Dg93, a Nodule-Abundant mRNA of *Datisca glomerata* with Homology to a Soybean Early Nodulin Gene[1]

Patricia A. Okubara[2], Nancy A. Fujishige, Ann M. Hirsch, and Alison M. Berry*

Department of Environmental Horticulture, University of California, Davis, California 95616 (P.A.O., A.M.B.); and Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, California 90095–1606 (N.A.F., A.M.H.)

We have isolated a 590-bp full-length cDNA clone designated *Dg93*, an mRNA that is highly expressed in symbiotic root nodules of the actinorhizal host *Datisca glomerata*. *Dg93* mRNA encodes a deduced polypeptide of 105 amino acids with significant identity (74%) to the soybean (*Glycine max*) early nodulin (ENOD) gene *GmENOD93* (Kouchi and Hata, 1993). *Dg93* mRNA is abundant in nodules at 4 weeks post inoculation, the earliest time assayed, and steady-state mRNA levels remain elevated 11 weeks after inoculation. Spatial patterns of *Dg93* mRNA expression are complex, with transcript accumulation in the nodule lobe meristem, early infection zone, periderm, and cells of the vascular cylinder, but not in the surrounding uninfected cortical cells. *Dg93* is encoded by a small gene family in *D. glomerata*. To our knowledge, this is the first report of a gene from an actinorhizal host that is expressed in the nodule meristem and that shares sequence homology with an early nodulin gene from a legume.

Organogenesis of symbiotic root nodules involves the induction of multiple developmental processes that are initiated by a host-endosymbiont interaction and that result in a functional exchange of nitrogen and carbon that supports both host and endosymbiont. Both legume and actinorhizal hosts share common aspects of nodule development, including the infection process and nitrogen assimilatory pathways (for review, see Pawlowski and Bisseling, 1996; Hirsch and LaRue, 1997; Pawlowski, 1997). Likewise, molecular components of nitrogen assimilation and oxygen partitioning, such as Gln synthetase, nitrogenase, and leghemoglobin, are synthesized in both legume and actinorhizal nodules.

Datisca glomerata nodules are modified lateral roots, indeterminate, generally multi-lobed, with a periderm four to eight cells thick (Benson and Silvester, 1993). Each nodule lobe contains an infected area, visible as a crescent covering about two-thirds of the nodule when viewed in transverse section, and a surrounding uninfected region. The nodule is traversed by a central vascular cylinder, which is enclosed by a multi-layered pericycle. Eventually, the nodule meristem grows out to form an ageotropic nodule root.

The expression of early nodulin (ENOD) genes has been well characterized in several legume species. Based on their biochemical attributes and expression patterns, they are postulated to have roles in cell structure (see Franssen et al., 1992; Küster et al., 1995; Greene et al., 1998), in the control of nodule ontogeny by the degradation of Nod factor (Goormachtig et al., 1998a), and in carbon metabolism (Coba de la Peña et al., 1997). Some ENODs are proposed to be involved in oxygen partitioning (van de Wiel et al., 1990), but the findings of Wycoff et al. (1998) suggest that ENOD2 is not a part of the oxygen exclusion system. The induction of *ENOD40* (Fang and Hirsch, 1998), *ENOD2* (Silver et al., 1996; Goormachtig et al., 1998b), *ENOD12* (Bauer et al., 1996), carbonic anhydrase (Coba de la Peña et al., 1997), and other *ENOD* genes by Nod factor or cytokinin indicates a hierarchy of action in nodule organogenesis. In general, *ENOD* genes exhibit complex spatial and temporal expression patterns, indicating that they are required at various times and in various cell types throughout nodule development.

In actinorhizal hosts, genes associated with nodule morphogenesis have been primarily expressed in mature nodules (e.g. Pawlowski et al., 1993; Ribeiro et al., 1995; Gherbi et al., 1997; Franche et al., 1998; Jacobsen-Lyon et al., 1995). One *Alnus* gene of unknown function is specifically expressed in cells of the infected region and the pericycle (Pawlowski, 1997). There remains a paucity of information on genes expressed during the early stages of nodule development. We present evidence for the isolation and characterization of the first actinorhizal early nodulin.

MATERIALS AND METHODS

Plant Material

Datisca glomerata plants were grown in the greenhouse and inoculated with crushed, *Frankia*-infected nodules of *Ceanothus griseus*, as described previously (Liu and Berry, 1991). For RNA analyses, young leaves, developing fruits containing immature seeds, flowers (sepals, anthers, stamens, and styles), roots, and nodules at 4, 5, 7, and 11 weeks after inoculation were harvested into liquid nitrogen. For fixation, nodules were collected in water at 1 and 6 months post inoculation.

cDNA Isolation and RNA Analysis

Dg93 was originally identified as a chimeric nucleotide sequence adjoined to a full-length cDNA clone of Rubisco activase (P.A. Okubara and A.M. Berry, unpublished data). The chimeric clone had been obtained from a Lambda Zap II (Stratagene, La Jolla, CA) cDNA library representing mRNAs of *D. glomerata* nodules harvested 4, 5, and 7 weeks after inoculation (P.A. Okubara and A.M. Berry, unpublished data). To obtain plasmids containing only *Dg93* cDNA, 8×10^4 unamplified recombinant clones from the cDNA library were screened with a *Dg93*-specific probe obtained from the chimeric cDNA clone following digestion with *Eco*RI (Bethesda Research Laboratory, Gaithersburg, MD) and *Bsp*106I (Stratagene). One resulting *Dg93* clone was selected for all subsequent work.

Analysis of RNA expression in northern blots was carried out essentially as described in Pawlowski et al. (1994). *Dg93* cDNA was excised from the Bluescript phagemid vector and radiolabeled by incorporation of [α-^{32}P]dCTP (Amersham, Arlington Heights, IL) using the Multiprime DNA Labeling System (Amersham) to specific activities of 1.8 to 3.7×10^4 Bq μg^{-1} ($1–2 \times 10^6$ cpm ng^{-1}). Radiolabel was quantified from freshly washed nylon membranes using a phosphor imager (Storm, Molecular Dynamics, Sunnyvale, CA) and imaging plate (BAS III, Fuji, Tokyo), and analyzed with imaging software (Image QuanNT, version 4.00, Molecular Dynamics).

DNA Isolation and Southern-Blot Analysis

Total DNA was obtained from young leaves of *D. glomerata* as described in Ribeiro et al. (1995). DNA was transferred to Zeta Probe nylon membranes (Bio-Rad, Hercules, CA) following treatment with *Hin*dIII or *Eco*RI restriction enzymes (Sambrook et al., 1989). Hybridization of blots was carried out as recommended by the manufacturer, using the probes described below.

In Situ Hybridization

One- and 6-month-old nodules were fixed in 14.3% (w/v) paraformaldehyde, 0.25% (w/v) glutaraldehyde in phosphate buffer, rinsed in buffer, dehydrated, and stored in 80% (v/v) ethanol at $-20°C$. Fixed nodules were shipped on ice prior to dehydration in the *tert*-butyl alcohol series and Paraplast embedding medium (McKhann and Hirsch, 1993). Sections were cut at 10 μm and mounted with water onto Vector bond-treated slides (Vector Labs, Burlingame, CA). The in situ hybridization protocol of McKhann and Hirsch (1993) was carried out except that the slides were pretreated at 37°C with increased amounts of proteinase K (5 μg mL^{-1}) and acetic anhydride (0.5%, w/v). *Dg93* cDNA was used to generate antisense and sense riboprobes labeled with digoxigenin-11-UTP (Boehringer Mannheim, Indianapolis). Color development was carried out for 12 to 16 h. The slides were not counterstained. Photographs were taken with Ektachrome Tungsten 160 slide film (Eastman Kodak, Rochester, NY) on a microscope (Axiophot, Carl Zeiss, Thornwood, NY). The slides were scanned into a computer and processed with Photoshop software (Adobe Systems, San Jose, CA).

Computerized Sequence Analyses

Nucleotide and deduced amino acid sequences of *Dg93* were compared to GenBank database entries using BLASTN and BLASTX algorithms, respectively (Altschul et al., 1997). Amino acid sequence alignments were carried out with ClustalW 1.7 (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html) and the Shading Utility program of GeneDoc (http://www.cris.com/~ketchup/genedoc.html, and refs. therein). Nucleotide and amino acid sequence identity values were obtained using Align (http://dot.imgen.bcm.tmc.edu:9331/seq-search/alignment.html). Protein folding was examined on the Baylor College of Medicine Protein Secondary Structure Prediction website (http://dot.imgen.bcm.tmc.edu:9331/pssprediction/pssp.html, and refs. therein). Protein motifs were analyzed using the Baylor College of Medicine PSITE and NNPREDICT programs (http://www.cmpharm.ucsf.edu/~nomi/nnpredict, and refs. therein). Consensus signal sequences were analyzed using PSORT (http://psort.nibb.ac.jp, and refs. therein).

RESULTS

Molecular Characterization of *Dg93*

As a part of our interest in the molecular basis of organogenesis of actinorhizal nodules, we have isolated a highly expressed, nodule-specific cDNA (*Dg93*) from symbiotic root nodules of *D. glomerata*. Using a probe obtained from a chimeric *Dg93*-Rubisco activase clone, we screened 8×10^4 unamplified recombinant phage and identified about 50 clones hybridizing to the *Dg93* probe. The retrieval frequency of one clone in 1.6×10^3 indicated that *Dg93* was abundant in our cDNA library.

One strongly hybridizing *Hin*dIII fragment of 1.65 kb and one strongly hybridizing *Eco*RIII fragment of 5.0 kb were detected in a Southern blot of total *D. glomerata* DNA hybridized to the *Dg93* cDNA (Fig. 1). One weakly hybridizing *Hin*dIII fragment and one weakly hybridizing *Eco*RI fragment were also observed in the autoradiograph. The sequence of the *Dg93* cDNA does not contain a *Hin*dIII or an *Eco*RI restriction site. Therefore, our data indicate the presence of one or two copies of *Dg93* in the *D. glomerata* genome.

In database searches using the BLASTN and BLASTX algorithms (Altschul et al., 1997), *Dg93* was highly similar (P[N] = 1.3×10^{-35} for amino acid sequences) to a soybean ENOD, *GmENOD93* (Kouchi and Hata, 1993). A nucleotide sequence comparison yielded 83% identity. Deduced polypeptides of both genes were 105 amino acids in length and shared 74% identity (Fig. 2). Dg93 also showed 44% and 40% amino acid sequence identity to polypeptides encoded by two rice cDNA clones, *OsENOD93a* and *OsENOD93b*, respectively (Reddy et al., 1998). Twenty-two residues (21%) of the putative Dg93 polypeptide were Ala, rendering it particularly rich in this amino acid. Protein-folding algorithms predicted that the Dg93 polypeptide is
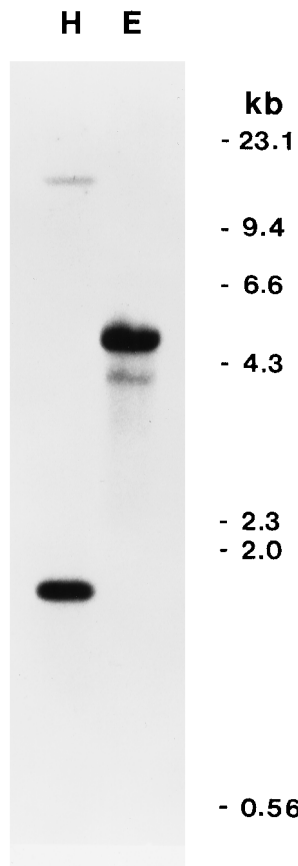
Figure 1. Southern blot of total DNA of *D. glomerata* treated with *Hin*dIII (H) or *Eco*RI (E) and hybridized to radiolabeled *Dg93* cDNA probe. Autoradiography was carried out at −80°C for 5 d.

comprised mainly of α-helical secondary structure, with putative sites for *N*-glycosylation, protein kinase phosphorylation, and myristoylation.

Dg93 mRNA was abundant in nodules at 4 to 11 weeks after *Frankia* inoculation, but transcripts were not detectable in leaves, flowers, developing fruits, or roots of *D. glomerata* (Fig. 3). Thus, *Dg93* appears to be nodule specific. Quantitation of radiolabel on freshly washed nylon membranes indicated that the steady-state levels of mRNA in the nodule remained constant over the 7-week sampling period (data not shown).

Spatial Patterns of *Dg93* Transcript Expression

To localize *Dg93* transcripts within the *D. glomerata* nodule, we examined nodules harvested 1 and 6 months after *Frankia* inoculation. The expression pattern of *Dg93* message, indicated by the dark blue color of precipitated digoxigenin conjugate, was complex. At 1 and 6 months after inoculation, *Dg93* transcripts were expressed in the nodule lobe meristem, closely associated with meristematic cells destined to form the vascular cylinder (Fig. 4, A, C, and E). *Dg93* transcripts were also detected in cells within the phloem tissue, distal to the apical meristem (Fig. 4, A, B, and E). At 6 months post inoculation, a lower level of *Dg93* expression was also observed in the multi-layered
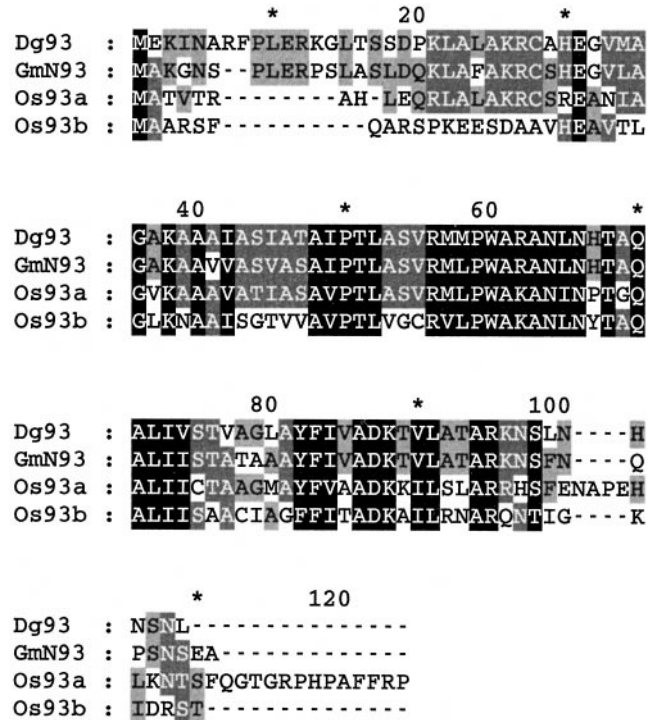

Figure 2. Comparison of the deduced amino acid sequences of *Dg93*, GmENOD93 (GmN93; Kouchi and Hata, 1993), and two rice homologs (Os93a and Os93b; Reddy et al., 1998) obtained from analysis of a ClustalW alignment with the shading program of GeneDoc. The black-boxed portions indicate identical or conserved amino acids found in all four sequences; lighter shadings indicate conservation among three or fewer sequences.

pericycle (Fig. 4B). Cytoplasm-containing cells of the periderm also accumulated *Dg93* transcripts, whereas the uninfected, starch-filled cortical cells surrounding the infection zone were completely devoid of *Dg93* message (Fig. 4, C and D).

In the *Frankia*-infected zones of the nodule at 6 months post inoculation, we observed differential accumulation of blue color in bands across the infected region, as shown in
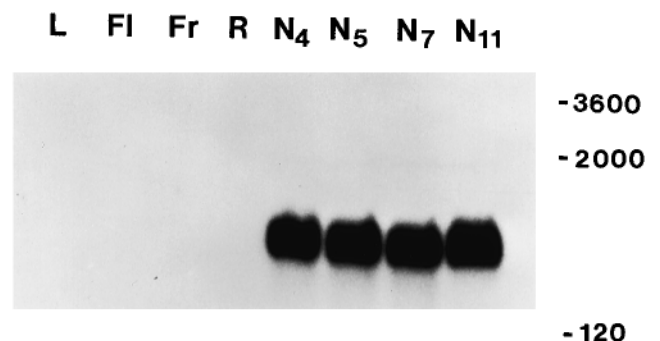

Figure 3. Expression of *Dg93* mRNA in various organs of *D. glomerata*. Total RNA samples (5 μg per lane) from young leaves (L), flowers (Fl), developing fruits (Fr), roots (R), and nodules harvested 4 (N_4), 5 (N_5), 7 (N_7), and 11 (N_{11}) weeks after inoculation were hybridized to radiolabeled *Dg93* cDNA in a northern blot. Autoradiography was conducted at 25°C for 21.5 h.
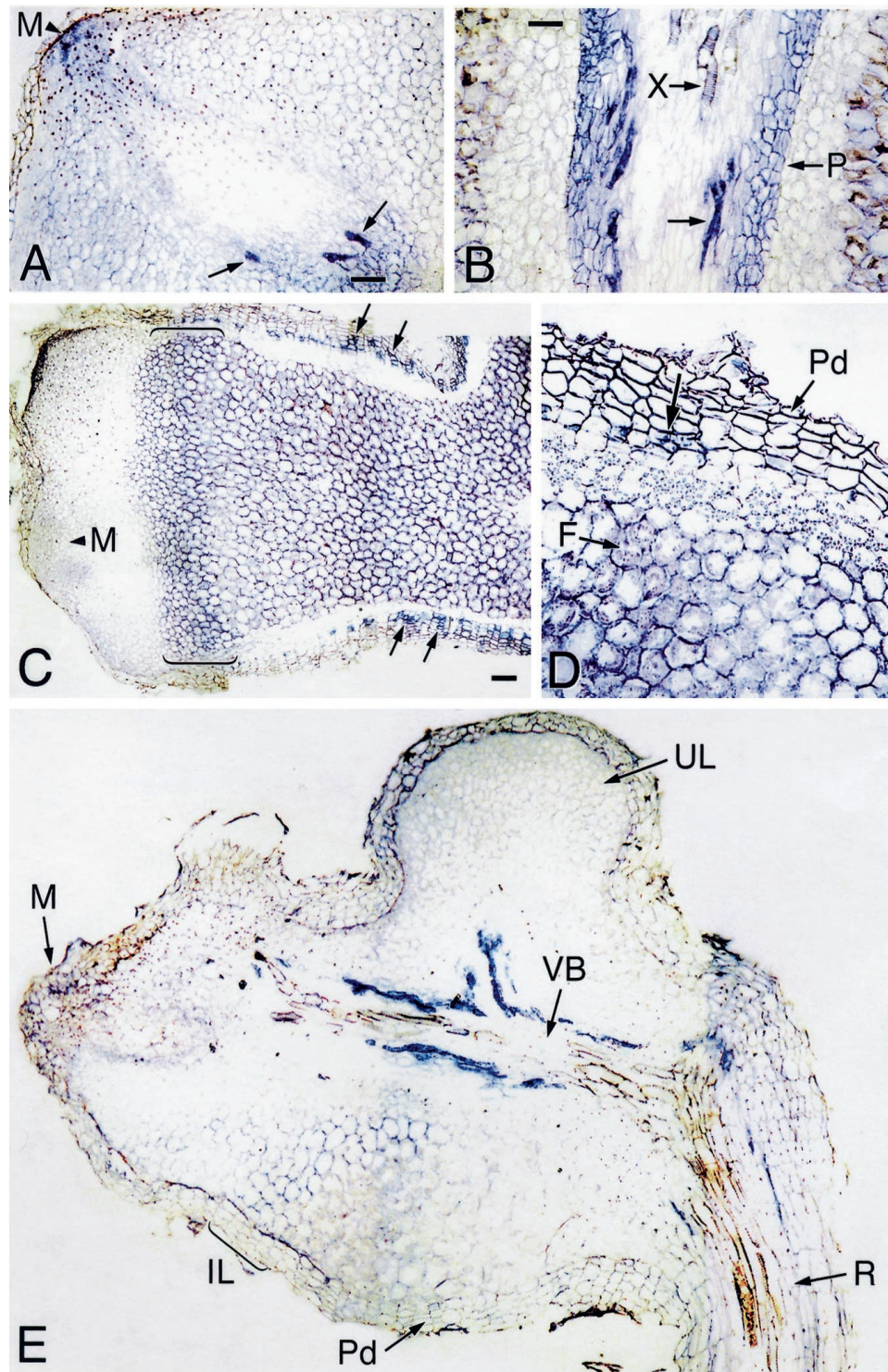
Figure 4. In situ localization of *Dg93* transcripts in longitudinal sections of *D. glomerata* nodules harvested 6 months (A–D) and 1 month (E) after inoculation with *Frankia*. A, Dark blue color indicating *Dg93* gene expression is present in the nodule apical meristem (M) and is associated with the vascular cylinder. Arrows indicate cells in the phloem that contain *Dg93* transcripts. Bar = 20 μm. B, Enlargement of the infected-cell region distal to the apical meristem. *Dg93* transcripts are present in the phloem tissue (arrow) and in the multi-layered pericycle (P). X, Xylem vessel. Bar = 20 μm. C, A mature lobe containing infected cells. *Dg93* transcripts are found in the meristem (M), in the periderm (arrows), and in the infected central region of the nodule. Brackets indicate a region of the infection zone where *Dg93* transcripts accumulate to a high level. The infected tissue appears banded due to varying levels of *Dg93* gene expression. Bar = 40 μm. D, Enlargement of the distal
(Legend continues on facing page.)

Figure 4C. *Dg93* transcripts accumulated to high levels in a band of cells proximal to the nodule meristem, some of which contained visible *Frankia* (Fig. 4, C and D). A region of less-intense blue can be seen below the first zone (Fig. 4, C and D). The peripheral, doughnut-shaped configuration of *Frankia* within cells of this second zone is typical of mature, vesicle-containing tissue in *D. glomerata* (Benson and Silvester, 1993; Okubara et al., 1999). Below this region, further into the mature-infected zone, digoxigenin staining increased in intensity again.

In nodules at 1 month post-inoculation, as shown in Figure 4E, *Dg93* mRNA was detected in the nodule meristem (M) and in a band of early-infected cells (IL), but not in the mature, vesicle-containing tissue just basal to this zone. As in the 6-month post-inoculation nodules, blue color indicating the presence of *Dg93* mRNA was especially prevalent in the phloem, and was also observed in the periderm (Pd). Very few transcripts were found in the cells of the pericycle at this stage. The uninfected region of this nodule lobe was devoid of *Dg93* transcripts except in the innermost layer of the periderm.

Nodule sections incubated with the sense *Dg93* transcript showed no detectable color (data not shown).

DISCUSSION

Dg93 has several characteristics in common with *GmENOD93*, a highly homologous gene from soybean (Kouchi and Hata, 1993). Both mRNAs are about 600 nt in length, produce a putative polypeptide of 105 amino acids and are coded by single-gene or small gene families. Like *GmENOD93* (and the rice homologs), the deduced amino acid sequence of *Dg93* is Ala rich, although its Ser content is not as high as its soybean counterpart. Both mRNAs are abundant in nodules but not detectable in other organs of the host plant, including leaves and roots. These shared features suggest that *Dg93* is an actinorhizal homolog of *GmENOD93*.

The spatial expression pattern of *Dg93* in *D. glomerata* nodules may be compared to that of *GmENOD93* in soybean nodules. In the early stages of soybean nodule development, *GmENOD93* is expressed in dividing cells or in the nodule meristem. In mature nodules, *GmENOD93* is only expressed in infected cells (Kouchi and Hata, 1993). In *D. glomerata*, *Dg93* expression is first observed in the nodule lobe meristem and in cells undergoing *Frankia* infection, then is detectable to a lesser degree in mature *Frankia*-containing cells. In contrast to *GmENOD93*, *Dg93* transcripts accumulate to higher levels in the young infected cells, in cells of the vascular cylinder, and in cytoplasm-

containing periderm cells. This pattern of expression more closely resembles that of ENOD2 in *Sesbania rostrata* nodules (Goormachtig et al., 1998b), and of ENOD40 in soybean nodules (Yang et al., 1993).

In soybean, *GmENOD93* transcripts were detectable as early as 3 d following incubation with *Bradyrhizobium*, and mRNA levels were examined up to 17 d post inoculation. Our earliest assay point was approximately 4 weeks post inoculation, or 1 week after formation of visible nodules on *D. glomerata* roots. The presence of an early nodulin transcript in mature, 11-week-old *D. glomerata* nodules can be attributed in part to the indeterminate nature of these nodules, in which an active meristem is maintained, and a developmental gradient of infection is present over a period of months following initial infection by *Frankia*. Since *Dg93* transcripts also accumulated in tissues of mature nodules, the term "early nodulin" does not entirely accurately describe this gene.

Computerized secondary protein structure analyses predict one major, Ala-rich α-helix of 28 amino acids (MKLA-LAKRCAHEGVMAGAKAAAIASIATAI), four phosphorylation sites, three *N*-myristoylation sites, and targeting to the endoplasmic reticulum membrane. Dg93 lacks an apparent N-terminal signal sequence and other peptide motifs of known function. The high Ala content of the deduced polypeptide is expected to confer hydrophobicity, a characteristic that might have a role in protein aggregation or membrane protection. The Alas are generally distributed throughout the Dg93 polypeptide, unlike the PASSA repeat motif found in Ser/Ala-rich structural proteins from lower eukaryotes (described by Goldman et al. [1994]). Ala richness is also a characteristic of some cold-regulated genes, including KIN1 (Kurkela and Franck, 1990) and *cor6.6* (Gilmour et al., 1992) from Arabidopsis and *Wcs19* (Chauvin et al., 1993) from wheat.

Until now, actinorhizal homologs of legume early nodulins have not been found, possibly due to differences in the biochemical nature of the host-microbe interface (Pawlowski, 1997). The actinorhizal nodule arises from cell divisions of the pericycle and, although the prenodule formed in many actinorhizal roots originates from cortical cells, these nodules are clearly modified roots (for review, see Berry and Sunell, 1990). The legume nodule originates in cortical tissues and does not bear obvious resemblance to lateral roots (for review, see Hirsch and LaRue, 1997). Differences in the developmental origins and cellular architecture of the two nodule types might account for the apparent lack of actinorhizal ENODs. For example, ENOD2 is expressed in the legume nodule parenchyma (van de Wiel et al., 1990; Goormachtig et al., 1998b) for which there

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Figure 4. (Legend continued from facing page.)
part of the nodule. The *Dg93* transcripts seen in the band of young infected cells in brackets in C is to the right of this micrograph. Transcripts are also seen in the inner cells (arrow) of the periderm (Pd) and in the host cells surrounding the *Frankia* (F). Magnification same as B. E, Off-median section in which *Dg93* gene expression is most evident in the central vascular bundle (VB). Some blue color is apparent in the meristem (M) and in the early-infected region of the nodule lobe (IL), but not in mature infected cells, where the host cytoplasm containing *Frankia* surrounds a small central vacuole in a doughnut shape. The innermost cells of the periderm (Pd) are also labeled. Uninfected regions of the nodule lobe (UL) and parent root (R) cortex show no detectable *Dg93* expression. Magnification same as C.

is no cellular equivalent in the actinorhizal nodule. Alternatively, biological function might be based on overall protein structure or conformation rather than on specific amino acid sequences. For example, *ENOD40* genes from soybean and alfalfa share 79% nucleotide sequence homology (Asad et al., 1994). At the amino acid level, however, the degree of homology between the putative homologs is significantly lower (39%). Likewise, the *ENOD12* gene from *Medicago truncatula* shares 65% nucleotide sequence identity with *ENOD12B* from pea, yet the amino acid sequences are 50% identical. It is also possible that nucleotide sequence divergence among legume and actinorhizal functional homologs might reflect the significant evolutionary distance between the legume family and the various actinorhizal groups (Soltis et al., 1995), and might account for the lack of detection of homologs in hybridization experiments.

As in the case of the hemoglobin genes from *Casuarina* (Jacobsen-Lyon et al., 1995), the nodule-expressed *Dg93* might be a member of a family of divergent genes. The presence of a weakly hybridizing fragment in our genomic Southern blot raises the possibility that a divergent family member is expressed in non-nodule tissues and is not detectable in our stringent northern blots. Alternatively, *Dg93* might be expressed in non-symbiotic tissues of *D. glomerata*, but at levels below the threshold of detection in our northern blots.

ENOD-like mRNAs are expressed in organs and tissues of non-nodulating plants. The presence of *OsENOD93a* mRNA in dividing cultured rice cells suggests that some *ENOD*-like genes are involved in cell division or dedifferentiation (Reddy et al., 1998). *OsENOD93b* mRNA was most abundant in rice roots, but was also detected at reduced levels in etiolated and green leaves. In legumes, *ENOD12* is also expressed in the stem and flower (Govers et al., 1991). We noted that the Dg93 deduced polypeptide shares identity with small polypeptides encoded by mRNAs expressed in tomato ovaries (58% identity, accession no. AI487889), maize endosperm (50% identity, accession no. AI664823), tomato shoots (44% identity, accession no. AI491189), pepper leaves (44% identity, accession no. AA840637), and cotton fibers (44% identity, accession no. AW108647). Comparing the most conserved 77 amino acids, identity to Dg93 was as high as 73% for the maize polypeptide, and 55% to 58% for polypeptides from the other species. Reddy et al. (1998) postulate that early nodulin genes have a general role in the development of multiple organs of both nodulating and non-nodulating plants, and that these genes have been recruited for nodule organogenesis in the legumes. The expression of *Dg93*-like mRNAs in numerous organs of diverse plant species is consistent with this hypothesis.

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