Localization of a Nod Factor-Binding Protein in Legume Roots and Factors Influencing Its Distribution and Expression[1]

Gurpreet Kalsi and Marilynn E. Etzler*

Section of Molecular and Cellular Biology, University of California, Davis, California 95616

The roots of the legume *Dolichos biflorus* contain a lectin/nucleotide phosphohydrolase (Db-LNP) that binds to the Nod factor signals produced by rhizobia that nodulate this plant. In this study we show that Db-LNP is differentially distributed along the surface of the root axis in a pattern that correlates with the zone of nodulation of the root. Db-LNP is present on the surface of young and emerging root hairs and redistributes to the tips of the root hairs in response to treatment of the roots with a rhizobial symbiont or with a carbohydrate ligand. This redistribution does not occur in response to a non-symbiotic rhizobial strain or a root pathogen. Db-LNP is also present in the root pericycle where its level decreases upon initiation of nodule formation. Maximum levels of Db-LNP are found in 2-d-old roots, and the expression of this root protein is increased when the plants are grown in the absence of NO_3^- and NH_4^+. These results support the possibility that Db-LNP is involved in the initiation of the *Rhizobium* legume symbiosis.

The establishment of the nitrogen-fixing symbiosis between rhizobia and legumes is a multistep process that involves the differentiation of root cortical cells to form a new organ (called the root nodule), the adhesion of the rhizobia to root hairs, and the subsequent internalization and transport of the rhizobia to the root nodule along infection threads produced by the plant (for reviews see Mylona et al., 1995; Schultze and Kondorosi, 1998). Lipochitooligosaccharides (called Nod factors) produced by the rhizobia function as signals in the initiation of the plant responses that lead to nodule formation and rhizobial entry (for reviews see Dénarié et al., 1996; Long, 1996). Differences among Nod factors produced by various rhizobial strains and the abilities of different leguminous species to perceive these signals confer a host-strain specificity on this symbiosis (Dénarié et al., 1996).

Within a few minutes after application of picomolar amounts of purified Nod factors to the roots of an appropriate legume species a number of plant responses are elicited, including plasma membrane depolarization (Ehrhardt et al., 1992; Felle et al., 1995), fluctuations in concentration of intracellular free calcium in root hair cells (Ehrhardt et al., 1996; Gehring et al., 1997), and rearrangements of the cytoskeleton (Cardenas et al., 1998; Timmers et al., 1998). Within several hours these changes are followed by root hair deformation (Heidstra et al., 1994), cortical cell dedifferentiation and mitosis (Spaink et al., 1991; Truchet et al., 1991; Relic et al., 1993), and the expression of genes such as *ENOD12* (Bauer et al., 1994; Journet et al., 1994) and *rip1* (Cook et al., 1995) in the differentiating root epidermis. These findings have implied the existence of Nod factor receptors on the roots of the plant and the presence of a signal transduction mechanism. Recent studies with pharmacological agents have suggested that this mechanism might be mediated by G-proteins and coupled to the activation of a phosphoinositide and calcium second messenger pathway (Pingret et al., 1998).

Although extensive studies have been conducted on a number of Nod factors and the *nod* genes that encode the enzymes involved in their synthesis (Carlson et al., 1995; Dénarié et al., 1996; Long, 1996), little is known about the ability of the plant to perceive these signals. Several studies suggest the existence of multiple receptors that differ from one another in stringency of recognition and may trigger different response pathways (Ardourel et al., 1994; Felle et al., 1996; Minami et al., 1996a). Both high- and low-affinity Nod factor-binding sites have been identified in plasma membrane-enriched fractions from legume roots and cell cultures (Bono et al., 1995; Niebel et al., 1997, 1999; Gressent et al., 1999).

Recent studies in our laboratory have shown that a lectin we previously isolated from the roots of the legume *Dolichos biflorus* (Quinn and Etzler, 1987) is a Nod factor-binding protein and is also an enzyme that catalyzes the hydrolysis of phosphoanhydride bonds of nucleoside diphosphates and triphosphates (Etzler et al., 1999). The amino acid sequence of this lectin has no significant homology to any lectin sequence reported to date, and the properties of this protein show that it clearly represents a completely

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* Corresponding author; e-mail meetzler@ucdavis.edu; fax 530–752–3085.

different category of lectin. We have renamed this lectin Db-LNP to reflect its lectin and nucleotide phosphohydrolase activity (Etzler et al., 1999) and recently found that homologs of this new category of LNP lectins exist in other legumes (Roberts et al., 1999).

Db-LNP is present on the surface of the root hairs and treatment of *D. biflorus* roots with antiserum against Db-LNP was found to inhibit root hair deformation and nodule formation upon exposure to symbiotic rhizobia (Etzler et al., 1999). These properties suggest that Db-LNP might play a role, perhaps as a receptor, in the initiation of the rhizobium-legume symbiosis. Such a possibility is supported by the present study in which we further examine the localization of this lectin in the roots and the factors that affect its distribution and expression.

RESULTS

Differential Distribution of Db-LNP along the Surface of the Root Axis

Previous experiments established that Db-LNP is present on the surface of the root hairs and other epidermal cells of young roots (Etzler et al., 1999). The surface distribution of this lectin along the entire root axis was studied by immunofluorescence confocal microscopy of whole mounts of 6-d-old *D. biflorus* roots that had been fixed prior to staining. Db-LNP is particularly prominent on the surface of the newly emerging (Fig. 1F) and young (Fig. 1D) root hairs and is also present on the surface of epidermal cells in these regions of the root. The level of this surface protein diminishes along the root axis from the region of young root hairs to the zone of root hair maturation. No fluorescence was detected on the surface of root hairs or epidermal cells in the mature root hair zone (Fig. 1B) nor was it detected in the zone at the tip of the roots that is devoid of root hairs (Fig. 1H). This differential pattern of Db-LNP detection on the surface of the fixed root whole mounts cannot be attributed to differences in accessibility of surface Db-LNP to the antibodies due to variations in cell wall deposition because the same variations in root surface Db-LNP are found in immunohistochemical comparisons of transverse sections through different regions of the root (data not shown). The decrease in level of surface Db-LNP with root hair maturation correlates with the decreased susceptibility to nodulation by rhizobia that has been found to occur as root hairs mature (Bhuvaneswari et al., 1981).

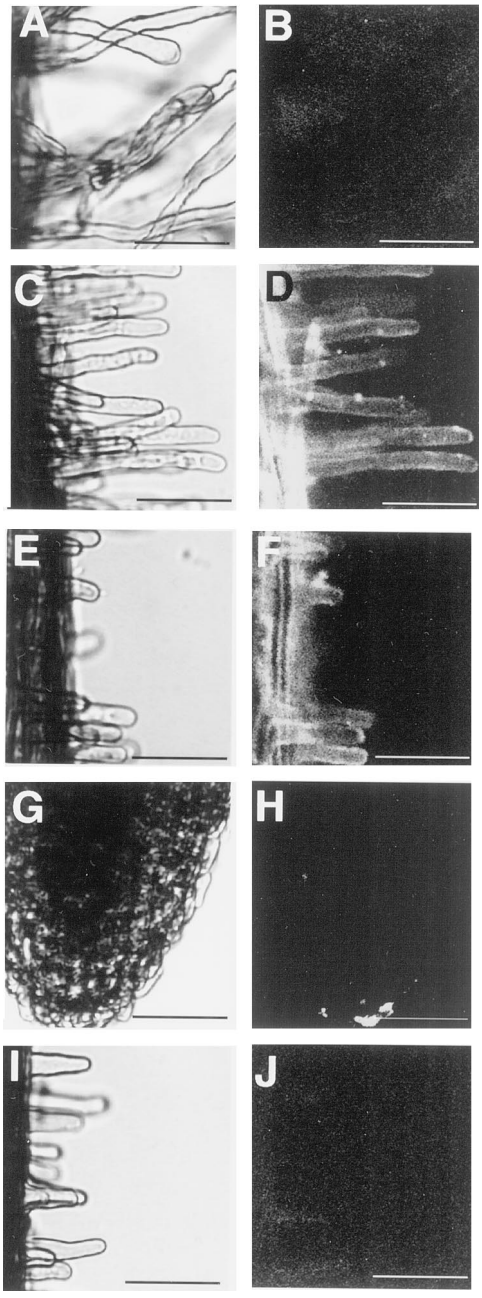

Figure 1. Differential distribution of Db-LNP along the surface of the root axis. Fixed whole mounts of 6-d-old *D. biflorus* roots were treated with antiserum prepared against recombinant Db-LNP (A–H) or with preimmunization serum (I and J) and viewed by bright field (A, C, E, G, and I) and immunofluorescence confocal microscopy (B, D, F, H, and J). Each image is a composite of five optical sections. A and B, Mature root hair region 18 mm from the root tip. C and D, Region containing young root hairs 6 mm from the root tip. E, F, I, and J, Emerging root hair region 3 mm from the root tip. G and H, Root tip. Scale bars = 60 μm.

Db-LNP Is Also Present in the Root Pericycle

Initial attempts using immunofluorescence microscopy to determine if Db-LNP is associated with cells in the root interior were inconclusive due to nonspecific fluorescence associated with the vascular tissue of the root. We therefore investigated transverse sections of *D. biflorus* roots utilizing an immunohistochemical approach, employing colloidal gold-conjugated secondary antibodies. In addition to confirming the finding that the epidermal surface

localization of Db-LNP is confined to the regions of newly emerging and young root hairs, this study showed that this lectin is also present in pericycle cells and perhaps an occasional parenchyma cell in the region opposite the xylem poles in the emerging, young, and mature root hair zones (Fig. 2, B and D). The pericycle cells were distinguished from the endodermis by counterstaining the Casperian strips of endodermal cells with Sudan IV (data not shown). Whether Db-LNP is present on the inside or outside of the pericycle cell membrane cannot be resolved using the conditions employed in this study.

A comparison of immunoblots of extracts of the young and emerging root hair region and mature root hair region of 6-d-old roots showed that the Db-LNP extracted from the mature root hair zone has the same electrophoretic mobility as the 46-kD lectin extracted from the young and emerging root hair region as well as Db-LNP extracted from isolated root hairs (Fig. 3E). Because Db-LNP in the mature root hair zone is found only in the pericycle, these data suggest that the pericycle Db-LNP might be identical to the Db-LNP on the root surface.

Root Surface Db-LNP Is a Peripheral Membrane Protein

The localization of Db-LNP on the root hair and epidermal surface in the young and emerging root hair regions of the root and its presence in an isolated root hair preparation suggested that this protein is associated with the membranes or cell walls of these cells. Db-LNP was found in microsomal fractions of 6-d-old roots, and the lectin was released from this

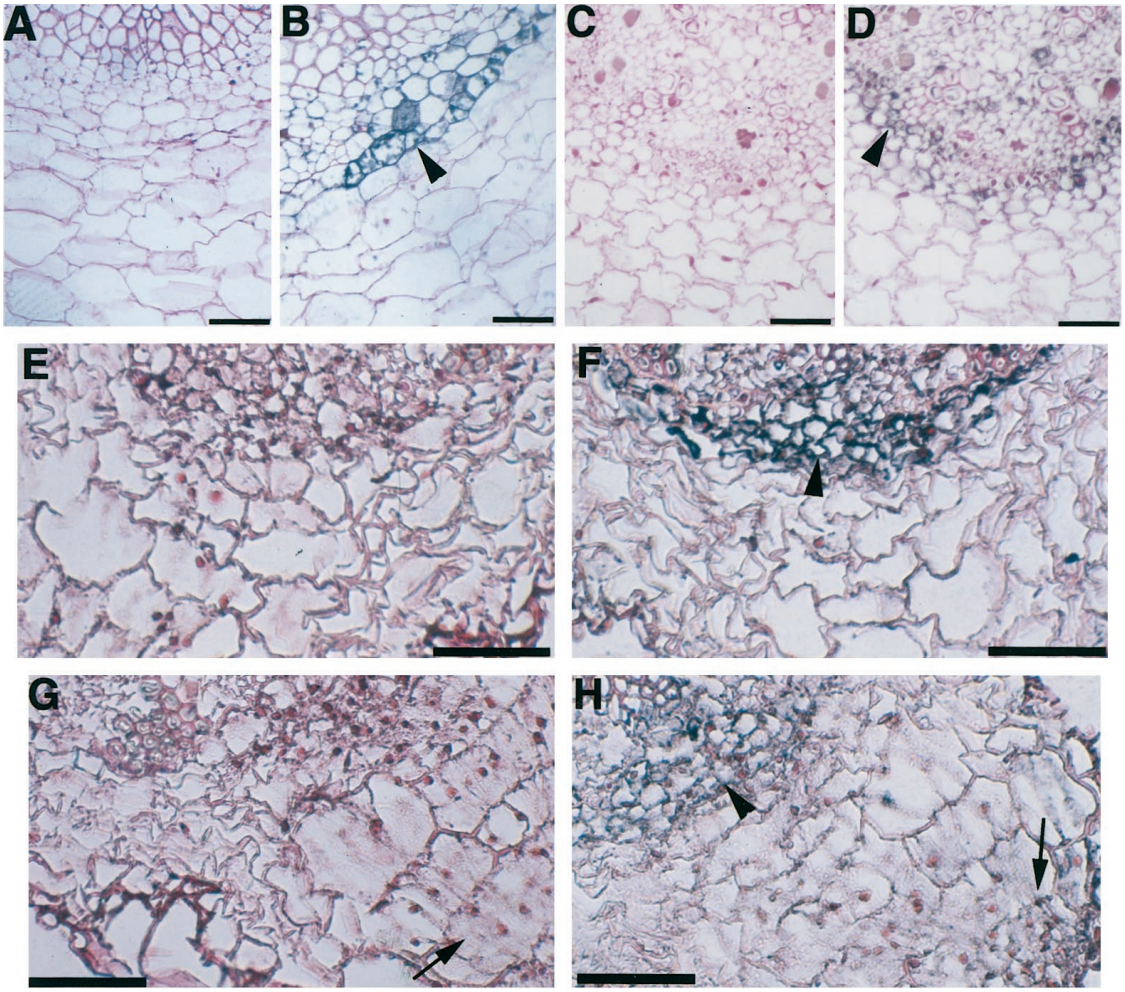

Figure 2. Immunohistochemical localization of Db-LNP in transverse sections of *D. biflorus* roots. Paraffin sections were made of the mature (A and B) and young, emerging (C and D) root hair regions of a 6-d-old *D. biflorus* root and of the nodulation zone of *D. biflorus* roots at 2 d (E and F) and 5 d (G and H) after inoculation with the symbiont, *Bradyrhizobium* sp. 24A10. The sections were treated with preimmunization serum (A, C, E, and G) or antiserum prepared against recombinant Db-LNP (B, D, F, and H) and processed by the immunogold assay as described in the text. The sections were counterstained with basic fuchsin. The arrowheads designate Db-LNP associated with the pericycle. Arrows show the area of cortical cell division. Note that the level of Db-LNP in the pericycle decreases after the initiation of cortical cell division. Scale bars = 50 μm.

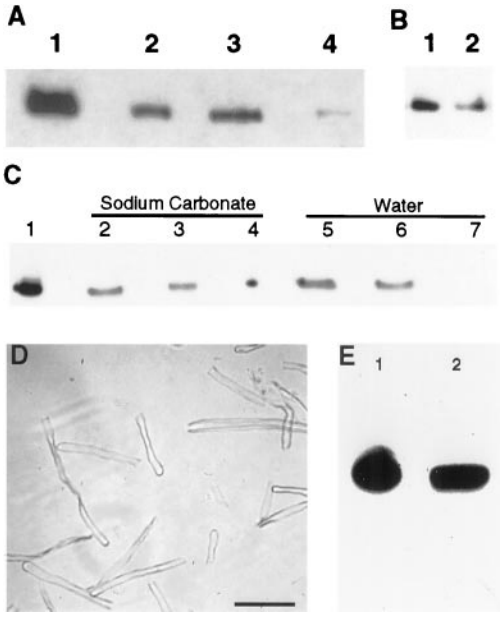

Figure 3. Db-LNP is a peripheral membrane protein and is present in isolated root hairs. A, Immunoblot showing Db-LNP in microsomal fraction from 6-d-old *D. biflorus* roots (lane 2), supernatant (lane 3), and pellet (lane 4) after extraction of an equivalent amount of this microsomal fraction with Na_2CO_3 as described in the text. An affinity-purified 46-kD Db-LNP standard is in lane 1. B, Immunoblot of Db-LNP in isolated plasma membranes from 6-d-old *D. biflorus* roots (lane 2). Lane 1 contains affinity-purified Db-LNP. C, Immunoblot of Db-LNP extracted from 6-day-old *D. biflorus* roots. Whole roots were first extracted with Na_2CO_3 (lane 4) or water (lane 7) as described in the text. The roots were then divided into two regions, homogenized, and extracted with sample buffer to determine the amount of Db-LNP remaining in the region of young and emerging root hairs (lanes 2 and 5) or the mature root hair region (lanes 3 and 6). Lanes 2, 3, 5, and 6 each contain extract from 20 mg of root tissue. An affinity-purified Db-LNP standard is in lane 1. D, Light microscopic view of root hairs isolated from 6-d-old *D. biflorus* roots. Scale bar = 50 μm. E, Immunoblot of Db-LNP extracted from isolated root hair preparation shown in D. The electrophoretic mobility of Db-LNP extracted from the root hairs (lane 2) is identical to that of an affinity purified Db-LNP standard (lane 1).

membranous fraction by treatment for 30 min at 4°C with 0.1 M Na_2CO_3 (pH 10.5) (Fig. 3A). Similar results were obtained with plasma membrane fractions (Fig. 3B). Such alkaline extraction procedures are commonly used to dissociate peripheral membrane proteins (Thrift et al., 1991). Db-LNP was also released from the surface of intact roots of 6-d-old *D. biflorus* plants by this same procedure (Fig. 3C). Following this release of surface Db-LNP, an immunoblot comparison of extracts of the mature root hair region with the young and emerging root hair region showed similar levels of Db-LNP remaining per unit weight of tissue (Fig. 3C, lanes 2 and 3); this remaining Db-LNP probably represents the lectin in the pericycle of these root regions. Immunohistochemical analysis of transverse sections of the young root hair region after treatment of whole roots with Na_2CO_3 showed Db-LNP remaining only in the pericycle (data not shown). Control roots immersed in water for the same length of time did not release Db-LNP into the medium; immunoblot analyses of extracts of

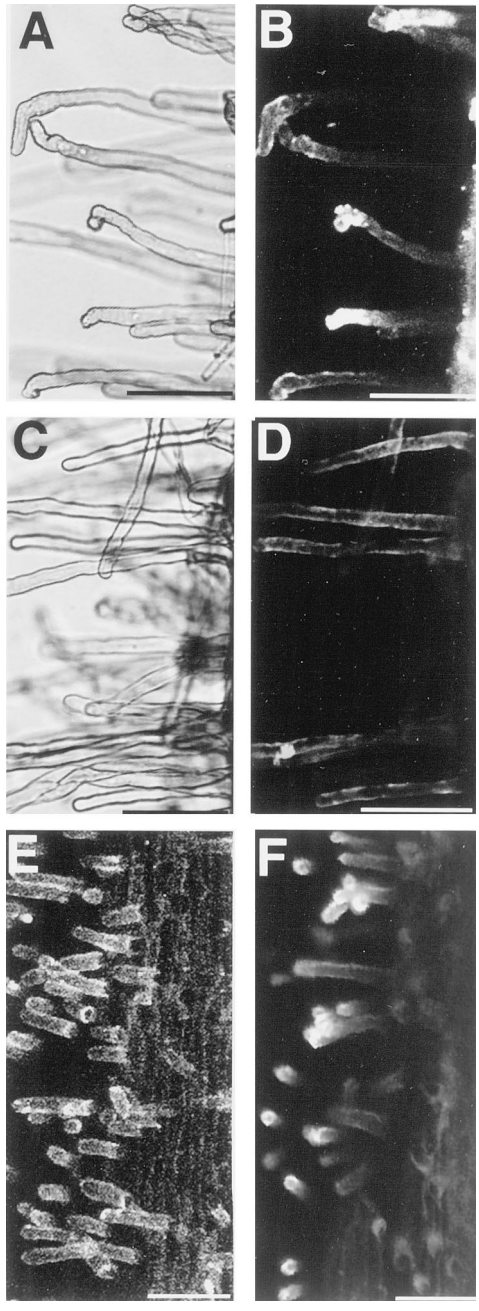

Figure 4. Redistribution of Db-LNP on root hair surface upon treatment with symbiotic rhizobia or a carbohydrate ligand. Bright field (A and C) and immunofluorescence confocal (B and D–F) microscopy was conducted on fixed whole mounts of *D. biflorus* roots 24 h after inoculation of 3-day-old roots with *Bradyrhizobium* sp. 24A10, a symbiont of the plant (A and B), or with *Sinorhizobium meliloti*, a non-symbiont of the plant (C and D). Three-day-old roots were also examined 30 min after immersion in water (E) or in a 100 μg/mL solution of hog blood group A + H substance (F), a carbohydrate ligand of Db-LNP. Each image is a composite of 5 (A–D) or 16 to 18 (E and F) optical sections. Scale bars = 60 μm.

these roots showed more Db-LNP in the young and emerging root hair region than in the mature root hair zone (Fig. 3C, lanes 5 and 6). The Db-LNP in the young and emerging root hair region of these control plants thus represents the combination of lectin present in the pericycle plus the lectin present on the epidermal surface of this region.

Redistribution of LNP in Response to Symbiotic Rhizobia

Within 24 h of inoculation of 3-d-old *D. biflorus* roots with *Bradyrhizobium* sp. 24A10, a symbiotic rhizobial strain, a distinct change in root hair morphology, characterized by branching, deformation, and curling of the root hairs (Fig. 4A) is observed in the young and emerging root hair regions that constitute the zone of the root that is susceptible to nodulation. At this stage surface Db-LNP was found to be localized primarily at the tips of these root hairs (Fig. 4B). This change in root hair morphology and Db-LNP localization did not occur when the roots were inoculated with *S. meliloti*, a non-symbiotic rhizobial strain (Fig. 4, C and D). It should be noted that the lower intensity of the signal obtained on the root hairs, as compared to that shown in Figure 1, D and F, probably reflects the decrease in LNP that has occurred during maturation of this area during the 24 h after inoculation. Treatment of the roots with *Phytophthora sojae*, a pathogen, also failed to promote the redistribution of surface lectin (data not shown), thus suggesting that the redistribution is not a defense response. Db-LNP was, however, redistributed to the tips of the root hairs after immersion of the roots for 30 min in 100 μg/mL hog blood group A + H substance (Fig. 4F), a ligand that interacts with the carbohydrate binding site of the lectin (Etzler et al., 1999). Immunoblot analyses showed no detectable Db-LNP release from the root surface with this ligand and no alteration in amount of Db-LNP in roots treated with symbiotic rhizobia (data not shown). The change in localization of Db-LNP in response to symbiotic rhizobia or carbohydrate ligand thus appears to be due to a redistribution of the lectin on the surface of the root hairs.

In an effort to obtain further information on Db-LNP redistribution, we utilized a double labeling approach in which we used *Bradyrhizobium* sp. 24A10 transformed with the green fluorescent protein (GFP) and secondary antibodies labeled with rhodamine. As shown in Figure 5, the redistribution of the lectin on the root hair surface correlates with the localization of the rhizobia on the surface. The lectin remains uniformly distributed on the surface of a root hair to which no rhizobia are bound.

Immunohistochemical examination of transverse sections of roots obtained at different times after infection with symbiotic rhizobia shows that Db-LNP is still present in the pericycle at 2 d after inoculation (Fig. 2F). By 5 d postinoculation, the amount of Db-LNP in the pericycle is decreased (Fig. 2H); this stage corresponds to the period in which cortical cell divisions are occurring prior to the appearance of the nodule. No Db-LNP was seen on the epidermal surface, the developing nodule, or in the pericycle in transverse sections of roots at the zone of nodulation at later stages of nodule development.

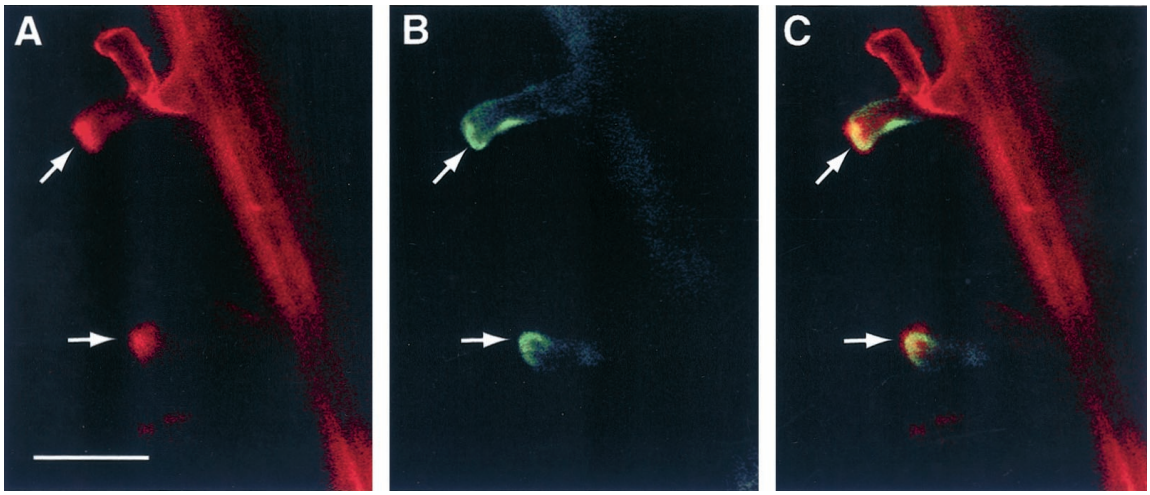

Figure 5. Colocalization of Db-LNP and symbiotic rhizobia on the surface of root hairs of *D. biflorus*. A 3-d-old plant was inoculated with *Bradyrhizobium* sp. 24A10 transformed with a plasmid encoding the GFP. A fixed whole mount of the roots was prepared 2 d after inoculation, treated with anti-Db-LNP and then with rhodamine-conjugated secondary antibody, and examined by confocal fluorescence microscopy. Scale bar = 50 μm. A, Immunolocalization of Db-LNP, shown in red, on the surface of root hairs and epidermal cells. The arrows show the redistribution of the lectin to the tips of two of the root hairs. B, The same portion of the root as shown in A showing the binding of the rhizobia in green. Note that the rhizobia are bound only to the two root hairs designated by the arrows. C, An overlay of A and B showing that the redistribution of Db-LNP has occurred only on those root hairs to which the rhizobia are bound.

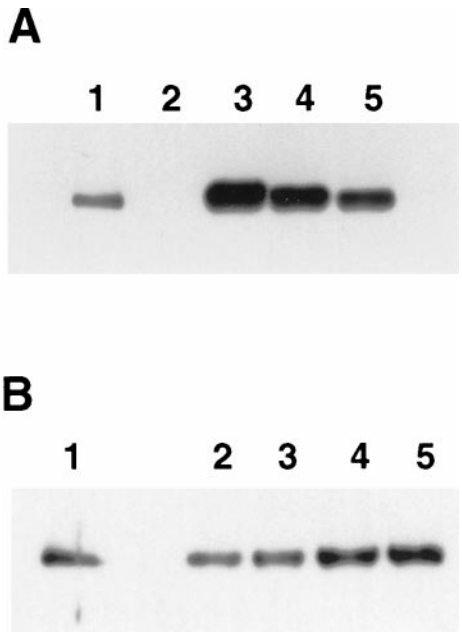

Figure 6. Expression of Db-LNP. A, Levels of Db-LNP at early stages of root development. The radicles from imbibed seeds (lane 2) and roots from 2-d-old (lane 3), 4-d-old (lane 4), and 6-d-old (lane 5) *D. biflorus* seedlings were extracted and analyzed for Db-LNP by immunoblot as described in the text. Lanes 2 through 5 each contain 50 μg of root extract protein. Lane 1 contains an affinity-purified Db-LNP standard. B, Effect of NO_3^-/NH_4^+ and K^+ on the expression of Db-LNP. *D. biflorus* plants were grown for 6 d as described below and their roots were then extracted and assayed for Db-LNP by immunoblot. An affinity-purified Db-LNP standard is in Lane 1. Lane 2 contains root extract from plants grown in medium containing K^+, NO_3^-, and NH_4^+. Lane 3 contains root extract from plants grown in medium containing NO_3^- and NH_4^+ but lacking K^+. Lane 4 contains root extract from plants grown in medium containing K^+ but lacking NO_3^- and NH_4^+. Lane 5 contains root extract from plants grown in medium lacking all three of the above nutrients. Lanes 2 through 5 each contain 31 μg of protein.

Expression of Db-LNP

Immunoblot analyses of *D. biflorus* roots at different stages of development show maximal amounts of this protein in 2-d-old roots (Fig. 6A). Because whole roots were used for these analyses, it must be recognized that the apparent decrease in the amount of Db-LNP after the 2nd d may reflect the diminishing proportion of the nodulation zone of the root to the total amount of root tissue.

Growth of the plants in medium deficient in NH_4^+ and NO_3^-, conditions that have been found to promote root hair deformation and nodulation of legumes in response to Nod factors and rhizobia (Thornton, 1936; Malik et al., 1987; Carroll and Mathews, 1990; Heidstra et al., 1997), resulted in an increase in the level of Db-LNP (Fig. 6B). We cannot exclude the possibility that this increase may represent a higher relative proportion of Db-LNP containing cells. Such an increase does not occur if the plants are deprived of K^+ (Fig. 6B), suggesting that the elevation of Db-LNP levels is not a general stress response.

DISCUSSION

For many years it has been hypothesized that lectins might play a role in the early events leading to the establishment of the rhizobium-legume symbiosis (Hamblin and Kent, 1973; Bohlool and Schmidt, 1974). Most of the work in this area has focused on the well-characterized lectins found in the seeds of legumes (for reviews see Etzler, 1986, 1998; Brewin and Kardailsky, 1997). Recent transgenic studies have shown that hairy root transformation of one legume species with a gene encoding a seed lectin from another legume species enabled the transformants to form nodules in response to symbiont rhizobial strains of the donor species (Diaz et al., 1989; van Rhijn et al., 1998). Although the carbohydrate binding sites of the seed lectins used in these studies were found to be required for their effect on nodulation, these lectins have not been found to bind to the Nod factor signals produced by the rhizobia and studies with mutant rhizobial strains indicate that these lectins are probably binding to the rhizobial lipopolysaccharides or exopolysaccharides (Diaz et al., 1995; van Rhijn et al., 1998).

The Db-LNP root lectin that is the subject of the present investigation represents a completely new category of lectin with no sequence homology to the conventional legume seed lectins. In contrast to the conventional *D. biflorus* seed lectin, Db-LNP binds to the Nod factors produced by rhizobia that nodulate this plant (Etzler et al., 1999). As shown above, the expression of Db-LNP is enhanced by growth of the roots in the absence of NO_3^- and NH_4^+, the deprivation of which have been found to promote root hair deformation and nodulation in response to Nod factors and rhizobia (Thornton, 1936; Malik et al., 1987; Carroll and Mathews, 1990; Heidstra et al., 1997).

The two sites of localization of Db-LNP in the roots support the possibility that this protein might play a role in the early events leading to the initiation of the rhizobium-legume symbiosis. The presence of this lectin on the surface of root hairs and epidermal cells would enable Db-LNP to be involved in initial interactions of the root with the Nod factor signal and/or rhizobia. It is of interest that this surface localization appears to be confined to that region of the root axis that is susceptible to nodulation. The presence of Db-LNP in the root pericycle is of interest in that it is within these cells that the early nodulin gene, *ENOD40*, is activated within several hours of exposure of the roots to rhizobial symbionts or to Nod factor (Kouchi and Hata, 1993; Vijn et al., 1993; Yang et al., 1993; Crespi et al., 1994; Minami et al., 1996b). These cells subsequently contribute to the formation of the nodule (for reviews see Mylona et al., 1995; Dénarié, et al., 1996). Whether the activation of the

pericycle cells is due to a direct interaction with transported Nod factor or a response to secondary messengers produced in a signal transduction cascade is not known.

In addition to the above correlative results, we have shown that infection of the plants with rhizobia results in the redistribution of Db-LNP to the tips of the root hairs. This redistribution occurs only with symbiotic rhizobial strains and does not occur upon infection of the plant with a pathogen. This redistribution resembles the patching/capping response of many animal cell surface components upon exposure to ligand or antibodies and is also achieved upon exposure of the root to hog blood group A + H substance, a ligand that binds to the carbohydrate binding site of Db-LNP. The subsequent disappearance of Db-LNP from the surface of the root hairs and also from the pericycle might be associated with its utilization during the initiation of a plant response or perhaps represent a down regulation of the protein in response to the rhizobia or Nod factor.

Work in progress in our laboratory suggests that at least two Db-LNP genes are present in the *D. biflorus* genome and that these genes are very similar to one another. The extent to which the Db-LNP at the two different sites in the root tissue represents the expression of more than one gene is not known. It should be noted, however, that the identical electrophoretic mobilities of the Db-LNP obtained from each site suggest that the Db-LNP is undergoing identical modifications in each cell type. These modifications include the removal of a signal peptide and a 19-amino acid segment immediately downstream of this signal as well as glycosylation at at least one of two consensus *N*-glycosylation sites (Quinn and Etzler, 1987; Etzler et al., 1999). It should also be pointed out that the antiserum used in this investigation does not react with a second, related nucleotide phosphohydrolase found in legumes as well as in other families of plants (Roberts et al., 1999).

Although we do not yet know the subcellular localization of the pericycle Db-LNP, the presence of Db-LNP in isolated root hairs and root plasma membranes and its release from these fractions as well as from whole roots by treatment with 0.1 M Na_2CO_3 suggests that the root surface Db-LNP is a peripheral membrane protein. Hydropathic plot analysis of the sequence of this protein predicted the presence of only a single transmembrane domain, which is the signal peptide (Etzler et al., 1999). The identical electrophoretic mobility of the membrane protein to that of the isolated lectin, which does not contain this signal peptide (Quinn and Etzler, 1987), provides further evidence that Db-LNP is not anchored to the membrane by this signal peptide. Experiments are in progress to identify the membrane constituent(s) with which Db-LNP might be associated. The interaction of such components with Db-LNP would be anticipated should this protein function in a signaling or transport process.

The identification of Nod factors as signals for the initiation of nodule formation in the rhizobium-legume symbiosis (Lerouge et al., 1990) predicts that plant Nod factor-binding proteins might function as receptors or transporters for these signals. The results of the present investigation support our previous findings (Etzler et al., 1999) that suggest Db-LNP could play such a role. Such a possibility has also been strengthened by our finding that homologs of this type of LNP appear to be confined only to leguminous plants (Roberts et al., 1999). However, it is also possible that this protein might play a role in recognizing endogenous Nod factor-like signals that have been proposed to act in the regulation of plant growth and organogenesis (Spaink et al., 1993). Transgenic experiments are in progress in an effort to obtain further information on the role of this protein and its mode of action.

MATERIALS AND METHODS

Plant Material, Microorganisms, and Antiserum

Dolichos biflorus seeds were purchased from F.W. Schumacher (Sandwich, MA). The seeds were surface sterilized as previously described (Etzler et al., 1999) and germinated overnight in Hoagland nitrogen-deficient medium (Hoagland and Arnold, 1950) unless specified otherwise. The germinated seeds were transferred to sterile growth pouches containing this same medium and the plants were grown with a 16-h-light/8-h-dark cycle. Root hairs were isolated from 6-d-old roots by a freeze-fracture procedure (Gloudemans et al., 1989). Microsomal and plasma membrane fractions of the roots were prepared as described by Widell et al. (1982).

Phytophthora sojae was obtained from Dr. Brett Tyler (University of California, Davis) and grown in vegetable juice agar medium (Morris et al., 1998). *Sinorhizobium meliloti* 445 was obtained from Dr. Donald Phillips (University of California, Davis) and grown in TY medium (Rosenberg et al., 1981). *Bradyrhizobium* sp. 24A10 was obtained from Lipha Tech (Milwaukee, WI) and grown in RDY medium (Nieuwkoop et al., 1987). A GFP derivative of *Bradyrhizobium* sp. 24A10 was constructed by electroporation (Seidman et al., 1997) with the pGFP plasmid (CLONTECH Laboratories, Palo Alto, CA) encoding the GFP under the control of the *lac* promoter.

The preparation of the anti-Db-LNP serum used in this investigation was previously described (Etzler et al., 1999). This antiserum reacts with only a single band (46 kD) in immunoblots of *D. biflorus* root extracts.

Inoculation of Roots with Rhizobia

Bradyrhizobium sp. 24A10 or *S. meliloti* were grown to mid-log phase and suspended (1×10^7 cells/mL) in nitrogen-deficient Hoagland's medium. Three-day-old *D. biflorus* plants were inoculated with 100-μL suspensions of

these rhizobia and grown in sterile growth pouches for the times stated in the text. The positions of the tips of the roots were marked on the growth pouches at the time of inoculation. Control plants were not inoculated with rhizobia.

Immunolocalization Assays

Confocal immunofluorescence microscopy was conducted as previously described (Etzler et al., 1999) on whole mounts of fixed roots. After fixation, the roots were treated for 20 min with 1:250 dilutions of preimmunization serum or antiserum (anti-Db-LNP serum) prepared against recombinant Db-LNP (Etzler et al., 1999), washed, and then treated for 20 min with fluorescein-labeled or tetramethylrhodamine isothiocyanate-labeled goat anti-rabbit IgG (Sigma Chemical Co., St. Louis). After washing, the roots were examined with a Leica TCS NT confocal microscope (Leica, Wetzlar, Germany) using a 488-nm excitation laser line and 520-nm barrier filter for fluorescein or a 568-nm excitation laser line and 630-nm barrier filter for rhodamine. Confocal images were reconstructed using IMAGESPACE 3.2 software (Molecular Dynamics, Inc., Sunnyvale, CA).

Immunohistochemistry was conducted on 8-μm transverse sections of root tissue that had been fixed for 2 or 6 h at 4°C in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS; 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl), washed with PBS, passed through an ethanol dehydration series, and embedded in paraplast (Fischer Scientific, Pittsburgh). The sections were deparaffinized, passed through an ethanol hydration series, and incubated 2 to 12 h at room temperature in 10% (w/v) bovine serum albumin (BSA) in PBS. The sections were then treated for 1 h with 1:250 dilutions of preimmunization serum or anti-Db-LNP serum prepared in PBS containing 0.1% (w/v) BSA. The sections were washed extensively with 0.1% (w/v) BSA/PBS over a period of 30 min and treated with a 1:100 dilution of 5 nm of colloidal gold-conjugated goat anti-rabbit IgG (Sigma Chemical Co.) for 1 h at room temperature. After washing extensively for 30 min with 0.1% (v/v) Tween 20 in PBS, the sections were washed with water and treated for 10 min with silver enhancement reagent (Sigma Chemical Co.). The sections were counter stained with 0.05% (w/v) basic fuchsin.

Immunoblot Assays

SDS-urea PAGE was conducted as previously described (Carter and Etzler, 1975). Whole roots or various regions of the roots were ground in liquid nitrogen and the powder was weighed and suspended in gel sample buffer and incubated at 65°C for 20 min. The extracts were then microfuged at 8,160*g* for 5 min and the supernatants were assayed for protein using the bicinchoninic acid method (Smith et al., 1975). Bromphenol blue, mercaptoethanol, and dithiothreitol were then added to the samples at final concentrations of 0.0012% (w/v), 1.96% (v/v), and 0.15% (w/v), respectively. These three reagents were included in the initial incubation buffer used for cell fractions that were not subjected to protein determination. Affinity-purified Db-LNP (Quinn and Etzler, 1987) was used as a standard. After electrophoresis the proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA) as previously described (Bunker and Etzler, 1994). The membrane was treated overnight at 4°C with BLOTTO (Johnson et al., 1984) and then incubated in a 1:500 dilution of anti-Db-LNP serum in BLOTTO for 1 h at room temperature. After extensive washing, the membrane was incubated for 1 h at room temperature with a 1:24,000 dilution of horse radish peroxidase-conjugated goat anti-rabbit IgG (Sigma Chemical), washed, and developed with a chemiluminescence assay (Schneppenheim et al., 1991).

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