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Proteins Associated with Root-Hair Deformation and Nodule Initiation in *Vigna unguiculata*

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Developmental changes in the synthesis of root-hair proteins were followed in the symbiosis between the promiscuous legume *Vigna unguiculata* and the broad host-range *Rhizobium* sp. NGR234. Comparison of the two-dimensional electrophoresis patterns of proteins isolated from root hairs inoculated with wild-type or hair-deformation minus mutants revealed 12 symbiosis-specific proteins. Synthesis of three of these proteins was repressed 4 days after inoculation with *Rhizobium*. The remaining nine proteins were induced by *Rhizobium* 1, 2, or 4 days after inoculation. As three of these (15-, 31-, and 44-kDa hadulins) were specifically and transiently expressed in root hairs during the deformation process, we have named them hadulins (*hair-d*eformation specific proteins). Five proteins (including 15- and 31-kDa hadulins) were first observed 24 hr after inoculation. Of these, only the 15-kDa hadulin was not induced by *R. fredii* USDA257S1 Nod$^+$, Fix$^+$ on *V. unguiculata*). Two days after inoculation, three additional proteins became apparent, while another (44-kDa hadulin) appeared on day 4. All 12 proteins seem to be associated with root-hair deformation and nodule development.

Additional keywords: developmental changes, early nodulins, two-dimensional gels.

Bacteria belonging to the genera *Azorhizobium, Bradyrhizobium*, and *Rhizobium* symbiotically associate with the roots of legumes. This often leads to the formation of highly organized structures, termed nodules, in which atmospheric nitrogen may be fixed. Nodules develop in a complex series of steps, during which root hairs play an important role in most legumes (for review, see Dart 1977; Long 1989; Nap and Bisseling 1990). Initially, rhizobia multiply in the rhizosphere, where some of them attach to the root hairs. Homologous plant hosts (i.e., the legume from which the original *Rhizobium* was isolated) respond with curling of root hairs followed by formation of infection threads through which the bacteria enter the plant. Mitotic activity in the root cortex is induced in front of the inwardly growing infection thread. After release of the bacteria from the infection thread, the fully differentiated nodule has the potential to fix nitrogen.

During nodule development, both the microorganism and the host plant exchange signals to coordinate the expression of genes specific for nodulation. Flavonoids secreted by the legume represent one class of signals (Firmin *et al.* 1986; Peters *et al.* 1986; Redmond *et al.* 1986). In homologous bacteria (i.e., rhizobia that are Nod$^+$, Fix$^+$ on the particular host), the flavonoids induce the synthesis and release of a rhizobial signal molecule that provokes root-*hai*r *d*eformation (Had) and *c*urling (Hac). In *R. meliloti* the Had factor was shown to be an acylated and sulphated oligosaccharide of β-1,4-*N*-acetyl-D-glucosamine (Lerouge *et al.* 1990). Broughton *et al.* (1991) demonstrated that the Had factors of the broad host-range *Rhizobium* sp. NGR234 belong to the same class of compounds.

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Symbiosis-specific genes involved in this unique interaction include the bacterial *nod* and *hsn* genes (for review, see Martinez *et al.* 1990), while the products of the host genes have been referred to as nodulins (for review, see Gloudemans and Bisseling 1989; Verma *et al.* 1991). Nodulins are differentially and sequentially expressed during nodule development (Le Gal *et al.* 1989; Scheres *et al.* 1990b; Trese and Pueppke 1991) and can be grouped into early or late nodulins according to the time of their synthesis.

So far, early nodulins have been described in four different legumes (Dickstein *et al.* 1988; Franssen *et al.* 1987, 1988; Gloudemans *et al.* 1989; Scheres *et al.* 1990a, 1990b; Trese and Pueppke 1990, 1991; van de Wiel *et al.* 1990a, 1990b), but the precise symbiotic functions of the proteins are largely unknown.

Five cDNA clones of early nodulins have been isolated from *P. sativum*. Recent data indicate that two of the five clones may direct the synthesis of cell wall proteins (Enod2 and Enod12; Govers *et al.* 1990; Scheres *et al.* 1990a; van de Wiel *et al.* 1990b), whereas one (Enod5) seems to code for a membrane or an extracellular protein (Scheres *et al.* 1990b). Amino acid sequences encoded by the open reading frames of Enod3 and Enod14 suggest that they may bind metal ions (Scheres *et al.* 1990b). Transcription of the three genes Enod3, Enod5, and Enod14 is restricted to infected cells, while Enod12 is induced in both infected and uninfected cells (Scheres *et al.* 1990a). The expression of Enod2 on the other hand occurs in the nodule parenchyma (van de Wiel *et al.* 1990a, 1990b).

As the first visible responses to inoculation with *Rhizobium* involve deformation of root hairs, it seemed logical to examine changes in protein synthesis in root hairs not masked by contamination with massive amounts of root material. With two exceptions (Gloudemans *et al.* 1989; Trese and Pueppke 1990, 1991) all early nodulins were in fact identified/cloned from proteins/mRNA isolated

from nodule material. A shortcoming of this approach is that genes expressed only during hair deformation, or those whose synthesis is repressed following inoculation, will not be represented at the mRNA level in nodules.

To overcome this difficulty, we investigated the very early changes in protein synthesis in the promiscuous legume *V. unguiculata* (L.) Walp. (Lewin *et al.* 1987) following inoculation with the broad host-range *Rhizobium* sp. NGR234 (S. G. Pueppke and W. J. Broughton, unpublished). This plant was chosen because: 1) it is one of the most promiscuous known legumes (Lewin *et al.* 1987); 2) it responds rapidly (Lewin *et al.* 1990) and massively to inoculation (Danso and Owiredu 1988; Wong Kah-Lin and W. J. Broughton, unpublished); 3) it produces large amounts of root hairs (about 2 mg dry weight per 100 plants; A. Krause, unpublished); 4) it grows rapidly and well under artificial conditions (Broughton *et al.* 1978; Broughton 1979); and 5) it can be transformed with *Agrobacterium tumefaciens* (Smith and Townsend) Conn based vectors (Garcia *et al.* 1987; D. P. S. Verma, unpublished).

Here we report that changes in the patterns of root-hair proteins of *V. unguiculata* are first visible 24 hr after inoculation with *Rhizobium* sp. NGR234. Further differences were detected 2 and 4 days after inoculation. These changes include both repression and induction of protein synthesis.

MATERIALS AND METHODS

Bacterial strains. Strains used in this study are listed in Table 1. For inoculation, bacteria were cultured in TY medium (Beringer 1974) to an A_{600nm} of about 1.7. After centrifugation, bacteria were resuspended in H_2O to a concentration of about 10^9 cfu ml^{-1}.

Plant growth and inoculation. *Vigna unguiculata* 'Red Caloona' seeds were obtained from Wright Stephenson & Co. (Seven Hills, N.S.W., Australia). Seeds were surface sterilized by immersion in 70% (v/v) ethanol for 10 min, washed twice with H_2O and then kept in 5% (v/v) H_2O_2 for 10 min. After washing six times in H_2O, the seeds were placed on 1.5% B+D agar (Broughton and Dilworth 1971) and incubated at 28° C. Three days later, the seedlings were inoculated by spraying the inoculum directly onto the roots (1 ml per 30 plants). Root hairs were isolated 1, 2, and 4 days after inoculation. In every experiment, four seedlings were randomly selected and transferred to Magenta jars (Lewin *et al.* 1990) to verify the nodulation phenotype.

Isolation of root hairs. Root hairs were isolated from batches of 200 seedlings as described by Röhm and Werner (1987) and stored at −70° C until needed. Root-hair preparations harvested this way were virtually free of contaminating pieces of root, cortex, epidermis, and root-cap cells.

Protein isolation. Membrane proteins were extracted from isolated root hairs or from total roots stripped of root hairs with phenol, precipitated with ammonium acetate (Hurkman and Tanak 1986), and resuspended in a urea buffer. Protein concentrations were determined using the Bio-Rad protein assay (Richmond, CA).

Two-dimensional gel electrophoresis of proteins. Two-dimensional analysis of proteins was performed essentially as described by O'Farrell (1975). Three hundred micrograms of protein (the normal yield of root-hair proteins from 200 plants) was separated per gel. Proteins were focused in the first dimension with ampholytes ranging in pH from 3.5 to 10 (Pharmacia LKB Biotechnology, Uppsala, Sweden). Separation of the proteins in the second dimension was accomplished in 12% polyacrylamide gels. Proteins in the gel were visualized by the sensitive urea-silver stain method (Chaudhuri and Green 1987). SDS-PAGE low standards (Bio-Rad, Richmond, CA) were used as molecular weight markers.

RESULTS

Plant growth conditions. Growth conditions of the seedlings have a strong influence on the production and size of root hairs. Seeds of *V. unguiculata* 'Red Caloona' grown on 1.5% B+D agar not only produced the most root hairs, but the roots grew on the surface of the agar, facilitating their removal without damaging the root hairs.

Under these conditions, the primary root emerged 2 days after sowing, whereas secondary roots appeared on day 4. After inoculation with *Rhizobium* sp. NGR234 on the third day, root-hair deformation was visible on day 4 (i.e., 1 day after inoculation), while root-hair curling could be detected on the fifth day (i.e., 2 days after inoculation). The first nodules were visible 11 days after sowing (i.e., 8 days after inoculation).

Root-hair specific proteins. In preliminary experiments, the patterns of proteins extracted from roots were compared

Table 1. Bacterial strains used in this study and their characteristics

Designation	Characteristics[a]	Phenotype on *Vigna*[b]	Reference
Rhizobium sp. NGR234			
NGR234	Rif^r derivative of NGR234	Nod^+	Lewin *et al.* 1990
NGR234NodD1$^-$	NGR234 mutant with a Ω insertion in *nodD1*, Rif^r, Sp^r	Had^- Nod^-	A. Lewin[c]
NGR234NodABC$^-$	NGR234 mutant with a 4.2 kb deletion of *nodABC*, Rif^r, Sp^r	Had^- Nod^-	A. Lewin[c] and B. Relic[d]
R. fredii			
USDA257S1	*Tn5*-insertion derivative of USDA257, Km^r	Nod^+	Heron *et al.* 1989
USDA257B3	USDA257S1 mutant with a deletion of *nodABC*, Km^r	Had^- Nod^-	Heron *et al.* 1989
R. meliloti			
GMI357	RCR2011 mutant with deletion of *nodABC*, Rif^r, Nm^r	Had^- Nod^-	Debellé *et al.* 1986

[a] Rif = rifampicin; Sp = spectinomycin; Km = kanamycin; Nm = neomycin; ^{r} = resistant.
[b] Nod = nodulation; Had = hair deformation.
[c] L.B.M.P.S.
[d] L.B.M.P.S.

with those of root hairs (Fig. 1). Proteins were extracted from both roots stripped of root hairs and the root hairs themselves. The patterns of proteins were highly reproducible between different preparations and always included polypeptides with molecular masses of up to 200 kDa.

At least 12 root hair-specific proteins (indicated by arrows in Fig. 1) were reproducibly detected. In addition, some proteins were expressed at higher levels in root hairs than in roots. On the other hand, some proteins were not visible in root-hair preparations, while they occurred in roots (shown by arrowheads in Fig. 1).

Repression of protein synthesis following inoculation. To monitor whether *Rhizobium* sp. NGR234 induced changes in protein synthesis in root hairs, duplicate batches of root hairs were harvested 1, 2, and 4 days after inoculation and proteins were extracted and separated on two-dimensional gels.

Synthesis of three proteins was reproducibly repressed during the early phases of nodule development (Fig. 2). Repression occurred 4 days after inoculation (i.e., 2 days after root-hair curling first became visible). Molecular masses of the repressed polypeptides ranged from 17 to 44 kDa. One of these three proteins (spot *a*) belongs to the family of 12 root hair-specific proteins (see Fig. 1), whereas the other two were detected in both roots and root hairs. Interestingly, protein *a* also disappeared after treatment of seedlings with a *Rhizobium* sp. NGR234NodABC$^-$ mutant (Fig. 2). Changes in the expression of the other two proteins were specifically induced by *Rhizobium* sp. NGR234.

Induction of the synthesis of symbiotic root-hair proteins by *Rhizobium* sp. NGR234. To analyze changes in the synthesis of proteins in root hairs induced by *Rhizobium* sp. NGR234, two-dimensional protein patterns were compared with those of various controls at different times after inoculation. As controls, duplicate batches of seedlings were inoculated with *Rhizobium* Had$^-$ mutants (mutants incapable of provoking root-hair deformation). Comparison of the two-dimensional patterns of proteins isolated from root hairs inoculated with Had$^+$ or Had$^-$ strains of *Rhizobium* allowed differentiation between those proteins that are part of the general plant response to rhizobia and those specific to *Rhizobium* sp. NGR234 (Table 2). An example of these data is shown in Figure 3.

Twenty-four hours after inoculation with *Rhizobium* sp. NGR234, five proteins were detected that were not visible

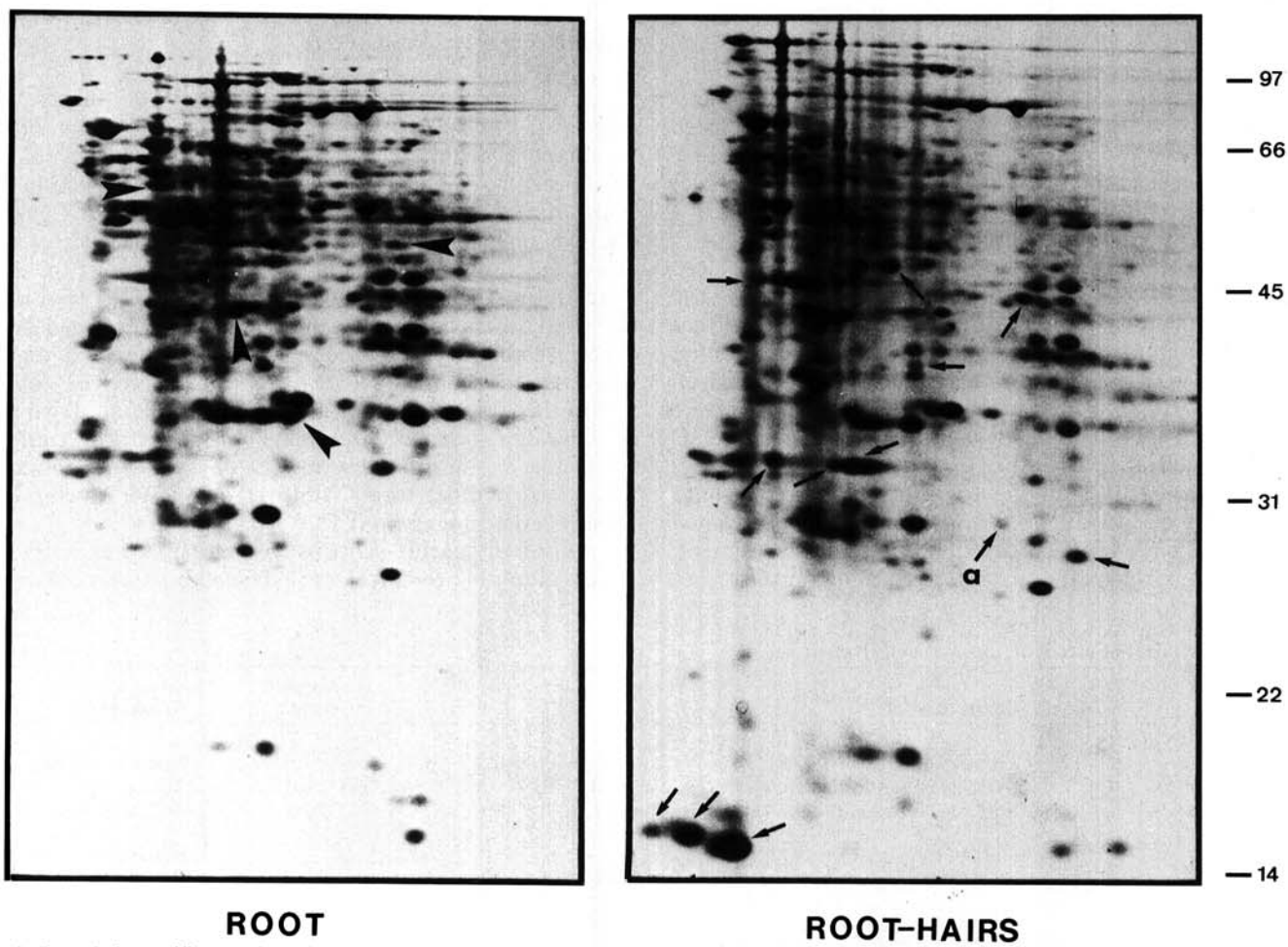

Fig. 1. Root hair-specific proteins of *Vigna unguiculata*. Silver-stained two-dimensional gels of proteins extracted from 4-day-old roots and root hairs (uninoculated). Arrows indicate the root hair-specific proteins, arrowheads show proteins specific to roots. A protein that plays a role in the interaction between legumes and *Rhizobium* (see Fig. 2) is labeled "a." The pH ranges from 3.7 to 6.5, left to right. Numbers refer to the molecular mass (in kilodaltons) of the marker proteins.

after inoculation with any Had$^-$ mutant (Table 2). This period coincides with root-hair deformation but not curling. Two days after inoculation, three additional proteins were observed (Fig. 3), corresponding to the onset of root-hair curling. An additional protein appeared on day 4 (data not shown). These nine proteins range in size from 15 to 55 kDa. Two of the proteins visible on day 1 as well as the protein that appeared on day 4 were transiently expressed (Table 2).

Comparison of symbiotic proteins induced by *Rhizobium* sp. NGR234 and *R. fredii* USDA257S1. Symbiotic proteins were further analyzed by comparing the patterns of proteins from root hairs inoculated with *Rhizobium* sp. NGR234 with those produced by treatment with *R. fredii* USDA275S1 (Nod$^+$, Fix$^+$ on *V. unguiculata*, Fig. 4). As a control in this experiment, a Had$^-$ mutant of *R. fredii*, in which the *nodABC* genes have been deleted (= USDA257B3), was used. Root hairs were harvested 24 hr after treatment with *Rhizobium*.

Synthesis of four additional proteins was induced by *R. fredii* over those produced by the Had$^-$ mutant. An extra protein (spot 6) was observed in one of two experiments. Again, these proteins ranged in size from 31 to 55 kDa (Fig. 4). Interestingly, the two-dimensional protein patterns of root hairs inoculated with either *Rhizobium* sp. NGR234 or *R. fredii* USDA257S1 were similar. This includes both root-hair and symbiosis-specific proteins. The only exception was spot 1, whose synthesis may be specifically induced by *Rhizobium* sp. NGR234. There was, however, no visible spot characteristic for inoculation with *R. fredii* USDA257S1.

DISCUSSION

Ultimately, our aim is to elucidate the molecular basis of recognition between legumes and rhizobia in nodule formation. As the symbiosis begins with attachment of rhizobia in the rhizosphere to the legume root hairs, it

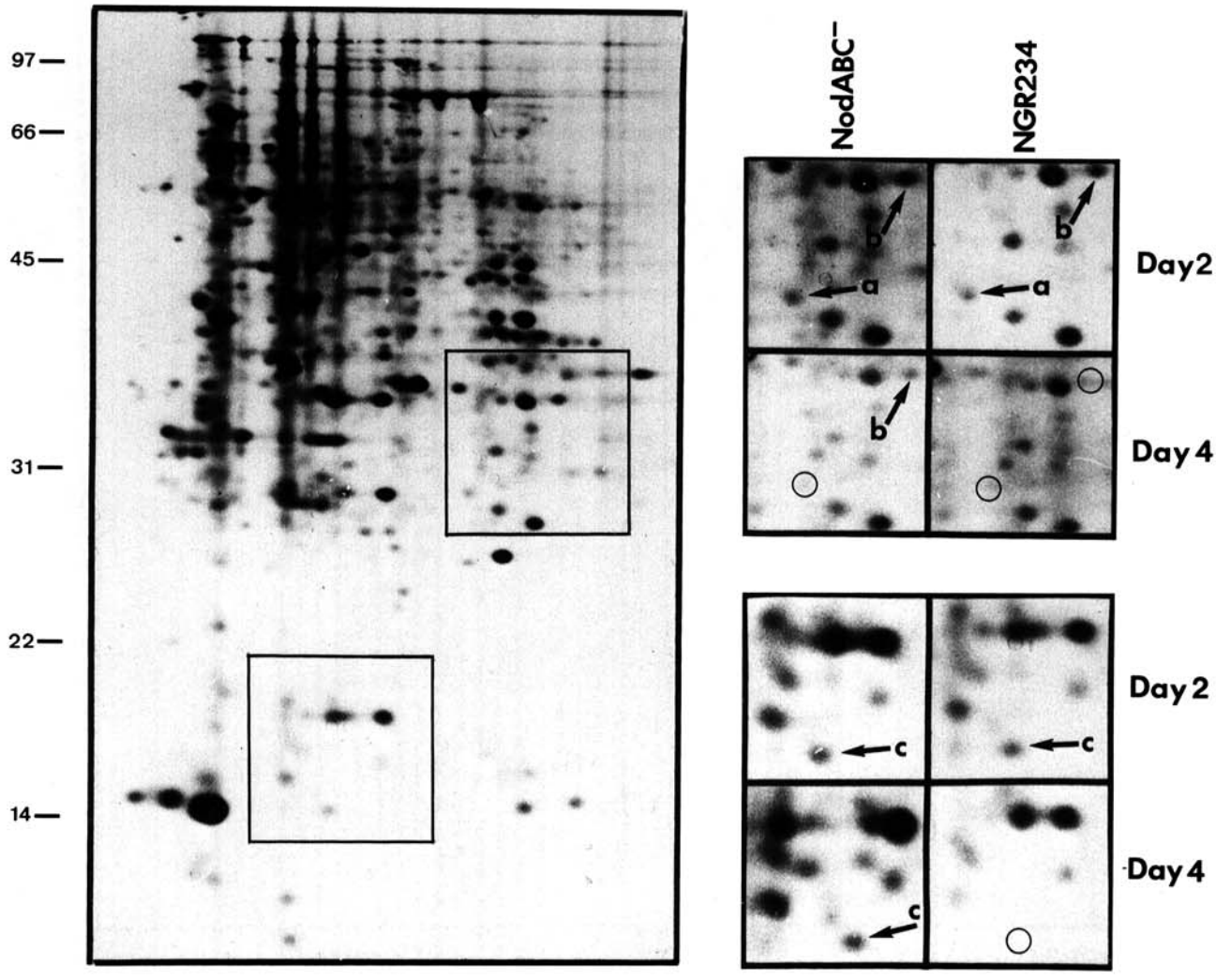

Fig. 2. Disappearance of proteins following inoculation. Silver-stained two-dimensional gels of proteins extracted from root hairs isolated 2 or 4 days after inoculation with *Rhizobium* sp. NGR234 or NGR234NodABC$^-$. Only those parts indicated by the rectangles in the two-dimensional gel on the left are shown on the right. The pH ranges from 3.7 to 6.5, left to right. Numbers indicate the molecular mass (in kilodaltons) of the marker proteins. Arrows highlight proteins that disappeared reproducibly during nodule initiation. For ease of reference, the locations of responding proteins are indicated by empty circles in corresponding gels. A protein that belongs to the family of root hair-specific proteins (see Fig. 1) is labeled "a."

Table 2. Symbiotic root-hair proteins of *Vigna unguiculata* induced by *Rhizobium* sp. NGR234

Spot /kDa[a]	in Root hairs: + NGR234 Day 1	+ NGR234 Day 2	+ NGR234 Day 4	+ Had⁻ mutants[a] NodABC⁻	+ Had⁻ mutants[a] NodD1⁻	+ Had⁻ mutants[a] GMI357	in Nodules[b]
1/15	+	+	+/−	−	−	−	−
2/31	+	+	+	−	−	−	−
3/37	+	+	+	−	?	−	+
4/38	+	+	+	−	−	−	+
5/37	+	+	+	−	−	−	+
6/55	+/−	+	+	−	ND	ND	+
7/36	−	+	+	−	ND	ND	+
8/38	−	+	+	−	ND	ND	+
9/44	−	−	+	−	ND	ND	−

[a] Proteins isolated 24 hr after inoculation; NodABC⁻ = NGR234NodABC⁻; NodD1⁻ = NGR234NodD1⁻; GMI357 = *R. meliloti nodABC*⁻; ND = not determined; ? = not clear; and Day = days after inoculation.
[b] Proteins isolated 4 wk after inoculation.

seemed obvious that changes in the pattern of gene expression in root hairs must represent the first detectable signs of the interaction. Surprisingly, many reports on "early nodulins" exist (for reviews see Gloudemans and Bisseling 1989), but they were, with only two exceptions (Gloudemans *et al.* 1989; Trese and Pueppke 1990, 1991), based on cDNA clones/proteins isolated from nodule material. Northern blotting methods were then used to monitor the expression of selected nodulins "backwards" to the root hairs (e.g., Scheres *et al.* 1990a, 1990b).

By analyzing the products of genes expressed in root hairs, we were able to identify a series of symbiosis-specific proteins. Apparently two classes of these proteins exist. The first class contains three proteins, whose synthesis was repressed 4 days after inoculation (Fig. 5). One of these proteins belongs to the family of root hair-specific proteins. If rhizobia are really "intelligent pathogens" that have

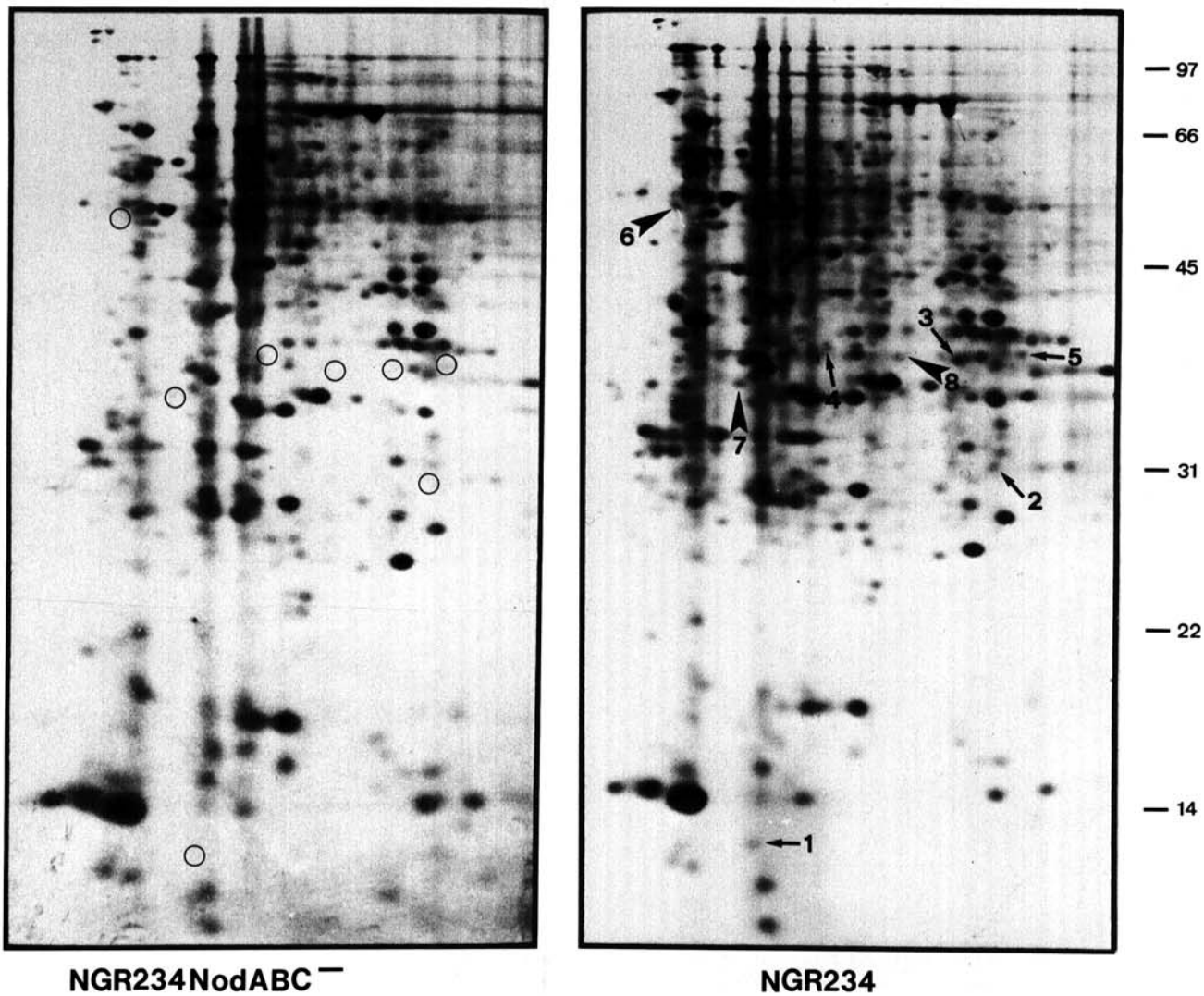

Fig. 3. Changes in protein patterns after inoculation with *Rhizobium* sp. NGR234. Silver-stained two-dimensional gels of proteins extracted from root hairs isolated 2 days after inoculation with *Rhizobium* sp. NGR234 (or as a control with NGR234NodABC⁻). Arrows indicate protein spots already present 24 hr after inoculation; arrowheads highlight proteins that appeared 2 days after inoculation with *Rhizobium* NGR234. For ease of reference, the location of responding proteins is indicated by empty circles in the corresponding gel of the control. The pH ranges between 3.7 to 6.5, left to right. Numbers refer to the molecular mass (in kilodaltons) of the marker proteins. This figure forms part of the results shown in Table 2.

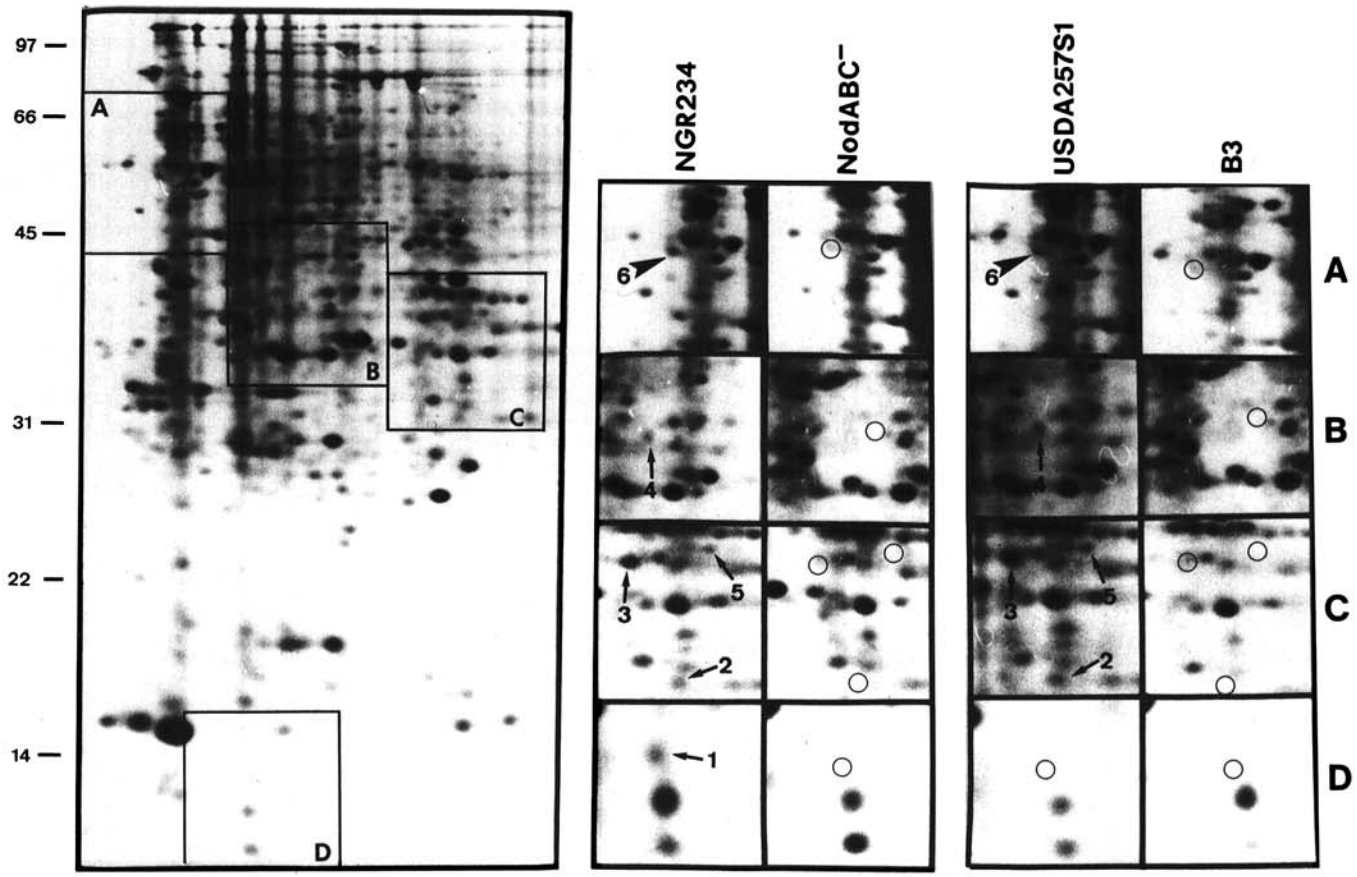

Fig. 4. Comparison of the symbiotic root-hair proteins induced by *Rhizobium* sp. NGR234 with those induced by *R. fredii* USDA257S1. Photograph shows silver-stained two-dimensional gels of proteins extracted from root hairs isolated 24 hr after inoculation with *Rhizobium*. As controls, roots were inoculated with the Had⁻ mutant (NGR234NodABC⁻ or USDA257B3). Arrows indicate protein spots already present 24 hr after inoculation with *Rhizobium* sp. NGR234 or *R. fredii* USDA257S1. For ease of reference, the location of responding proteins is indicated by empty circles in the corresponding gels. Only those parts indicated by the rectangles in the two-dimensional gel on the left are shown on the right. The pH ranged from 3.7 to 6.5, left to right. Numbers indicate the molecular mass (in kilodaltons) of the marker proteins. NodABC⁻ = *Rhizobium* sp. NGR234NodABC⁻, B3 = *R. fredii* USDA257B3.

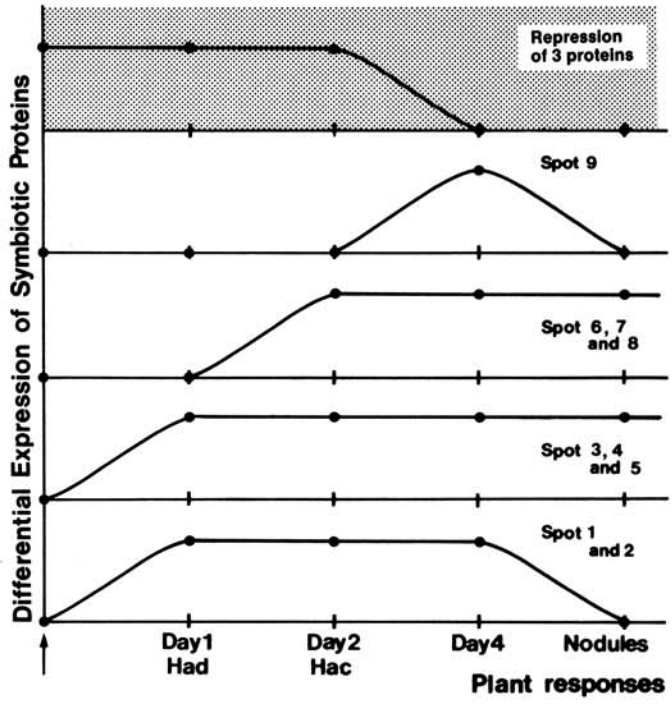

Fig. 5. Schematic representation of changes in the expression of symbiosis-specific proteins accompanying root-hair deformation and nodule initiation of *Vigna unguiculata*. Shaded areas show proteins that disappeared during nodule development; curves on a white background represent the appearence of proteins following inoculation of *V. unguiculata* by *Rhizobium* sp. NGR234. The arrow marks the time of inoculation. X and Y axes in arbitrary units, Spot no. = spot, Day = day(s) after inoculation, Had = root-hair deformation, Hac = root-hair curling, Spot 3 = 15-kDA hadulin, Spot 5 = 31-kDa hadulin,and Spot 23 = 44-kDa hadulin.

acquired genes for regulating the recognition and defense responses of a host plant (Rolfe and Gresshoff 1988), then repression of the synthesis of these three proteins might be the key step that allows rhizobial entry into the host plant. We are currently investigating this hypothesis.

The second class comprises nine proteins, which are visible after inoculation with *Rhizobium* sp. NGR234 (Fig. 5), but not after inoculation with any of the Had⁻ mutants. Synthesis of five proteins (spots 1–5) appeared as early as 24 hr after inoculation, whereas synthesis of three additional proteins (spots 6–8) started on day 2 and another protein (spot 9) began on day 4.

To the best of our knowledge, this is the first report of a series of unique proteins apparently involved in root-

hair deformation and nodule initiation. Although Trese and Pueppke (1990) reported the synthesis of two early nodulins in *V. unguiculata* (first visible 2.5 days after inoculation with *R. fredii*), and Gloudemans *et al.* (1980) showed that the synthesis of one protein in *P. sativum* was induced by *R. leguminosarum* 20 hr after inoculation, these studies were based on the products of *in vitro* translated mRNA. For this reason, direct comparison of their proteins with our results is not possible.

Three of the nine symbiotic proteins of *V. unguiculata* (spots 1, 2, and 9) were only expressed in root hairs. Since these proteins were not synthesized in nodules (and therefore cannot be considered as "early nodulins"), and as two of them appear at the same time as hair deformation first became visible, we will refer to them as "hadulins" (i.e., *ha*ir *d*eformation-specific proteins). Protein 9 (i.e., 44-kDa hadulin) is particularly interesting since it was first observed on day 4 and disappeared before nodules were formed. It is thus a short-lived "late hadulin."

To further study the function of these symbiosis-specific proteins, we have isolated mRNA from infected root hairs and used it to prepare a cDNA library. Symbiosis-specific clones will be isolated by differential screening.

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