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Plant gene expression in effective and ineffective root nodules of alfalfa (*Medicago sativa*)

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Abstract

Expression of plant genes involved in the symbiosis between alfalfa (*Medicago sativa*) and *Rhizobium meliloti* has been studied by comparing root and root nodule mRNA populations. Two-dimensional gel electrophoretic separation of the *in vitro* translation products of polyA$^+$ RNA isolated from either roots or effective root nodules has allowed us to identify thirteen nodule-specific translation products, including those corresponding to the leghemoglobins (Lb). These translation products, representing putative nodulin mRNAs, are first detected between 9 and 12 days after inoculation, a result which has been confirmed for Lb mRNA by Northern blotting and hybridization with a Lb cDNA probe. Analysis of three different types of ineffective root nodules arrested in different stages of development has led to the following conclusions. (i) The transcription of eleven nodule-specific genes, including the Lb genes, is independent of nitrogen-fixing activity. (ii) Differentiation of the primary nodule structure does not require the transcription of any of these genes but can be correlated with a dramatic reduction in the level of at least five transcripts present in the root. (iii) There is enhanced expression of certain plant genes in the case of nodules elicited by an *Agrobacterium* strain carrying the symbiotic plasmid of *R. meliloti*.

Introduction

Leguminous plant species can develop symbiotic associations with nitrogen-fixing bacteria of the genus *Rhizobium*. This interaction leads to the formation of specialized organs, root nodules, and involves the co-differentiation of both symbiotic partners. Considerable progress has been made in recent years towards identifying bacterial genes involved in this process [2, 20]. Whilst parallel studies on the host plant have advanced more slowly, both genetic [16] and molecular [5, 29] approaches have nevertheless underlined the essential role played by plant genes in the development of the nitrogen-fixing nodules. Proteins specific to the nodule that are encoded by the plant genome have been termed nodulins [15, 27]. Amongst the few nodulins whose functions have so far been identified are the oxygen-binding proteins leghemoglobin (Lb) [1] and the nodule-specific forms of two enzymes involved in ammonium assimilation, uricase [3] and glutamine synthetase [8, 22].

In the symbiotic relationship between alfalfa (*Medicago sativa*) and *Rhizobium meliloti*, Lang-Unnasch [14] and Vance [26], using immunological techniques, have reported respectively the detection

of nine and nineteen nodule-specific polypeptides *in vivo*. We have used an alternative and complementary methodology by which plant gene expression during nodule development has been studied at the mRNA level. Thirteen nodule-specific plant mRNA translational activities, including those for the Lb family, have been detected by *in vitro* translation of nodule polyA$^+$ RNA, followed by two-dimensional (2-D) gel electrophoretic separation of the translation products. Furthermore, we have followed the kinetics of Lb mRNA induction by means of Northern blotting and hybridization with a Lb cDNA probe. Finally, we have compared the expression of the genes encoding nodule-specific polypeptides in mature effective nodules with that in three different types of nodules elicited by non nitrogen-fixing bacteria. We conclude that, whilst all except two of the alfalfa nodule-specific plant genes that we have detected are expressed independently of nitrogenase synthesis, their expression is not a prerequisite for nodule organogenesis.

Material and methods

Plant material

Cuttings from an alfalfa clone (*Medicago sativa*, cv. hybrid 11 × 8, from Dr Dattée, Orsay, France) were grown in aeroponic conditions at 20 °C, with a relative humidity of 75% and a 16 h light/8 h dark photoperiod. The composition of the nutritive solution was a modification of that described by Coic *et al.* [7]: 5.5 mM potassium phosphate (pH 7.0), 0.52 mM K_2SO_4, 0.25 mM $MgSO_4$, 1 mM $CaCl_2$, 50 μM $FeSO_4$, 50 μM Na_2EDTA, 30 μM H_3BO_3, 10 μM $MnSO_4$, 0.7 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 1 μM Na_2MoO_4, 0.04 μM $CoCl_2$, 5 mM NH_4NO_3. Two days before inoculation, the plant medium was changed for a low-nitrogen nutritive solution (0.5 mM NH_4NO_3). The nitrogen-fixing activity of the nodules was measured by the acetylene reduction method [25].

Bacterial strains

The Nod$^+$ Fix$^+$ *R. meliloti* strain is a Rif^r derivative of RCR 2011 [22]. *R. meliloti* 1354 is a Sm^r Nod$^+$ Fix$^-$ derivative of RCR 2011 carrying a Tn5 insertion in the *nifA* regulatory gene [23]. *R. meliloti* EJ355 is a spontaneous acidic exopolysaccharide-deficient mutant of a Sm^r Rif^r derivative of RCR 2011 [10]. The *Agrobacterium* strain (GMI9013 (pGMI27)) is an Ery^r Cm^r derivative of C58, cured of its Ti plasmid, and carrying the symbiotic plasmid (pSym) of RCR 2011 [24].

Extraction of RNA

Roots, inoculated roots and hand-picked root nodules were stored in liquid nitrogen. Frozen tissue was ground in a mortar to a fine powder under liquid nitrogen. To 1 g fresh weight of tissue, 2 ml 0.2 M Tris-HCl (pH 7.2), 20 mM EDTA, 100 mM NaCl, 1% SDS and 2 ml phenol:chloroform:isomyl alcohol (24:24:1) were added. After shaking and centrifugation of this suspension, the aqueous phase was reextracted three times with 2 ml phenol:chloroform:isomyl alcohol and then twice with the same volume of chloroform:isoamyl alcohol (24:1). Total RNA was precipitated overnight at −20 °C in the presence of 0.3 M sodium acetate and 70% ethanol. The polyA$^+$ RNA was purified by passing twice through an oligodT cellulose column (Collaborative Research) [19].

In vitro *translation and gel electrophoresis*

In vitro translation of polyA$^+$ RNA was carried out in a rabbit reticulocyte lysate system (Genofit) and polypeptides were labeled with (^{35}S) methionine (Amersham). Typically 1 μg of polyA$^+$ RNA was translated during 30 min at 30 °C in a 50-μl incubation mixture containing 1 mCi/ml (^{35}S) methionine. Approximately 3×10^5 cpm of *in vitro* translation products was analysed by two-dimensional gel electrophoresis as described by O'Farrell [17]. For the isoelectric focusing 1.6% ampholines pH 5–7 and 0.4% ampholines pH 3.5–10 (L.K.B.) were used and SDS electrophoresis was performed in 12.5% polyacrylamide slab gels. After protein fixation the gels were dried, treated with Amplify (Amersham) and

exposed at −70 °C to Kodak X-Omat S film for fluorography [6].

Immunoprecipitation

The translation products were immunoprecipitated, according to Fuller *et al.* [11], with rabbit antisera raised against either alfalfa or *Sesbania rostrata* purified leghemoglobin preparations (kindly given by Dr Bogusz, Dakar, Senegal) and separated by 2-D gel electrophoresis.

Northern blotting and hybridization

Ten μg of total RNA was separated under denaturing conditions in 1.2% agarose gels containing 6% formaldehyde. After eletrophoresis the RNA was blotted onto GeneScreen membrane (New England Nuclear Corp.) according to manufacturer's instructions. The membrane was hybridized, at 42 °C, in the presence of 50% formamide and 10% dextran sulfate as described in the GeneScreen protocol. After hybridization the filter was washed at 65 °C in 2×SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 1% SDS, then at room temperature in 0.1×SSC, and exposed for autoradiography. Lb mRNAs were identified using a plasmid (pNL154) consisting of a 600-bp long alfalfa Lb cDNA insert cloned in the PstI of pBR322 (confirmed by sequencing; manuscript in preparation). For hybridization, a 100-bp fragment of the insert was ^{32}P-radiolabeled using the random oligodeoxynucleotide priming method of Feinberg and Vogelstein [9].

Results

In vitro *translation of mRNAs from effective root nodules*

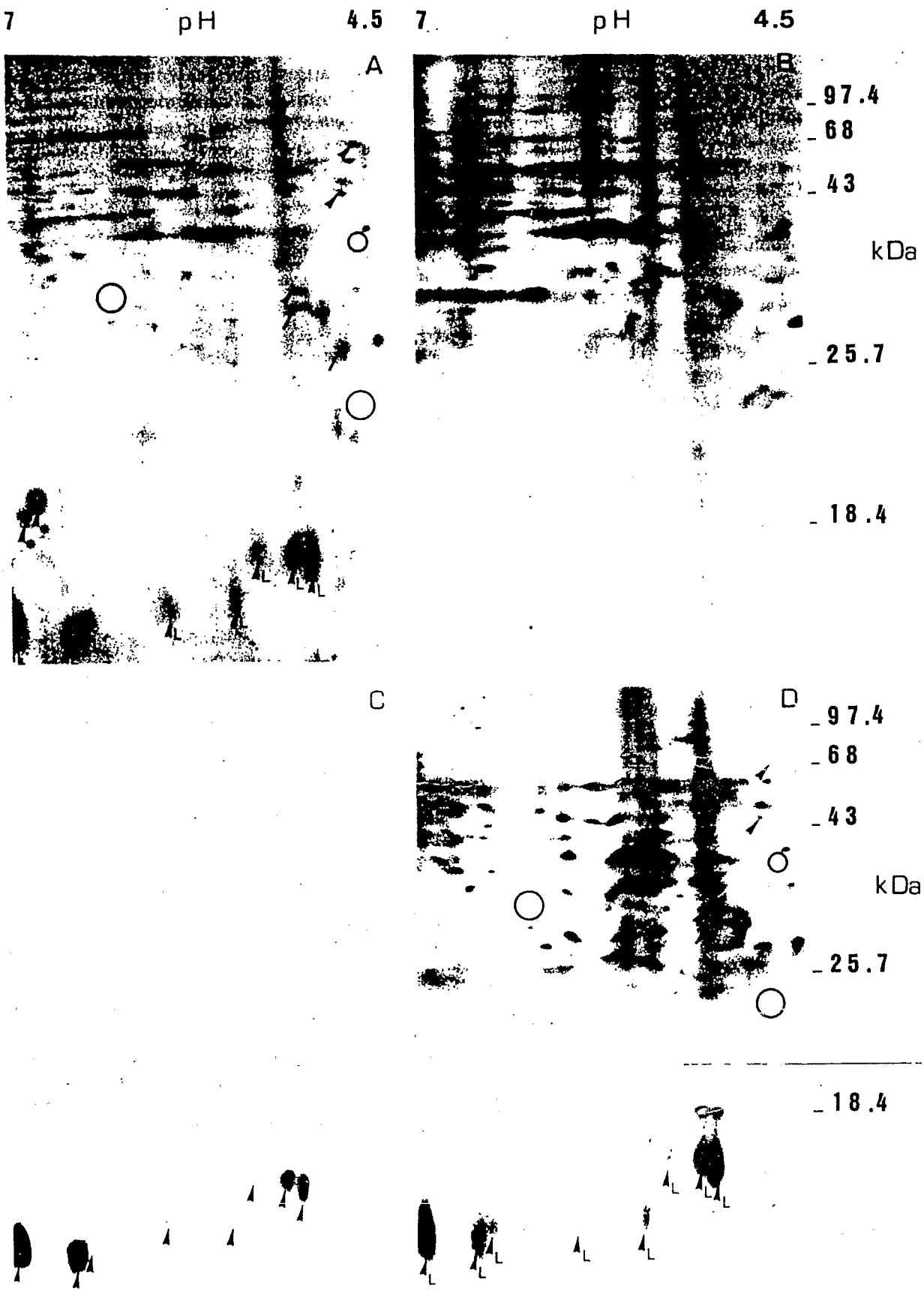
The expression of plant genes during nodule development has been analysed by 2-D gel electrophoretic separation of the *in vitro* translation products of polyA$^+$ RNA. This technique has allowed us to detect 200−300 polypeptides in the range 10 to 100 kDa molecular mass in a reproducible manner.

The comparison of the 2-D pattern of translation products from mature effective root nodules, elicited by *R. meliloti* 2011, with that obtained from uninoculated alfalfa roots (Fig. 1A and 1B) shows that whilst the majority of polypeptide spots are present in the patterns of both roots and nodules, there are, however, a number of quite clear differences. Approximately fifteen root polypeptides show reduced levels in the nodule pattern, and at least five of these, with apparent molecular masses of 22 kDa, 23 kDa, 32 kDa, 33 kDa, 39 kDa, are undetectable even after lengthy exposure of the fluorograph. On the other hand, ten polypeptides are more abundant in the 2-D gel pattern of the *in vitro* translated nodule mRNAs compared with that from root.

In addition, our data show unambiguously that thirteen polypeptides are specific to the effective nodule and hence might represent the expression of nodulin genes. Eight of these (four major and four minor spots with molecular masses of around 15 kDa) have been identified as leghemoglobins (Lb) because they can be immunoprecipitated by an alfalfa anti-Lb serum (Fig. 1C). Furthermore, an antiserum raised against Lbs from the tropical legume *Sesbania rostrata* shows cross reactivity with all eight of these alfalfa polypeptides (data not shown). The approximate molecular masses of the other 5 nodulins are 19 kDa, 20 kDa, 32 kDa, 43 kDa, 60 kDa.

Expression of nodulin genes during the development of effective root nodules

Under our particular growth conditions, alfalfa nodules first appear 1 week after inoculation with *R. meliloti* 2011, but are too small to be hand-picked until approximately 1 week later. Thus, in order to compare nodulin gene expression throughout nodule development we were obliged to work with nodulated root material instead of isolated nodules. The resulting dilution of the nodule-specific mRNAs led us to study the induction of Lb gene expression by means of Northern hybridization analysis and making use of an alfalfa Lb cDNA clone (see Material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and methods). For this purpose, total RNA was extracted from roots of *M. sativa* which had been harvested at regular intervals up to 28 days following inoculation with *R. meliloti* 2011. Figure 2A shows that Lb mRNAs (ranging from 650 to 700 bp in size) are absent in uninoculated roots, and first appear around 9 days after inoculation. There is a dramatic increase in Lb mRNA levels during the following 3 days, and this correlates well with the observed onset of nitrogen fixation around 11 – 12 days after inoculation. The 2-D gel patterns of *in vitro* translated polyA$^+$ RNA extracted from nodulated roots had confirmed these results for the Lbs, but the other nodulins are barely detectable even 12 days after inoculation (data not shown). Because of the dilution of the signal we are unable to deduce the precise moment during nodule development when these other genes are induced.

Root nodules defective in a late stage of their development

Having established that certain alfalfa genes are subject to programmed expression during the establishment of the nitrogen-fixing nodule, we wanted to examine if this expression could be correlated with that of *Rhizobium* symbiotic genes. For this we used a

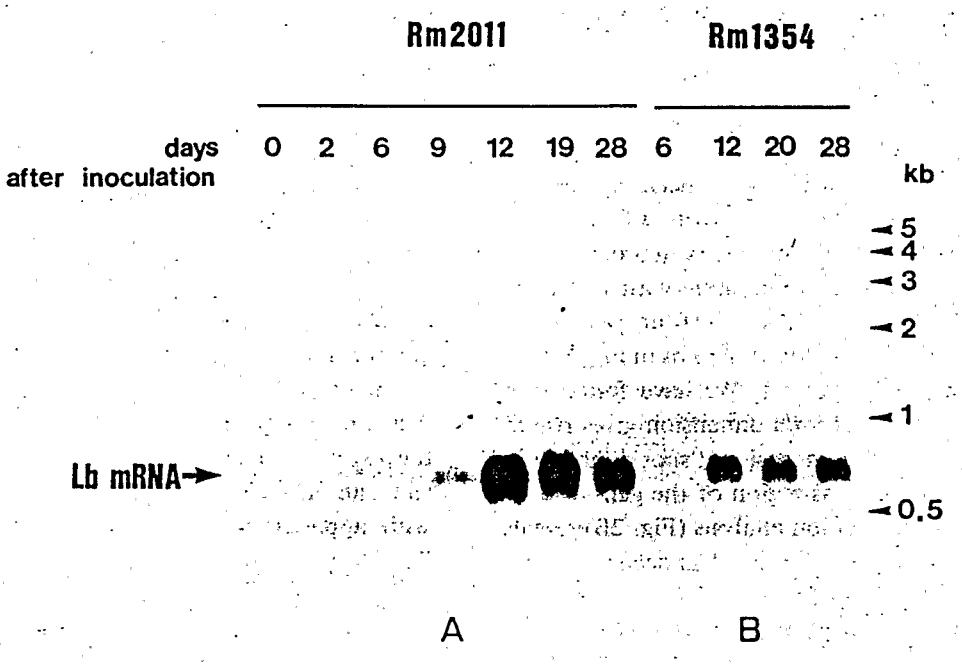

Fig. 2. Autoradiograph representing Northern blot of total RNA isolated from inoculated roots at different stages during the development of (A) effective nodules induced by *R. meliloti* 2011 (Rm2011) and (B) ineffective nodules induced by RM1354. The probe used for hybridization was a ^{32}P labeled fragment of the insert of pNL154, an alfalfa Lb cDNA clone.

Fig. 1. Fluorographs of 2-D gels of *in vitro* translation products, labeled with (^{35}S) methionine, from polyA$^+$ RNA isolated from (A) effective alfalfa root nodules induced by *R. meliloti* 2011, 15 days after inoculation, (B) uninoculated alfalfa roots, and (D) ineffective alfalfa root nodules induced by the *nifA* regulatory mutant of *R. meliloti* (Rm1354), 28 days after inoculation. In (C) the *in vitro* translation products from effective root nodule polyA$^+$ RNA were immunoprecipitated with alfalfa anti-Lb serum and the precipitate was then separated on a 2-D gel. Circles indicate the positions of root polypeptides which decrease in nodules, – the positions of those which increase and ► the nodule-specific polypeptides. The nodule-specific polypeptides not detected in Rm1354-induced nodules are marked by *. L indicates polypeptides immunoprecipitated with the alfalfa anti-Lb serum. Molecular mass markers included ^{14}C-methylated β lactoglobulin (18.4 kDa), α chymotrypsinogen (25.7 kDa), ovalbumin (43 kDa), bovine serum albumin (68 kDa) and phosphorylase B (97.4 kDa).

mutant of *R. meliloti* (Rm1354) which carries a Tn5 insertion in the *nifA* regulatory gene required for nitrogenase expression in *Rhizobium* [23]. Plant cells in nodules elicited by this mutant contain released bacteria as in the case of effective nodules, but there is no nitrogen fixation.

The 2-D gel electrophoretic pattern of *in vitro* translation products of polyA$^+$ RNA isolated from these ineffective nodules is very similar to that from nodules induced by *R. meliloti* 2011 (Fig. 1D). In particular, the levels of the same five root polypeptides are again dramatically reduced, and the levels of certain others are increased. The Lbs and all except two of the other nodulins (19 kDa, 20 kDa) are also present in these ineffective nodules, and this shows that, for the most part, the induction of nodulin gene expression is independent of nitrogen-fixing activity. Since the Lbs (see below) and certain of the other nodulin mRNAs are present at significantly lower levels in nodules induced by this mutant, the fluorograph shown in Fig. 1D has been exposed for considerably longer than that shown in Fig. 1A (effective nodules). This was necessary in order to confirm the absence of the two nodulins with molecular masses of 19 kDa and 20 kDa. The four spots which migrate just behind certain of the Lbs in Fig. 1D are not additional polypeptides. We have found that slow running of the second dimension gives rise to frequent smearing and occasional spot doubling in the low molecular mass region of the gel.

Northern hybridization analysis (Fig. 2B) reveals that the appearance of Lb mRNAs occurs roughly at the same time as in effective nodules; however, the level of this mRNA is approximately 4-fold lower than in effective nodules.

Root nodules defective in early stages of their development

Since the majority of nodulin genes are still expressed in the nodules elicited by the nitrogenase-deficient mutant we extended our studies to ineffective nodules arrested in earlier stages of development.

The first of these is induced by a spontaneous acidic exopolysaccharide-deficient mutant of *R. meliloti* (EJ355 [10]) and the second by an *Agrobacterium* strain, cured of its Ti plasmid but carrying the symbiotic plasmid (pSym) of *R. meliloti* 2011 (GMI9013 (pGMI27) [24]. In these nodules there there are no infection threads and bacterial penetration is intercellular. The central tissue of the nodule is therefore devoid of bacteria and we should thus be able to distinguish between those molecular events involved in nodule organogenesis and those concerned with the differentiation and functioning of both the bacteria and the central tissue of the host in the nodule.

Apparently none of the nodule-specific transcripts can be detected in nodules elicited either by the exopolysaccharide-deficient mutant of *Rhizobium* or the *Agrobacterium* strain carrying the symbiotic plasmid of *R. meliloti* 2011, as shown in Figs. 3A and 3B. Furthermore, even with the highly sensitive Northern hybridization technique we were unable to detect Lb mRNA in either of these two types of ineffective nodule (data not shown). However, it is important to note that, in common with effective nodules, the formation of these two types of ineffective nodules is accompanied by a reduction in the levels of the same five mRNAs described previously, which are normally present in roots.

Finally, in the 2-D gel pattern derived from nodules induced by the *Agrobacterium* strain carrying the pSym of *R. meliloti,* numerous polypeptide spots show an increased intensity and in particular those with apparent molecular masses of 40 kDa and 70 kDa (Fig. 3B). This response has not been observed for any other type of nodule that we have studied and does not occur when roots are in contact with the *Agrobacterium* reference strain lacking the symbiotic plasmid (results not shown).

Discussion

In studying the symbiotic association between alfalfa and *Rhizobium meliloti,* we have chosen an approach in which gene expression is analysed at the transcriptional level by means of *in vitro* translation of nodule polyA$^+$ RNA followed by 2-D gel electrophoresis of the translation products. Only mRNAs of plant origin should be present in the

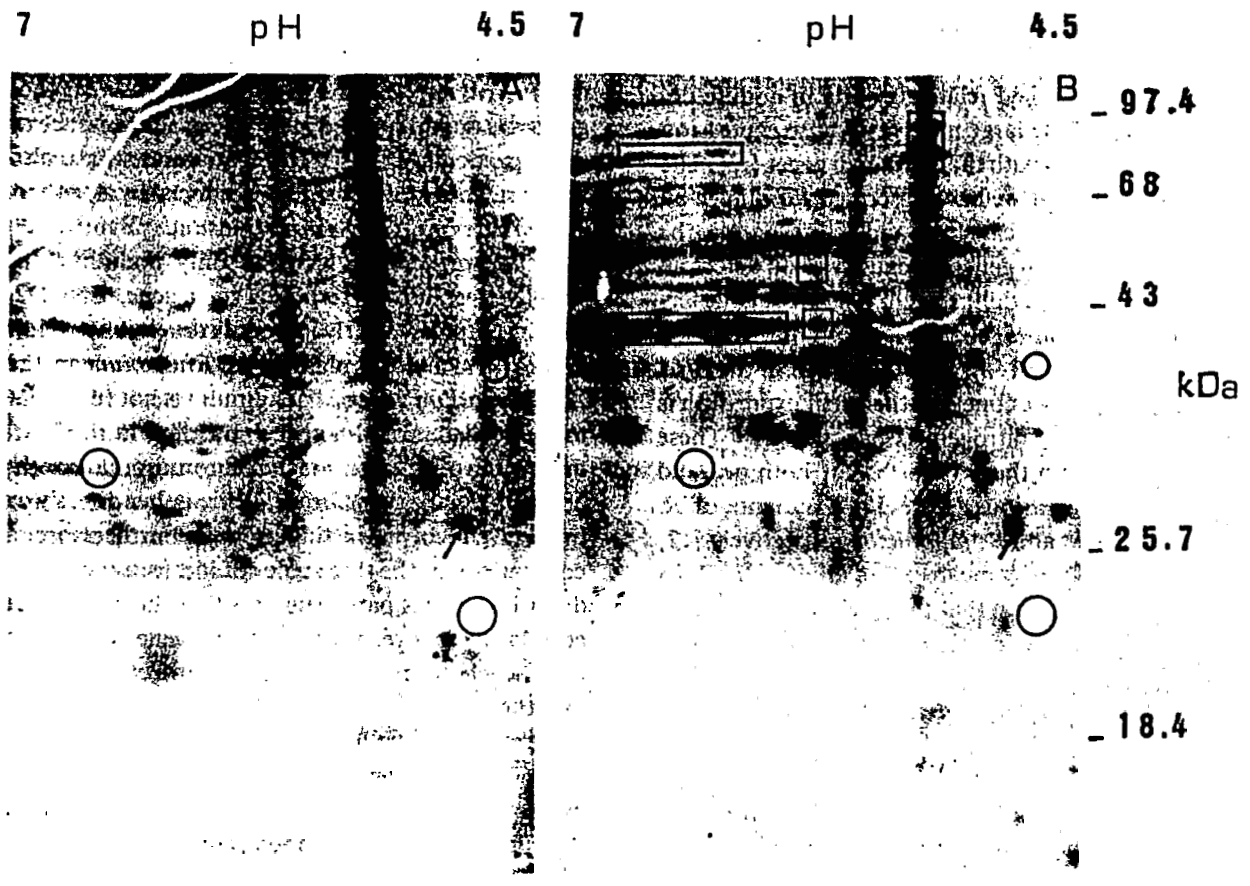

Fig. 3. 2-D gel analysis of *in vitro* translation products from polyA$^+$ RNA isolated from (A) ineffective nodules formed by a spontaneous acidic exopolysaccharide-deficient mutant of *R. meliloti* (EJ355), 28 days after inoculation and (B) ineffective nodules formed by an *Agrobacterium* strain carrying the symbiotic plasmid of *R. meliloti* 2011 (GMI9013) (pGMI27), 28 days after inoculation. For circles and ►, see Fig. 1. Rectangles indicate translation products which specifically increase in nodules elicited by the *Agrobacterium*/pSym strain.

polyA$^+$ RNA fraction and hence all nodule-specific polypeptides detected in this manner should be derived from nodulin genes.

This method has allowed us to detect indirectly thirteen plant mRNAs which are present in effective nodules but not in uninoculated roots. The SDS gel electrophoretic mobilities of the polypeptides resulting from *in vitro* translation of these nodule-specific mRNAs are difficult to correlate with those described for alfalfa proteins detected *in vivo* using nodule-specific antibodies [14, 26]. These differences can probably be accounted for by the fact that these latter studies were unable to discriminate between plant- and *Rhizobium*-encoded proteins, and secondly, that some of these proteins may be subject to post-translational modification. Nevertheless, eight of the low molecular mass nodulins can be identified as Lbs because they were immunoprecipitated by an anti-alfalfa Lb serum, and also with an antiserum raised against the Lbs of the distantly related legume *Sesbania rostrata*. Taken together, the *in vivo* and *in vitro* approaches suggest that there are fewer highly expressed nodulins in alfalfa than in either pea or soybean, for which the detection of twenty to thirty nodule-specific polypeptides has been reported [4, 15]. The precise significance of this observation is at present unclear and may simply reflect the need for techniques of greater sensitivity to reveal additional alfalfa nodulins.

In the studies carried out on the pea-*R.*

leguminosarum symbiosis, it has been possible to classify nodule-specific genes between those few which are transcribed relatively early in nodule development and the large majority whose expression broadly coincides with that of the Lb genes [12]. The nodulin genes that we have described for alfalfa all fall into the latter category and will therefore be termed late nodulin genes. Because we can detect all except two of the nodulins, including Lbs, in ineffective alfalfa nodules elicited with a nitrogenase-deficient strain of *R. meliloti* (defective in the *nifA* regulatory gene) we conclude that their expression is independent of nitrogen-fixing activity. These results agree with those reported for both pea and soybean, using various Nod^+ Fix^- mutants of *R. leguminosarum* and *R. japonicum* respectively [12, 28]. Furthermore, by means of an alfalfa Lb cDNA probe, we have shown that the level of Lb mRNA is significantly lower in these ineffective nodules as compared with normal nitrogen-fixing nodules. This is consistent with the reduction in the amount of Lb proteins found in alfalfa nodules induced by the same mutant [14, 30].

Alfalfa root nodules are elongated and cylindrical in shape, and being indeterminate in character, contain at all times regions in various stages of symbiotic development [18]. Hence, the lower than normal expression of Lb genes in the nitrogenase-deficient ineffective nodules could simply be the result of a reduction in the proportional size of the zone in which these genes are actively transcribed. This may be correlated with the cytological observation that these nodules contain bacteria blocked in an immature stage of their normal differentiation (G. Truchet, personal communication). Alternatively, the inability to establish a normal nitrogen-fixing symbiosis with the bacteria may lead to impaired induction of Lb gene expression or perhaps to an increased degradation of Lb transcripts.

We have examined two other types of ineffective nodules formed when alfalfa is inoculated either with an acidic exopolysaccharide mutant of *R. meliloti*, or an *Agrobacterium* strain carrying the *R. meliloti* symbiotic plasmid. In both cases, root nodule development is perturbed at a relatively early stage because there are no infection threads and as a consequence bacteria are not released into the host cells. Since these nodules do not contain detectable levels of any of the nodulin mRNAs identified by us in effective nodules, the process of nodule organogenesis is apparently independent of the presence of these particular nodulins. Similar results have also been presented for pea nodules induced by *Agrobacterium* carrying the symbiotic plasmid (pSyml) of *R. leguminosarum,* in which the only nodulin transcript detectable corresponds to a gene, ENOD2, which is expressed early in nodule development [13]. Thus the symbiotic plasmid of *Rhizobium* in the *Agrobacterium* genetic background is not sufficient to induce late nodulin gene expression in the host, and so one must conclude that chromosomal or other plasmid genes of *Rhizobium* are either directly or indirectly involved in this process. Furthermore, a comparison of the three types of ineffective nodules described in this paper suggests that there may be a correlation between late nodulin gene expression and the release of bacteria from the infection threads into the plant host cells. Clearly, additional types of mutants will have to be studied in order to throw more light on the interdependence of these two events.

In parallel with the induction of the nodule-specific mRNAs we have also observed a striking reduction in the expression of certain root genes during the development of the nitrogen-fixing alfalfa nodule. We have confirmed that this effect is confined to the nodule by carrying out a parallel analysis of portions of the root which are not nodulated (results not shown). Furthermore, the same root mRNAs are undetectable in all three of the ineffective nodule types that we have examined. Thus, the change in the levels of transcription of these particular root genes is presumably one of the events associated with nodule organogenesis, and unlike late nodulin gene expression appears to be independent of the presence of released bacteria in the central tissue of the nodule.

On the other hand, an effect that has only been observed in the case of nodules formed by the *Agrobacterium* carrying the symbiotic plasmid is a large increase in expression of certain plant genes different from those involved in normal nodule formation. Since contact with the *Agrobacterium* strain alone does not elicit the same reaction, one possible

explanation for this effect is that the intercellular localization of this normally non-symbiotic bacterium [24] induces a defensive reaction on the part of the plant. Such a possibility is now being further examined in this interaction by studying the expression of various plant genes known to be implicated in the general defense response.

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