The EMBO Journal Vol.2 No.6 pp.961 – 966, 1983

Nodule-specific host proteins in effective and ineffective root nodules of *Pisum sativum*

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Communicated by K.A.Marcker
Received on 14 March 1983

Nodule-specific root proteins – so called nodulins – were identified in root nodules of pea plants by an immunological assay. Nodulin patterns were examined at different stages of nodule development. About 30 nodulins were detectable during development. Some were preferentially synthesized before nitrogen fixation started, whereas the majority were synthesized concomitantly with leghaemoglobin. Some of the nodulins were located within the peribacteroid membrane. Ineffective *Rhizobium* strains (a natural nod$^+$fix$^-$ and a pop$^-$fix$^-$) appeared to be useful in studying the expression of nodulin genes. Synthesis of some nodulins was repressed in ineffective root nodules, indicating that nodulins are essential for the establishment of nitrogen fixation. In both types of ineffective root nodules, leghaemoglobin synthesis was not completely repressed. Low amounts of leghaemoglobin were always detected in young ineffective root nodules whereas in old nodules no leghaemoglobin was present.

Key words: nodulins/leghaemoglobin/*Pisum sativum*/root nodules/host-*Rhizobium* interaction/*Rhizobium*

Introduction

Upon infection with *Rhizobium*, legumes develop highly differentiated root nodules in which bacteria and the host closely interact to fix nitrogen. Nodule development starts with rhizobia invading the legume roots *via* root hairs by inducing the formation of an infection thread which surrounds the bacteria and penetrates into the root cortex cells. As a result, small groups of inner cortical cells start to divide and de-differentiate into meristematic tissue. Rhizobia are released from the infection thread into the cytoplasm of these host cells and may develop into the characteristic bacteroid form of *Rhizobium*, which is capable of nitrogen fixation (Libbenga and Bogers, 1974). The differentiation of *Rhizobium* into bacteroids is not, however, the only prerequisite for nitrogen fixation. The plant cells of, for example, pea and clover, invaded by rhizobia differentiate into non-dividing cells and a number of plant genes specific for bacteroid-containing nodules are expressed. The most well-known examples of such plant genes are those for leghaemoglobin which is essential for nitrogen fixation and is only found in root nodules. It is plausible that, in addition to the genes for leghaemoglobin, a number of plant genes are involved in the formation of effective root nodules. Classical genetic analysis of the pea has revealed that several host genes play an important role in the formation of root nodules in this plant (Lie *et al.*, 1981). With soybean it has been shown that indeed several genes for nodule-specific host polypeptides, the so-called nodulins, are expressed during nodule formation and development. Legocki and Verma (1979, 1980) have found, using nodule-specific antisera, that besides leghaemoglobin ~20 nodulins can be detected in soyabean root nodules. Furthermore, Auger and Verma (1981) have shown that in the class of moderately abundant mRNA molecules from soyabean root nodules, 20–40 mRNAs are nodule specific. The work on soyabean root nodules thus shows that, in addition to the family of leghaemoglobin genes, various plant genes (nodulin genes) are specifically expressed during nodule formation.

Here we describe nodulins in pea root nodules formed by infection with *Rhizobium leguminosarum* and their changes throughout nodule development. Root nodules produced by *nod$^+$fix$^-$ R. leguminosarum* strains, which form nodules in which nitrogen fixation does not occur, were analysed to determine whether nodulins are involved in the nitrogen fixation process.

Results

Specificity of the anti-nodulin serum

To detect nodulins in pea root nodules, an anti-nodulin serum was prepared by titrating an antiserum prepared against soluble nodule proteins with root proteins. The nodulins in root nodule cytoplasm were subsequently identified with the anti-nodulin serum using the protein blotting procedure.

Soluble plant proteins from uninfected pea roots and pea

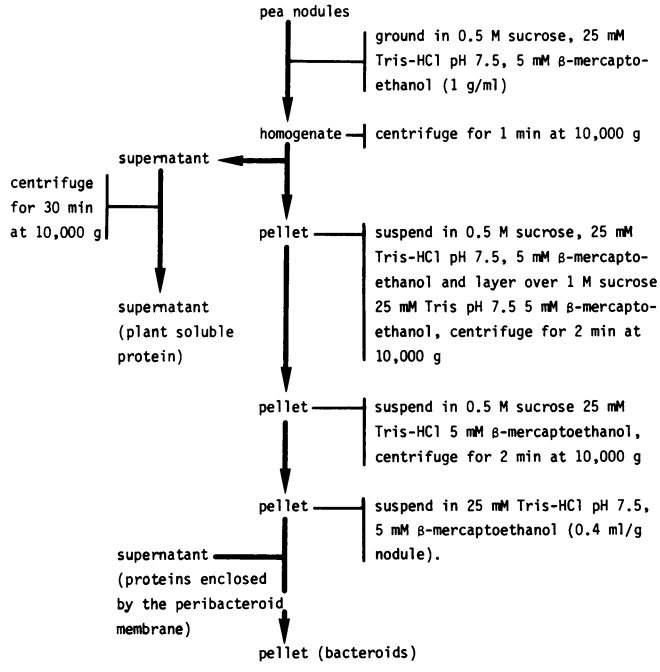

Fig. 1. Protocol for the preparation of soluble cytoplasmic (plant) proteins and proteins enclosed by the peribacteroid membrane.

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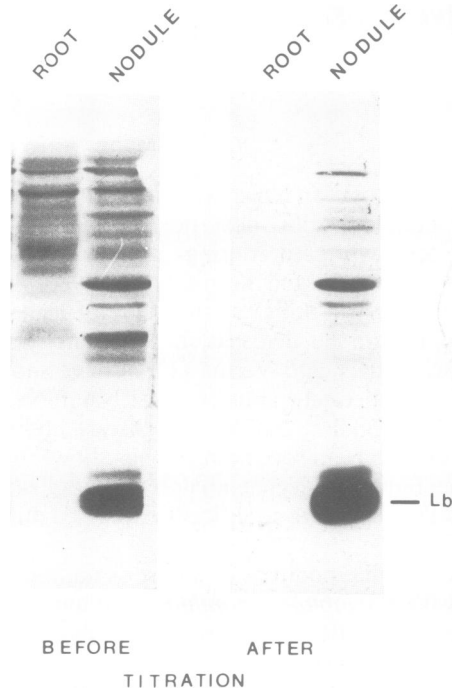

Fig. 2. Detection of nodule-specific proteins with anti-nodule serum before and after titration with root proteins: autoradiograph of two protein blots of soluble pea polypeptides from uninfected roots (root) and root nodules of pea and *R. leguminosarum* strain PRE (nodule). One of the blots was incubated with the antiserum against soluble nodule protein before titration and the other after titration with root protein. Finally the blot was incubated with [^{125}I]protein A.

root nodules formed by *R. leguminosarum* strain PRE were separated by electrophoresis in SDS-polyacrylamide gels and blotted onto nitrocellulose filters. One blot was incubated with antiserum against soluble nodule proteins before titration with root proteins and the other blot with anti-nodulin serum. The autoradiographs of these blots after incubation with ^{125}I-labeled protein A are shown in Figure 2. The antiserum before titration reacted with a number of polypeptides from uninfected roots as well as from root nodules. After titration, the antiserum gave only a very faint reaction with root proteins, whereas in the protein pattern from root nodules several polypeptides other than leghaemoglobin still showed a clear reaction. Thus, the absorption of the antiserum with soluble root proteins resulted in a nodule-specific anti-nodulin serum.

Pea nodulins during nodule development

Under the conditions of our experiments, the first root nodules become visible on the eleventh day after inoculation. Nitrogenase activity measured as acetylene reduction can be detected on day 12; the activity increases rapidly reaching its maximal rate between 3 and 4 weeks after sowing and then the nitrogenase activity decreases (Bisseling *et al.*, 1979).

Root nodules were harvested at different stages of root nodule development, the fraction with the soluble plant nodule proteins was isolated and analysed by SDS-gel electrophoresis and protein blotting for the occurrence of nodulins. Nodulins visualised with the anti-nodulin serum at different stages of nodule development are shown in Figure 3. About 30 different polypeptides could be detected with mol. wts. ranging from 120 K to 15 K. The nodulins were numbered in order of size, number one (N-1) being the largest polypeptide detected. The smallest nodulin, N-30, is leghaemoglobin since this polypeptide specifically reacted with anti-leghaemoglobin serum (result not shown).

Some nodulins e.g., N-3, N-6, N-8, N-11, N-14 and N-24 were mainly found in nodules at an early stage of root nodule development, i.e., at 11 and 12 days after sowing. Other nodulins appeared to accumulate in the course of nodule development as is illustrated by leghaemoglobin; leghaemoglobin was first detected at day 12 and the amount of leghaemoglobin increased till ~day 21. N-17 behaved in a similar way, but the synthesis of N-17 was detectable before that of leghaemoglobin.

The polypeptides 10 and 23 were also detected in uninoculated roots, although the bands were more intense in root nodules. These may represent proteins of which the synthesis is stimulated in nodules or the bands are composed of polypeptides occurring in uninoculated roots and nodule-specific polypeptides.

Nodulins in ineffective root nodules

To examine whether nodulins have a role in the establishment of an effective nitrogen fixing nodule, the nodulins in nodules produced by *nod*$^+$*fix*$^-$ strains of *R. leguminosarum* were analysed. Two types of mutants were used: P8, a naturally occurring *fix*$^-$ variant of *R. leguminosarum* and a mutant (116) of *R. leguminosarum* 1062. This mutant is *pop*$^-$ which is manifested by the accumulation of porphyrin. Mutant 116 apparently has a defect in the biosynthesis of haem and therefore the bacteroids of 116 excrete a low amount of haem (Nadler, 1981).

The soluble host proteins from ineffective nodules formed by strain P8 were isolated at different stages of root nodule development and analysed as described above. The pattern of nodulins visualized after protein blotting with anti-nodulin serum was compared with the nodulin pattern of effective nodules produced by *R. leguminosarum* (PRE) (Figure 4). The anti-nodulin serum preparation used in these experiments was from a different titration of antiserum against soluble plant nodule protein with root proteins than the anti-nodulin serum used in the experiments of Figures 2 and 3. The anti-nodulin serum used for the experiments of Figure 4 showed not only reaction with polypeptides 10 and 23 found in uninfected roots but also with root proteins co-migrating with polypeptides 7 and 9. The anti-nodulin serum preparation used in the experiment of Figure 3 does not react with root proteins co-migrating with the polypeptides 7 and 9. We conclude, therefore, that in the experiment of Figure 4, bands 7 and 9 are a mixture of N-7 and N-9 respectively and root proteins co-migrating with these bands.

The nodulin pattern from the effective root nodules produced by strain PRE was similar to the pattern shown in Figure 2 but, in addition, N-16 not visible in Figure 3 was detected.

In P8-induced root nodules, the number of detectable nodulins was less than in effective nodules produced by PRE. The only early nodulin detectable in P8 root nodules at day 13 is probably N-6. Other early nodulins, for example N-8, were not detected in young P8 nodules. P8 nodules 15 or 17 days after sowing contained a small amount of leghaemoglobin. Four days later (i.e., 21 days after sowing) leghaemoglobin was no longer detectable in P8 root nodules. Other nodulins like N-15, N-17, N-19, N-25 and N-27 were not detected in P8-induced root nodules at any stage of development. Polypeptides 7, 9 and 23 which were also found in uninfected

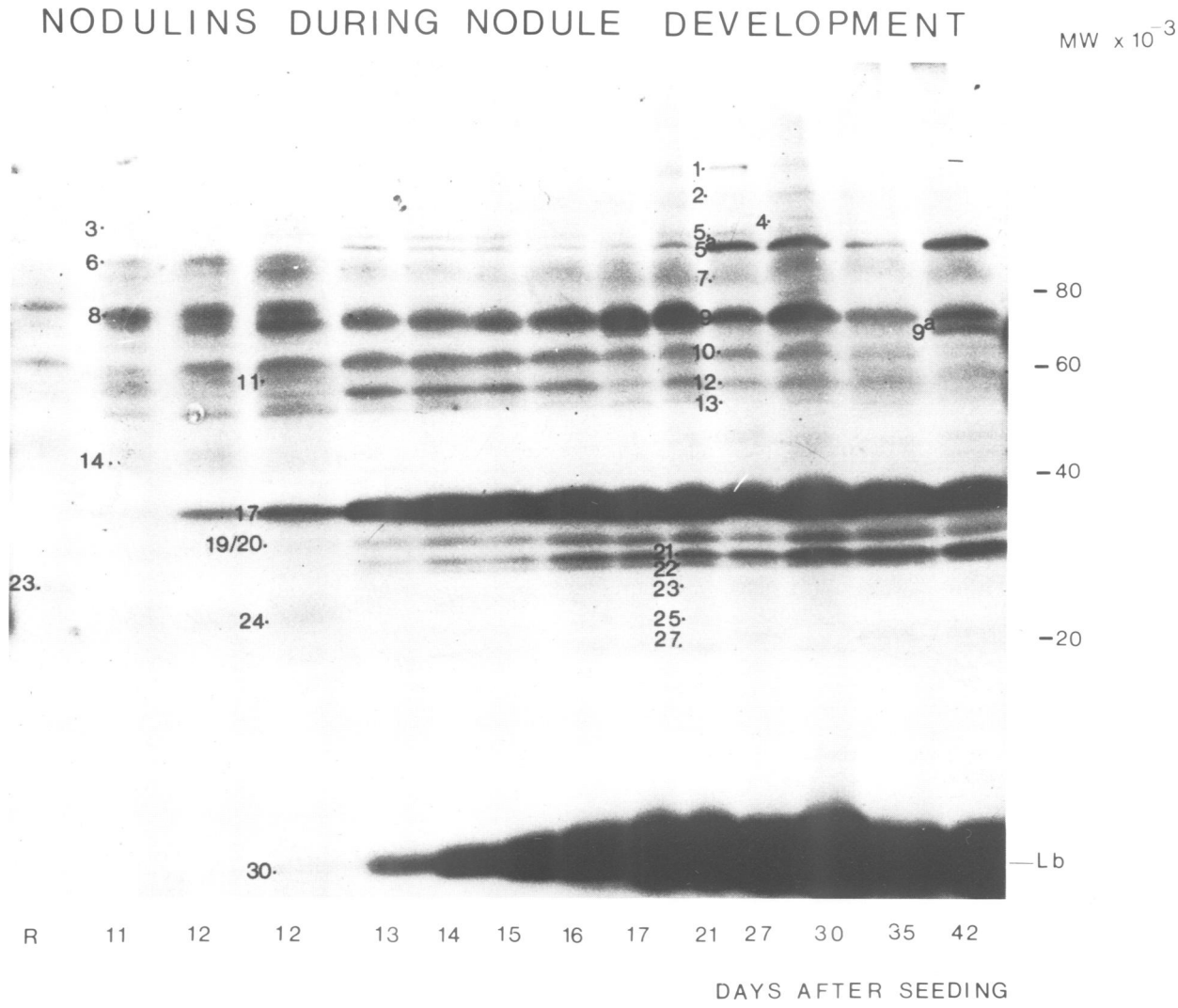

Fig. 3 Pea nodulins during nodule development: autoradiograph of a protein blot of soluble pea proteins from uninfected roots (R) and root nodules of pea and *R. leguminosarum* (PRE) harvested at different times after seeding, as indicated. The blot was incubated with anti-nodule serum and [^{125}I]protein A.

roots could be detected in P8-induced root nodules at all stages of development. Polypeptides 7, 9 and 23 which were also found in uninfected roots could be detected in P8-induced nodules until 17 days after sowing. After that they obviously disappeared as did leghaemoglobin. N-12, N-20 and N-22 were detected in all stages of P8 root nodules analysed and the synthesis of N-22 was stimulated in older P8-induced nodules (i.e., 21 days after sowing).

The nodulin pattern found in the effective nodules formed on pea roots by *R. leguminosarum* 1062 was almost identical to that by strain PRE. The only difference was that N-16 was absent amongst nodulins formed by strain 1062 (Figure 5). This nodulin does not appear to be essential for the establishment of nitrogen fixation. In Figure 5 the nodulins of root nodules from 21-day-old plants formed by the fix$^-$pop$^-$ mutant (116) of *R. leguminosarum* strain 1062 are also shown. The changes in the nodulin pattern were less than was the case with the ineffective strain P8. Only the amounts of N-15 and N-17 were lower compared with those in the effective root nodules. The amount of leghaemoglobin decreased to ~3% of that in effective root nodules as determined by a radioimmunoassay (Bisseling *et al.*, 1980; Nadler, 1981).

Localization of nodulins

It was of interest to examine whether some nodulins are specifically localized within the peribacteroid space. Such nodulins might play a role in the communication between bacteroids and host cells. Equal amounts of protein fractions from the host plant cytoplasm, the peribacteroid space and bacteroids were separated on a polyacrylamide gel and a protein blot of this gel was incubated with anti-nodulin serum. The antiserum was raised against soluble root nodule proteins which include proteins from the plant cytoplasm and from the peribacteroid space. The plant cytoplasm protein fraction contains the same nodulins as described above (Figure 6). Polypeptides co-migrating with the cytoplasmic N-17, N-19, N-20, N-22, N-25 and N-27 are found in the peribacteroid space. The peribacteroid protein fraction contained only a minor amount of leghaemoglobin, the vast majority being in the cytoplasmic protein fraction.

Anti-nodulin serum contained antibodies that recognize bacteroid polypeptides. This is probably because the antiserum was raised against soluble nodule proteins from root nodules of plants 21 days after sowing. As a result of the

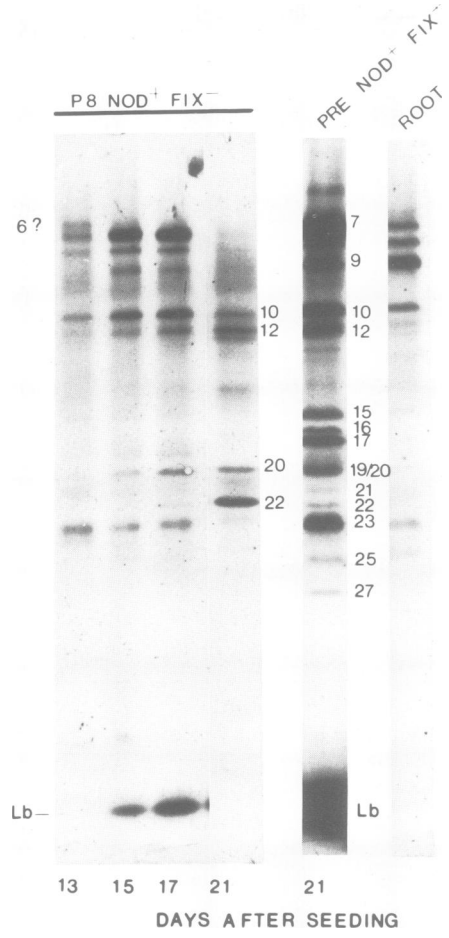

Fig. 4. Nodulin pattern of ineffective 'P8 nodules': autoradiograph of a protein blot of soluble pea protein from uninfected roots (root) and root nodules of pea and *R. leguminosarum* strain PRE and strain P8, harvested at different times after seeding. The blot is incubated with anti-nodulin serum and [^{125}I]protein A.

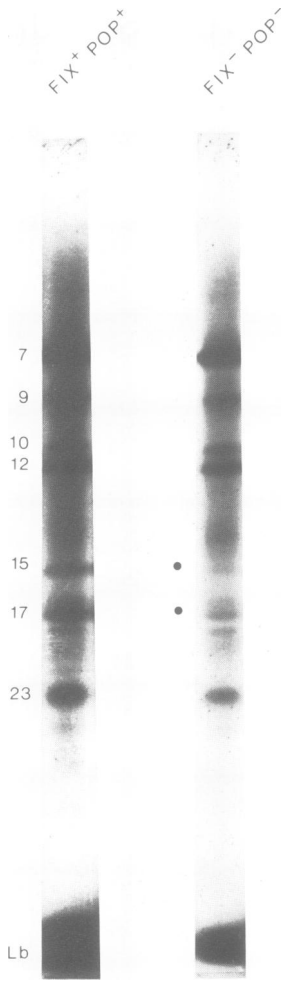

Fig. 5. Nodulin pattern of ineffective 'pop$^-$ nodules': autoradiograph of a protein blot of soluble pea proteins from root nodules of pea and *R. leguminosarum* strain 1062 (*fix*$^+$, *pop*$^+$) and mutant 116 (*fix*$^-$, *pop*$^-$) of this strain.

small zone of senescent tissue (van Brussel, 1973; Kijne, 1975) the anti-nodulin serum may contain antibodies against bacteroid proteins. The two polypeptides from the bacteroid protein fraction which showed the most intense reaction with the anti-nodulin serum are found just below N-5 and between N-9 and N-10 (Figure 6). Small amounts of these polypeptides were also detected in the peribacteroid protein fraction. This suggests that some bacteroids are lysed during the osmotic shock used for liberating the proteins from the peribacteroid space. Other bacteroid polypeptides for which antibodies are present in the anti-nodulin serum seem to occur in the per-bacteroid protein fraction in amounts below the level of detection. The two bacteroid proteins that showed the strongest reaction with anti-nodulin serum co-migrated with 5a and 9a (Figure 3) in the soluble nodule protein fraction (results not shown). Thus polypeptides 5a and 9a are presumably not nodulins but bacteroid proteins. Other polypeptides detected in the bacteroid protein fraction were not detectable in the soluble nodule protein fraction.

Discussion

An antiserum was developed against proteins from nodules 21 days after sowing when nitrogen fixing activity is maximal. It is remarkable that with such antisera, nodulins (which we refer to as early nodulins because they were only detectable in early stages of root nodule development) were detected, whereas these nodulins were not detectable in 21-day-old root nodules. Pea root nodules from 21-day-old plants have a zone of tissue that is still meristematic as well as a zone of senescent tissue in the oldest parts of the nodule (van Brussel, 1973; Kijne, 1975). Apparently, early nodulins occur in older nodules and are highly antigenic but their concentration appears low. Therefore, it is desirable to also use antisera against protein from nodules at different stages of development. The occurrence of a large number of specific polypeptides in root nodules (~30) but not in uninfected root tissue clearly illustrates the differentiation of plant cells during root nodule formation.

Whether or not the nodulins we have identified are plant encoded is not proven by our results. The approach Legocki and Verma (1980) used to identify soyabean nodulins better indicated the genomic origin of the nodulins, since they studied the run-off products of plant polysomes. We have not used this approach, however, since only nodulins with mol. wts. of ~20 000 daltons were identified, even soyabean N-35 a prominent nodulin in soyabean nodules (Legocki and Verma, 1979) was not detectable. With our method, it was possible to identify nodulins with mol. wts. up to 120 000. More recent work of Verma *et al.* (1982) demonstrated that, by

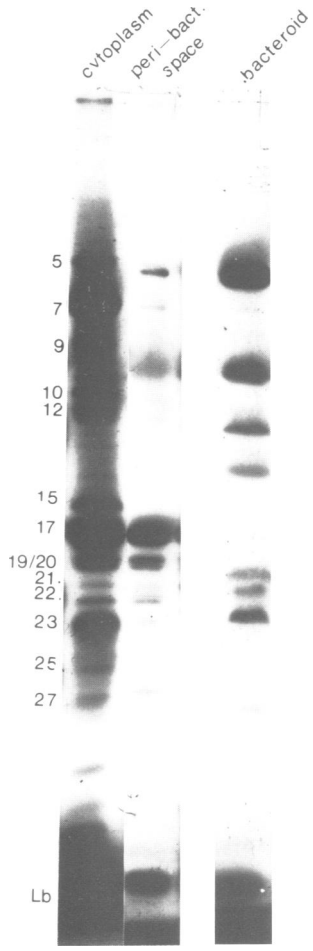

Fig. 6. Localization of nodulins: autoradiograph of a protein blot of root nodule proteins (pea x *R. leguminosarum* PRE). Soluble cytoplasmic pea proteins (cytoplasm), peribacteroid space (peri-bact. space) and bacteroids were analysed. The blot was incubated with anti-nodulin serum and [^{125}I]protein A. The same antiserum preparation was used as in Figure 3 and 4.

hybrid-released translation experiments also in soyabean nodules, nodulins with higher mol. wts. are detectable.

Since the pea nodulins are detectable on gels they must belong to the population of abundant proteins (Davidson and Britten, 1979) and therefore the corresponding mRNA sequences will probably belong to the (moderately) abundant mRNA population. Studies of Auger and Verma (1981) on the moderately abundant mRNA population of soyabean root nodules revealed that within this population 20–40 sequences are nodule specific; our observation, that ~30 nodule-specific polypeptides are synthesized in pea root nodules, are in good agreement with these data.

Nodulins 17, 19, 20, 25 and 27 can be identified in the peribacteroid space as well as the host cytoplasm. When nodules are fractionated into cytoplasm, and peribacteroid space some of the peribacteroid membranes break during fractionation. As a result, the cytoplasmic fraction will be contaminated with peribacteroid space protein. Therefore, it remains unclear whether N-17, 19, 20, 25 and 27 are localised in the cytoplasm as well as in the peribacteroid space, or solely in the peribacteroid space. The fact that the peribacteroid protein fraction contained only a minor amount of leghaemoglobin, confirms the observation of Robertson *et al.* (1978) and Verma *et al.* (1978) that leghaemoglobin is localized in the cytoplasm of the plant nodule cells.

It will be of great interest to determine the function of the different nodulins in the root nodule. Among the nodulins, there may be proteins which reflect the formation of nodule tissue while other nodulins may be essential for nitrogen fixation. Nodulins whose synthesis is repressed in ineffective nodules are probably directly involved in the nitrogen fixation process, whereas the others might have a function in nodule tissue formation. With P8 the changes were more marked than with the *pop*$^-$ mutant. In P8 nodules only rhizobia that retained their bacterial shape could be seen. Traces of leghaemoglobin could be detected in P8 nodules, however. This indicates that P8 rhizobia induce leghaemoglobin synthesis, but that they are not able to maintain the 'transformed' state of the nodule cell.

In the nodules induced by the *pop*$^-$ strain, bacteroids with a normal shape are present and no other morphological abnormalities are observed (Nadler, 1981). In the 'pop$^-$ nodules' leghaemoglobin synthesis is also reduced although more leghaemoglobin can be found than in 'P8 nodules'. The fact that apo-leghaemoglobin is detectable in the 'pop$^-$ nodules' implies that leghaemoglobin synthesis is not solely regulated by the excretion of haem from *Rhizobium*. Most nodulins can be detected in 'P8 nodules', while some like N-22 are stimulated. N-15 and N-17 genes are also repressed in the 'pop$^-$ nodules'. The repression of N-15 and N-17 genes in both types of ineffective root nodules is a strong indication that these polypeptides are involved in nitrogen fixation and are not the result of, for example, a defence mechanism of the plant against bacteria. Furthermore, the repression of nodulin genes shows that rhizobia influence the expression of host genes.

Materials and methods

Root nodules

Pea plants (*Pisum sativum* var. Rondo) were cultured and inoculated with *R. leguminosarum* as described earlier (Bisseling *et al.*, 1978).

Preparation of soluble nodule and root protein

Root nodules were ground in a mortar with 50 mM Tris-HCl pH 7.5 (1 ml/g). After filtering the homogenate through Miracloth (Calbiochem-Behring Corp.) bacteroids were removed by centrifugation for 10 min at 10 000 *g* and 4°C. The resulting supernatant contained the soluble cytoplasmic proteins and the proteins enclosed by the peribacteroid membrane. Cytoplasmic proteins from pea roots were prepared in a similar way using 5-day-old seedlings or roots from 14-day-old uninoculated plants. Protein concentrations were determined as described by Bisseling *et al.* (1978).

Fractionation of root nodules

Root nodules were ground with buffer containing 0.5 M sucrose to isolate the bacteroids surrounded by the peribacteroid membrane. The homogenate was fractionated by centrifugation into soluble cytoplasmic plant proteins and peribacteroid membrane enclosed bacteroids. The bacteroids were released from the peribacteroid membranes by resuspending in a buffer with a low osmotic potential. The method used is derived from the procedure described by Robertson *et al.* (1978). The procedure for fractionating root nodules is described in Figure 1. The bacteroids were lysed by boiling for 2 min in 2% SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, and the lysate was clarified by centrifugation (10 min, 10 000 *g*).

Protein blotting

SDS-polyacrylamide gels (15% acrylamide, 0.088% bis-acrylamide) were run as described by Zabel *et al.* (1982). After electrophoresis, polypeptides were transferred to nitrocellulose (BA 85; Schleicher and Schuell) by a modification of the method of Bowen *et al.* (1980) as described by Zabel *et al.* (1982). The nitrocellulose sheets were dried and autoradiographed with Cronex 4 X-ray film.

Protein A (50 μg) from *Staphylococcus aureus* (Pharmacia Fine Chemicals) was labeled with Na ^{125}I (1 mCi) (to a specific activity of 10 μCi/μg) using the solid-phase lactoperoxidase-glucose oxidase system (Enzymobeads, Bio-Rad).

Preparation of nodule-specific antiserum (anti-nodulin serum)

Antiserum against soluble plant nodule proteins of 21-day-old pea plants was prepared by injecting New Zealand white rabbits i.v. with 1 mg of soluble plant proteins in 0.9% NaCl; 6 and 8 weeks later this was followed by s.c. injections with 10 mg of soluble plant proteins in 0.9% NaCl mixed with an equal volume of Freunds complete adjuvant (Difco, Detroit, MI). Rabbits were bled through the jugular ear vein 6 days after the third injection. This antiserum was made nodule-specific (anti-nodulin serum) by titration with root proteins prepared from 5-day-old seedlings and 16-day-old roots from uninfected pea plants. The root protein preparations from 5-day-old seedlings and 16-day-old roots from uninfected pea plants were mixed in the ratio 1:1. The antiserum (200 μl) was incubated successively with 10, 20, 40, 80 and 160 μl of the root protein preparation (1.2 mg/ml), each incubation lasted 8 h at 4°C, after which the immunoprecipitates were removed by centrifugation (5 min, 10 000 *g*).

Acknowledgements

The authors thank Mr.Houwers for culturing nodulated plants, Mr.Madern for making the illustrations and Mrs.M.J.van Neerven for typing the manuscript. This investigation was supported by the Niels Stensen Stichting (T.Bisseling) and a grant from the U.S. Department of Agriculture Competitive Research Grant Program (# 5901-0410-8-0100) (K.Nadler).

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