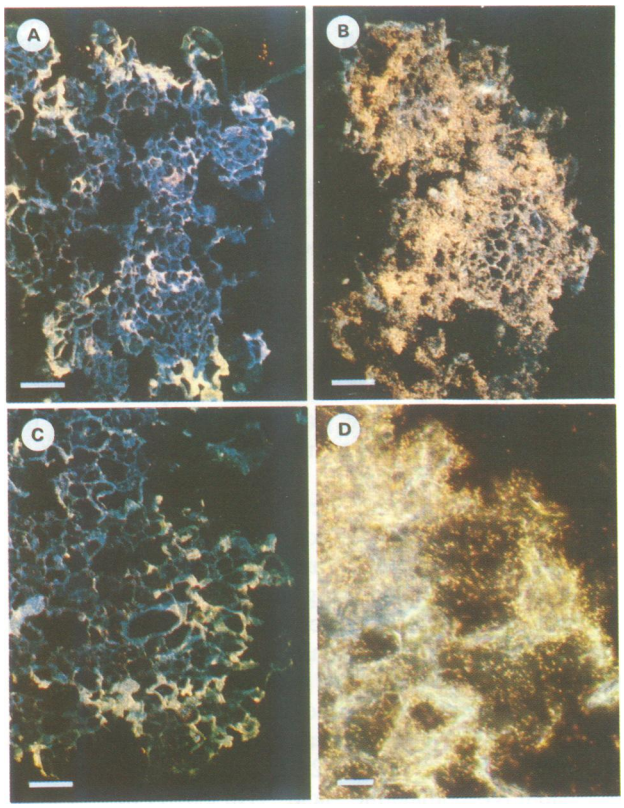

Fig. 7. *In situ* localization of microcalli with a ^{35}S-labelled antisense *IFR* RNA probe. The microcalli were treated for 5 h with water (**A**) or with 10^{-6} M (**B** and **D**) or 10^{-9} M (**C**) NodRm-IV(C16:2,S). In panels A–C, bars = 10 μm; in panel D, bar = 3 μm.

cultures the expression of the cell cycle marker genes identified so far from *Medicago*, some of which are key elements in cell cycle regulation, are influenced by the major *R.meliloti* Nod factor. It has to be recalled that the flow cytometric analysis of nuclei, the Northern hybridizations and the [^{3}H]thymidine incorporation and p34^{cdc2} kinase assays were carried out on different batches of cell cultures, varying sometimes by a few hours in the start of the Nod factor treatment after subculturing. Although we worked with non-synchronized cultures, it seems that a portion of cells cycled synchronously, probably due to regular subculturing. Therefore, a few hours' difference in the start of the experiments may shift the distribution and availability of cycling cells in the different phases so the time points are not directly comparable between different experiments. Nevertheless, our data indicate that NodRm-IV(C16:2,S) acts on the cell cycle machinery rapidly since both concentrations of the factor (10^{-6} M and 10^{-9} M) were able to enhance the level of transcription of the *cdc2Ms* gene after a 2 h treatment. Rapid accumulation of the *cdc2* transcripts during auxin-induced proliferation has already been reported (Magyar *et al.*, 1993). In separate experiments, a significant increase of nuclei in S phase was detected after treatment of cells with the Nod factor for 5 h, again indicating a rapid response of the cells. When the cells were treated with 10^{-9} M Nod factor for 24 h, increases in thymidine incorporation, expression of histone *H3*-1 and activation of the p34^{cdc2}-related protein kinase were observed. The differences in the distribution between the control and Nod factor-treated cells were maintained even during a 3 day growth period. The reduced number of G_1 nuclei, corresponding to a lower number of quiescent cells, indicates a higher rate of proliferation in cells treated with the cognate Nod factor compared with the water-treated cells or cells treated with the non-sulfated Nod signal molecules.

We suggest that a similar effect of Nod factor on the expression of these cell cycle genes may take place in roots during nodule induction. In fact, high histone *H3*-1 transcript levels were detected in young nodules, indicating that, at least, this *H3*-1 variant may indeed be involved in nodule development.

The *in situ* localization of the histone *H3*-1 transcript in MCS showed that only certain cells were able to respond to the Nod factor. The strong hybridization signal in these calli suggests reactivation of quiescent cells committed to cell division. This phenomenon is reminiscent of somatic cells at the early stage of embryogenesis where, after hormonal induction, only selected cells were able to reprogram gene expression required for embryogenesis (Dudits *et al.*, 1991).

When a 1000-fold higher concentration (10^{-6} M) of the Nod factor was used, a transient inhibitory effect on the expression of the histone *H3*-1 gene was observed after 5 h. This might be explained by a more rapid transition of cells from G_1–S to G_2 phase; the early appearance of *cycMs2* transcript seems to lend some support to this suggestion. Flow cytometric analysis of nuclei showed that treatment of MCS with 10^{-6} M Nod factor for 5 h resulted in an

increase of S phase nuclei relative to the control cells and even to those treated with low concentration of the Nod factor. Interestingly, in the embryogenic culture, treatment with high concentration of the Nod factor for 5 h was less effective in incrementing S phase nuclei than addition of Nod factor at low concentration.

All these changes in the cell cycle progression depended on the presence of the cognate Nod factor; the hydrogenated Nod factor was less active and the non-sulfated form was inactive.

We found that treatment of cells with 10^{-6} M NodRm-IV(C16:2,S) and NodRm-IV(C16:0,S) induced rapid (<2 h) and high expression of *IFR*, a marker gene of the isoflavonoid pathway. The kinetics of transcript accumulation after Nod factor treatment was comparable to that of the accumulation of the same transcript after treatment of *Medicago* cells with baker's yeast elicitor (Paiva *et al.*, 1991). Based on the fact that unmodified chito-oligosaccharides can act as potent elicitors (Barber *et al.*, 1989; Ren and West, 1992; Yamada *et al.*, 1993), it is likely that the expression of the *IFR* gene induced by the modified chito-oligosaccharide Nod factors is an elicitor-like effect evoking a plant defence response. Alternatively, compounds produced in this pathway may influence cell division via the alteration of the phytohormone balance or sensitivity in the cells. Nodule-like structures can be induced by polar auxin transport inhibitors, such as 2,3,5-triiodobenzoic acid and *N*-(1-naphthyl)-phthalamic acid (Hirsch *et al.*, 1989). Perturbation of the endogenous hormone balance and of auxin-sensitivity by transformation of *Medicago* with the *rolA*, *rolB* and/or *rolC* genes of *Agrobacterium rhizogenes* affect nodulation ability (Kondorosi *et al.*, 1992). Moreover, it has been shown that certain flavonoids act as auxin transport inhibitors (Jacobs and Rubery, 1988). Activation of the phenylpropanoid pathway by the Nod factors may lead to the production of compounds which disturb the hormonal balance by interfering with the endogenous plant hormones required for mitotic activity. Further experiments are needed to answer whether in the root the phytoalexin biosynthesis pathway is induced during nodule development and whether, at high concentration, the Nod factor induces also other defence responses characteristic of pathogenic bacteria or whether responses to the Nod factor modify the pattern of flavonoid production to trigger a unique developmental program, nodule organogenesis.

The early nodulin *MsEnod12* transcript was not detected in the Nod factor-treated cell suspension by Northern analysis, although NodRm-IV(C16:2,S) was shown to elicit the expression of this gene within 24 h in root epidermal cells of transgenic *Medicago* (Pichon *et al.*, 1993). It is possible that a root specific *trans*-acting factor is also required for the expression of this nodulin gene. It can also be that the perception and the transduction of the signal in calli and in roots are not identical and only a part of the nodule organogenesis program can be switched on in cultured cells. Moreover, recent data by Csanádi *et al.* (1994) demonstrate that mutant *Medicago* plants lacking *MsEnod12* genes produce wild type nodules indicating that either MsENOD12 is not required for nodule development or its function is substituted by another proline-rich protein.

In these studies, the *Medicago* suspension cultures provided a useful system for elucidating the molecular responses elicited by Nod signal molecules, allowing the demonstration that the NodRm-IV(C16:2,S) factor was recognized by cultured cells and that cell division was triggered. We propose that alfalfa suspension cultures could be used for the identification of certain genes regulated by the Nod factor and not requiring additional root-specific *trans*-acting factors for activation, and also for the elucidation of the Nod signal transduction pathway.

It has been shown that the presence of a sulfate group at the reducing sugar residue, the fatty acid chain and the length of the oligosaccharide are determinants of host specificity and biological activity (Roche *et al.*, 1991a; Spaink *et al.*, 1991; Schultze *et al.*, 1992). Overall, our data using different Nod factors correlate with these findings and establish a close relationship between the presence of the active Nod factor NodRm-IV(C16:2,S) and activation of the cell cycle, suggesting that this molecule plays a major role in triggering the developmental pattern of cell proliferation leading to nodule organogenesis. However, how the host-specific lipo-oligosaccharide signal molecules activate the cell cycle machinery and, at high concentration, the isoflavonoid pathway, remains an open question.

Materials and methods

Plant material

Microcallus cell suspension cultures (MCS) derived from alfalfa (*Medicago sativa* subsp. *sativa*, genotype RA3) callus tissues were maintained and propagated in the presence of plant hormones as described by Dudits *et al.* (1991). Somatic embryogenesis of MCS was induced by an auxin shock with 100 μM 2,4-D for 5 h. The culture was then washed twice in hormone-free medium and subcultured twice a week usually for 5 weeks in order to obtain embryos at different developmental stages. The non-differentiated MCS and the embryogenic cultures were treated with the purified signal molecules NodRm-IV(C16:2,S), the hydrogenated NodRm-IV(C16:0,S) (Schultze *et al.*, 1992) or the non-sulfated NodRm-IV(C16:2) (Truchet *et al.*, 1992; Baev *et al.*, 1992) at different concentrations ranging from 10^{-6} M to 10^{-10} M or with an equal volume of sterile water as control on the third day after the transfer into fresh medium. The cells used for isolation of RNA for *in vitro* translation and for Northern analysis were harvested by vacuum filtration on Whatman 3MM filter paper and were frozen rapidly in liquid nitrogen.

Nodulation assay was done on *M.sativa* cv. Sitel seedlings grown in nitrogen-free medium and inoculated with wild type *R.meliloti* strain 41 as described earlier (Allison *et al.*, 1993). After 12 days of infection, white (immature) and pink (nitrogen-fixing) nodules were harvested for RNA isolation. For [^{3}H]thymidine incorporation and kinase assays, MCS were treated with water or with 10^{-6} or 10^{-9} M NodRm-IV(C16:2,S) for 2, 5, 9, 12, 15, 18, 21, 24 or 27 h before harvesting.

Total RNA isolation

Total RNA was isolated according to Chomczynski and Sacchi (1987) by grinding the plant material in a mortar under liquid nitrogen. The powder was then added to the guanidinium thiocyanate extraction buffer. Cell debris was removed by centrifugation and the supernatant was loaded onto a CsCl cushion and centrifuged for 16 h at 150 000 *g*. The RNA pellet was redissolved in water, extracted with phenol–chloroform (1:1) and then with chloroform and precipitated with 0.3 M sodium acetate at −20°C for 1 h. After centrifugation and washing the pellet with 70% ethanol, the RNA was dissolved in DEPC-treated sterile water and stored at −70°C.

In vitro translation of total RNA

20 μg of total RNA was translated *in vitro* using the rabbit reticulocyte lysate kit (Promega) and labelled with [^{35}S]methionine. The amount of [^{35}S]methionine incorporated into proteins was determined according to the manufacturer's protocol.

Two dimensional gel electrophoresis and analysis of the polypeptide patterns

500 000 c.p.m. of the *in vitro* translation products were separated by two dimensional gel electrophoresis according to O'Farrell (1975) with minor modifications. For the first dimension, the composition of the isoelectric focusing gel was 4.5% acrylamide, 0.25% bisacrylamide, 9.2 M urea, 2.2% Nonidet P-40 and an ampholine solution containing 0.44% 2D-Pharmalyte 3-10 (Pharmacia LKB), 0.22% Biolyte 3-10 and 1.8% Biolyte 5-7 (Bio-

Rad). The second dimension was accomplished in an 11.25% polyacrylamide running gel (0.1% SDS) with a 4% stacking gel (0.1% SDS). ^{35}S-labelled polypeptides were visualized by fluorography using Kodak films.

Image analysis of the 2D-gel patterns was done using the Bio-Image software package (Millipore). To minimize gel variability, at least two replicates were used for each condition and only reproducible quantitative differences were taken into account. Around 850 well defined spots were detected and quantified per gel. Each spot of an image was matched to its corresponding spot in the reference image and an arbitrary match number was given to it. All the images were normalized to each other and the pattern of spots from treated cells was always compared with that of the appropriate control. Beside the qualitative changes, those quantitative changes were taken into account whose intensity ratio between two matched spots was >3, indicating an error of 5% for a variance test.

Thymidine incorporation and kinase assay

The MCS cells were pulse-labelled with 37 kBq/ml [^{3}H]thymidine by incubation for 2 h on a rotary shaker. Thymidine incorporation was measured according to Kapros *et al.* (1992). For the histone H1 kinase assays, cells were collected, homogenized and assayed according to Magyar *et al.* (1993). Total protein was isolated from water- and Nod factor-treated cells at defined time points. Isolation of p34^{cdc2} complexes was carried out by incubating equal amount of proteins with p13^{suc1}–Sepharose beads overnight at 4°C on a rotary shaker. Histone kinase activity was revealed by autoradiography after separation by SDS–PAGE.

Flow cytometric analysis

MCS and embryogenic cultures were treated with water or with 10^{-6} M and/or with 10^{-9} M concentrations of Nod factors. The nuclei were isolated according to Sgorbati *et al.* (1986). Flow cytometric analysis of nuclei stained with DAPI was carried out using an EPICS V cytofluorometer (Coulter, FL) with an argon laser (Spectra-Physics 2025-05) and doublet elimination by pulse height–area analysis. Cell cycle profiles were analysed with the MULTICYCLE computer program (Phoenix Flow Systems, San Diego, CA).

Northern blot analysis

Total RNA samples were separated on a 1.2 % agarose gel using denaturing conditions with 2.2 M formaldehyde. The RNA was blotted onto a Hybond-N membrane (Amersham) and hybridized according the manufacturer's protocol. The ^{32}P-labelled DNA probes were prepared by random primer labelling (Boehringer kit) to a specific activity of at least 5×10^8 c.p.m./μg. The Northern blots were hybridized at 42°C in 50% formamide, 5 × Denhardt's solution containing 200 μg/ml denatured salmon sperm DNA with the 3′-end of the *MsEnod12* gene (Allison *et al.*, 1993), the complete *cdc2Ms* cDNA (Hirt *et al.*, 1991), the *Eco*RV–*Pst*I fragment of *H3c*-1 (Wu *et al.*, 1988; Kapros *et al.*, 1992), the coding region of the *cycMs2* cDNA clone (Hirt *et al.*, 1992) and the entire *Eco*RI insert of pIFRalf11 (Paiva *et al.*, 1991). Filters were washed according to the manufacturer's protocol. The RNA in the samples was quantified by staining the Northern blots with 0.04% methylene blue in 0.5 M sodium acetate (pH 5.2).

In situ hybridization

In situ hybridization and partial hydrolysis of the RNA probes were carried out according to Grosskopf *et al.* (1993). Microcalli were fixed with 2% *p*-formaldehyde, 0.5% glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.0). Dehydration was performed in ethanol and xylene series and plant tissues were embedded in Paraplast. Sections of 8 μm were fixed to poly-L-lysine-coated slides. Paraffin was removed from sections with xylene and rehydrated through a graded ethanol series.

The insert of pH3c-1 and the pIFRalf1 were cloned in pBlueScript SK+ (Stratagene) and antisense RNA was transcribed using an *in vitro* transcription kit (Boehringer) with the T3 or T7 RNA polymerase and labelled with [^{35}S]UTP (1000–1500 Ci/mmol) as the radioactive nucleotide.

Acknowledgements

We thank Dr R.Esnault for fruitful discussion, Dr A.Dixon (Plant Biology Division, The Samuel Roberts Noble Foundation, Ardomore, OK) for providing the isoflavone reductase cDNA clone and Dr J.Leung for critical reading of the manuscript. A.S. was supported by a CIFRE contract (no. 518/90) between ANRT and Bertin et Cie and M.S. by the A.von Humboldt foundation (F.Lynen Fellowship). This work was supported by a grant from the European Economic Community, BIOT-900159-C, by a CNRS–Hungarian Academy of Sciences collaboration programme and by a Max Planck Research Award to A.K.

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Received on June 1, 1993; revised on November 26, 1993