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Expression of plant genes during the development of pea root nodules

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The expression of plant genes involved in the pea-*Rhizobium* symbiosis was studied by analysing mRNA from root nodules. The RNA was translated *in vitro* and the translation products were separated by two-dimensional gel electrophoresis. The results show differential expression of nodulin genes during root nodule development. One gene encoding N-40′ is expressed at a significant level 5 days before the leghemoglobin genes. Most other nodulin genes are expressed more or less concomitantly with the leghemoglobin genes whereas the N-21 mRNA is only present late during the development. In the development of ineffective root nodules induced by infection with different *nod*$^+$*fix*$^-$ mutants of *R. leguminosarum* all nodulin genes are expressed except for the N-21 gene. The results suggest that neither bacteroid development, heme excretion nor nitrogen fixation are essential for the induction of nodulin gene expression in the host plant. Further, it appears that the amount of leghemoglobin in ineffective nodules is regulated at a post-transcriptional level.

Key words: ineffective root nodules/leghemoglobin/nodulin gene expression/pea-*Rhizobium* symbiosis/*Rhizobium*/root nodule development

Introduction

Symbiotic nitrogen fixation involving *Rhizobium* and legumes depends on genetic properties of both the bacteria and the host plant. The *Rhizobium* genes involved in host specificity, nodulation and nitrogen fixation are located on a large plasmid, the so-called *sym*-plasmid and the genes concerned are the subject of intensive study (Ausubel, 1982; Pühler *et al.*, 1984; Rolfe and Shine, 1984). Classical genetic experiments have revealed that plant genes play an important role in the symbiotic process from the pre-infection stage to the assimilation of ammonia in a nitrogen-fixing root nodule (for reviews, see Vincent, 1980; Nutman, 1981). The nature and number of plant genes which are essential for symbiotic nitrogen fixation have hardly been studied. Recently, molecular hybridization and immunological techniques have been used by Verma and co-workers (Auger *et al.*, 1979; Legocki and Verma, 1980; Auger and Verma, 1981; Fuller *et al.*, 1983; Fuller and Verma, 1984) for the identification of mRNAs and polypeptides specifically synthesized in soybean root nodules, whereas Bisseling *et al.* (1983) used a nodule-specific antiserum preparation for the detection of nodule-specific proteins in pea root nodules (for reviews, see Bisseling *et al.*, 1984b; Verma and Long, 1983). Nodule-specific proteins that are encoded by the plant genome, are called nodulins (Van Kammen, 1984). Over 20 different nodulins have been detected so far. Among the nodulins are leghemoglobin (Appleby, 1984) and a nodule-specific form of uricase (n-uricase) (Bergmann *et al.*, 1983), but the possible function of most nodulins is as yet not clear. Nodulins may have specific functions in the formation of nodule tissue after de-differentiation and proliferation of cortical cells, in the transport of substrates to the bacteroids, in assimilation of ammonia excreted by the bacteroids or even in the senescence of the nodule tissue. In view of the specific interaction between *Rhizobium* and its host plant it appears likely that the expression of nodulin genes is controlled in some way by *Rhizobium*. However, the molecular mechanisms of this regulation are completely unknown.

Previously, we have reported the detection of 20–30 nodule-specific proteins in pea root nodules using a nodule-specific antiserum preparation and the appearance of these proteins during the development of the nodule (Bisseling *et al.*, 1983). Although this approach proved valuable for our understanding of root nodule development, the method has several limitations. It has been shown that *Rhizobium* bacteroids excrete some proteins into the cytoplasm of the plant cells and the nodule-specific antiserum preparation does not discriminate between host plant and *Rhizobium* encoded proteins (Bisseling *et al.*, 1984b; and in preparation). Further, the titer of the antibodies raised against each protein varies depending on the antigenicity of the protein and, therefore, variation in reaction with the complex nodule-specific antiserum preparation did not necessarily reflect the relative amounts of the proteins present in root nodules. Relatively abundant but less antigenic proteins may have remained undetected.

Here we report our analysis of nodule mRNA at different stages of nodule development by *in vitro* translation of the RNA followed by two-dimensional separation of the translation products on polyacrylamide gels. The data give information on the relative amounts of mRNA present for different nodulins and the rate of expression of the genes. Furthermore, we have studied the expression of nodulin genes in three different types of ineffective, i.e., not nitrogen-fixing, pea root nodules produced by different mutants of *R. leguminosarum*. The results of these experiments have led us to hypothesize on the regulation of nodulin gene expression in the *Rhizobium*-host plant interaction.

Results

Nodulin mRNAs

Total RNA isolated from mature, wild-type pea root nodules was translated in a reticulocyte lysate and the translation products were separated by two-dimensional (2-D) gel electrophoresis (Figure 1A). Using this method ~500 polypeptides could be identified in a reproducible manner. When bacteroid RNA was translated in the same eukaryotic translation system no detectable polypeptides were synthesized, indicating that all *in vitro* translation products derived from total nodule RNA and visualized by this method are plant encoded. This was confirmed by translation and analysis of nodule polyA^+ RNA which resulted in a polypeptide pattern identical to total RNA. The polypeptides

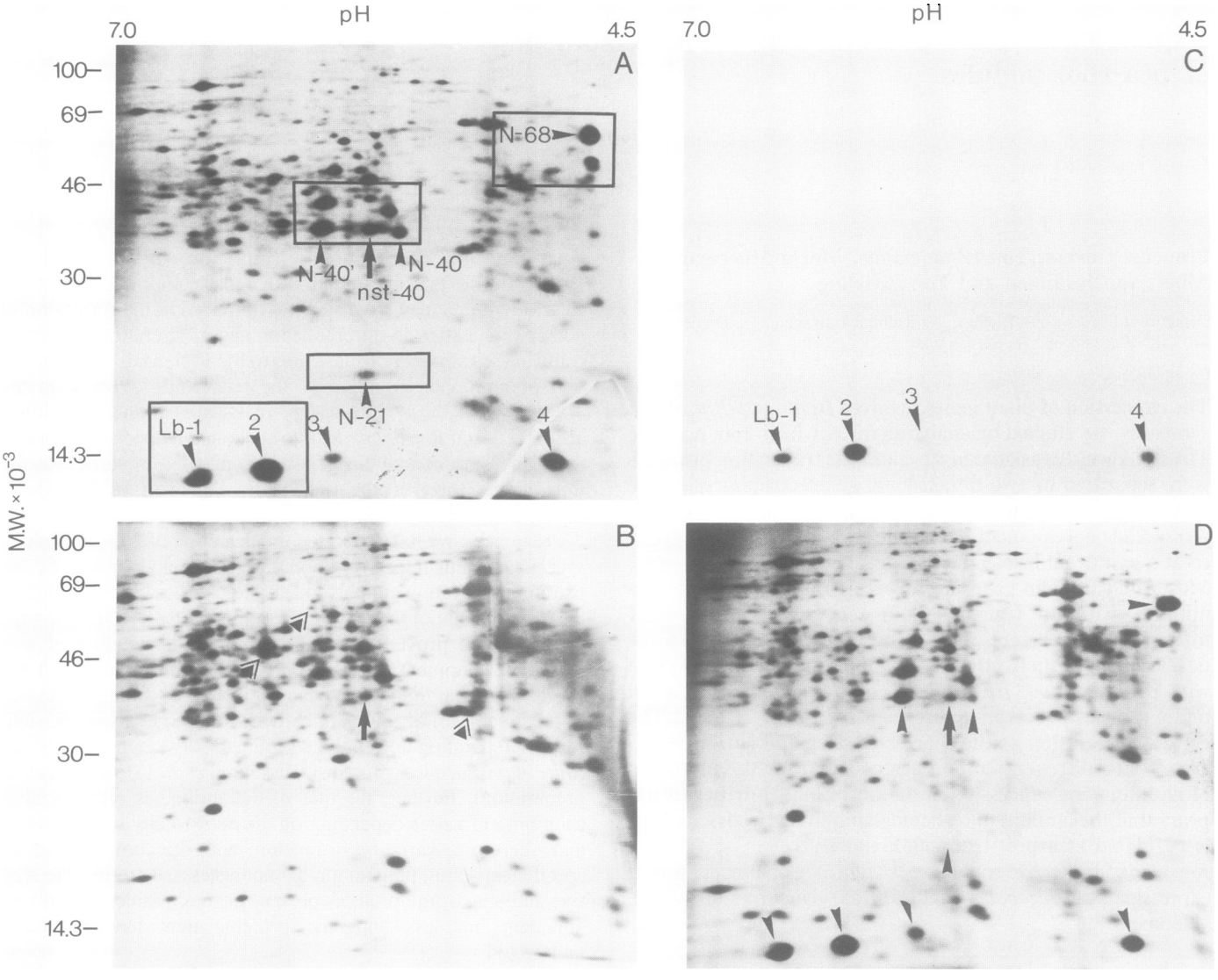

Fig. 1. Identification of nodulin mRNAs. Fluorographs of two dimensional (2-D) gels of *in vitro* translation products from total RNA isolated from **(A)** wild-type effective pea root nodules induced by *R. leguminosarum* PRE, 15 days after sowing, **(B)** 3-day-old uninfected pea roots and **(D)** ineffective pea root nodules induced by *R. leguminosarum* (PRE)2(Tn5::*nifD*), 15 days after sowing. In **(C)** the *in vitro* translation products from RNA isolated from effective pea root nodules were immunoprecipitated with anti-Lb serum and the precipitate was separated on a 2-D gel. The major nodule-specific spots, N-68, N-40′, N-40, N-21, Lb-1, Lb-2, Lb-3 and Lb-4 are indicated by ►, the nodule stimulated spot, nst-40, by ⇀ and some root stimulated spots by ▻. Mol. wt. markers included ^{14}C-methylated phosphorylase b (100 000 and 92 500), bovine serum albumin (69 000), ovalbumin (46 000), carbonic anhydrase (30 000) and lysozyme (14 300).

detectable in this way reflect the abundant and middle abundant mRNAs present in the total RNA population (Davidson and Britten, 1979). The RNA concentration used for *in vitro* translation was the same in all experiments and was chosen in the range of a linear relationship between the amount of RNA added and the incorporated radioactivity ([^{35}S]methionine). The intensity of a particular spot will therefore be directly proportional to the relative amount of mRNA present for that particular polypeptide. Changes in the intensities of spots during nodule development can be interpreted as increases or decreases in the expression of the corresponding plant genes, assuming that the translation efficiencies of the mRNAs remain at the same level during nodule development.

Comparison of the 2-D patterns of nodule polypeptides with those obtained from 3- and 8-day-old uninfected pea roots showed that the majority of proteins was present in both roots and nodules (Figure 1A and 1B). However, 21 spots were only observed in the pattern of polypeptides obtained after translation of nodule mRNA and presumably are nodulins. The mol. wts. of these nodulins vary between 15 000 and 80 000 and the group can be divided in 13 minor and eight major spots (the arrowheads in Figure 1A point to the major spots). Four of the conspicuous nodulin spots are leghemoglobin, as shown by immunoprecipitation of the *in vitro* translation products with anti-leghemoglobin serum, and are indicated as Lb-1, Lb-2, Lb-3 and Lb-4 (Figure 1C). The four other major nodulins have apparent mol. wts. of 68 000, 40 000, 40 000 and 21 000 and are indicated as N-68, N-40, N-40′ and N-21, respectively, in agreement with the notations proposed recently (Van Kammen, 1984).

Eleven other polypeptides occur both in the pattern from uninfected roots and root nodules, but these spots are more intense in the pattern from root nodules, indicating that the relative amounts of the corresponding mRNAs have increased in the nodules. A clear example of such a nodule-stimulated (nst) polypeptide is nst-40 with a mol. wt. of 40 000 (Figure 1A). Further comparison of the 2-D gel patterns showed that 13 proteins *in vitro* translated from root mRNA are present at a much lower concentration or are even below the level of detection

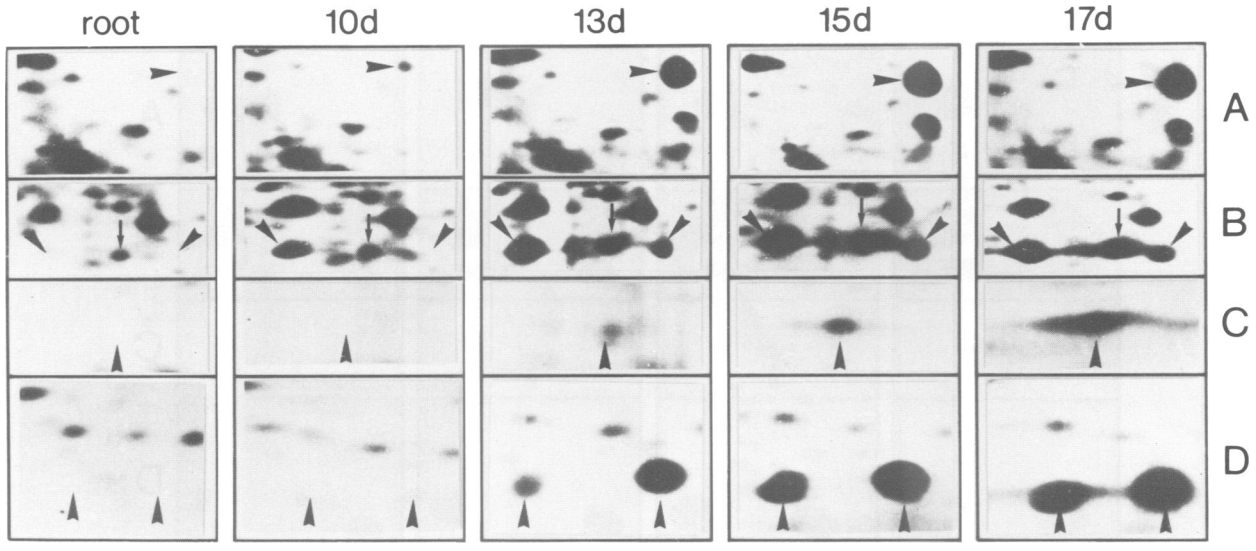

Fig. 2. Expression of nodulin genes during development of nitrogen fixing pea root nodules. Fluorographs of *in vitro* translation products from total RNA isolated from uninfected pea roots and from root nodules, 10, 13, 15 and 17 days after sowing and inoculation. Only the parts of the gels within the squares indicated in Figure 1A are shown as these contain the major nodulin spots. Nodule-specific and nodule-stimulated polypeptides are indicated by ► and →, respectively. The appearance after infection is shown for N-68 in **(A)**, N-40′, nst-40 and N-40 from left to right in **(B)**, N-21 in **(C)** and Lb-1 and Lb-2 from left to right in **(D)**.

among the translation products from root nodule mRNA (Figure 1B).

These observations indicate that a number of plant genes are expressed in root nodules but not in uninfected roots, whereas the expression of some genes is increased and of some others decreased.

Nodulin mRNAs during nodule development

To study whether the expression of all nodulin genes starts at the same time after infection of the roots with *Rhizobium* or alternatively whether some nodulin genes are expressed early and others later, we followed the appearance of the nodulin mRNAs during development of a nitrogen-fixing root nodule.

In the pea-*R. leguminosarum* symbiosis nodules appear, under our growth conditions, only on a restricted part of the main root. The first nodule-like structures are visible 10 days after sowing and inoculation. At day 12 leghemoglobin is present and one day later nitrogenase can be detected (Bisseling *et al.*, 1980). After this the nitrogen-fixing activity increases rapidly and reaches its maximum between 3 and 4 weeks after sowing whereupon it starts to decrease (Bisseling *et al.*, 1979). At day 7, 8, 9 and 10 after sowing and inoculation we collected 2.5 cm pieces of roots where nodules normally appear and at day 13, 15 and 17 we harvested root nodules. From these tissues RNA was isolated, translated and analysed on 2-D gels. We focused our analysis on the major nodulin spots N-68, N-40′, N-40, N-21, Lb-1, Lb-2, Lb-3 and Lb-4 and on the major nodule-stimulated spot nst-40 (Figure 2). On 2-D gels from 8-day-old infected tissue N-40′ was the only detectable nodule-specific translation product (data not shown). The mRNA of this nodulin increased in amount and produced a rather intense spot at day 10. At that time N-68 appeared as a minor spot (Figure 2A, B). The concentration of N-40′ mRNA further increased during the development until day 15, after which it remained constant. N-68 changed from a minor spot at day 10 to a major spot at day 13, so its mRNA concentration increased drastically during these 3 days. During the next 2 days, N-68 further increased only slightly. Nodule-specific spots which were still absent at day 10 but present in 13-day-old nodules are N-40 and the four *in vitro* translation products from Lb mRNAs (Figure 2B, D; Lb-3 and Lb-4 not shown). The amounts of mRNA for both N-40 and nst-40 rapidly increased between day 13 and 15, but then the increase slowed down and the amounts reached a maximum at day 17. At day 13 all four Lbs occurred in the 2-D gel pattern, but Lb-1 and Lb-3 as minor spots compared with Lb-2 and Lb-4.

The intensities of the Lb-1, Lb-2 and Lb-4 spots strongly increased from day 13 to day 15 whereas the Lb-3 spot did not display a comparable increase, neither did it reach the same intensity as those of the other Lbs (data not shown). At a certain spot intensity it is no longer possible to estimate visually the rate of increase of the intensities of the different nodulin spots. Therefore we also determined the relative mRNA concentrations for each of the major nodulins during nodule development by measuring the radioactivities in equal-sized pieces cut from the 2-D gels (data not shown). This allows a more precise estimation of the period during nodule formation in which the concentration of a nodulin mRNA rapidly increases. For Lb-1, Lb-2 and Lb-4, which are found at well-separated positions on the gels, we used these data to determine the beginning of the expression of the respective Lb genes by extrapolating to the time of zero incorporation. It appeared that expression of the genes coding for Lb-2 and Lb-4 starts before that of Lb-1. A comparable differential appearance of Lb components has also been found in soybean root nodules (Fuchsman and Appleby, 1979; Verma *et al.*, 1979) and expression studies at the RNA level confirmed this observation (Marcker *et al.*, 1984). Finally N-21 is hardly visible in the 2-D pattern from 13-day-old nodules; it is a minor spot at day 15 and a major spot at day 17 (Figure 2C), so the rapid increase in concentration of N-21 mRNA occurs at least 2 days later in comparison with the other nodulin mRNAs.

In summary, the group of eight major nodulins comprises one early nodulin, N-40′ and one late nodulin, N-21. N-40 and the Lbs appear and increase during a 4-day period from day 11 to day 15, whereas the appearance of N-68 is intermediate between the early nodulin N-40′ and the Lbs. The minor nodule-specific spots, which have not been studied in detail, also appear and increase from day 11 to 15. These results indicate that there is dif-

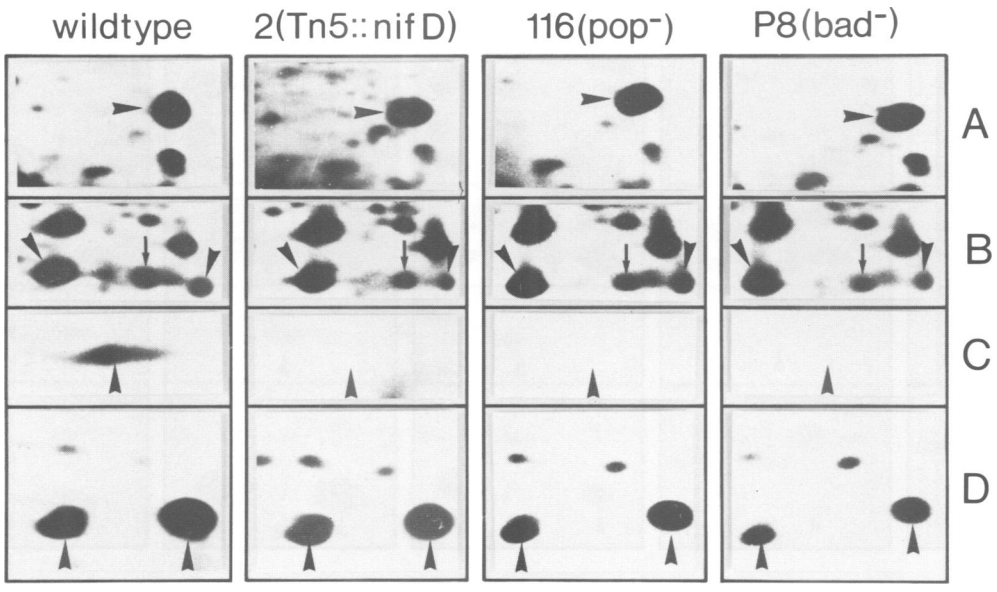

Fig. 3. Expression of nodulin genes in ineffective root nodules. A comparison of *in vitro* translation products from total RNA isolated from effective and ineffective pea root nodules, 15 days after sowing and inoculation with either the effective *R. leguminosarum* strain PRE or the ineffective strains (PRE)2(Tn5::*nifD*), (1062)116(*pop*$^-$) and P8(*bad*$^-$). Only the parts of the gels within the squares indicated in Figure 1A are shown as these contain the major nodulin spots. The comparison is shown for N-68 in **(A)**, N-40′, nst-40 and N-40 from left to right in **(B)**, N-21 in **(C)** and Lb-1 and Lb-2 from left to right in **(D)**.

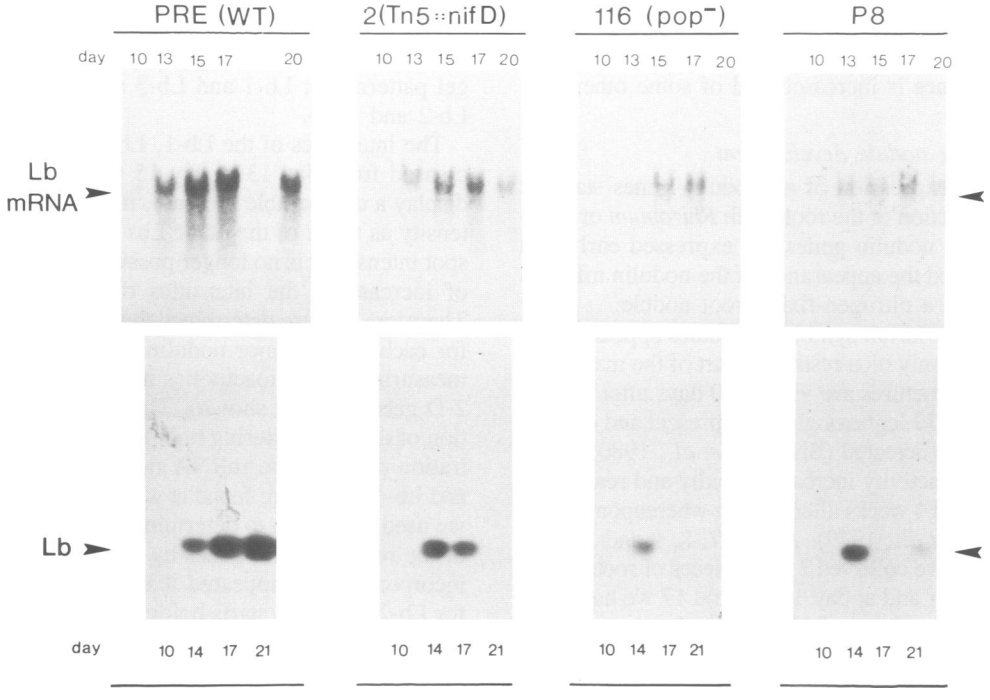

Fig. 4. The occurrence of Lb and Lb-mRNA in pea root nodules induced by infection with the effective strain *R. leguminosarum* PRE and the ineffective strains (PRE)2(Tn5::*nifD*), (1062)116(*pop*$^-$) and P8(*bad*$^-$). **(A)** Autoradiographs of Northern blots containing RNA isolated from effective and ineffective pea root nodules at different stages during the development and hybridized with ^{32}P-labeled pPsLb101 as a probe. The predominant band corresponds to a mRNA of ~700 bases in length. **(B)** Autoradiographs of Western blots containing cytoplasmic proteins from effective and ineffective pea root nodules at different stages of nodule development and incubated with antiserum raised against purified pea leghemoglobins and ^{125}I-labeled protein A to detect immune complexes. The band on the autoradiographs corresponds to a 14 000 mol. wt. protein.

ferential expression of nodulin genes during nodule development.

It seems likely that the first signal that activates expression of (a) nodulin gene(s) will be derived from the infecting rhizobia. Whether further signals that lead to expression of nodulin genes are derived from plant-encoded genes or also from the *Rhizobium* symbiont is a matter of speculation. If such signals are related to the expression of symbiotic genes of *Rhizobium*, one or more nodulin genes may not become active in nodules induced by rhizobia mutated in one of the known symbiotic genes. We have therefore analysed nodulin gene expression in ineffective nodules

produced by different *nod*$^+$*fix*$^-$ *Rhizobium* mutants.

Nodulin mRNAs in ineffective root nodules

Three mutant *R. leguminosarum* strains were used to obtain ineffective root nodules. (PRE)2(Tn5::*nifD*) is a *nod*$^+$*fix*$^-$ mutant of the wild-type *R. leguminosarum* PRE used in our experiments to produce effective nodules. This mutant has a Tn5 insertion in *nifD*, one of the structural genes of nitrogenase and consequently (PRE)2 bacteroids lack component I of nitrogenase (Schetgens *et al.*, 1984). (1062)116(*pop*$^-$) is a *nod*$^+$*fix*$^-$ mutant of *R. leguminosarum* 1062. The *pop*$^-$ mutation is manifested by the accumulation of porphyrin. (1062)116 apparently has a defect in the biosynthesis of heme and therefore the bacteroids excrete a low amount of heme (Nadler, 1981). The third *nod*$^+$*fix*$^-$ *R. leguminosarum* used, is a wild isolate referred to as P8(*bad*$^-$). P8 bacteria are released from the infection threads and invade root nodule cells, but appear not to differentiate into characteristic Y-shaped bacteroids as do the wild-type PRE and the other two mutants. It seems plausible that these three *nod*$^+$*fix*$^-$ mutants disturb the development of an effective root nodule at different stages. (PRE)2 is clearly defective in one of the last steps before nitrogen fixation can start.

Since leghemoglobin (Lb) synthesis precedes the synthesis of nitrogenase (Bisseling *et al.*, 1980; Bergersen and Goodchild, 1973) mutant (1062)116, which has a defect in the synthesis of heme required for functional Lb, may disturb nodule development at an earlier stage than (PRE)2. The differentiation of *R. leguminosarum* bacteria into Y-shaped bacteroids normally occurs shortly after the bacteria have entered into the plant cells and therefore P8 may disturb root nodule development at a rather early stage.

When total RNA from effective nodules and from the three types of ineffective nodules was translated *in vitro*, the incorporated radioactivity was in all cases about similar indicating that the available amount of translatable mRNA is the same in both effective and ineffective. The 2-D gel patterns of the *in vitro* translation products from the ineffective nodules were for the greater part similar to those from effective nodules. An example is shown in Figure 1D and a composition of details showing the major nodulins in Figure 3. In 15-day-old nodules induced by each of the three *nod*$^+$*fix*$^-$ mutants all major nodulins, except the late nodulin N-21, are present. Even in still older nodules this translation product remained below the level of detection. The minor nodulin spots were also detectable in the three types of ineffective nodules. This is not clearly shown in Figure 3, but the presence of a minor nodulin spot with a somewhat lower mol. wt. than N-68 and the same isoelectric point (Figure 3A) may serve as an example. Whereas the results demonstrate that all but one of the nodulin genes are expressed in ineffective nodules the major nodulin spots had less intensity in the 2-D pattern from ineffective nodules than the corresponding spots in the pattern from effective nodules. Measuring the radioactivity incorporation in individual nodulin spots confirmed this observation.

The radioactivity in the major nodulin spots was reduced to 15 – 30% of that in the corresponding nodulins translated from RNA of effective nodules, indicating that the relative amount of translatable nodulin mRNA is considerably decreased in ineffective nodules in comparison with effective nodules. Although it is tempting to assume that nodulin gene expression is less active in ineffective nodules the observed relative decrease in mRNA is probably due to a smaller zone of bacteroid containing cells or a lower ratio of infected to uninfected cells within this zone in ineffective nodules (Bisseling *et al.*, unpublished data; Newcomb *et al.*, 1977).

Post-transcriptional regulation of leghemoglobin

In contrast with previously published results (Bisseling *et al.*, 1983, 1984a) which showed that in protein preparations from ineffective nodules Lb is found at very reduced levels, the analyses presented here indicate the presence of a considerable amount of mRNA capable of directing the synthesis of Lb. Therefore we compared the relationship between the amount of Lb mRNA and leghemoglobin proteins during development of effective and ineffective nodules by Northern and Western blotting, respectively (Figure 4). In effective nodules induced by *R. leguminosarum* PRE, Lbs and Lb mRNA were detected in 13-day-old nodules and both increased in amount till 17 days after inoculation whereupon both Lb mRNA and the protein remained at a constant level during further nodule development. In ineffective nodules induced by mutants (1062)116 or P8, Lb mRNA was found in 13-day-old nodules and increased in amount till day 17. Densitometric scanning of the autoradiographs showed that the amount of Lb mRNA in P8-induced nodules reached 30% of the amount found in effective nodules; in nodules induced by strain (1062)116 Lb mRNA reached 60% of the amount present in effective nodules.

In both types of ineffective nodules Lb was detectable in 14-day-old nodules but at day 17 and following days the amount of Lb appeared strongly reduced, mostly below the detection level, although mRNA was detectable even until day 20 after inoculation. The same phenomenon occurred in ineffective nodules induced by mutant (PRE)2, with the difference that the amount of Lb mRNA now reached 70 – 80% of the level in effective nodules and that the protein decreased in amount after day 17.

These results confirm the previously published observations of strongly reduced amounts of Lb in ineffective nodules (Bisseling *et al.*, 1983, 1984b) and demonstrate that the continuous synthesis of Lb mRNA in ineffective nodules does not result in accumulation of Lb in the nodules as is the case in effective nodules. It is clear that the synthesis of Lb is not only regulated at the level of transcription or processing of primary transcripts to mature mRNA but also at later stages, presumably at the post-transcriptional level.

Discussion

To elucidate the molecular mechanisms of the development of nitrogen-fixing root nodules as a result of the symbiotic interaction between *Rhizobium* bacteria and leguminous plants, it is as important to analyse the plant genes involved in this process as it is to study the concerned *Rhizobium* genes. We have tried to survey the expression of plant genes involved in pea root nodule development using *in vitro* translation of mRNA and analyses of the *in vitro* translation products by two-dimensional gel electrophoresis. In this way we were able to detect the specific expression of 21 different plant genes during nodule development and, in addition, increased and decreased activities of plant genes which are also expressed in non-infected root tissue. Previously, 20 – 30 nodule-specific proteins have been detected by using a nodule-specific antiserum preparation (Bisseling *et al.*, 1983) but in that study no clear distinction could be made between pea and *Rhizobium* encoded proteins, between the relative amounts of the different proteins and between the rate of expression of different nodulin genes. Here we have shown that eight genes, encoding nodulins N-21, N-40, N-40′, N-68 and four Lbs are strongly expressed. Among these nodulins the Lbs have a well-

defined function in O_2-transport in nodules (Appleby, 1984) whereas the function of the four other nodulins is as yet unknown. The appearance of the eight major nodulins showed that there is differential expression of specific plant genes during nodule development and this result may therefore help in the selection of plant genes worth isolating for further study of their structure and regulation of expression.

Our results indicate that the N-40′ gene is strongly expressed from the very beginning of the development of nodule tissue, even before nodule-like structures are visible and that N-40′ might be involved in the formation of nodule meristem. Since we have not been able to detect N-40′ mRNA in the meristematic tissue of root tips, N-40′ may be a specific marker for nodule meristem formation. The expression of the N-40′ gene is not repressed during further nodule development which is in agreement with the fact that pea nodules have a zone of actively dividing cells. The expression of the N-68 nodulin gene appears to start ~2 days later than that of N-40′ but is clearly prior to the expression of most of the other nodulin genes such as the genes for Lbs, N-40 and many nodulins occurring in minor amounts. This suggests that N-68 marks a further specialization of the nodule cells while the Lbs, N-40 and the other nodulins might be involved in creating an appropriate environment for nitrogen fixation, since the stage at which the genes for these nodulins are expressed is characterized by the beginning of symbiotic nitrogen fixation. N-21, the gene of which is expressed rather late during nodule development, is distinct from the other nodulins. A zone of senescent tissue has been observed in pea root nodules ~20 days after inoculation of the roots (Kijne, 1975) and it might therefore be argued that N-21 is involved in the senescence process. This appears, however, very unlikely since the expression of the N-21 gene is repressed in ineffective nodules which show signs of senescence at an earlier stage than effectively nitrogen-fixing nodules (Bisseling *et al.*, unpublished data; Newcomb *et al.*, 1977). A common feature of the three types of ineffective nodules used for our analyses, is their inability to fix nitrogen and one might assume that N-21 is involved in the assimilation or transport of ammonia.

Our observations that nodulin genes are expressed in ineffective root nodules are in agreement with those of Fuller and Verma (1984), who showed that in two kinds of ineffective soybean nodules the Lb genes and the genes of four other nodulins were expressed. The amounts of the relative nodulin mRNAs in such ineffective soybean nodules was considerably reduced compared with the level in effective nodules. Likewise in ineffective pea root nodules the expression of the nodulin genes is reduced. Since pea root nodules are composed of different types of cells (Newcomb, 1976) and specific nodulin genes might only be expressed in one cell type, the decrease of nodulin mRNAs in ineffective nodules may be due to a change in the ratio of different cell types resulting in, for example, less bacteroid-containing cells in ineffective nodules.

In ineffective nodules the amount of some nodulins appears to be regulated by a mechanism other than transcriptional control of the corresponding genes as is illustrated here by the amount of Lbs and Lb mRNA found at different stages of nodule development. We demonstrated that Lb mRNA is present in ineffective nodules in considerable amounts from about day 13 till day 20 after inoculation of the roots, which was the latest stage tested. In contrast, Lbs were detected in ineffective nodules only during the first 3–4 days of that period but were virtually undetectable from day 17 after inoculation. On the other hand, in effective nodules both the amount of detectable Lb and the relative amount of Lb mRNA remained at a constant level under comparable conditions. Therefore, it appears that the amount of Lb in root nodules is regulated at a post-transcriptional level. The translation experiments with RNA isolated from nodules demonstrate that there is no change in *in vitro* translatability of the Lb mRNA. If this reflects the situation *in vivo* the disappearance of the Lbs in ineffective nodules can only be explained by rapid turnover of synthesized Lbs. A similar post-transcriptional regulation for Lb does not seem to exist in soybean nodules. In cases where strongly reduced amounts of Lbs are found in ineffective soybean nodules it is low throughout the development and is in approximate proportion to the amount of Lb mRNA present. A marked decrease of the protein compared with the mRNA at later stages of ineffective nodule development has not been detected (Verma *et al.*, 1981). Whether this reflects an essential difference between ineffective soybean and pea nodules is unknown.

Not only Lb but also other nodule-specific proteins or nodule-stimulated proteins have been found in decreased amounts in ineffective nodules as compared with effective nodules. In ineffective pea nodules several nodule-specific proteins were decreased or even missing (Bisseling *et al.*, 1983, 1984b). In ineffective soybean nodules the activity of glutamate synthase was not detectable and that of glutamine synthetase considerably decreased (Sen and Schulman, 1980). Ineffective *Phaseolus* root nodules contained only trace amounts of nodule-specific glutamine synthetase activity whereas the normal glutamine synthetase activity was reduced (Lara *et al.*, 1983). Such decreases may be due to decreases in the relative amounts of mRNAs such as we have observed here in ineffective pea nodules and Fuller and Verma (1984) in ineffective soybean nodules. On the other hand it may also be a matter of increased turnover of some nodule-specific proteins in ineffective nodules.

The results of our experiments show that there is successive expression of different nodulin genes during pea root nodule development indicating that the expression of all nodulin genes is not induced at the same time. Since the expression of nodulin genes is effected by interaction with infecting rhizobia it seems plausible to assume that signals from *Rhizobium* play a role at an early stage of nodule development for example in the induction of early pea nodulin genes like N-40′. Such signals might be generated by information encoded by the *nod* genes on the *sym* plasmid of *R. leguminosarum*, which are essential for the induction of nodule formation on the pea roots (Ausubel, 1982; Rolfe and Shine, 1984). It is not clear whether at later stages of root nodule development, when *Rhizobium* bacteria differentiate into nitrogen-fixing bacteroids, the bacteria again give signals to the pea genome for further nodulin gene expression. We used three different mutated rhizobia, which produced ineffective root nodules, but no differential effect on the expression of most nodulin genes was observed. We can therefore conclude that neither nitrogen fixation, heme secretion nor bacteroid development are essential for the expression of these genes. In all three types of ineffective, not nitrogen-fixing root nodules the late nodulin gene N-21 was not expressed. This suggests that the expression of the N-21 gene is in some way controlled by the nitrogen fixation process since the expression of the gene is only started concomitantly with or after the onset of symbiotic nitrogen fixation.

Further studies with *R. leguminosarum* mutants with defects in symbiotic genes other than those in the mutants used in the present experiments, are in progress to establish whether or not the expression of *R. leguminosarum* genes is essential for the induction of nodulin genes at different stages of pea root nodule development.

Materials and methods

Root nodules

Pea plants (*Pisum sativum* var. Rondo) were cultured and inoculated with wild-type and mutant *R. leguminosarum* strains as described by Bisseling *et al.* (1978). At early stages during the development, 2.5 cm pieces where nodules normally appear were cut from the main root whereas at day 13, 15 and 17 root nodules were removed. Uninfected plants were cultured in the same way and total roots or pieces from the main root were harvested 3 and 8 days after sowing. The roots and nodules cultured for the isolation of RNA, were immediately frozen in liquid nitrogen and stored at −70°C. Freshly harvested material was used for isolation of cytoplasmic proteins.

Isolation of total RNA

Total RNA was isolated essentially as described by De Vries *et al.* (1982). Frozen tissue was ground in a mortar to a fine powder under liquid nitrogen. Portions of >4 g were ground in a Waring Blender. To 1 g of material a mixture of 2.5 ml 0.2 M sodium acetate, pH 5.0, 1% SDS, 10 mM EDTA and 2.5 ml distilled phenol containing 0.1% 8-hydroxyquinoline was added. After vigorously shaking for 5 min, 2.5 ml chloroform (chloroform:isoamylalcohol = 24:1) was added and the suspension was shaken for another 5 min. After centrifugation the aqueous phase was removed, re-extracted twice with phenol:chloroform (1:1) and chloroform, respectively, and 8 M LiCl was added to a final concentration of 2 M. The RNA was precipitated overnight at 4°C, collected by centrifugation, washed once with 2 M LiCl and twice with 70% ethanol. The dried pellet was dissolved in double-distilled water and stored in portions at −70°C.

In vitro translation

Total RNA isolated from effective and ineffective pea root nodules and uninfected pea roots was translated in a mRNA-dependent rabbit reticulocyte lysate. Typically 2 μg total RNA was translated during 60 min at 30°C in a 6 μl incubation mixture containing cell-free rabbit reticulocyte lysate with additives according to standard procedures (Pelham and Jackson, 1976) [^{35}S]Methionine (6 μCi per 6 μl reaction mixture) was used as radioactive amino acid. The radioactivity incorporated in translation products was counted after precipitation of 1 μl reaction mixture in 10% trichloroacetic acid on Whatmann 3 MM filters.

Immunoprecipitation

For immunoprecipitation of *in vitro* translation products with antiserum raised against purified Lb components (Bisseling *et al.*, 1979), 4 μg RNA isolated from 15-day-old nodules was translated in a 12 μl incubation mixture. After translation, 10 μl anti-Lb serum and 500 μl RIA buffer (10 mM Tris-HCl pH 7.5, 0.9% NaCl, 1% BSA, 0.05% Triton X-100) was added and incubated for 16 h at 4°C. Subsequently, 50 μl of a 10% (w/v) suspension of *Staphylococcus aureus* cells (IgGsorb from the Enzyme Center Inc., Boston, MA) in RIA buffer was added and incubation at 4°C was continued for 1 h by constantly shaking on a rotary mixer. Samples were then centrifuged through a sucrose cushion consisting of a 0.5 ml layer of 1 M sucrose and a 0.2 ml layer of 0.5 M sucrose both in RIA buffer, at 17 000 *g* for 20 min. The precipitate was washed twice in RIA buffer and finally resuspended in sample buffer for isoelectric focusing.

Two-dimensional gel electrophoresis

The *in vitro* labeled translation products were separated in two dimensions, the first according to isoelectric point and the second according to mol. wt. The procedure was performed essentially as described by O'Farrell (1975). Amounts of protein corresponding to 20 000 − 300 000 c.p.m. in the trichloroacetic acid-precipitable fraction were applied to the gel. For the isoelectric focusing, 1.6% ampholines pH 5 − 7 and 0.4% ampholines pH 3.5 − 10 were used and for the SDS-electrophoresis, 12.5% polyacrylamide slab gels. The translation products were visualized by fluorography using preflashed Kodak XAR-5 films.

Northern blotting and hybridization

Total RNA isolated from root nodules was separated under denaturing conditions on 1.5% agarose gels containing 6% formaldehyde. To each lane 25 μg total RNA was applied. After electrophoresis the RNA was blotted onto Gene Screen paper (New England Nuclear Corp.). The electrophoresis and diffusion blotting procedure were performed as described in detail in the Gene Screen information booklet. The RNA was hybridized to pPsLb101, a plasmid consisting of a 400-bp insert of Lb cDNA in the *Eco*RI site of pBRH2. ^{32}P-Labeling of pPsLb101 was performed by nick translation. The Northern blot was hybridized with the denatured DNA probe at 42°C in a hybridization mixture containing 50% formamide, 10 x Denhardt's solution, 50 mM Tris-HCl pH 7.5, 1 M NaCl, 0.1% sodium pyrophosphate, 0.1% SDS, 10% dextran sulfate and 100 μg/ml denatured salmon sperm DNA. After hybridization the filter was washed twice for 30 min at 65°C in 2 x SSC, 0.5% SDS and 30 min at room temperature in 0.1 x SSC, 0.1% SDS and exposed to Kodak XAR-5 film.

Western blotting and immunological detection

Cytoplasmic proteins from root nodules were isolated and separated by SDS-gel electrophoresis in 15% polyacrylamide gels. The proteins were blotted onto nitrocellulose and this was incubated with anti-Lb serum and [^{125}I]protein A. All procedures were performed as described previously by Bisseling *et al.* (1983).

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