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Cloning of Cowpea (*Vigna unguiculata*) Genes that Are Regulated During Initiation of Nodulation

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Taproots of cowpea plants inoculated with *Rhizobium fredii* USDA257 produce numerous nodules that are clustered in a region approximating the position of the root tip at the time of inoculation. Inoculation with the Nod$^-$ mutant strain, 257B3, does not induce symbiosis specific responses, but it should have the same general effects on roots. We have used these strains to inoculate seedlings and have isolated poly(A)$^+$ RNA from root segments 6 days after inoculation. A cDNA library was constructed from poly(A)$^+$ RNA that had been extracted from USDA257-inoculated root segments, and screened by differential hybridization with homologous cDNA probes and cDNA probes from 257B3-inoculated roots. Two clones that hybridize preferentially to nodulating root cDNA were isolated, VuA and VuB. Two others, VuC and VuD, hybridized preferentially to cDNA from nonnodulated roots. Northern gel analysis of total RNA confirmed differential regulation of the mRNA transcripts corresponding to these clones. The VuA clone hybridized to two transcripts that are independently regulated, and to several restriction fragments of cowpea genomic DNA, suggesting that it is a member of a small gene family. Hybridization of VuA with the coding region of pGmENOD2 suggests that VuA represents the cowpea homologue of this early nodulin clone. The VuB homologous transcript is differentially expressed within 1.5 days, and the VuC and VuD clones represent genes whose expression is altered within 2.5 days of inoculation. This rapid response suggests that their regulation is triggered by some early signal(s) from nodulation-competent rhizobia.

Inoculation of leguminous plants with compatible rhizobia initiates a series of developmental processes that culminate in a morphologically and metabolically unique symbiosis. The benefit provided the plant is nitrogen, fixed in the form of ammonia, while the bacteria benefit from a protected environment and direct nutrient supply. In the case of soybean (Turgeon and Bauer 1982) and cowpea (Pueppke 1983), nodule development begins with the induction of root hair deformation and cell division in the hypodermis. Shortly thereafter, infection threads develop in some curled root hairs, associated with discrete foci of cortical cell divisions. Further cell division is coordinated with the elongation of infection threads, which eventually ramify throughout the meristematic center of the developing nodule. Once the bacteria are released from the intracellular infection threads, they are transformed into bacteroids, capable of nitrogen fixation (Werner and Morscel 1978).

Several aspects of this process are of particular interest in terms of plant-microbe interactions. How does the plant perceive the presence of bacteria at the root surface and respond by cell division within the cortex? How do the bacteria induce the elaboration of an infection thread without triggering a defense response by the plant cells? How are the bacteria released into the plant cells and allowed to function there on a very intimate level without adverse responses from the host? Additionally, what are the controls that limit and define the growth of both the host tissue and the bacterial cells?

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The answers to these questions must involve regulation of gene expression by both plant and microbe. Our knowledge of the bacterial genetics is increasing, with many of the genes involved in these steps identified. In particular, *nodDABC* are required for the earliest symbiosis-specific responses: root hair curling, infection thread formation, and cortical cell division (Long 1989). Plant gene regulation, on the other hand, has been studied primarily during later stages of the interaction. Much of this work has focused on plant genes that are expressed exclusively in nodule tissue, producing proteins termed nodulins (Delauney and Verma 1988; Vance *et al.* 1988; Verma and Delauney 1988). The majority of these genes are induced concomitantly with nitrogenase, and are grouped as the late nodulins. Several others, the early nodulins, are expressed well before the onset of nitrogen fixation, as early as 6 days after inoculation (Govers *et al.* 1987; van de Wiel *et al.* 1990). However, by 6 days many of the steps crucial to establishing a compatible interaction have been completed (Calvert *et al.* 1984). Understanding these early events will require identification and characterization of plant genes whose regulation is involved in nodule intitiation.

Here we report the construction of a cDNA library corresponding to mRNA isolated from nodulated cowpea root segments harvested 6 days after inoculation. After differential screening of the library to identify clones that were either induced or repressed during early nodule development, four selected clones were further characterized by northern gel analysis.

MATERIALS AND METHODS

Bacterial strains. *Rhizobium fredii* Scholla & Elkan USDA257 (hereafter designated 257) and *Bradyrhizobium*

japonicum (Buchanan) Jordan USDA110 were from the U.S. Deptartment of Agriculture (Beltsville, MD) The Tn*5*-containing Nod$^-$ mutant, 257B3, known to lack *nodDABC* (Heron *et al.* 1989; H. B. Krishnan and S. G. Pueppke, unpublished observations), was used for inoculation of control roots. Bacteria were cultured as described (Trese and Pueppke 1990). Briefly, 2 L of inoculated yeast-extract mannitol medium (YEM) (Vincent 1970) was incubated on a rotary shaker (100 rpm) at 28° C, until the cultures reached mid log phase. The mutant strain was grown under identical conditions, except that starter cultures (100 ml) were grown under antibiotic selection to ensure genetic uniformity, whereas large-scale cultures were antibiotic-free to prevent undesirable plant responses to the medium.

Plant growth and inoculation. Cowpea seeds (*Vigna unguiculata* (L.) Walp. 'Pink Eye Purple Hull') were obtained from Hastings Seeds, Atlanta, GA. Soybean seeds (*Glycine max* (L.) Merr. 'Williams') were purchased from Missouri Foundation Seed, Columbia, MO. The seeds were surface-sterilized (Pueppke 1983) and planted in large plastic trays (50 × 28 × 15 cm deep) filled with vermiculite. After 48 hr of growth in the dark at 28° C, at which time the taproots had grown to approximately 4 cm, the plants were inoculated. Inoculum for cowpeas consisted of a 1:1 dilution of mid log phase cultures with Jensen's nitrogen-free nutrient solution (Vincent 1970). Control treatments included sterile YEM, diluted as above, and deionized water. Each tray was saturated with 4 L of inoculum and allowed to drain freely. Within 36 hr after inoculation, a 2- to 3-cm segment of each taproot developed a slightly swollen appearance. The center of this region was previously shown to roughly correspond to the position of the root tip at the time of inoculation and, fortuitously, to include the highest concentration of nodule initiation sites (Trese and Pueppke 1990). This slight thickening of the root allowed us to select actively responding root tissue well before nodules were visible. The Nod$^-$ mutant, 257B3, produces the same visible response, but none of the the symbiosis-specific responses such as root hair curling, cell division, or infection thread development. Culture supernatants and diluted YEM also induced root thickening, indicating that this is a nonspecific response. For soybean inoculations *B. japonicum* cells were pelleted and resuspended to 1×10^8 cells per milliliter of half-strength Jensen's solution. Trays of soybean seedlings were saturated with inoculum as above.

Tissue harvest and nucleic acid isolation. To harvest root tissue 1.5–6 days after inoculation, lateral roots were detached from the taproot, and the slightly thickened segments were excised. An equivalent segment was estimated in the case of control roots treated with Jensen's solution. Emerging nodules were visible on 257-inoculated roots by 6 days, and only densely nodulated root segments were collected. Nodules were picked from cowpea roots at 12 and 16 days after inoculation. With soybean, root segments that had clusters of emerging nodules were selected at 6 days, and comparable segments were chosen from control root tissue. All plants were harvested individually, and the selected tissue was quickly frozen in liquid nitrogen.

For isolation of cortex-enriched tissue, segments were collected as above from plants at 1.5–3.5 days after inoculation. Each segment was then gently crushed by rolling over it with the wooden handle of a dissecting needle. This partially softened the root tissue, allowing the stele to be pulled free. The hollow cylinder of cortical and epidermal tissue was immediately frozen in liquid nitrogen.

Total RNA was isolated by the method of Rochester *et al.* (1986), including two precipitations from 2 M LiCl. Oligo(dT)-cellulose chromatography was used to enrich for poly(A)$^+$ RNA (Aviv and Leder 1972). Cowpea genomic DNA was isolated by a mini-prep procedure (Dellaporta *et al.* 1983).

Library construction and screening. A cDNA library was constructed in λ-gt10 (Huynh *et al.* 1985) from poly(A)$^+$ RNA isolated from 6-day nodulated roots by the method of Gasser *et al.* (1989), including the addition of *Eco*RI linkers to the cDNA. The library was screened by hybridization of duplicate plaque lifts with cDNA probes (Gasser *et al.* 1989) prepared from poly(A)$^+$ RNA from 6-day-old roots that had been inoculated with strain 257 or mutant 257B3. Plaques that hybridized preferentially with either probe were selected as clones representing differentially expressed genes. These plaques were isolated, replated at a lower density, and screened a second time to allow isolation of single plaques. As a third screen, the clones were digested with *Eco*RI, the inserts separated by electrophoresis, and duplicate Southern blots were prepared. These filters were hybridized with cDNA probes from 6-day root tissue that had been inoculated with either 257 or 257B3. A second pair of equivalent filters was hybridized to cDNA probes from poly(A)$^+$ RNA isolated from cortical cylinders that had been harvested 2.5 days after inoculation of roots with 257 or 257B3.

Four selected cDNA inserts were subcloned into pGEM7Zf+ (Promega Biotech, Madison, WI). Isolated insert DNA was labeled with a random primer kit from Boehringer-Mannheim (Indianapolis, IN).

Northern and Southern blots. RNAs for northern blots were denatured with formaldehyde and separated in 1.0% agarose gels as described by Perbal (1984). The gels were blotted to nitocellulose, baked, and hybridized as for Southern blots (Maniatis *et al.* 1982). An RNA ladder from Bethesda Research Laboratories (Gaithersburg, MD) was included in each experiment to determine transcript sizes. Hybridization was performed at 68° C, and the final washing conditions were 0.3× SSPE, 0.1% SDS, for 1 hr at 68° C.

Cowpea genomic DNA was digested with *Eco*RI or *Hin*dIII and separated in 1% agarose gels. Southern blots were performed as described in Maniatis *et al.* (1982), with final washes in 0.3× SSC and 0.1% SDS at 68° C for 30 min. A subclone of pGmENOD2 (2pH2) (Franssen *et al.* 1987) generously supplied by H. Franssen (Wageningen, The Netherlands) was used to test for homology with the four cDNA inserts. The Southern hybridization and final washes were at 58° C. The 2pH2 and VuA clones were also used to probe replicate northern gel transfers of 6-day-old nodulating root total RNA.

RESULTS

Selection of nodulation-regulated clones. cDNA probes were prepared from poly(A)$^+$ RNA from root segments

harvested 6 days after inoculation with 257 or 257B3. Differential screening of roughly 16,000 recombinant plaques with these probes identified 28 clones that preferentially hybridized to one set of probes. After second and third screenings, we selected four promising clones that showed differential expression both with the initial probes and with cDNA probes synthesized from poly(A)$^+$ RNA from cortical cylinders harvested 2.5 days after inoculation with 257 or 257B3. Two of the cloned cDNA inserts preferentially hybridized to probes from 257-inoculated roots and have been designated clones A and B. The remaining two, clones C and D, preferentially hybridized to probes from 257B3-inoculated roots. Each clone was subcloned into pGEM7Zf+ and the recombinant plasmids designated pVuA, pVuB, pVuC, and pVuD. The cDNA insert sizes are 1,100, 1,300, 600, and 1,150 base pairs (bp), respectively.

Analysis of mRNA levels in root segments. Figure 1A illustrates the levels of pVuA-homologous transcripts present in total RNA samples isolated from root segments or nodules, 1.5–16 days after inoculation. Two transcripts hybridize to this clone. An mRNA of approximately 2,000 bp appears in 257- and 257B3-treated roots at 2.5 and 3.5 days after inoculation. It is also present in control roots 6 days after inoculation with 257B3, or treatment with either Jensen's solution or YEM. This transcript does not appear to be differentially expressed during nodulation.

A smaller transcript, of 1,400 bp, is detectable in 257-inoculated segments by 3.5 days after inoculation. Expression of this transcript in 257-inoculated roots is more pronounced at 6 days, with further increases at 12 and 16 days. This transcript is absent from analogous 257B3-inoculated samples and other control treatments, with the exception of a faint band present in the YEM inoculated roots. A homologous transcript of similar size is present in soybean root segments, harvested 6 days after inoculation with *B. japonicum* USDA110, but not in control roots inoculated with Jensen's solution. As in cowpea, soybean control roots treated with Jensen's solution have a small amount of a 2,000-bp transcript.

The pattern of hybridization with the insert from pVuB to RNA samples is distinctly different (Fig. 1B). In all comparisons the homologous 1,800-bp transcript is present only in roots inoculated with 257. Differential expression of this transcript is clear within 2.5 days after inoculation. The transcript is most abundant in root segments after 6 days, and then declines through 12 and 16 days. Nodulating soybean roots contain a similar transcript, which is absent in control root tissue. The larger transcripts visible in 257B3-inoculated roots at 6 days were not seen in other experiments and are probably artifacts.

The parent clone of pVuC was originally selected because it preferentially hybridized with cDNA probes from 257B3-inoculated root segments collected at both 2.5 and 6 days after inoculation. When labeled pVuC insert was used to probe northern gels, hybridization to a transcript of 800 bp was detected (Fig. 1C). Expression of this transcript was higher in 257B3-inoculated roots, particularly at 2.5 and 3.5 days. After 6 days, the level of this transcript was similar in 257B3 and Jensen's inoculated roots, while the YEM treatment produced somewhat smaller amounts. There was a trace of hybridization to a larger transcript in RNA from nodulated root segments at 6 days, but no

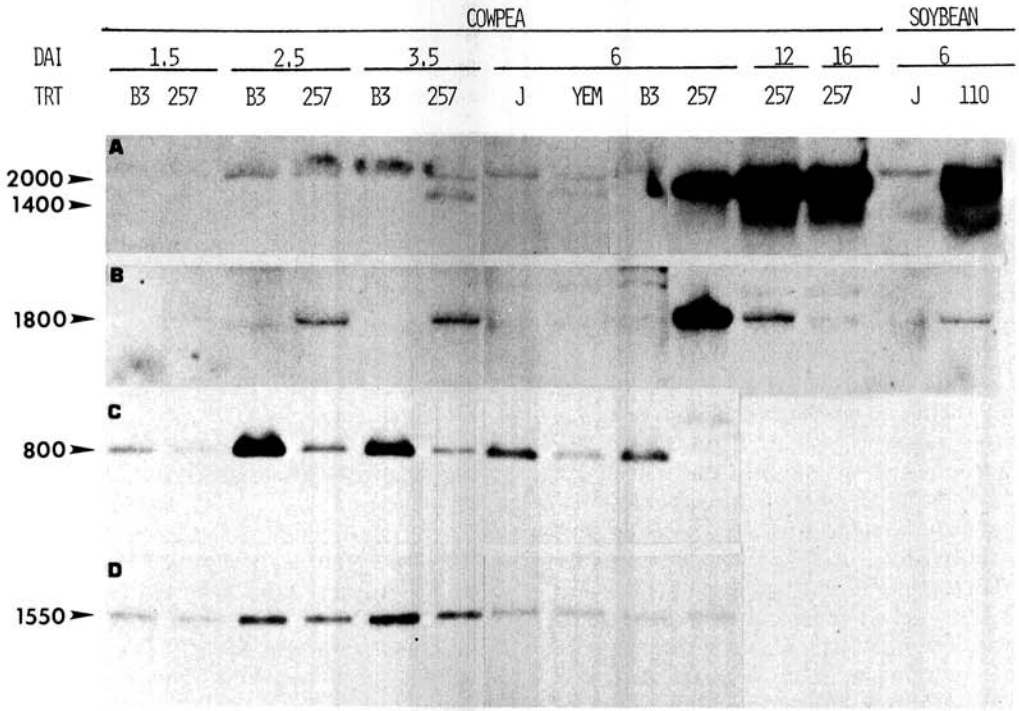

Fig. 1. Hybridization of total RNA isolated from cowpea and soybean root segments, or nodules, with probes from four cDNA inserts. cDNA clones used were: **A,** pVA; **B,** pVuB; **C,** pVuC; and **D,** pVuD. Each lane contains 16 μg of RNA. DAI, days after inoculation; TRT, bacterial strain used to inoculate plants: 257, *Rhizobium fredii* 257; B3, Nod$^-$ mutant *R. fredii* 257B3; J, Jensen's nutrient solution; YEM, yeast extract-mannitol medium; 110, *Bradyrhizobium japonicum* USDA110. Nodules were removed individually at 12 and 16 days after inoculation. Arrows on the left indicate sizes of transcripts, in base pairs.

detectable transcript at 800 bp.

The fourth clone, pVuD, hybridized to a 1,550-bp transcript (Fig. 1D). There was modest differential expression of this transcript at 2.5 and 3.5 days. At 6 days the transcript levels were nearly identical in all treatments.

Analysis of mRNA levels in cortical tissue. In an earlier report we have shown that most of the root cells responding to inoculation with *R. fredii* 257 are cortical cells and their derivatives. Further, clear differences were noted in the *in vitro* translation products of mRNA, isolated from cortical cylinders, within 2.5 days after inoculation with 257 or 257B3 (Trese and Pueppke 1990). Consequently, we have followed the accumulation of transcripts homologous to pVuA and pVuB in total RNA isolated from such cylinders. Whereas the expression of the VuA homologous transcripts was essentially the same as seen in Figure 1A, differential induction of the VuB transcript can be seen within 1.5 days (Fig. 2).

Genomic organization. The four cDNA clones were labeled and used to probe cowpea genomic DNA that had been digested with *Eco*RI or *Hin*dIII (Fig. 3). The insert DNA from pVuB, pVuC, and pVuD strongly hybridized to a single band in each instance, indicating that there are probably single copies of these genes in the genome. The insert from pVuA, however, hybridized to two bands of *Eco*RI-digested, and three bands of *Hin*dIII-digested, genomic DNA.

Homology to GmENOD2. Under moderately stringent conditions the insert from pVuA hybridized to the pGmENOD2 subclone, 2pH2 (Fig. 4). No other clone had detectable homology to this probe. When the pGmENOD2 subclone and VuA were used as probes in duplicate northern blots of 6-day nodulating root RNA they both hybridized to an abundant 1,400-bp transcript, as well as two larger transcripts (Fig. 5).

DISCUSSION

Because many questions about the symbiotic relationship between legumes and rhizobia concern early stages in the interaction, we and others are interested in plant genes whose regulation is altered during nodule initiation. To date, studies of plant genes involved in nodulation have concentrated on proteins produced uniquely in the nodule, the nodulins (Vance *et al.* 1988; Verma and Delauney 1988). The majority of the 40 or so nodulins cloned from seven different genera of legumes (Delauney and Verma 1988) are induced concomitantly with, or after, leghemoglobin. These are referred to as the late nodulin class (Govers *et al.* 1987). Only a few of the cloned nodulins are expressed before, or during, bacterial release, and belong to the early nodulin class (Govers *et al.* 1987). In soybean, mRNA homologous to a cDNA clone, pGmENOD2, is induced after 6 days (van de Wiel *et al.* 1990), and nodulin N-40′ is induced after 8 days in pea (Govers *et al.* 1985). In alfalfa root nodules lacking infection threads and bacteroids, two other early nodulins, N-38 (Norris *et al.* 1988) and Nms-30 (Dickstein *et al.* 1988), are induced within 3 wk.

Nodule initiation and early development, events that occur before infection thread release, certainly require

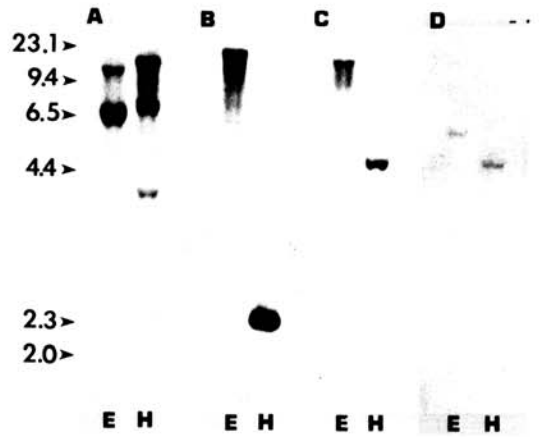

Fig. 3. Hybridization of four cDNA clones with restiction enzyme digested genomic cowpea DNA (10 μg/lane). Probes were prepared from inserts in recombinant plasmids **A**, pVA; **B**, pVuB; **C**, pVuC; **D**, pVuD. E = *Eco*RI; H = *Hin*dIII.

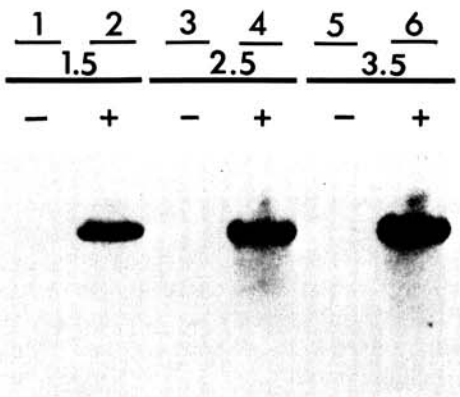

Fig. 2. Hybridization of VuB cDNA insert to total RNA isolated from selected cortical cylinders from inoculated cowpea roots. Each lane was loaded with 16 μg of RNA. DAI, days after inoculation; lanes 1, 3 and 5 (–) inoculated with the Nod⁻ mutant *Rhizobium fredii* 257B3, lanes 2, 4 and 6 (+) inoculated with *R. fredii* 257.

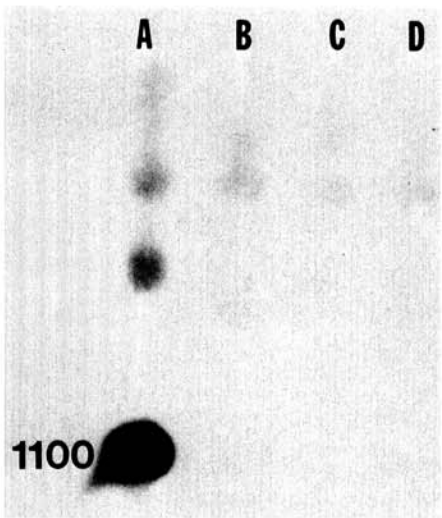

Fig. 4. Hybridization of pENOD2 subclone 2pH2 to *Eco*RI digested cDNA clones, under moderately stringent conditions. Lanes A–D, clones VuA, VuB, VuC, and VuD, respectively. Band at 1,100 bp is the insert of VuA. Each lane contained 0.2 μg of DNA.

active regulation of additional plant genes. The primary constraint on efforts to isolate such genes is the disperse nature of nodule initiation events. The proportion of host cells that respond to inoculation is relatively small, and any changes in their mRNA or protein pools is diluted by the greater volume of nonresponding tissue.

By combining a host-symbiont pair that produces up to six times as many nodules per taproot as soybean (Bhuvaneswari *et al.* 1988), a Nod^- mutant for control inoculations, and a system of careful tissue selection, we have previously demonstrated changes in plant gene expression within 2.5 days after inoculation of cowpea with *R. fredii* 257 (Trese and Pueppke 1990). Because the number of differentially expressed genes increased as nodule development progressed, we chose to construct a cDNA library from mRNA of nodulating roots harvested 6 days after inoculation. This library was screened for sequences that were either induced or repressed during nodule initiation. Four clones were selected, and the temporal regulation of their homologous transcripts has been examined. These clones have been identified simply as VuA, VuB, VuC, and VuD, where Vu represents *V. unguiculata*.

The cDNA insert of clone pVuA hybridizes to two transcripts in inoculated cowpea root RNA. The smaller, 1,400-bp transcript represents an early nodulin, induced during nodule initiation and expressed in nodules through 16 days after inoculation. A transcript of approximately the same size was detected in nodulating soybean roots at 6 days after inoculation, but not in control roots.

The pVuA clone has significant homology to the coding region of GmENOD2, an early nodulin induced in soybean nodules (van de Wiel *et al.* 1990). VuA and GmENOD2 both hybridize to the same mRNA transcripts in northern gels, suggesting that VuA is the cowpea homologue of GmENOD2. ENOD2-homologous genes have been described in pea (Franssen *et al.* 1987), vetch (Moerman *et al.* 1987), alfalfa (Dickstein *et al.* 1988), and *Sesbania rostrata* Brem. & Oberm. (Strittmatter *et al.* 1989). Expression of ENOD2, a proline-rich, putative cell wall protein, has been localized to the inner cortex (nodule parenchyma) of both developing and mature nodules of soybean and pea (van de Weil *et al.* 1990). Furthermore, it has been proposed that ENOD2 may be involved in restricting oxygen diffusion into the nodules. In cowpea, the homologous gene was first detected in nodulating root segments 3.5 days after inoculation, as dividing cortical cells begin to organize into a meristem and before bacteria are released from ramifying infection threads.

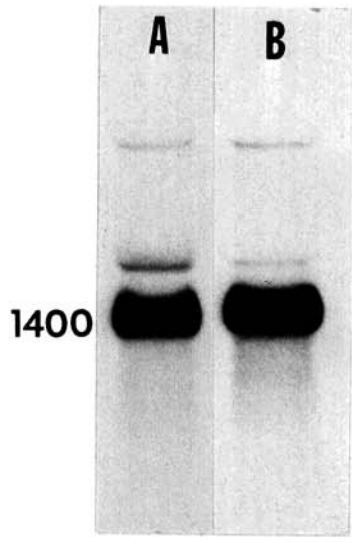

Fig. 5. Hybridization of total RNA from 6-day nodulating roots with **A,** coding region of GmENOD2 and, **B,** cDNA insert of pVuA. Each lane contains 9 μg of RNA. The 1,400-bp transcript represents the cowpea homologue of ENOD2.

The fact that VuA hybridizes to several genomic DNA fragments suggests that this is a member of a small gene family. A second homologous transcript, of 2,000 bp, is regulated independently from the ENOD2 homologue. This transcript may code for another cell wall associated protein. Hong *et al.* (1989) and Datta *et al.* (1989) have described the independent developmental regulation of several proline-rich cell wall proteins in soybeans, where expression was specific to tissue type and tissue age.

The VuB-homologous transcript is present only in nodulating cowpea roots, and not in roots exposed to 257B3, YEM, or Jensen's solution. Therefore, it represents a cowpea early nodulin. By selecting cortical tissue enriched for nodule initiation events we were able to demonstrate that this transcript is induced within 36 hr. At this time the nodule initiation centers consist of a small number of dividing cortical cells (unpublished observations). Thus, induction of this nodulin must result from some early signal(s) produced by nodulation competent *R. fredii* 257. In addition, the amount of transcript increased through 6 days, decreased considerably by 12 days, and was much reduced by 16 days. This pattern suggests that the gene product may be involved in cell division or infection thread formation, processes that are initiated and decline across a similar time sequence.

In molecular studies of nodulation there has been a tendency to focus on plant genes, the expression of which increases during development of the symbiosis. However, repression of some host genes may be equally important. In particular, cortical cell dedifferentiation and induction of meristematic growth may require localized gene repression in the root cortex. Genes involved in secondary wall synthesis, or lignification, are possible examples of such host genes. In the first screen of our library we found that roughly half of the differential clones were expressed more abundantly in the 257B3-inoculated roots. Two such clones were used to examine relative reductions in gene expression at early stages of nodule initiation.

The VuC-homologous transcript represents a gene that is strongly repressed in nodulating roots, relative to 257B3 inoculated control roots. Differential expression is greatest at 2.5 days, and then declines through 3.5 and 6 days. The fact that nodulation dramatically reduces expression of this transcript suggests a widespread suppression throughout the root tissue, rather than highly localized effects at nodule initiation sites. The VuD clone represents a gene that is moderately repressed in nodulating roots at 2.5 and 3.5 days. However, this may reflect substantive gene regulation, localized to sites of nodule initiation. *In situ* hybridization studies with these clones will help to clarify their spatial regulation during nodule initiation.

Three of the four clones described here, VuB, VuC, and VuD, are differentially expressed most dramatically during early nodule development. This suggests that nodule initiation may represent a discrete stage in the symbiosis, requiring rapid regulation of plant genes not involved in later stages of nodule development and function. It should now be possible to define the roles these genes might play in nodule initiation and their regulatory controls. Additionally, these clones should provide molecular markers to further study the role of bacterial nod genes in initiating host responses.

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