Modulation of Host Gene Expression during Initiation and Early Growth of Nodules in Cowpea, *Vigna unguiculata* (L.) Walp. 1

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ABSTRACT

Inoculation of 2-day-old cowpea (*Vigna unguiculata* [L.] Walp.) seedlings with *Rhizobium fredii* USDA257 results in proficient nodulation of the tap root. The most abundant nodulation occurs in a region roughly corresponding to the position of the root tip at the time of inoculation. We have examined plant gene expression in this region, after inoculation with either USDA257 or a nonnodulating mutant, 257B3. After isolation of mRNA and in vitro translation, the protein products were separated by two-dimensional gel electrophoresis. Seven proteins are induced within 2.5 days after inoculation with USDA257. One additional induced protein is detectable by 3.5 days after inoculation, and three more appear by day 6. Three of the proteins that are differentially expressed at 2.5 and 3.5 days after inoculation are produced at equivalent levels after 6 days, indicating transient induction of these genes during early stages of nodule development. Several proteins were more abundant in translations of mRNA from roots that had been inoculated with the nonnodulating mutant. This was particularly true after 6 days, when nine proteins were in this class. Thus, altered plant gene expression in carefully selected, highly responsive tissue can be detected 2 days before emerging nodules are visible on the roots, and 6 to 7 days before acetylene reduction is detectable. Additionally, comparisons of ionically bound cell wall proteins isolated 6 days after inoculation revealed four that were unique to nodulating roots, suggesting that some of the nodulation-induced genes may code for structural proteins.

The interaction of plants in the legume family with bacteria in the genera *Rhizobium* and *Bradyrhizobium* results in the development of a morphologically, physiologically, and biochemically unique symbiosis. The most visible response of the plant is the production of a specialized organ, the nodule. The process of nodule development consists of multiple steps, where microbe and host must exchange signals (for review, see 17). This coordinated interplay starts with bacterial gene induction in the presence of host root exudates and host responses to the presence of compatible bacteria in the environment. In the case of soybean and cowpea, the host responses include root hair curling, cell division in the hypodermis and cortex, and infection thread formation. In concert with continued infection thread growth, further cell division causes the nodule to erupt through the epidermis. As the globular meristem matures into a nodule, bacteria are released from the infection threads, differentiate into bacteroids, and begin fixing nitrogen.

Successful nodule development and function requires regulated expression of both *Rhizobium* and plant genes. The bacterial genes include the nod, nif, and hsn translation products. Nodule-specific host proteins are referred to as nodulins. Most of the nodulins described to date are induced at approximately the same time as leghemoglobin (9, 10). However, several nodulins are induced before leghemoglobin in soybean and pea (8). Translatable mRNA for nodulin N-75 is first detectable when soybean nodules are emerging through the epidermis, at 7 d after inoculation. Nodulin N-40' can be detected in pea roots 8 d after inoculation, 4 to 5 d before the onset of nitrogen fixation (10).

Certainly, the process of nodule initiation and early development requires changes in the expression of additional host genes, but these have been difficult to characterize. The primary constraint lies in the fact that the cells involved in initiation events represent a small fraction of the total root tissue. Changes in the protein or mRNA pools of responding cells may be obscured by the preponderance of nonresponding cells. Second, many of the plant genes involved in nodule morphogenesis may be expressed at some level in uninoculated roots. Localized induction or repression of these genes would appear as quantitative changes in protein or mRNA pools, rather than on-off responses. In both cases, nodulation-induced changes in gene expression would become more apparent as the concentration of responding cells in the sample increases.

We have chosen to use cowpea (*Vigna unguiculata* [L.] Walp.) in an effort to identify host genes involved in nodule initiation and early development. Cowpea produces up to six times as many nodules as soybean in the initially infectible zone of the taproot. This region is restricted to the root tissue that was positioned just proximal to the root tip at the time of inoculation (2, 13). Furthermore, the increased number of nodules appears to reflect an increased number of infection events (2). We selected *Rhizobium fredii* USDA257 as inoculant because it elicits abundant nodulation on cowpea, and because a symbiotically null mutant, 257B3 (12), was available for inoculation of control plants. We have developed a
tissue selection system that greatly enriches for actively responding plant cells. Differences in mRNA in vitro translation products can be detected within 2.5 d following inoculation. Additional differences are apparent at 3.5 and 6 d postinoculation, and include both increased and decreased gene expression. Most of these are quantitative changes, rather than on-off responses.

MATERIALS AND METHODS

Bacterial Strains

The bacterial strains used were Rhizobium fredii USDA257 (hereafter designated 257) from H. H. Keyser (U.S. Department of Agriculture, Beltsville, MD) as the wild type, and a nonnodulating mutant, designated 257B3. This mutant was selected after Tn5 mutagenesis of strain 257 and is known to have a deletion in the common nod genes independent of the site of Tn5 insertion (12). For inoculation, starter cultures of 257B3 in 100 mL of YEM broth (13) containing 50 μg/mL kanamycin were prepared. Fifty mL of this culture was used to inoculate 2 L of YEM broth without antibiotic in a Fernbach flask. R. fredii 257 was cultured similarly, in the absence of kanamycin, and all cultures were grown on a rotary shaker (100 rpm) at 28°C.

Plant Growth and Inoculation

Cowpea seeds (Vigna unguiculata [L.] Walp. cv Pink Eye Purple Hull), obtained from Hastings Seeds, Atlanta, GA, were surface-sterilized as described by Pueppke (23). The seeds were planted in large plastic trays (50 × 28 × 15 cm deep) with drainage holes, filled with medium grade vermiculite. Approximately 300 seeds were planted per tray. The trays were watered at planting with 4 L of deionized water, and with 1 L of Jensen’s N-free solution (13) on the following day. Seeds were germinated for 48 h in dark, until the tap roots were approximately 4 cm long, before inoculation. Inoculum consisted of mid-log phase rhizobia in YEM broth diluted with Jensen’s solution at a ratio of 1:1, to give a final concentration of approximately 5 × 10⁶ cells per mL. Four L of this solution were poured over each tray. Plants inoculated in this manner developed a slight thickening of the tap root, perceptible within 36 h, in the region that would roughly correspond to the root tip at the time of inoculation. Microscopic enumeration of nodule initiation sites in this area (23) indicated that this was approximately the center of the most responsive root tissue. Thus, we were able to use this visible marker to locate and harvest root segments having the highest concentration of initiation sites at time points well before nodule emergence. Cultures of the mutant 257B3 induced the same swelling of the tap root, permitting harvest of root segments equivalent to those inoculated with strain 257.

Microscopy

Root segments from plants were harvested at 2.5, 3.5, and 6 d after inoculation with R. fredii 257. The segments were fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). The tissue was postfixed for 3 h in 1% osmium tetroxide and embedded in Spurr’s resin. Semithin sections (3 μm) were stained with 1% toluidine blue and examined by bright field microscopy.

Acetylene Reduction Assays

Acetylene reduction assays were performed by the method of Schwinghamer et al. (26). Nodulated root pieces were used for the assay, with each 20 mL vial containing a single root. Three root segments were used for each time point and the experiment was done twice.

RNA Isolation and in Vitro Translation

Root segments were harvested 2.5 and 3.5 d after inoculation. All lateral roots were detached from the tap root, the slightly thickened root zone (about 2–3 cm long) was selected, and the stele then was removed from the segment. This was accomplished by gently rolling the wooden handle of a dissecting needle over each root segment, after which the stele could easily be removed with forceps. The remaining hollow cylinder of cortical and epidermal cells was immediately frozen in liquid nitrogen. Samples collected 6 d after inoculation with strain 257 consisted of visibly nodulated segments, while the slight swelling was used to select segments from plants inoculated with strain 257B3. The stele was not removed from 6 d samples.

Total RNA was extracted by the method of Rochester et al. (24) and precipitated twice from 2 mL LiCl. Poly(A⁺) RNA was isolated by oligo-dT cellulose chromatography (1). Cell-free translations were done with a pretreated rabbit reticulocyte system from Promega Biotec (Madison, WI), in the presence of [³⁵SJ-L-methionine (New England Nuclear, 1151 mCi/mmole). One to 2 μg of poly(A⁺) RNA were translated in each case, and incorporation determined by precipitation with trichloroacetic acid, according to the protocol recommended by Promega Biotec.

Two-dimensional analysis of the translation products was carried out essentially as described by O’Farrell (22). We used 5 to 7 × 10⁵ cpms of incorporated label per gel. Equal counts were loaded onto each gel for pairwise comparisons. Proteins were focused in the first dimension with a 3:2 mixture of LKB pH 5 to 7 and pH 3.5 to 10 ampholytes, at a total concentration of 2% (v/v). The measured pH range of the gels was 3.9 to 6.4. Proteins were separated in the second dimension on a 12% polyacrylamide gel. The gels were treated with EN3HANCE (New England Nuclear) prior to drying and exposure to X-ARP film (Kodak). The mol wt markers were BRL prestained standards.

Cell Wall Proteins

Six d after inoculation with either 257 or 257B3, root segments were harvested as above, but without freezing. Salt-extractable cell wall proteins were isolated as described (29). After concentration with 10K Centricron microconcentrators (Amicon, Lexington, MA), protein concentration was determined by the method of Lowry et al. (18), with BSA as the standard. The proteins were separated on a 12% polyacryl-
amide gel by the method of Laemmli (16), in comparison to Sigma prestained standards. The gel was stained with silver by the method of Morrissey (20).

RESULTS AND DISCUSSION

Nodule Initiation and Development

The interaction of cowpea and R. fredii 257, under our experimental conditions, results in two measurable responses. One is the coordinated induction of numerous nodule initials in the most susceptible region of the tap root, that area which is immediately above the root tip at the time of inoculation. This restricted localization causes mature nodules to develop as dense clusters in this region, as seen in Figure 1. Fortuitously, the second response is a slight thickening of the same root zone, which develops within 36 h after inoculation. This provides a convenient marker for selecting root segments possessing the highest concentration of initiation events, well before nodules are visible.

To assess which cell types or tissues are involved in the early stages of nodule development, thickened segments were fixed at 2.5, 3.5, and 6 d after inoculation. At 2.5 d post-inoculation, several hundred cortical cells are involved in each nodule initiation site (Fig. 2A). The cortical cells closest to the point of infection have concentrated, dense cytoplasm and are dividing in many planes. A globular nodule meristem, consisting of many highly cytoplasmic cells, has formed in the outer cortex by 3.5 d (Fig. 2B). It appears that most of the host cells involved in early nodule development are cortical cells, or their derivatives. Thus, the early development of nodules in cowpea is quite similar to that reported for soybean (4).

Transverse sections of 6-d-old nodules (Fig. 2C) show that infection threads (large arrowheads) have now spread throughout a larger meristematic center, and that some bacteria have been released into the host cell cytosol (small arrowheads). These results indicate that bacterial release occurs earlier in cowpea than in soybean, where bacterial release begins 8 to 10 d after inoculation (30). Furthermore, longitudinal sections of 6-d-old nodules (not shown) indicate that nodule development now involves more than cortical cells, as the nodule is attached to the stele through a developing vascular system.

Figure 3 shows the temporal induction of acetylene reduction capacity in inoculated cowpea roots. Acetylene reduction is first detectable 9 d after inoculation. It increases linearly during the next 3 d and then levels off. Nitrogenase activity was detectable in our system several days earlier than reported for soybean, at d 9 as compared to d 11 (27).

The above studies of nodule development indicated that it would be possible to enrich our tissue samples for responding cells by removing the steles from 2.5 and 3.5 d samples. Although this may have removed some responding cells we reasoned that by harvesting cortical and epidermal cells only, thereby reducing the complexity of the selected tissue, we would improve the chances of detecting differences in gene expression.

The diluted cultures used as inoculum are likely to cause changes in gene expression not directly related to nodulation. For this reason, control roots were inoculated with diluted cultures of an R. fredii 257 mutant lacking genetic determinants to initiate nodule development. Cultures of this mutant induced the same thickening of the tap root but did not induce any symbiotic specific responses, i.e. root hair curling, cell division, or infection thread formation (data not shown). Root thickening also was induced by culture supernatants, and thus is a nonspecific response. Caetano-Anolles and Bauer (3) reached the same conclusion after studying a similar, although more drastic, response induced on alfalfa by inoculation with high doses of Nod+ Rhizobium meliloti cells or treatment with culture fluids.

Analysis of mRNA Populations

Differences in mRNA populations were examined by in vitro translations and two-dimensional separation of the labeled protein products. Replicate gels were run for each time point, and we were able to closely reproduce each comparison. The highlighted protein products are numbered (Fig. 4), and nodulation-induced versus nodulation-depressed proteins are distinguished by right or left facing arrows, respectively.

As early as 2.5 d after inoculation, clear differences were visible between mRNA populations isolated from root segments treated with 257 and 257B3. As expected, the majority of proteins were translated at essentially the same level, but at least seven proteins were more abundant in the 257-inoculated tissue (Fig. 4A), while the amount of one protein was elevated in the 257B3-treated sample (Fig. 4B). At this time the most noticeable response in 257-inoculated roots is cortical cell dedifferentiation and division. Similar protein patterns were evident when the translation products from samples harvested 3.5 d after inoculation were compared, but there were important differences. One new protein was more abundant in the 257-inoculated tissue (Fig. 4C, >8), and one
Additional protein was more abundant in the nonnodulating sample (Fig. 4D, <2). Nodule morphology also was similar at 2.5 and 3.5 d in that the cells involved are cortical cell derivatives. The main distinction is the presence of a discernable globular meristem, composed of many highly cytoplasmic cells.

Protein differences were more numerous in 6-d-old samples. At least three additional proteins were more abundant in the tissue sample containing nodules (Fig. 4E, >9, >11, >12). Additional differences detected at this time point represent proteins which were expressed more abundantly in the 257B3-inoculated sample; there were now at least nine proteins in this class (Fig. 4F, arrows 1–9). Most of these proteins could be detected at some level in the nodulating root sample, with the exception of those designated <6 and <9. Nodule development at 6 d includes infection thread ramification throughout a distinct meristem and release of some bacteria. Additionally, the root segments compared here included stele tissue.

The nodulins described to date have been divided into two major classes (9, 11). Early nodulins are expressed before, or during, bacterial release. These include N-40’ (10) and three others, N-75 (8), N-38 (21), and Nms-30 (7), which also are transcribed in pseudonodules devoid of intracellular bacteria. The late nodulins are expressed concomitantly with, or after, leghemoglobin, and are only found in nodules that have a mature structure. It has been suggested that bacterial release acts as a signal to the host, which initiates the transcription of many of these nodulins, including leghemoglobin (5, 21).

By careful selection of root tissue that has a high concentration of nodule initiation sites, we have detected changes in expression of 20 host genes, before or during bacterial release. As summarized in Table I, the number of differentially expressed genes increases between 2.5 to 6 d, as the nodule initiation centers develop. This may be due to increased gene induction, or repression, within the population of responding cells or it may reflect an increase in the proportion of responding cells within the nodulating sample. By 6 d, the clusters of emerging nodules on 257-inoculated roots make up at least 10% of the tissue mass. Furthermore, because 6 d samples included stele tissue, some of the differences first detected at this time may reflect stele-specific gene regulation responses. Only two proteins appear to be unique to 257-inoculated roots (Fig. 4, >1 and >7), and thus might qualify as nodulins.
Figure 4. Nodulation induced changes in gene expression. Fluorographs of two dimensional gels of in vitro translation products of isolated poly(A+)* RNA. A, C, and E, roots 2.5, 3.5, and 6 d after inoculation with *Rhizobium fredii* USDA 257; B, D, and F, the same times after inoculation with a Nod" mutant, 257B3. Differentially expressed protein products are numbered; those noted at early time points are given lower numbers, and differences that appear later are given sequentially higher numbers. Right and left facing arrows distinguish 257-induced from 257-repressed products, respectively. For ease of reference, the locations of responding proteins are indicated by empty circles, or squares, in corresponding gels. The pH range was 3.9 to 6.4, left to right.
Table I. Summary of Changes in the Level of in Vitro Translation Products Directed by mRNA Isolated from Inoculated Roots, as Determined by Two-Dimensional PAGE

Differentially expressed proteins are marked with the symbol, *, coincident with their first appearance. Proteins that are no longer differentially expressed at 6 d are designated with the symbol, =.

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Otherwise, the differences represent plant genes that are expressed in both samples, yet respond to nodulation by increased or decreased expression. At 2.5 and 3.5 d, most differences represent increased gene expression in nodulating roots. These genes, although not nodulins, are of particular interest because they are induced during the initiation of nodule development. They must respond, directly or indirectly, to some early signal produced by nodulation-competent *Rhizobium fredii*. By 6 d after inoculation, more of the differences reflect reduced expression in modulating roots, relative to 257B3 roots. Although others have reported root-enhanced or nodule-repressed genes during late nodule development, these genes have received little attention (9, 19).

However, considering the developmental reversal required to initiate meristematic division of cortical cells, gene repression may be crucial to early nodule development. Proteins <1 and <2 are of particular interest in this regard because they are repressed during initiation of cortical cell division.

Three nodulation-stimulated proteins, >2, >4, and >5, appear to be transiently regulated in response to inoculation. Protein >2 is induced in cortical cylinders at 2.5 and 3.5 d, but is very low in both samples at 6 d. Proteins >4 and >5 are differentially induced at 2.5 and 3.5 d, but are equally abundant in both samples at 6 d. This transient induction suggests that initiation of nodulation may represent a distinct phase of development.

Changes in Cell Wall Proteins

Following inoculation of soybean or pea, the earliest detectable change in gene expression is induction of the nodulin
termed ENOD2 (8). ENOD2 is developmentally induced at approximately the same time as bacteria are released from infection threads, and it begins to decline 14 to 21 d after inoculation. A cDNA corresponding to ENOD2 hybridized to a mRNA of 1200 nucleotides in length, with a coding capacity for a protein of not more than 45 kD. However, translation of hybrid-released mRNA produced two protein products with apparent mol wt of 75 kD. The cDNA sequence indicated the presence of 20 heptapeptide repeating units, and a protein product containing 45% proline, but lacking methionine. It has been suggested that this gene may represent a new class of structural proteins, related to, but distinct from, a group of hydroxyproline-rich glycoproteins, the cell wall bound extensins.

Because we used [35S]L-methionine in our in vitro translations, we would not have detected an analogous protein product in our studies. Therefore, we compared cell wall-bound, salt-extractable proteins isolated from tissue samples 6 d after inoculation with R. fredii 257 or 257B3 (Fig. 5). Four proteins with mol wt of 31, 40, 55, and 108 kD were unique to the inoculated root segments. The protein at 55 kD was present as a diffuse band, which may be due to glycosylation. The extracted cell wall proteins from 257B3 inoculated roots included three (27, 30, and 37 kD) that were present at relatively greater abundance. We did not find any differences in proteins near 75 kD, the in vitro translation size of mRNA hybridizing to ENOD2. Govers et al. (10) have shown that during pea nodule development the first detectable change in in vitro translation products is the appearance of a protein of MW 40 kD, N-40'. It was first detectable at 8 d after infection, increased until d 15, and then remained constant. Whether the cell wall-bound, 40 kD protein we have detected is related to N-40’ has not been determined, but it is clear that within 6 d after inoculation, several cell wall-associated proteins have accumulated selectively in root segments containing developing nodules. These differences in cell wall proteins reflect differences in mRNA populations to the extent that nodulation induces both increases and decreases relative to 257B3 inoculation.

A group of hydroxyproline rich glycoproteins are associated with plant cell walls and have been implicated in plant defense responses, among other roles (14, 25, 28). One such protein, related to the wound-inducible p33 of carrot, is known to be developmentally regulated in soybean (29). Additionally, Casab (6) has shown that hydroxyproline-rich proteins accumulate in soybean nodule cell walls as the nodules mature from 2 to 10 weeks in age. Further study of the cell wall proteins induced in cowpea nodules should determine whether they are members of this protein class or other structural proteins, their regulation, and what role they play during nodule development.

CONCLUSION

Through a system of tissue enrichment, we have been able to demonstrate induction of 11 host proteins during the early growth and development of cowpea nodules. Two nodule-specific and five nodulation-stimulated proteins are discernible within 2.5 d after inoculation. In addition, several root proteins decline in the inoculated samples in the first few days after inoculation. This is the first report of changes in host gene expression that occur when nodule initiation centers are just beginning to form an organized meristem. This rapid regulation suggests that these genes may respond to some early signals produced by nodulation-competent R. fredii. Because these changes reflect differences in mRNA populations, it will be feasible to construct and screen cDNA libraries, and select clones that represent these genes. Further work examining their regulation, particularly in host-symbiont interactions blocked at early stages of development, may help determine their functions and increase our understanding of host specificity.

LITERATURE CITED

HOST GENE EXPRESSION DURING NODULE INITIATION OF COWPEA


