Symbiosis, 3 (1987) 219–232
Balaban Publishers, Philadelphia/Rehovot

Nodule Specific Polypeptides of Broadbean (*Vicia faba* L.)

S.S. MOHAPATRA[1], A. PERLICK and A. PÜHLER

Lehrstuhl für Genetik, Fakultät für Biologie
Universität Bielefeld, D-4800, Bielefeld-1, F.R.G.

Received July 20, 1987; Accepted August 13, 1987

Abstract

Several plant gene products have been implicated in the development of nitrogen-fixing root nodules in legumes. In this study, two approaches *in vivo*, namely immunoblotting and labeling *in vivo* as well as the analyses of translation products *in vitro* were used to identify the nodule specific polypeptides in *Vicia faba* L. Fourteen polypeptides ranging in molecular weight from 13 to 82 kd appeared to be specific to the root nodule in an immunoblotting experiment. These polypeptides are detected in the root nodules at various stages of nodule growth. Most of these polypeptides are synthesized at about the same week as the most abundant leghemoglobin, with the exception of 2 polypeptides that are detectable 1 to 2 weeks later. SDS-PAGE analysis of root and nodule polypeptides selectively labeled *in vivo* indicates 4 early nodule specific polypeptides are present in addition to 6 others detected later in nodule development. Finally, comparative analysis of translation products *in vitro* by two-dimensional polyacrylamide gel electrophoresis shows about 25 nodulin polypeptides specific to the nodule tissue.

Keywords: *Vicia*, nodule-specific polypeptides, nodulins

Abbreviations: SDS-PAGE = Sodium dodecylsulfate-polyacrylamide gel electrophoresis, 2-D = two-dimensional, IEF = isoelectric focusing, PMSF = phenylmethyl sulfonylfluoride

[1]Present address: Centre for Plant Molecular Biology, Department of Biology, McGill University, 1205 Docteur Penfield Ave., Montreal, PQ H3A 1B1, Canada

1. Introduction

A successful interaction between a legume host and an invadi ıg *Rhizobium* strain leads to the development of a nitrogen-fixing root nodule. This involves specific and/or amplified synthesis of several polypeptides by b ɔth plant and bacterium. Molecular studies of the genes involved in nodulati ɔn and nitrogen fixation have advanced rapidly for various *Rhizobium* speci s recently. In contrast, knowledge at the molecular genetic level of the contr ɔution of the host plant to the process of symbiosis has been limited to soy ɔean (Verma et al., 1986) and pea (Govers et al., 1985).

In soybeans, immunoprecipitation of translation products f om root and nodule mRNA *in vitro* with nodule-specific antisera led to the dentification of 18 to 20 nodule-specific polypeptides, termed "nodulins" Legocki and Verma, 1980). Analysis of cDNA clones made from nodule mI NA revealed 5 unique sequence species (Fuller et al., 1983) specific to th e nodule tissue. The structure and function of 2 of the soybean nodulins, l ghemoglobin (Marcker et al., 1984) and n-uricase (Nguyen et al., 1985) have been characterised in detail. Two other soybean nodulin genes, nodulin-23 Maura et al., 1985) and nodulin-24 (Katinakis and Verma, 1985) have been se uenced. The function of these and other nodulins remains unknown. Bisselin et al. (1983) used immunoblotting technique to identify 30 nodulins in p a. However, comparative analyses by two-dimensional gel electrophoresis f translation products *in vitro* from RNA of root and nodule tissue showed 21 nodulins, which included 8 major and 13 minor polypeptides (Govers et al, 1985). Recently, nodulins have been studied in phaseolus (Lara et al., 1983) and in alfalfa (Lang-Unnasch and Ausubel, 1985; Vance et al., 1985; M ɔhapatra and Pühler, 1986). Western blotting analyses (Vance et al., 1985) h ıve indicated that only a few nodulins, including leghemoglobins, are conse ved across a diverse range of legumes.

Vicia faba is becoming increasingly important in Europea ı agriculture because of its nitrogen fixation yield per hectare and protein yield. Thus, studies involving its association with *Rhizobium* are timely (riefer et al., 1985). In addition, *Rhizobium* strains which infect *V. faba*, constitute a subgroup of *Rhizobium leguminosarum*. Thus, *V. faba* noduli s may differ from those of pea, the other legume host infected by this *Rhizo ium* species. *V. faba* is also very distantly related to the well studied soybean. So nodulins common to these legumes would be of evolutionary interest wi h respect to nodulation and nitrogen fixation.

As part of a study on the regulation and expression of *Vicia faba* nodulin genes in symbiosis (Kuhse et al., 1985; Kuhse and Pühler, 1987), we report here the detection of several nodule-specific polypeptides as useful background information for future molecular genetic studies. Immunoblotting and labeling *in vivo*, as well as the analysis of translation products *in vivo* were used to identify the nodule-specific polypeptides.

2. Materials and Methods

Plant culture and nodule collection

Vicia faba L cv. Kleine Thuringer seeds were surface-sterilised, inoculated with effective *Rhizobium leguminosarum* strain Vf39 (Priefer et al., 1985), planted in pots containing sand and grown in a glass house. In the case of young seedlings, less than 7 days old, 1–2 cm pieces of the infected zone were cut from the main root. In plants older than 7 days the infected zone containing root nodules was collected. Uninfected plants were cultured in the same way and 7–8 day old roots were harvested. For isolation of proteins, the roots and nodules were freshly harvested. For collection of RNA, the tissues were frozen in liquid nitrogen and stored at –70° C.

Extraction of proteins and separation by gel electrophoresis

The root or nodule tissue was homogenized with mortar and pestle in an extraction buffer containing Tris-HCl 25 mM pH 7.5, KCl 10 mM, $MgCl_2$ 20 mM, β-mercaptoethanol 5 mM and 0.2 g/g f.w. of tissue of insoluble PVP. The insoluble fraction was pelleted by centrifugation at $14,000 \times g$ for 2 min. The bacteroids were obtained as a pellet after another centrifugation at $40,000 \times g$ for 30 min. The bacteroid pellet was washed several times in extraction buffer and finally the cells were lysed in a buffer containing Tris-HCl pH 6.8, SDS 2% (w/v) and β-mercaptoethanol 5 mM. Equal amounts of root and nodule polypeptides were separated by electrophoresis on 7.5–17.5% linear gradient SDS-polyacrylamide gel (Lammelli, 1970). The gels were stained in 0.2% (w/v) coomassie brilliant blue R (Sigma) in a solution containing 30% v/v methanol and 7% (v/v) acetic acid. The gels containing translation products formed *in vitro* or labeled products formed *in vivo*, following treatment with "Amplify" (NEN), were dried and exposed to x-ray film (Kodak RP-x-omat) for fluorography.

Molecular weight standards for polyacrylamide gel electrophoresis were purchased from Bethesda Research Laboratories GmbH, West Germany. The proteins included in these are: cytochrome c (12.3 kd), β-lactoglobulin

(18.4 kd), α-chymotrypsinogen (25.7 kd), ovalbumin (43 kd), bovine serum albumin (68 kd), phosphorylase B (97.4 kd) and myosin H (200 kd).

Preparation of antisera

The plant cytoplasmic proteins (the supernatant from 40,000×g centrifugation) from 25 day old root nodules of *V. faba* were injected into the rabbit subcutaneously. About 1 mg of proteins in 1 ml of solution containing 10 mM Tris-HCl pH 7.5 and 0.9% (w/v) NaCl was mixed with an equal amount of Freund's complete adjuvant (Difco Laboratories, Detroit) for the first injection. Three weeks later this was followed by an intravenous injection of 3 mg of similarly prepared soluble proteins. After a further 2 weeks, the rabbits were bled through the ear veins and antisera were collected. Before use the IgG fraction of the antiserum was normally enriched by ammonium sulfate fractionation (the 0 to 33% of saturation fraction was used after dialysis). The antiserum prepared was made "nodule specific" by absorption with uninfected root proteins and proteins from the nodule bacteria (Legocki and Verma, 1980; Bisseling et al., 1983).

Immunoblotting procedure

The proteins were transferred from the SDS-PAGE gel onto nitrocellulose (BA85 Schleicher and Schüll) by electroblotting (Brittner et al., 1980). Following blotting, the nitrocellulose filter was quenched in a buffer containing 0.01 M Tris-HCl pH 7.5, 0.35 M NaCl, 0.1 mM phenymethylsulfonylfluoride (PMSF) and 3% (w/v) bovine serum albumin (BSA) for 5 hr with slow shaking at room temperature. The filter was then incubated for 16 hr at 4° C in an assay buffer containing 0.01 M Tri-HCl pH 7.5, 0.15 M NaCl, 1% (v/v) Triton X-100, 0.5% sodium-deoxycholate, 0.1% (w/v) SDS, 0.1 mM PMSF and 1% (w/v) BSA and the appropriate antisera (10 μl/ml of the buffer). Following the antibody reaction the filter was washed three times in the assay buffer without BSA, 20 min each time, by shaking at 50 rpm on a gyratory shaker. Subsequently, the filter was incubated in the assay buffer containing affinity-purified [^{125}I]-labeled Protein A (Amersham Corp., Illinois) at about 10^6 cpm/ml of the buffer. The filter was washed 6 times 10 min each in the assay buffer without Protein A, dried and autoradiographed.

Labeling in vivo

For labeling *in vivo*, the infected zone containing nodules and the equivalent zone from 2 to 3 week-old uninfected roots were cut into 3 to 5 mm pieces. The tissues were incubated in an Eppendorf tube containing 100 μl of sterile water, 200 μg/ml streptomycin and 75 μCi [^{35}S]-methionine (1100 Ci/mmol) and/or cysteine (600 Ci/mmol) (Dupont de Nemours GmbH, NEN) for 3 hr on a shaker at room temperature. The radioactivity incorporated was measured by scintillation counting as for translation products *in vitro* (see below).

Isolation of mRNA

Twenty or 30 g of root or nodule tissue were ground in liquid nitrogen to a fine powder. The powder was thawed to 4° C and to this were added 4 ml/g tissue extraction buffer containing 200 mM Tris-HCl, pH 9.0, 400 mM KCl, 10 mM EDTA, 2% (v/v) Triton X100 and 5 mM β-mercaptoethanol. After removal of plant debris and/or bacteroids by centrifugation, the supernatant was extracted twice with phenol-chloroform mix (1 part phenol, 1 part chloroform:isoamyl alcohol and 0.1% (w/v) 8-hydroxy quinoline) and once with chloroform-isoamylalcohol (24:1 v/v). To the aqueous phase 10 M LiCl was added to a final concentration of 2 M. The RNA was precipitated overnight at 4° C on ice, collected by centrifugation and washed once with 2 M LiCl and once with 70% (v/v) ethanol. The poly(A)$^+$ RNA was then separated by oligo dT-cellulose-chromotography (Aviv and Leder, 1972).

Translation in vitro

Poly(A)$^+$ RNA isolated from uninfected roots and root nodules was translated in a mRNA-dependent wheat germ cell-free translation system (Amersham Corp., Illinois). The protocol provided by the supplier was followed. Translation reactions were carried out in 30 μl volumes and contained 0.5 to 1.0 μg poly(A)$^+$ RNA, 15 μCi/assay [^{35}S]-methionine (1100 Ci/mmol) (Dupont de Nemours GmbH, NEN) and other standard additives. The RNA concentration used for translation *in vitro* was chosen in the linear range so that the intensity of a spot was directly proportional to the amount of translatable mRNA. The reaction was run at 30° C for 1 hr and was terminated by placing the tubes on ice. The reaction mixture was treated with 10 μg of RNase for 15 min. A 2 μl aliquot was precipitated on Whatman GF/C filter. The filter was subsequently washed 3 times for 2 min in 5% TCA, once in 95% ethanol for 5 min and dried. Its radioactivity was determined by liquid scintillation spectrometry.

Two-dimensional gel electrophoresis

Translation products labeled *in vitro* from root and nodule RNA were separated in two dimensions; the first according to the isoelectric point, the second according to molecular weight following the O'Farrell procedure (O'Farrell, 1975). The pH range of the isoelectric focusing dimension was fixed from 5.8 to 7.2. The linear gradient was formulated with 1% ampholines pH 3.5–10 and 4% ampholines pH 5–7. The second dimension was subjected to SDS-PAGE in 10–20% gels. The gels were then fluorographed as described before.

3. Results

Detection of nodule specific proteins by immunoblotting

Analysis of the soluble proteins from uninfected roots and 4 week-old nodules of *V. faba* by SDS-PAGE (data not shown) showed that the majority of the polypeptides are common to both tissues. In addition to leghemoglobin (13 kd), two other polypeptides of apparent molecular weight 30 kd and 36 kd appeared to be formed specifically in the nodule tissue.

Nodule development is a complex process and it is thus likely that many other proteins are involved which are not detectable with one-dimensional SDS-PAGE. To detect nodule specific polypeptides, an immunoblotting procedure was used.

An antiserum was raised against proteins isolated from 21 day-old root nodules. The proteins extracted from uninfected root tissue and root nodules after 8, 9, 10, 11, 13, 17 and 19 days of planting, were separated by SDS-PAGE and transferred to nitrocellulose. The blot was incubated with the nodule antiserum. The polypeptides were visualized by incubating this blot with [^{125}I]-Protein A (Fig. 1A). The antiserum reacted with several root polypeptides. In addition to a strongly-reacting band with a molecular weight of 68 kd, some others were a strongly-reacting band *with a molecular weight* of 68 kd detectable in the uninfected root and the root nodule tissue, all throughout the time-course. About ten polypeptides (ranging from 13 kd to 82 kd) appeared gradually during the period of nodule development. These polypeptides are referred to in the text by following the suggested nomenclature (Van Kammen, 1984); i.e. by using the letter N followed by the molecular weight. The most intensely labeled low molecular weight polypeptide(s), apparently leghemoglobin, was detectable in the tissue between 9 to 10 days after planting (N13). Seven nodule specific polypeptides (N82, N58, N54, N37, N36, N28 and N14) were detectable at about the same time

as leghemoglobin and continued to accumulate in the tissue. The polypeptides N30 and N24 began to accumulate at 17 and 19 days after planting, respectively.

In order to increase the resolution of immuno-detection, the above nodule antiserum was made "nodule specific" by its absorption with root and bacteroid proteins. Proteins isolated from uninfected roots, 21 day old root nodules and bacteroids were separated by SDS-PAGE and electroblotted onto nitrocellulose. Fig. 1B represents an autoradiogram obtained after incubation of this nitrocellulose filter with the nodule-specific antiserum followed by its reaction with [^{125}I]-labeled Protein A. This antiserum did not react with the root polypeptides except for the 68 kd band that was detectable in root, nodule and bacteroid polypeptide pattern. About 14 different polypeptides, including the 10 identified in Fig. 1A reacted with this antiserum. These are designated as in Fig. 1A. An additional polypeptide of 43 kd was observed in 21 day nodules (Fig. 1B) and was not detected in the time course experiment (Fig. 1A). This may represent a late nodulin. The 66 kd polypeptide in Fig. 1A was not marked as a nodule specific polypeptide, since it was also observed in bacteroid protein profile (Fig. 1B). This may be the *R. leguminosarum* polypeptide reported previously (Lang-Unnasch and Ausubel, 1985) that is probably excreted to the nodule cell cytoplasm.

Labeling in vivo of root and nodule polypeptides

Results obtained by immunoblotting are dependent upon antigenicity of individual proteins; thus proteins that are less antigenic may remain undetected by this method. Therefore, another technique, labeling *in vivo* was used. Ten and 20 day-old tissue from the infected zone and the equivalent zone of the uninfected root after 10 days of planting were harvested and immediately labeled with [^{35}S]-methionine and -cysteine in presence of streptomycin (for details see Mohapatra and Pühler, 1986). Fig. 2 shows a fluorogram of these labeled products separated by SDS-PAGE. Of a total of 35 polypeptides, the majority was similar in both tissue types. Ten polypeptides ranging in molecular weight from 13 to 82 kd were specifically found in the nodule tissue. These are designated by their molecular weights. Four polypeptides (64 kd, 62 kd, 58 kd and 45 kd) appeared only in the tissue from 10 day-old plants. Other polypeptides except 13 kd and 14 kd were detectable in the tissue from 20 day-old nodules.

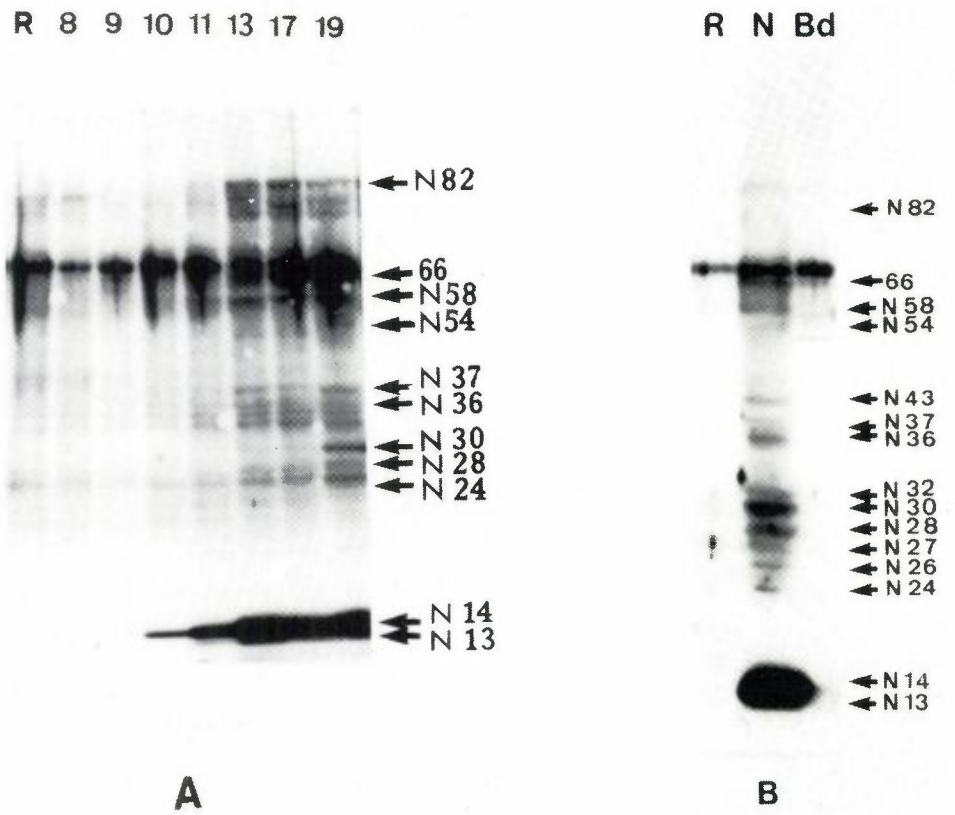

Figure 1A. Autoradiograph of an immunoblot containing proteins from uninfected root (R) and root nodules by *Rhizobium* strain V_f39 after different days of planting. The numbers above the lanes indicate the number of days after planting when the nodules were collected for the experiment. Equal amount (50 μg) of proteins were separated on a 7.5–17.5% linear gradient gel by SDS-PAGE, blotted onto nitrocellulose which was reacted first with nodule antiserum and then with [^{125}I]-labeled Protein A. Solid arrows indicate the nodule-specific polypeptides detected during the time course of nodule development. Using nomenclature suggested at the 5th International Symposium on nitrogen fixation (Van Kammen, 1984), the nodule-specific polypeptides are indicated by letter "N" followed by their molecular weight in kd.

Figure 1B. Autoradioigraph of a blot of proteins extracted from uninfected root (R), nodules induced by strain V_f39 (N) and bacteroids of these nodules (Bd). About 50 μg of protein were subjected to SDS-PAGE in a 7.5–17.5% gel and blotted onto nitrocellulose. The filter was incubated with nodule-specific serum (see methods) and then with [^{125}S]-labeled Protein A. Solid arrows indicate the position of the nodule-specific polypeptides which are marked as in Fig. 1A.

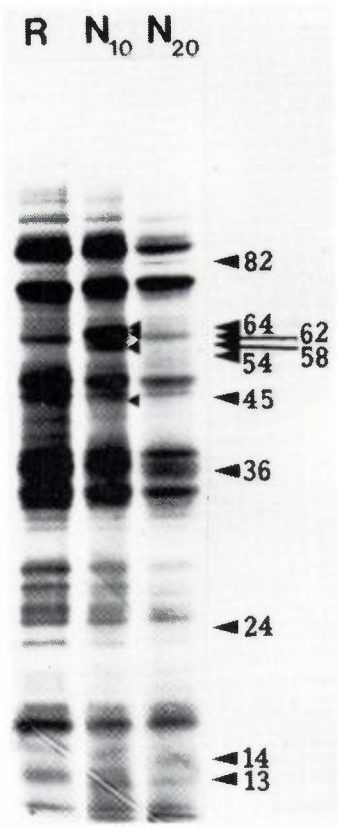

Figure 2. Fluorograph of products labeled *in vivo* from the uninfected root (R), 10 day-old root nodules (N10) and 20 day-old root nodules (N20). The tissues were labeled with 75 μCi each of [^{35}S]-methionine and -cysteine for 3 hr. Equal amounts of TCA precipitable radioactivity (100,000 cpm) were separated on a 7.5–17.5% gel by SDS-PAGE and fluorographed.

Analysis by translation in vivo

Poly(A)$^+$ RNA isolated from mature root nodules of *Vicia faba* and from uninfected roots were translated in a wheat germ cell-free system. The translation products were separated by 2-D PAGE and the autoradiogram is presented in Fig. 3. About 400 polypeptides ranging from 12 to 80 K in molecular weight can be identified from each translation in a reproducible manner. Comparison of the spot pattern of both root and nodule translation products showed that the majority of the polypeptides was present in both. However 11 major and 14 minor spots were only observed in the pattern of polypeptides obtained from nodule Poly(A)$^+$ RNA. In addition, at least 4 spots were detected in the nodule fraction that were present in the root but at a lower

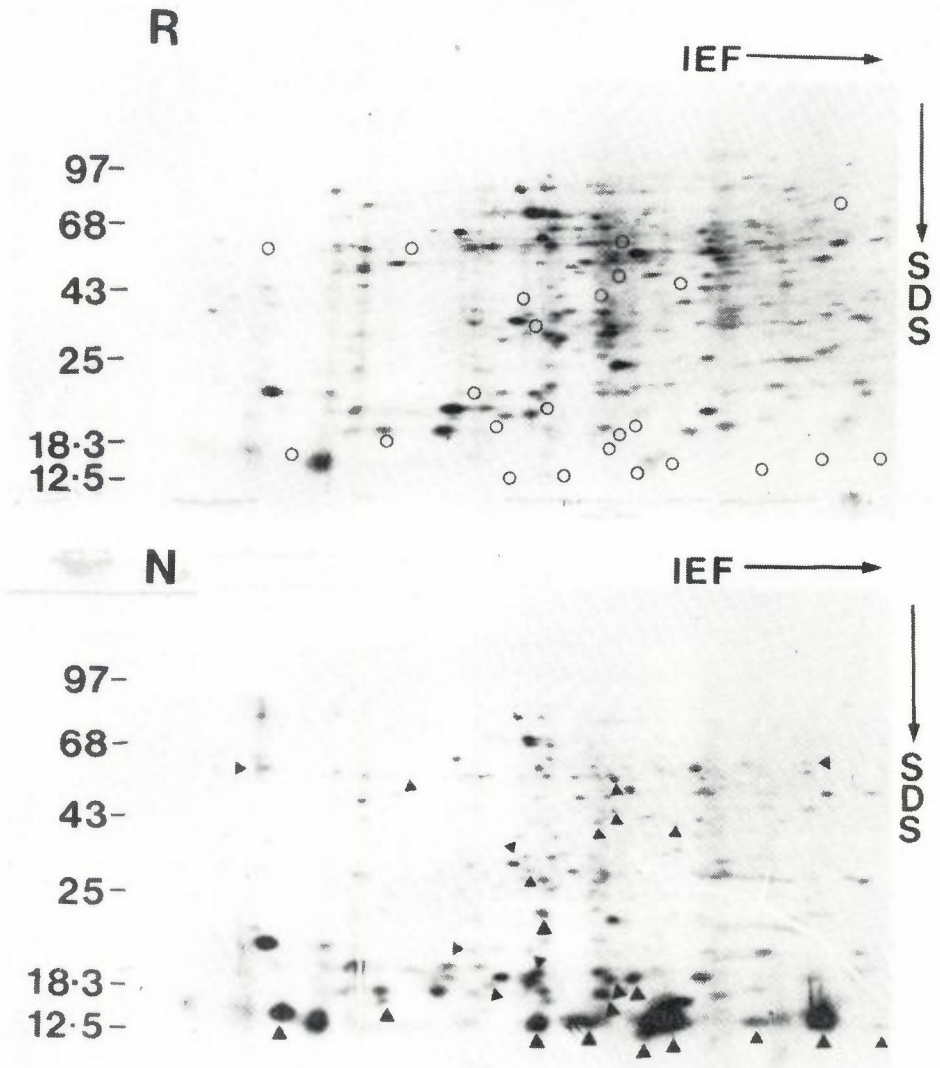

Figure 3. 2-D fluorographs of *in vitro* translation products of poly(A)$^+$ RNA isolated from uninfected root (R) and root nodule (N) tissue of *V. faba*. The RNA was translated using a cell-free wheat germ system. 500,000 cpm worth of translation products (measured by TCA precipitable radioactivity) were separated by IEF and SDS-PAGE (10–20%) gels and the gels were fluorographed as described in Materials and Methods. The solid arrows in "N" indicate polypeptide spots specific to the root nodule tissue. The empty circles indicate the approximate hypothetical position of these polypeptides in fluorographs of translation products from root RNA. The number on the left margin indicate positions, molecular mass in kd of subunit polypeptide bands of the marker proteins.

intensity. The nodulin polypeptides varied in their molecular weights from 13 to 65 kd with a majority ranging between 13 and 25 kd. Eight spots present in the pattern from uninfected roots were either absent or present at a lower intensity in the pattern from the nodules.

4. Discussion

The development of the root nodule follows infection by *Rhizobium* and includes a series of visible features in the infected part of the primary root. The first nodule origins in *V. faba* are noticeable at 7 to 8 days after planting the seeds. The nitrogenase activity appears 13 days after planting. The development of nodule specific polypeptides was assayed in this study by immunoblotting and labeling proteins *in vivo*. Appearance of several polypeptides including leghemoglobin after 10 days of planting (Fig. 1A) is consistent with the findings in other legumes (Bisseling et al., 1983; Lang-Unnasch and Ausubel, 1985; Mohapatra and Pühler, 1986). Labeling of root nodules *in vivo* provides limited resolution due to the low abundance and a low specific activity of the labeled polypeptides (for details see Legocki and Verma, 1980; Mohapatra and Pühler, 1986), particularly in matured nodules.

Additional [^{35}S]-cysteine in the labeling medium improved the general resolution and provided reproducible results. Using this technique, 4 polypeptides were detected specifically at 10 day-old root nodules which disappeared in 20 day-old root nodules (Fig. 2). These may represent the early nodulins of *V. faba*, similar to the ones described in pea system previously (Govers, et al., 1985). Thus, it appears that different polypeptides are induced at different stages of nodule development.

The *in vivo* approaches used here to catalogue nodule specific polypeptides in *V. faba* have their own merits and demerits. While immunoblotting procedure relies on antigenicity of proteins, labeling *in vivo* is dependent upon the presence of sufficient methionine and cysteins in the individual polypeptides. This study indicates that both approaches can be combined to obtain meaningful results. The results of labeling experiments *in vivo* cannot be directly compared with the results from that of the immunoblotting experiments, since post-translational modification and breakdown may occur in unlabeled versus labeled proteins. However, from the results in this report, the molecular weights of 7 nodule specific polypeptides (N82, N58, N54, N36, N24, N14 and N13) are identical as detected by both *in vivo* approaches (Fig. 1B and Fig. 2).

Leghemoglobin, the most abundant protein in the nodule tissue (also visualized in immunoblot Fig. 1A and 1B) was not labeled as efficiently *in vivo*. This is not surprising in view of the fact that only a few methionines and cysteines are present in leghemoglobins of *V. faba* (Kuhse and Pühler, 1987).

The *in vitro* translation products (visualized by spots) specific to nodule tissue are by definition nodulins (Van Kammen, 1984). A majority of the nodulins were predominant spots and may therefore be products of abundant and moderately abundant mRNA species (Davidson and Britten, 1979). Our results are comparable to those obtained in soybean (Legocki and Verma, 1980) and pea (Govers et al., 1985).

A detailed study of the regulation of these nodulin genes by translation of nodule RNA *in vitro* during the course of nodule development is currently in progress. Furthermore, in order to identify and characterise the nodulin genes of *V. faba*, a cDNA library was constructed in our laboratory (Kuhse et al. 1985; Kuhse and Pühler, 1987). It is hoped that the identification of nodule-specific polypeptides by immunoblotting and labeling *in vivo* as well as the analysis of the *in vitro* translation products presented in this report coupled with the analysis of the *V. faba* nodule-specific cDNA will enhance the knowledge of the role of plant genes in symbiosis.

Acknowledgements

We wish to thank Dr. A. Radunz for his help in raising antiserum against *Vicia* root nodule proteins. S. Mohapatra was a recipient of the Alexander von Humbolt Research Fellowship. Ms. L. Byrne is thanked for typing the manuscript. The experiments were financially supported by an EEC research grant.

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