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Plant gene expression during effective and ineffective nodule development of the tropical stem-nodulated legume *Sesbania rostrata*

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Abstract

The expression of plant genes during symbiosis of *Sesbania rostrata* with *Rhizobium* sp. and *Azorhizobium caulinodans* was studied by comparing two-dimensional PAGE patterns of *in vitro* translation products of poly(A)$^+$ RNA from uninfected roots and stems with that of root and stem nodules. Both types of nodules are essentially similar, particularly when stem nodules are formed in the dark. We detected the specific expression of at least 16 genes in stem and root nodules and observed the stimulated expression of about 10 other genes in both nodules. Six of the nodule-specific translation products (apparent molecular masses around 16 kDa) cross-react with an antiserum raised against leghemoglobin purified from *Sesbania rostrata* stem nodules. During stem nodule development, most of the nodule-stimulated genes are expressed concomitantly with leghemoglobin at day 12 after inoculation. However, some genes are already stimulated at days 6–7, some others later in development (day 18), and some are transiently activated. Patterns of root nodules induced by either *Azorhizobium caulinodans* strain ORS571, capable of effective root and stem nodulation, or *Rhizobium* sp. strain ORS51, capable of effective root nodulation only, are very similar except for a specific 37.5 kDa polypeptide. Several types of ineffective stem and root nodules were studied; in every case the amount of leghemoglobin components appeared reduced together with most of the nodule-stimulated polypeptides.

Introduction

Nodulins are plant proteins specifically synthesized in the nodules, the specialized organs resulting from infection of legumes by *Rhizobium* ([31]; see for nomenclature [46]). Nodulins have been described in a number of root-nodulated legumes such as *Glycine max* [31], *Pisum sativum* [5], *Medicago sativa* [33, 29]. Some nodulins have been identified: the leghemoglobins, the oxygen-binding hemoproteins characteristic of nodules of legumes [2], a nodule-specific uricase in soybean [4] and a nodule-specific glutamine synthetase in *Phaseolus vulgaris* [7] and in soybean [43]. These nodulins have been immunocytochemically localized: leghemoglobin as well as glutamine synthetase in the cytoplasm of infected cells [40, 47, 48]; uricase in uninfected cells of the nodule [4, 34]. In addition several nodulins are associated with the peribacteroid membrane in soybean [18, 26, 27].

Together with *Neptunia oleracea* [42] and several *Aeschynomene* species [1], *Sesbania rostrata* [11] is

one of the few legumes capable of forming nodules on stems. Nitrogen fixation by *S. rostrata* is very efficient and *S. rostrata* has a real potential in tropical agriculture as green manure [14, 39]. Several bacterial strains have been isolated from stem and root nodules, which have been taxonomically classified in two different genera: *Azorhizobium caulinodans* and *Rhizobium* sp. [13]. *A. caulinodans* strain ORS571 has been studied at physiological [12, 28, 32] and genetic [9, 17, 35, 38, 45] levels. Ultrastructural studies have been carried out on stem and root nodule formation in *S. rostrata* [16, 37, 44]. Stem and root nodules differ in at least two aspects: (i) stem nodules are photosynthetic [16]; (ii) stem infection occurs via "crack entry" [44], while root infection occurs via root hair [37].

We measured the level of translatable mRNA present in each tissue by comparing two-dimensional polyacrylamide gel electrophoresis patterns of *in vitro* translation products of poly(A)$^+$ RNA from uninfected stems and stem nodules, uninfected roots and root nodules. We focused on (i) the comparison of plant gene expression in root nodules and in stem nodules; (ii) the identification of leghemoglobin components by immunoprecipitation of *in vitro* translation products with a specific antiserum; (iii) the developmental expression of nodule-specific genes in stem nodules; (iv) plant gene expression in non-photosynthetic stem nodules; (v) the influence of the endosymbiont, *Rhizobium* or *Azorhizobium*, and of different non-nitrogen-fixing mutant bacterial strains on plant gene expression in stem and root nodules.

Materials and methods

Plant material

Sesbania rostrata seeds were scarified and surface-sterilized as described by Dreyfus and Dommergues [11]. Plants were grown in a greenhouse (80% humidity; day at 25–35 °C, night at 22 °C; 16-hour photoperiod). Stem nodules were obtained by spraying stems of 2-month-old plants grown in pots (50% sand, 50% compost) with an *A. caulinodans* suspension in water. For root nodule production, seeds were germinated 2–3 days on 1% water agar in aseptic conditions. Plants were then transferred to an aeroponic culture system [33] and inoculated after one month growth. Under these conditions, nodules could be visible after 3–4 days and acetylene-reducing activity [25] was observed after 8–9 days. Stem nodules developed in the dark were obtained by covering portions of stems with aluminium foil. Uninfected roots and stems were cut off from 7-day-old uninfected plants. All plant materials were immediately frozen and stored in liquid nitrogen.

Bacterial strains and media

A. caulinodans strain ORS571 and *Rhizobium* strain ORS51 were from Dr Dreyfus, ORSTOM, Dakar, Senegal. Nitrogen fixation deficient mutant strains 5740 and 5795 were from Dr C. Elmerich, Institut Pasteur, Paris, France. Bacterial cultures were grown at 30 °C with shaking on a rotary shaker, strains ORS571, 5740 and 5795 in YL medium [11] and strain ORS51 in YM medium [49].

Poly(A)$^+$ RNA purification

Two to ten grams (fresh weight) plant tissue were ground with liquid nitrogen in a mortar and pestle and fine powder was suspended in 4 ml/g of 1 vol. 0.2 M Tris-HCl pH 7, 0.02 M EDTA, 1% SDS, 1 vol. phenol:chloroform:isoamyl alcohol (25:24:1). After shaking and centrifugation, the aqueous phase was reextracted 4 times with 1 vol. phenol: chloroform:isoamyl alcohol and then 5 times in 1 vol. chloroform:isoamyl alcohol (24:1). Total RNA was precipitated twice at −80 °C in the presence of 0.3 M sodium acetate and 70% ethanol. Poly(A)$^+$ RNA was purified by passing twice on to a column of oligo (dT) cellulose (BRL 5940 SA/SB or Collaborative Research) [41].

In vitro *translation and electrophoresis*

Poly(A)$^+$ RNA was translated in a mRNA-dependant rabbit reticulocyte lysate (Genofit). Typi-

cally, 1–2 μg poly(A)$^+$ RNA was translated during 30 min at 30 °C in a 50 μl incubation mixture according to the vendor's instructions. ^{35}S-methionine from Amersham (50 μCi per 50 μl reaction mixture) was used as radiolabelled precursor. Approximately 200000 cpm TCA-precipitable *in vitro* translation products were analyzed by two-dimensional gel electrophoresis [36]. For the isoelectric focusing, 1.6% ampholines pH 5–7 and 0.4% ampholines pH 3.5–10 (LKB) were used and SDS electrophoresis was performed in 2.5% polyacrylamide slab gels. After protein fixation in 7% acetic acid, the gels were dried, treated with Amplify (Amersham) and exposed at −70 °C to Kodak X-Omat film for fluorography [30].

Immunoprecipitation

The translation products were immunoprecipitated according to Fuller *et al.* [20], with a rabbit antiserum raised against *S. rostrata* purified leghemoglobin preparation, done essentially as described by Appleby and Bergersen [3] and kindly given by Dr Bogusz (ORSTOM, Dakar, Senegal).

Results

Two-dimensional PAGE of in vitro *Translation products of Poly(A$^+$) RNA from effective stem and root nodules induced by* Azorhizobium caulinodans

Poly(A)$^+$ RNA was purified from uninfected 7-day-old stems and roots and from 18-day-old nitrogen-fixing stem and root nodules induced by *A. caulinodans* ORS571 [13], and *in vitro* translated in a rabbit reticulocyte lysate. Translation products were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The comparison of the different patterns gives an estimate of the differential levels of endogenous mRNA in the various plant tissues [23].

200–300 polypeptides, in the range of 10 to more than 100 kDa molecular mass, are visible on each pattern and most of them are present in all tissues (Fig. 1). However, more than 50 polypeptide spots exhibit different intensities in stems, roots, stem nodules and root nodules, reflecting tissue-related plant gene expression.

We retain the nomenclature suggested by Van Kammen [46], "N" for "nodulin", "nst" for "nodule-stimulated", followed by the polypeptide apparent molecular mass in kDa.

At least 16 polypeptides synthesized from root nodule mRNA cannot be detected in uninfected roots, 10 are considerably amplified, while 12 others decrease or are below detection in root nodules.

Similarly, by comparison of stem nodules to uninfected stems, we observed at least 16 specific products in stem nodules. The intensity of 11 polypeptide spots is increased in the symbiotic state, and the intensity of 12 others is decreased or is below detection levels in stem nodules.

At least 14 major polypeptides appear in stem and root nodules and cannot be detected in uninfected tissues. In addition, 11 polypeptides are stimulated in both root and stem nodules, while 8 are depressed as compared to uninfected tissues.

Plant gene expression during stem nodule development

To determine the time by which specific plant genes are activated in the course of establishment of symbiosis, we followed their expression at every day after infection. For such studies, stem nodulation constitutes a good model: nodules occur at predetermined sites, directly observable, and their age is precisely known; moreover, one can easily obtain nodules in sufficient quantity. Under our conditions of plant growth, nodules can first be observed at days 3–4 after inoculation and acetylene-reducing activity at days 8–9, from which it increases until day 21, and then decreases. From 6 to 32 days after inoculation, we collected every day nodule-bearing epidermal fragments cut off from the stems with a blade, from which poly(A)$^+$ RNA was purified, *in vitro* translated and analysed on 2D-PAGE.

It appears that plant genes that are repressed in mature stem nodules as compared to uninfected stems are already below detection level at day 6, suggesting that repression occurs during the early stages

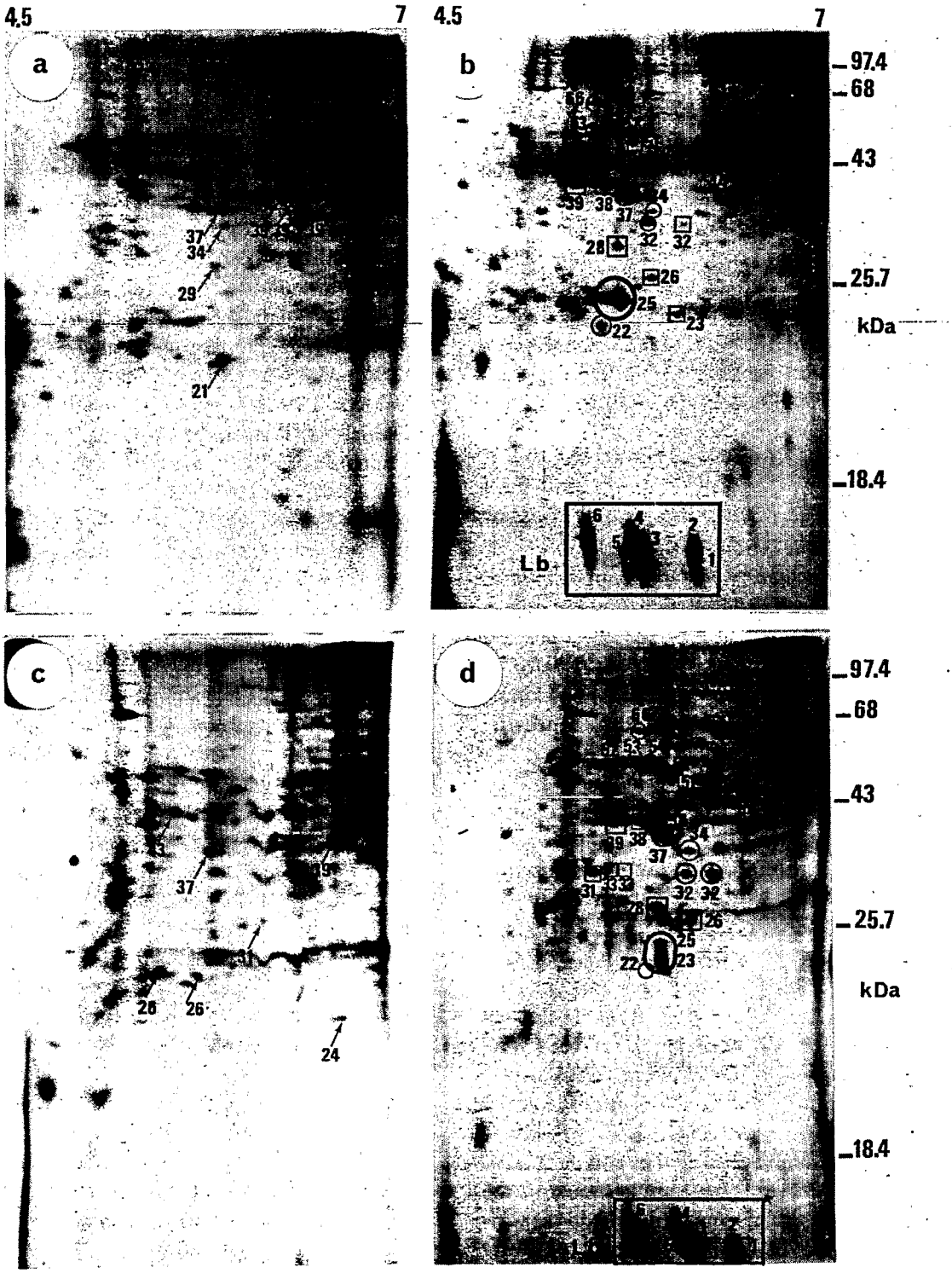

Fig. 1. Fluorographs of two-dimensional polyacrylamide gels of ^{35}S methionine-labeled *in vitro* translation products from poly(A)$^+$ RNA purified from: (a)7-day-old uninfected roots; (b)18-day-old ORS571-induced root nodules; (c)7-day-old uninfected stems; (d)18-day-old ORS571-induced stem nodules. □ nodule-specific polypeptide; ○ nodule-stimulated polypeptide; ⁄ uninfected root and/or stem stimulated polypeptide.

of nodule development. Nodule-stimulated genes are activated at different stages during nodule development and can be grouped in several classes (Fig. 2A, B, C): some are stimulated early, such as N44 and N38, detectable at day 6, or N28 and nst25, activated from day 7. Some polypeptides reach their maximum intensity and then remain more or less at the same level; this is the case for N38, N28, N26, nst66, nst53, nst52, nst37 and nst34 (plateau reached at day 12), and for N23 (plateau reached at day 14). Some, such as N51, nst52 and nst54 are stimulated late in development (day 18). Some others are transiently activated; N44 is very intense at days 6–7 and then decreases gradually; N33, nst31, nst25 and nst22 reach their maximum expression between days 12 and 16, then decreases.

All these observations indicate that plant genes are sequentially expressed during nodule development in *S. rostrata*. In the following studies we always compared nodules of the same age.

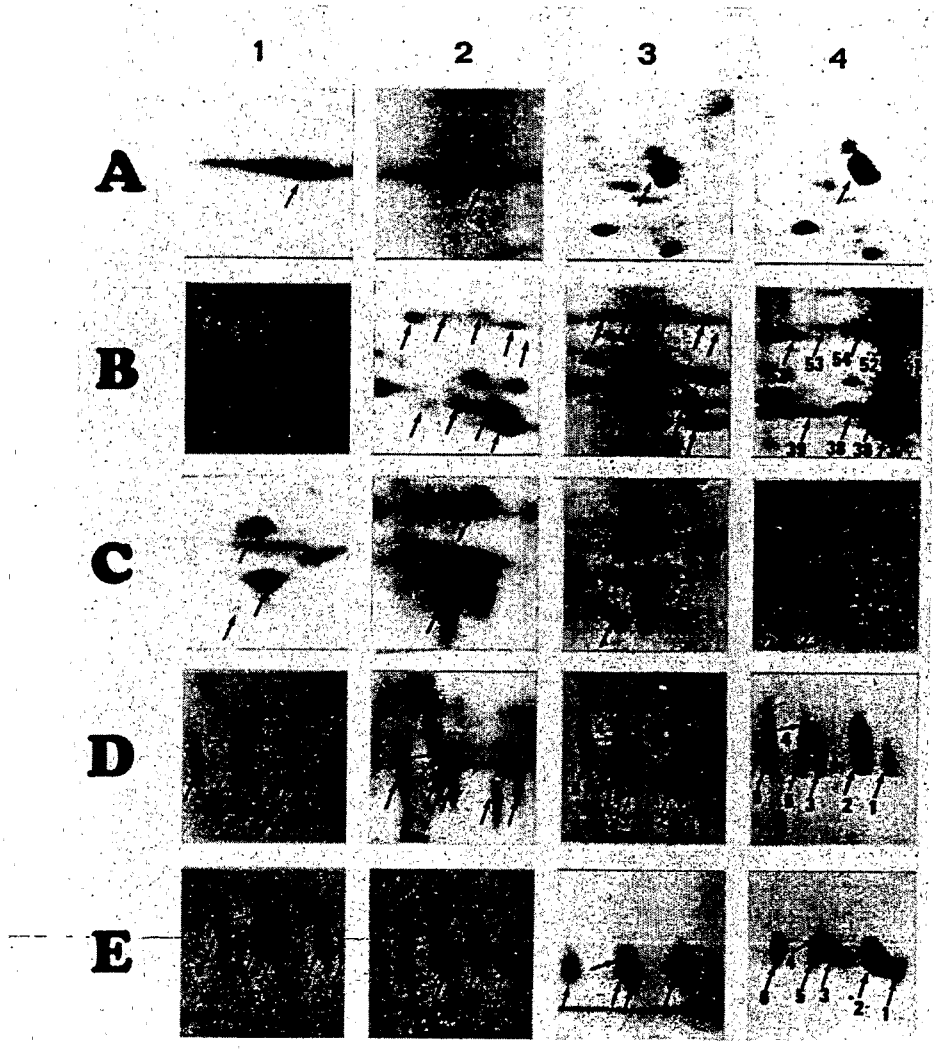

Fig. 2. Nodule-stimulated polypeptides during stem nodule development. A: N44; (1)7 days, (2)11 days, (3)18 days, (4)32 days.
B: nst37, N38, N38′, N39, N51, nst52, nst52′, nst53, nst54; (1)11 days, (2)14 days, (3)16 days, (4)18 days.
C: N28, nst25, nst22; (1)12 days, (2)14 days, (3)18 days, (4)32 days.
D: Leghemoglobin in stem nodules; (1)12 days, (2)14 days, (3)18 days, (4)32 days.
E(1–3): Leghemoglobin in root nodules; (1)18 days, (2)30 days, (3)45 days. E(4): immunoprecipitation of 32-day-old stem nodules translation products by anti-leghemoglobin serum.

Leghemoglobin

To identify leghemoglobin, we used an antiserum raised against leghemoglobin purified from *S. rostrata* stem nodules to immunoprecipitate the corresponding *in vitro* translation products, and run them on 2D-PAGE in the same conditions. None of the uninfected root or stem poly(A)$^+$ RNA translation products reacted with the antiserum (data not shown). On the other hand, at least six abundant polypeptides (apparent molecular masses around 16 kDa) belonging to the class of common root and stem nodulins mentioned above were immunoprecipitated from both 32-day-old stem and root nodules. According to their decreasing isoelectric points, we called them Lb1 to Lb6 (see Fig. 2E [4]).

During nodule development, leghemoglobin components are differently expressed: in stem nodules, from day 9, at which they are detectable at a low level, leghemoglobin components increase in intensity throughout our observation period (32 days), which can be correlated with the increase of nitrogen fixation during the same time. However, their relative proportions vary during nodule development (Fig. 2D): at day 12, Lb2, Lb3 and Lb6 are minor (of approximately the same intensity) while LB4–Lb5 are major, and Lb1 is not visible; after day 16, Lb2 and Lb6 become major products, Lb3, Lb4 and Lb5 are moderately abundant, and Lb1 appears slightly. After day 30, Lb5 is present at very low intensity (it could only be detected after immunoprecipitation, which resulted in the amplification of the signal; see Fig. 2E [4]). So it appears that Lb1 increases while Lb5 decreases. Root nodules exhibit the same leghemoglobin content and similar variation during nodule development, with some slight differences in their relative proportions; Lb2, for example, is minor in root nodules whereas major in stem ones at day 18 (Fig. 2D [3] and 2E[1]). Different relative proportions of leghemoglobin components in root nodules have been reported in soybean [19] and pea [24].

Nodules induced by the root-specific Rhizobium *sp. strain ORS51*

Bacteria capable of nodule induction on *S. rostrata* belong to two different genera: "stem strains" to *Azorhizobium caulinodans*, type ORS571, capable of effective stem and root nodulation; "root strains" to *Rhizobium* sp., type ORS51, capable of effective root nodulation only [13].

Patterns of *in vitro* translation products of poly(A)$^+$ RNAs from root nodules induced by either of the two bacterial species *Rhizobium* sp. or *A. caulinodans* differed by only a few spots (Fig. 1b, 4a): an extra spot corresponding to a 37.5 kDa polypeptide is observed in ORS51 nodules and three others (21, 26 and 31 kDa respectively) are more abundant than in ORS571 ones.

Nodules developed in the dark

Stem nodules on *S. rostrata* are dark-green in external appearance. The bacteroid zone is surrounded by cortical cells containing chloroplasts [16]. In order to know whether photosynthesis influences plant gene expression in nodules, we compared 3-week-old stem nodules protected from light and normally light-grown ones of the same age on the same plants. Nodules formed in the dark are white outside, pink inside; in addition, many of them are surmounted by a little root emerging from the nodule (Fig. 5). Their acetylene-reducing activity is comparable to that of light-growing stem nodules of the same age picked from the same plants.

Both types of nodules exhibit qualitatively the same patterns with several nodulins or nodule-stimulated polypeptides less abundant in nodules not exposed to light, such as nst32, nst32′, nst31, nst26, nst25, nst22 (Fig. 3a, b). We noticed several similarities between stem nodules formed in the dark and root nodules of the same age, such as nst32, nst31 and N38 (weakly expressed), N23 (very intense).

Ineffective nodules

To discriminate which of the nodule-stimulated polypeptides could be implied in nitrogen fixation or assimilation processes, we analyzed 2D-PAGE poly(A)$^+$ RNA translation product patterns of several types of ineffective stem and root nodules

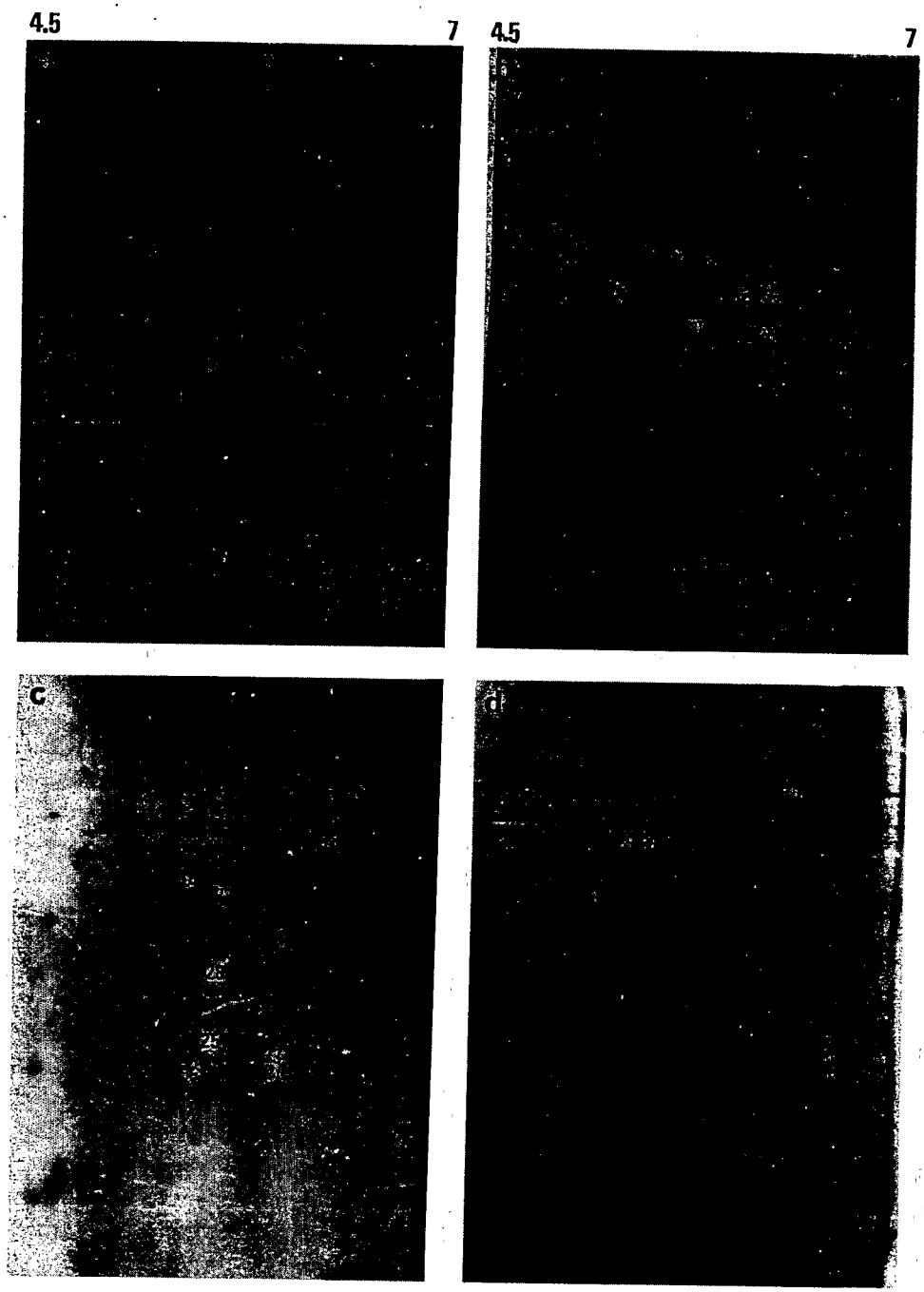

Fig. 3. Fluorographs of two-dimensional polyacrylamide gels of ^{35}S methionine-labeled *in vitro* translation products from poly(A)$^+$ RNA purified from: (a)25-day-old ORS571-induced effective stem nodules; (b)25-day-old ORS571-induced effective stem nodules in dark; (c)25-day-old 5740-induced ineffective stem nodules; (d)25-day-old 5795-induced ineffective stem nodules. □ nodule-specific polypeptide; ○ nodule-stimulated polypeptide.

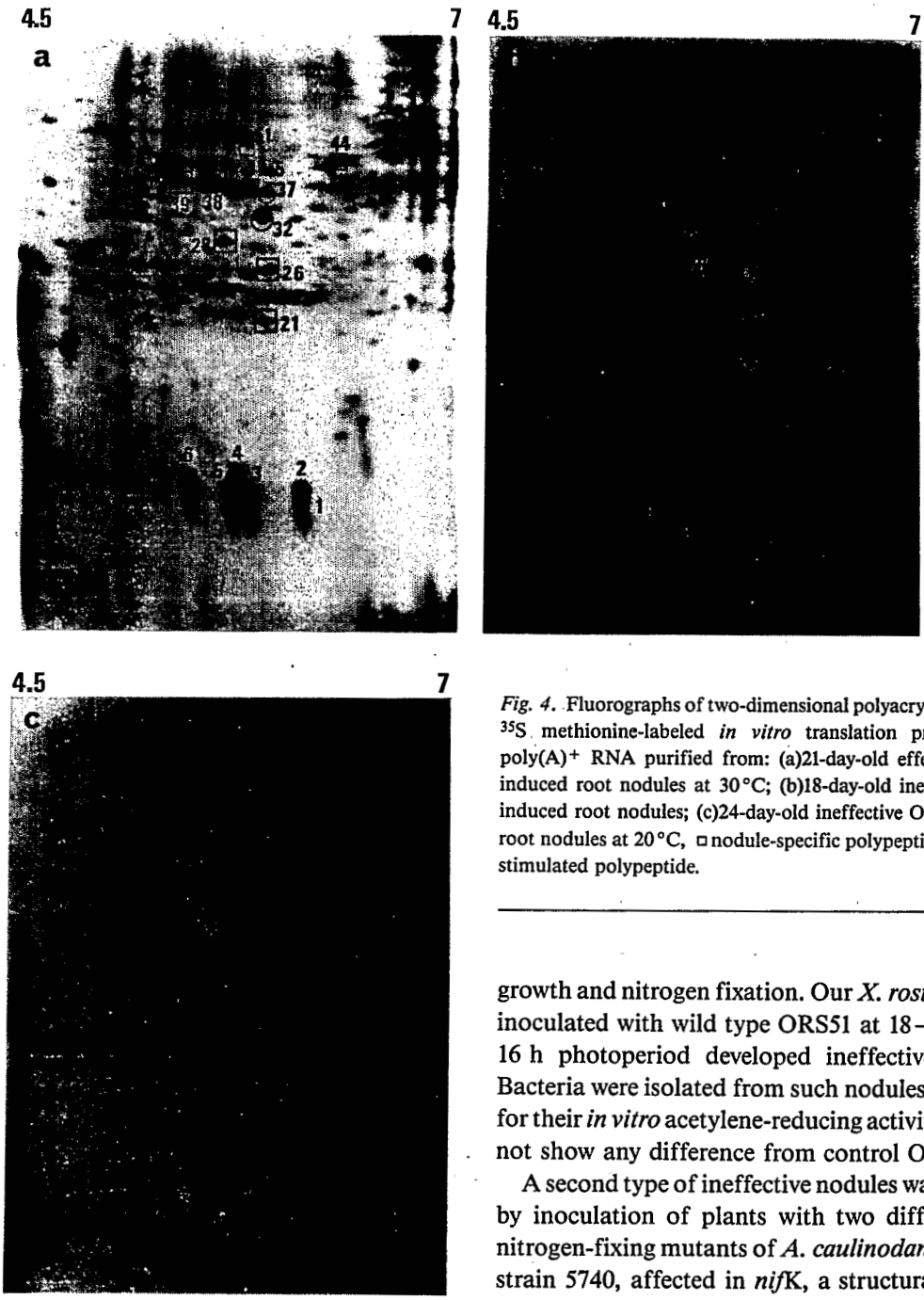

Fig. 4. Fluorographs of two-dimensional polyacrylamide gels of ^{35}S methionine-labeled *in vitro* translation products from poly(A)$^+$ RNA purified from: (a)21-day-old effective ORS51-induced root nodules at 30 °C; (b)18-day-old ineffective 5740-induced root nodules; (c)24-day-old ineffective ORS51-induced root nodules at 20 °C, □ nodule-specific polypeptide; ○ nodule-stimulated polypeptide.

(Figs. 3 and 4).

Dreyfus *et al.* [14] and Rinaudo and Moudiongui [39] reported that *S. rostrata* exhibits strict requirements regarding light (13 h photoperiod), temperature (28–30 °C) and humidity (70–100%) for its growth and nitrogen fixation. Our *X. rostrata* plants inoculated with wild type ORS51 at 18–20 °C and 16 h photoperiod developed ineffective nodules. Bacteria were isolated from such nodules and tested for their *in vitro* acetylene-reducing activity; they did not show any difference from control ORS51.

A second type of ineffective nodules was obtained by inoculation of plants with two different non-nitrogen-fixing mutants of *A. caulinodans* ORS571: strain 5740, affected in *nif*K, a structural gene for nitrogenase [17], and strain 5795, a regulatory mutant which does not produce any of the specific polypeptides synthesized by wild-type ORS571 in conditions of nitrogenase activity derepression [9].

In all cases, nodules appeared with a delay as compared with wild-type ones, were not pink inside (suggesting lack of leghemoglobin) and did not show any

Fig. 5. Three-week-old stem nodules developed in the dark.

acetylene-reducing activity. Nodules were harvested 22–25 days after inoculation since majority of the nodule-stimulated polypeptides pointed out are present in effective nodules of that age.

In every case, leghemoglobin translation products could be observed, but at a very low level compared to effective nodules of the same age (Figs. 3 and 4). Immunoprecipitation and overexposure of autoradiograms shows that all six leghemoglobin components are present at low level. This indicates that, like in soybean [21] and pea [24], *S. rostrata* leghemoglobin genes are activated in ineffective nodules, but their expression is reduced as compared to effective nodules.

No polypeptide could be found specific to ineffective stem nodules; on the other hand, almost all the polypeptides classified as "nodulins" or "nodule-stimulated" are present, but generally at a reduced intensity as compared to the effective nodules. On the other hand, several polypeptides typically depressed in effective nodules are expressed in ineffective ones like in uninfected tissues.

Discussion

By means of comparison of 2D-PAGE patterns of Poly(A)$^+$ RNA translation products of root and stem nodules, uninfected roots and stems, we could evidence a difference in transcriptional activity of plant genes during stem and root nodulation of *Sesbania rostrata*.

During nodule development in *S. rostrata*, activity of about 12 genes decrease while about 26 other genes (16 nodulin genes and about 10 nodule-stimulated ones) show amplified expression, most of these being identical in both root and stem nodules. These results are comparable to those obtained on soybean and pea, in which 20 to 30 nodulin genes have been identified [5, 23, 31]. Plant genes are almost identically expressed in root nodules induced by either *A. caulinodans* ORS571 or *Rhizobium* sp. strain ORS51, except for one additional specific nodulin gene in the latter. It should be of interest to determine what is the function of this nodulin.

We previously reported five leghemoglobin components in *S. rostrata* [8]. One of these (Lb4) was very abundant and, by further investigation, could be separated here as two spots. The six leghemoglobin components present in stem and root nodules exhibit different relative proportions similar to soybean and pea root nodules [19, 24]. Moreover, the relative abundances of the different transcripts vary during nodule development like in soybean [19]. These results are in agreement with the conclusions of Bogusz *et al.* [6], confirming the largest leghemoglobin family characterized so far. These authors purified leghemoglobin from stem and root nodules of *S. rostrata* that were resolved as six components by isoelectric focusing. Furthermore, they showed evidence for a seventh component by anion exchange chromatography and concluded, by amino terminal amino acid sequencing, that at least six of them were separate gene products.

As in other systems [5, 21, 23], the nodule-stimulated genes are sequentially activated during nodule development, some very early (day 6), that could be implied in the first steps of nodule formation, the majority concomitantly with leghemoglobin (day 12), most likely involved in the constitution of appropriate conditions for nitrogen

reduction and transport, and some later (days 16–18) suggesting late functions, for example in senescence. We found several genes with a transient stimulated expression. This was recently also described for soybean nodules [23], where expression of this class of nodulins was, by parallel cytological studies, correlated with nodule differentiation. Since no major difference in the patterns appears between days 18 and 32 of nodule development, one can argue that all important events for the nodule formation and function have occurred before day 18.

In stem nodules developed in the absence of light, plant gene expression is not dramatically perturbed; however, we noticed some similarities in the expression of genes like in root nodules. It could therefore be possible that such nodules acquire some characteristics of subterranean organs. This is not surprising since, anatomically, stem and root nodules are not of a completely different nature: stem nodulation occurs at a site where a root primordium pierces the stem epiderm and the meristematic zone of the nodule develops from this root tissue [15, 16]; moreover, root nodules developed under light are green, suggesting photosynthetic activity. It could have been interesting to compare such photosynthetic root nodules to stem ones.

Our results showing that the majority of the nodule-stimulated and specific polypeptides are common to root and stem nodules, and the observation of Bogusz *et al.* [6] that leghemoglobins are qualitatively identical in both cases, suggest that root and stem nodules are essentially similar.

In ineffective nodules, plant gene expression appears different from effective ones: some stimulated genes in effective nodules are not (or at a lower level) expressed in ineffective ones while some others appear strongly expressed. This suggests that at least some of these could be implied in either the nitrogen-fixing process, the fixed-nitrogen assimilation and transport system, or the nodule senescence. Presence of trace amounts of all the leghemoglobin components in ineffective nodules indicates that leghemoglobin genes are activated independently from the endosymbiont effectiveness, but that their level of expression is affected by the ability of these nodules to fix nitrogen. These results agree with those reported for soybean [21, 31] and pea [24] using various ineffective *R. japonicum* and *R. leguminosarum* strains respectively.

Acknowledgement

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