The Galactolipid Digalactosyldiacylglycerol Accumulates in the Peribacteroid Membrane of Nitrogen-fixing Nodules of Soybean and *Lotus*

The peribacteroid membrane (PBM) surrounding nitrogen-fixing rhizobia in the nodules of legumes is crucial for the exchange of ammonium and nutrients between the bacteria and the host cell. Digalactosyldiacylglycerol (DGDG), a galactolipid abundant in chloroplasts, was detected in the PBM of soybean (*Glycine max*) and *Lotus japonicus*. Analyses of membrane marker proteins and of fatty acid composition confirmed that DGDG represents an authentic PBM lipid of plant origin and is not derived from the bacteria or from plastid contamination. In Arabidopsis, DGDG is known to accumulate in extraplastidic membranes during phosphate deprivation. However, the presence of DGDG in soybean PBM was not restricted to phosphate limiting conditions. Complementary DNA sequences corresponding to the two DGDG synthases, DGD1 and DGD2 from Arabidopsis, were isolated from soybean and *Lotus*. The two genes were expressed during later stages of nodule development in infected cells and in cortical tissue. Because nodule development depends on the presence of high amounts of phosphate in the growth medium, the accumulation of the non-phosphorus galactolipid DGDG in the PBM might be important to save phosphate for other essential processes, i.e. nucleic acid synthesis in bacteroids and host cells.

Symbiotic nitrogen fixation in legumes takes place in specialized organs called nodules that develop from root cortical cells following contact with soil bacteria of the family Rhizobiaceae. The symbiosis is mutualistic; the plant receives a crucial supply of reduced nitrogen from the bacteria in exchange for reduced carbon and other nutrients (1). The interaction between legumes and rhizobia is generally specific; different rhizobial strains are capable of infecting one or a few legume species. For example, soybean (*Glycine max*) is preferentially infected by *Bradyrhizobium japonicum*, whereas *Mesorhizobium loti* establishes a symbiosis with *Lotus japonicus* (2). Apart from being an important crop plant, soybean has been employed as a model for biochemical and physiological studies of symbiotic nitrogen fixation for decades. *L. japonicus*, on the other hand, has emerged as a model species for genetics and genomics studies of symbiotic nitrogen fixation and other legume-specific processes (3–5).

Following a series of initial signal exchanges (6–8), rhizobia enter root hair and underlying cells via an infection thread (9, 10) from which they are eventually released into cortical cells via endocytosis. This series of events results in a unique cytoplasmic “organelle” called the symbiosome, which consists of bacteria surrounded by a membrane of plant origin, called the symbiosome or peribacteroid membrane (PBM) (11). The PBM is both the structural and functional interface between the plant and bacterial cells, and its lipid and protein composition contribute significantly to its biological roles. Growth and division of rhizobia, matched by synthesis of PBM, continues until thousands of symbiosomes, each containing one or more bacteria, pack the infected cells. Rhizobia within symbiosomes eventually differentiate into nitrogen-fixing bacteroids, in response to lowered oxygen concentrations and possibly other physiological changes that accompany nodule development. Nodulation is also accompanied by a reprogramming of plant metabolism, which becomes specialized for the provision of reduced carbon and other nutrients to the bacteroids and for the assimilation of ammonium and other nitrogen compounds from the bacteroids (1, 12, 13).

The lipid composition of the PBM has been analyzed on a qualitative level. The PBM contains saturated (16:0, palmitic acid; 18:0, stearic acid) and unsaturated fatty acids (16:1Δ9trans, palmitoleic acid; 18:1Δ9cis, oleic acid 18:2Δ9,12, linoleic acid; 18:3Δ9,12,15, α-linolenic acid) typically found in higher plants. In addition to phospholipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylglycerol (PG), and phosphatidylglycerol (PG) lipids and glycolipids of unknown structure were detected previously (14, 15). The origin of PBM lipids presumably is the endoplasmic reticulum, where they are synthesized and transported to the PBM via the Golgi apparatus (16).

Under normal growth conditions, galactolipids (monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG)) are restricted to chloroplast membranes in plants, where they are important in maintaining the structural integrity of photosynthetic complexes. The enzymes involved in galactolipid formation and turnover are encoded within the nuclear genome (17, 18). The lipid composition of the PBM has been analyzed on a qualitative level. The PBM contains saturated (16:0, palmitic acid; 18:0, stearic acid) and unsaturated fatty acids (16:1Δ9trans, palmitoleic acid; 18:1Δ9cis, oleic acid 18:2Δ9,12, linoleic acid; 18:3Δ9,12,15, α-linolenic acid) typically found in higher plants. In addition to phospholipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylglycerol (PG), and phosphatidylglycerol (PG) lipids and glycolipids of unknown structure were detected previously (14, 15). The origin of PBM lipids presumably is the endoplasmic reticulum, where they are synthesized and transported to the PBM via the Golgi apparatus (16).

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lactolipid synthesis (MGDG synthases and DGDG synthases) have been isolated from Arabidopsis thaliana (17–19). During phosphate deprivation, the amount of DGDG increases greatly in Arabidopsis (20, 21). Under these conditions, phospholipids are replaced by glycolipids to save phosphorus for other essential processes (for a review see Ref. 22). Interestingly, a fraction of DGDG synthesized during phosphate deprivation is localized to extraplastidic membranes, e.g., the plasma membrane (21, 23). However, under normal growth conditions, galactolipids are of very low abundance in extraplastidic membranes.

Rhizobia contain a different set of fatty acids as compared with higher plants, e.g., with double bonds at different positions or with different acylpropane groups. The predominant fatty acids in Rhizobia are phospholipids (PC, PE, PG, and cardiolipin; Refs. 24 and 25). A diglycosylglycerol lipid was detected in B. japonicum USDA110, which was believed to be structurally identical with DGDG of higher plants (26). Interestingly, the Bradyrhizobium lipid was restricted to the bacteroid form but was not detected when bacteria were grown in liquid culture. The occurrence of DGDG in rhizobia raised many important questions regarding its function and origin, because this glycolipid was believed to be restricted to higher plants and Cyanobacteria but absent from nonphotosynthetic bacteria. To improve our understanding of the role of membrane lipids in the PBM, subcellular fractions were isolated from soybean and Lotus, and the glycerolipid composition was determined on a quantitative level. From these studies, it became clear that DGDG is indeed localized to the symbiosomes of legume nodules. However, we obtained strong evidence that this glycolipid is derived from plant lipid biosynthesis and is localized to the PBM but not to the bacteroids. Furthermore, our data demonstrate that the accumulation of DGDG in extraplastidic membranes is not restricted to the plasma membrane nor to plants deprived of external phosphate.

**EXPERIMENTAL PROCEDURES**

**Plant Material and Growth Conditions—**Soybean seeds (G. max cv. Stevens) were incubated overnight in water for swelling. The seeds were transferred into silica sand and grown under controlled conditions (16 h of light/day; day and night temperatures, 25 and 20 °C, respectively) and 65% humidity. B. japonicum USDA110 was grown in liquid culture (YT medium; 5 g of tryptone, 3 g of yeast extract, 10 ml of 0.45 M CaCl₂ in 1000 ml of water) for 5 days at 28 °C and diluted 1:10 with water before inoculation of 7-day-old soybean seedlings.

Prior to germination, L. japonicus seeds were treated with liquid nitrobenzil and incubated overnight in water. The plants were grown in silica sand at 16 h of light/day with day and night temperatures of 21 and 17 °C, respectively. M. loti strain R7a was grown in liquid culture (YT medium) to an A₅₄₀ of 1.3 (5 days, 28 °C), and the cells were diluted 1:10 and used for the inoculation of 3-day-old Lotus seedlings.

After infection with bacteria, soybean and Lotus plants were watered two times/week with complete mineral mixture excluding KNO₃ (27). All of the soybean plants grown under +P, conditions were additionally watered with 100 ml of 2.5 m phosphate two times/week. For phosphate deprivation experiments, soybean plants were grown for 2 weeks without nodulation in the presence of complete mineral mixture including 5 m KNO₃ and phosphate (27). The plants were subsequently grown with mineral mix excluding phosphate for another 10 weeks before sampling.

**Isolation of Symbiosomes and Peribacteroid Membranes from L. japonicus and G. max—**Approximately 20 g of fresh nodules from 12-week-old soybean or L. japonicus USDA110 was grown in liquid culture (YT medium; 5 g of tryptone, 3 g of yeast extract, 10 ml of 0.45 M CaCl₂ in 1000 ml of water) for 5 days at 28 °C and diluted 1:10 with water before inoculation of 7-day-old soybean seedlings.

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**Lipid Extraction and Quantification of Polar Lipids, Fatty Acids, and Sugars—**The lipids were isolated from frozen plant material, rhizobia (isolated from liquid culture), symbiosomes, or peribacteroid membranes (30). The separation of the polar lipids was done by thin layer chromatography (31). For quantification by gas chromatography, the lipids were isolated from the plates and converted to fatty acid methyl esters (32). Pentadecanoic acid (15:0) was used as internal standard. The sugar head group composition of DGDG was determined after hydrolysis by quantifying alditol acetates by gas chromatography (33).

**Northern Analysis—**Total RNA was isolated from 1 g of soybean tissue (34). 10 μg of total RNA was separated on an agarose gel and blotted onto a neutral nylon membrane (Parablot Ny amp; Macherey-Nagel, Duren, Germany) in 10× SSC. The RiboScribe RNA probe synthesis kit (Epitomizer Technologies, Madison, WI) was used for labeling probes with [³²P]UTP (EST clones se-pk00352-ec and BE021559 representing soybean DGD1 and DGD2).

**Isolation of Full-length DGDG Synthase cDNAs from Soybean by 5'-RACE—**Rapid amplification of cDNA ends was done to obtain the 5'-ends of the partial cDNA clones of soybean DGDG synthases. Poly(A) mRNA was isolated from total leaf RNA by oligo(dT) affinity purification (PolyATract; Promega, Mannheim, Germany). The first part of the missing 5'-end of soybean DGD1 was amplified from cDNA synthesized with the SMARTII oligonucleotide (AAG CUG CAG TGG TAA CCA CGG GAT A) and the gene-specific primer PD227 route to phosphatase, 7TCC TGC GAC ACA ATG TTC TCT C), and the cells were diluted 1:10 to the 5'-end of soybean 4 nodules. Total phosphate in soybean tissues was determined after hydrolysis with 65% (v/v) nitric acid (Merck). The concentration of total phosphate was determined by inductively coupled plasma optical emission spectroscopy using IRIS Advantage Duo ER/S (Thermo Jarell Ash, Franklin, MA) (38).

**Quantification of Protein, Fatty Acids, and Inorganic Phosphate in Symbiosomes—**Protein was measured according to Bradford (37). Total fatty acids were determined using pentadecanoic acid (15:0) as an internal standard after transmethylation of lipids in whole tissues by GC. The yield of symbiosome isolation from nodules was calculated using 19:0 as a bacteroid-specific marker fatty acid.

Total phosphate in soybean tissues was determined after hydrolysis with 65% (v/v) nitric acid (Merck). The concentration of total phosphate was determined by inductively coupled plasma optical emission spectroscopy using IRIS Advantage Duo ER/S (Thermo Jarell Ash, Franklin, MA) (38).

**In Situ Hybridization**—**In situ** hybridization experiments were performed as previously described (39, 40). L. japonicus nodules harvested at 21 days post-infection with M. loti (17) were fixed in 4% paraformaldehyde supplemented with 0.25% glutaraldehyde in 10 mM sodium phosphate buffer, pH 7.4, for 4 h in a vacuum aspirator. Fixed nodules were block-stained in 0.5% safranin, dehydrated through ethanol series, and embedded in paraffin, and 8–10-μm-thick sections...
RESULTS

The Galactolipid DGDG Is a Major Constituent of the Peribacteroid Membrane of Soybean and Lotus Nodules—The nodules harbor large numbers of symbiosomes in the cytosol of the infected cells. As a consequence, the nodules contain more lipid/unit mass than other organs, and a large fraction of lipids is contained within symbiosomes (Fig. 1A). The glycerolipid composition of soybean nodules is similar to that of roots, with phospholipids (PC, PE, and PG) representing the most abundant lipid classes (Fig. 2A). Galactolipids (MGDG and DGDG), which are the predominant lipids in leaves, are less abundant in roots and nodules (Fig. 2A). The symbiosomes were isolated from soybean nodules and, after rupture by osmotic shock, separated into bacteroids and PBM. In addition to phospholipids (PC, PE, and PG), the PBM contained another lipid co-migrating with a leaf DGDG standard. This lipid stained with \( \alpha \)-naphthol and therefore tentatively identified as DGDG. It was absent from bacteroids (Fig. 2A) and was also not found in Bradyrhizobium cells grown in liquid culture (data not shown). The PBM fraction was devoid of MGDG, another galactolipid highly abundant in plastids. Therefore, it is unlikely that DGDG in the PBM was derived from contamination with plastid membranes. Quantification of membrane lipids in different soybean tissues demonstrated that the amount of DGDG in symbiosomes was \( \sim 7 \)–8 mol %, about three times as high as in roots (\( \sim 2.5 \) mol %; Table I).

To address the question of whether the presence of DGDG in the PBM is unique to soybean, we included a second legume species, L. japonicus, in our analysis. Lotus nodules were obtained after inoculation with \( M. \) loti and used for symbiosome and PBM preparation. Similarly to soybean, a lipid co-migrating with DGDG was detected by TLC of Lotus PBM fractions (Fig. 2B). This lipid was also stained with \( \alpha \)-naphthol and therefore tentatively identified as DGDG. It was absent from Mesorhizobium bacteria, indicating that similarly to soybean, DGDG is an abundant lipid in Lotus PBM, but not in bacteria or bacteroids (Fig. 2B).

DGDG Is an Authentic Plant-derived Lipid of the PBM and Does Not Originate from Bacterial Lipid Biosynthesis—The fact that DGDG but not MGDG was detected in the soybean PBM suggested that the PBM fraction was largely devoid of plastid contamination. Western blot analysis was done to confirm the absence of plastid membranes in the isolated PBM fraction (Fig. 3). The NOD26 protein, a marker for symbiosome membranes (41), was specifically detected in the PBM. Immunoblots with antibodies raised against membrane components of the plastid protein import apparatus (TIC40, inner envelope; TOC75, outer envelope) showed weak or no cross-reaction with the PBM fraction but strong bands in the plastid fractions (Fig. 3). Therefore, the PBM fraction was indeed devoid of contamination by plastid membranes.

To assess the purity of the PBM fraction with regard to possible contaminations by bacterial lipids, total fatty acid composition was determined by GC (Table II). In contrast to soybean tissues that are rich in unsaturated fatty acids (18:1\(^{9\text{cis}},12\text{cis}\), oleic acid; 18:2\(^{9\text{cis}},12\text{cis}\), linoleic acid; and 18:3\(^{9\text{cis}},12\text{cis},15\text{cis}\), \( \omega \)-linolenic acid), \( B. \) japonicum contains large amounts of the monounsaturated fatty acid 18:1\(^{11\text{cis}}\) (vaccenic acid) and of the cyclopropane fatty acid 19:0\(^{11\text{cis}},12\text{cyco}\) (Ref. 42 and Table II). Oleic acid, linoleic acid, and \( \alpha \)-linolenic acid were predominant fatty acids in the PBM, but no bacteria-specific fatty acids were detected (Table II). Therefore, the PBM was devoid of contamination by bacterial lipids. DGDG was isolated from soybean PBM and subjected to fatty acid and sugar analysis by GC. As shown in Table III, the PBM-derived DGDG contains large amounts of typical plant fatty acids (oleic acid, linoleic acid, and \( \alpha \)-linolenic acid) but no bacteria-specific fatty acids. Furthermore, galactose was the predominant sugar in the head group of this glycolipid. (Table III). In conclusion, these findings strongly suggest that the glycolipid detected in the PBM fraction of soybean nodules represents authentic DGDG originating from plant lipid metabolism.

The Presence of DGDG in Symbiosomes Is Independent of the Availability of Phosphate in the Soil—Galactolipids are highly abundant in chloroplasts of leaves, whereas phospholipids are predominant in extraplastidic membranes. Previously, it was shown that in plants grown under phosphate limiting conditions, the amount of DGDG is greatly increased, and a large fraction of DGDG is found in extraplastidic membranes, e.g. in the plasma membrane (21, 23). Therefore, it was important to address the question of whether the presence of DGDG in the PBM was caused by phosphate deficiency in nodules or whether this lipid represents an authentic component of the

![Figure 1: Symbiosomes in soybean nodules contain a large fraction of lipids, phosphate, and protein.](http://www.jbc.org/)

**Fig. 1.** Symbiosomes in soybean nodules contain a large fraction of lipids, phosphate, and protein. A, total fatty acids were quantified by GC of methyl esters. B, total phosphate concentration in different soybean organs was determined after hydrolysis of bound phosphate with HCl/HNO\(_3\). C, soluble protein. The values represent the means ± S.D. of three measurements. The total amounts of fatty acids, phosphate, or protein in symbiosomes (hatched bars) were calculated after determining the yield of symbiosome isolation using 19:0\(^{11\text{cis}},12\text{cyco}\) as a marker fatty acid.

It was absent from bacteroids (Fig. 2A) and was also not found in Bradyrhizobium cells grown in liquid culture (data not shown). The PBM fraction was devoid of MGDG, another galactolipid highly abundant in plastids. Therefore, it is unlikely that DGDG in the PBM was derived from contamination with plastid membranes. Quantification of membrane lipids in different soybean tissues demonstrated that the amount of DGDG in symbiosomes was \( \sim 7 \)–8 mol %, about three times as high as in roots (\( \sim 2.5 \) mol %; Table I).
The lipids were isolated from different soybean tissues, separated by TLC, and quantified by GC of fatty acid methyl esters. Nodulated plants were grown on sand with fertilizer without nitrogen and with (+Pi) or without phosphate (−Pi). The amounts of lipids were determined in mol, and lipid composition was calculated in mol%. The data represent the means ± S.D. from three measurements.

TABLE I
Polar lipid composition of soybean tissues

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Leaf +Pi mol %</th>
<th>Leaf −Pi mol %</th>
<th>Root +Pi mol %</th>
<th>Root −Pi mol %</th>
<th>Nodules +Pi mol %</th>
<th>Nodules −Pi mol %</th>
<th>Symbiosomes +Pi mol %</th>
<th>Symbiosomes −Pi mol %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGDG</td>
<td>45.0 ± 3.7</td>
<td>49.0 ± 2.0</td>
<td>6.5 ± 0.3</td>
<td>7.7 ± 1.6</td>
<td>10.4 ± 0.7</td>
<td>6.8 ± 2.4</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>PG</td>
<td>9.8 ± 1.6</td>
<td>3.5 ± 0.1</td>
<td>5.1 ± 1.4</td>
<td>5.2 ± 0.9</td>
<td>5.1 ± 0.4</td>
<td>3.5 ± 0.7</td>
<td>9.9 ± 1.5</td>
<td>11.4 ± 1.2</td>
</tr>
<tr>
<td>DGDG</td>
<td>20.9 ± 2.2</td>
<td>38.4 ± 2.8</td>
<td>2.5 ± 0.3</td>
<td>36.6 ± 2.9</td>
<td>3.4 ± 0.2</td>
<td>11.1 ± 1.4</td>
<td>7.6 ± 1.3</td>
<td>10.6 ± 2.3</td>
</tr>
<tr>
<td>SL/Pi</td>
<td>3.2 ± 0.3</td>
<td>4.4 ± 1.7</td>
<td>6.7 ± 0.4</td>
<td>9.9 ± 1.1</td>
<td>1.9 ± 0.4</td>
<td>5.9 ± 1.0</td>
<td>24.6 ± 1.4</td>
<td>26.7 ± 2.2</td>
</tr>
<tr>
<td>PE</td>
<td>9.6 ± 2.4</td>
<td>1.7 ± 0.2</td>
<td>41.4 ± 0.3</td>
<td>31.1 ± 1.6</td>
<td>41.4 ± 3.4</td>
<td>34.1 ± 1.6</td>
<td>27.1 ± 2.5</td>
<td>19.2 ± 3.0</td>
</tr>
<tr>
<td>PC</td>
<td>11.7 ± 3.0</td>
<td>4.0 ± 0.3</td>
<td>37.8 ± 2.0</td>
<td>28.5 ± 3.3</td>
<td>37.8 ± 4.6</td>
<td>38.7 ± 3.2</td>
<td>30.7 ± 0.9</td>
<td>32.1 ± 2.9</td>
</tr>
</tbody>
</table>

* ND, not detected.

Sulfolipid (SL) and phosphatidylinositol (PI) were not separated.

The PBM fraction from soybean is devoid of plastidic membranes. Membrane proteins isolated from plastids of leaves and roots or from PBM isolated from nodules were subjected to Western analysis with antibodies raised to TIC40 (marker for plastid inner envelope), TOC75 (marker for plastid outer envelope), and NOD26 (marker for PBM).

PBM under all growth conditions. Soybean plants that were used for PBM preparations and lipid analysis were always grown in the presence of phosphate. When soybean plants were raised in the absence of phosphate, growth was stunted, and the lipid composition in leaves was drastically altered, i.e., the relative amount of DGDG was increased (Table I). A similar strong increase in DGDG was observed for roots and nodules. The amount of DGDG in symbiosomes grown in the presence of phosphate was higher than in nodules or roots, and it increased only slightly in response to phosphate deprivation. Therefore, the occurrence of DGDG in symbiosomes was independent of the supply of phosphate in the growth medium.

The amount of total phosphate was determined in different soybean organs to directly address the question of whether nodule tissues experience phosphate limitation under normal condition (Fig. 1). The amount of phosphate in nodules was similar to leaves but much higher than in roots. A large fraction of phosphate in nodules was associated with symbiosomes (Fig. 1B). Similarly, a large fraction of total fatty acids and total protein in nodules was contained in symbiosomes (Fig. 1). Therefore, measurement of total phosphate indicated that nodulation of root cells results in a drastic increase of phosphate uptake and that a large fraction of the additional phosphate is localized to the symbiosomes of infected cells.

Two DGDG Synthases Are Expressed during Nodulation—Two genes encoding DGDG synthases are present in Arabidopsis (18, 19). Full-length cDNA clones were obtained for soybean and Lotus DGDG synthases based on EST clones deposited in data bases and by 5’-RACE PCR and used for sequence comparison (Fig. 4). Similarly to Arabidopsis, soybean and Lotus contain at least two DGDG synthases, DGD1, which contains a long N-terminal extension and a C-terminal glycosyltransferase-like part, and DGD2, which possesses only the glycosyltransferase-like domain. Expression of the two DGDG synthases was very low in soybean leaves and roots but was induced in plants grown in the absence of phosphate (Fig. 5). To address the question of whether expression of DGDG synthetases was induced during nodule development, total RNA was extracted from the growing part of soybean roots and from developing nodules and used for Northern analysis. Expression of DGD1 and DGD2 was very low in soybean roots and young nodules but increased in the late stages of nodule development. Because plants were continuously fertilized with phosphate during this experiment (see above), induction of DGDG synthase expression appeared to be a consequence of nodule development/differentiation rather than phosphate deprivation in the growth medium.
Lipids were obtained from soybean tissues (leaf, root, or nodule), from different fractions isolated from nodules (symbiosomes, PBMs, or bacteroids), or from Bradyrhizobium grown in liquid culture (bacteria). All of the plants were grown on sand and fertilized with phosphate. The lipids were transmethylated, and total fatty acid composition was determined by GC (means ± S.D., n = 3).

### Table II

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Leaf</th>
<th>Root</th>
<th>Nodule</th>
<th>Symbiosomes</th>
<th>PBMs</th>
<th>Bacteria</th>
<th>Bacteroids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mol %</td>
<td>mol %</td>
<td>mol %</td>
<td>mol %</td>
<td>mol %</td>
<td>mol %</td>
<td>mol %</td>
</tr>
<tr>
<td>16:0</td>
<td>11.0 ± 0.5</td>
<td>18.9 ± 2.4</td>
<td>12.9 ± 0.4</td>
<td>12.5 ± 0.7</td>
<td>34.3 ± 4.7</td>
<td>9.4 ± 1.6</td>
<td>9.9 ± 0.2</td>
</tr>
<tr>
<td>16:1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4 ± 0.2</td>
<td>1.3 ± 0.5</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>3.3 ± 2.1</td>
<td>2.3 ± 1.9</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0</td>
<td>0.3 ± 0.1</td>
<td>0.8 ± 0.3</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>18:1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.5 ± 0.4</td>
<td>2.4 ± 0.4</td>
<td>0.3 ± 0.1</td>
<td>1.7 ± 0.3</td>
<td>7.8 ± 1.6</td>
<td>1.1 ± 0.2</td>
<td>1.5 ± 0.1</td>
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<tr>
<td>18:2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.4 ± 0.5</td>
<td>2.0 ± 0.4</td>
<td>2.2 ± 0.4</td>
<td>51.4 ± 2.5</td>
<td>10.8 ± 0.7</td>
<td>80.5 ± 3.7</td>
<td>64.4 ± 0.1</td>
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<td>18:3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>10.6 ± 2.8</td>
<td>28.5 ± 1.9</td>
<td>30.5 ± 0.2</td>
<td>9.0 ± 2.5</td>
<td>22.8 ± 8.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>19:0&lt;sup&gt;f&lt;/sup&gt;</td>
<td>71.3 ± 3.4</td>
<td>44.7 ± 5.7</td>
<td>28.5 ± 0.1</td>
<td>4.6 ± 2.0</td>
<td>21.4 ± 1.5</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>19:1&lt;sup&gt;11,12-cyclo&lt;/sup&gt;</td>
<td>ND</td>
<td>3.4 ± 0.6</td>
<td>16.9 ± 0.9</td>
<td>ND</td>
<td>1.5 ± 1.3</td>
<td>22.5 ± 0.4</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> 17:0 and 17:0<sup>c</sup> are present in bacteria and bacteroids in trace amounts (below 1 mol%).
<sup>b</sup> Contains 16:1<sup>3oxo</sup> (derived from plant) and 16:1<sup>3oxo</sup> (from bacteria/bacteroids).
<sup>c</sup> ND, not detected.
<sup>d</sup> Contains 18:1<sup>3oxo</sup> (derived from plant) and 18:1<sup>11oxcyclo</sup> (from bacteria/bacteroids).

### Table III

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Leaf</th>
<th>Nodules</th>
<th>Root</th>
<th>Symbiosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mol %</td>
<td>mol %</td>
<td>mol %</td>
<td>mol %</td>
</tr>
<tr>
<td>16:0</td>
<td>11.7 ± 1.6</td>
<td>21.9 ± 1.3</td>
<td>29.4 ± 3.4</td>
<td>25.1 ± 4.0</td>
</tr>
<tr>
<td>18:0</td>
<td>3.9 ± 0.2</td>
<td>6.5 ± 0.4</td>
<td>6.8 ± 1.3</td>
<td>13.1 ± 8.5</td>
</tr>
<tr>
<td>18:1&lt;sup&gt;11-cyclo&lt;/sup&gt;</td>
<td>1.9 ± 0.1</td>
<td>12.2 ± 1.2</td>
<td>5.9 ± 1.6</td>
<td>22.3 ± 8.2</td>
</tr>
<tr>
<td>18:2&lt;sup&gt;9,12-cyclo&lt;/sup&gt;</td>
<td>4.8 ± 0.2</td>
<td>30.9 ± 2.1</td>
<td>33.4 ± 3.7</td>
<td>19.9 ± 5.1</td>
</tr>
<tr>
<td>18:3&lt;sup&gt;9,12,15&lt;/sup&gt;</td>
<td>74.8 ± 1.8</td>
<td>24.9 ± 1.8</td>
<td>22.0 ± 9.2</td>
<td>9.8 ± 2.1</td>
</tr>
<tr>
<td>Galactose</td>
<td>97.3 ± 1.5</td>
<td>83.6 ± 2.9</td>
<td>77.0 ± 3.7</td>
<td>87.5 ± 0.7</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.8 ± 1.5</td>
<td>16.3 ± 3.5</td>
<td>23.0 ± 4.6</td>
<td>12.5 ± 3.5</td>
</tr>
</tbody>
</table>

Galactolipids are highly abundant in plants and cyanobacteria, where they are required to support an optimal rate of photosynthesis (for a review see Ref. 22). Nonphotosynthetic bacteria were also shown to contain non-phosphorus lipids with sugars in their head groups. Glucose-containing lipids (mono- and diglucosyldiacylglycerol) represent abundant membrane lipids in many bacteria including *Bacillus subtilis*, *Staphylococcus aureus*, and *Acholeplasma laidlawii* (43, 44). Furthermore, sulfoquinovosyldiacylglycerol, a sulfolipid typically found in photosynthetic bacteria, is an abundant constituent of membranes in *Sinorhizobium meliloti*, and it was speculated that this non-phosphorus lipid might be critical for the symbiotic interactions with infected cells in legume nodules (45). However, a knock-out mutant of *Sinorhizobium* completely devoid of sulfolipid was not affected in root nodulation, demonstrating that this lipid is not essential for plant-host interac-

**DISCUSSION**
tions (45). Diglycosyldiacylglycerol lipids containing different sugars in their head groups are present in some rhizobia (46). Interestingly, high amounts of DGDG were found in Bradyrhizobium bacteroids, suggesting that this lipid is not restricted to photosynthetic organisms (26). However, during the present study, clear evidence was obtained that this lipid was not derived from the bacteroids but from the peribacteroid membrane and that it originates from plant lipid biosynthesis: (i) DGDG in symbiosomes was localized to the PBM, but it was absent from bacteroids; (ii) DGDG was also absent from bacteria isolated from liquid culture; and (iii) DGDG isolated from symbiosomes contained fatty acids typical for plant lipids but no bacterial fatty acids. In the present analysis, an additional step gradient centrifugation was employed for PBM and bacteroid separation. Thus, the bacteroid preparation by Tan and Hollingsworth (26) might have been contaminated with plant-derived PBM, explaining the presence of DGDG in this fraction.

The fact that DGDG, but not MGDG, its biosynthetic precursor, was detected in the PBM fraction indicates that the PBM was not contaminated with plastid membranes. However, this finding implies that DGDG must be transported from its site of synthesis to the extraplastidic membrane system. In Arabidopsis, the two DGDG syntheses DGD1 and DGD2 were localized to chloroplast envelope (47). Assuming that the two soybean DGDG syntheses are also plastid-localized, DGDG produced in the plastid must be transported to extraplastidic membranes such as the endoplasmic reticulum, the plasma membrane, or the PBM. The mechanism of such lipid trafficking remains unknown (for a review see Ref. 48).

The accumulation of DGDG in the PBM raises the question of its function within the infected plant cell. It has previously been shown that DGDG is found in extraplasmatic membranes (e.g. plasma membrane) during phosphate deprivation but that its content in these membranes is very low during normal growth conditions (21, 23). Therefore, it was crucial to determine whether or not the presence of DGDG in the PBM depends on the availability of phosphate in the growth medium. As shown in Table I, DGDG represents a major symbiosome lipid under normal as well as phosphate-limiting growth conditions. Furthermore, the two soybean genes DGD1 and DGD2

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**Fig. 5.** Expression of DGDG synthases DGD1 and DGD2 from soybean. Total RNA was isolated from different tissues and blotted onto nylon membranes. Under the hybridization conditions employed, no cross-reaction between DGD1 and DGD2 of soybean was observed (data not shown). *Top panels,* hybridization signal; *bottom panels,* 25 S rRNA stained with ethidium bromide. *A,* expression of DGD1 and DGD2 was induced in soybean leaves by phosphate deprivation. The plants were grown on sand with normal phosphate concentration or without phosphate as indicated. *B,* expression of DGD1 and DGD2 in leaves, roots, and mature nodules of soybean. *C,* nodulation induces expression of DGD1 and DGD2. The numbers indicate the days after inoculation with *Bradyrhizobium.* Total RNA was isolated from roots (day 0), from roots containing developing nodules (days 7 and 14), and from nodules without roots (days 14–50).

**Fig. 6.** *In situ* localization of *LjDGD1* and *LjDGD2* gene transcripts in *L. japonicus* root nodules. Transverse thin sections (7 μm) of nodules at 21 days post-infection with *M. loti* were hybridized with digoxigenin-11-UTP-labeled antisense RNA probe *in vitro* transcribed from *LjDGD1* and *LjDGD2* cDNA clones. The hybridization signal is visible as blue-purple precipitate. *A,* *LjDGD1* gene transcripts were mainly localized in the infected cells of the nodule central tissue (ct), whereas a weak hybridization signal was visible in the nodule inner cortical cells (ic) and the root central cylinder. *B,* strong *LjDGD2* expression was detected in the infected cells and the nodule vascular tissue. A weak hybridization signal was again visible in the nodule inner cortical cells and the root central cylinder. *C,* as a negative control, the sections were hybridized to sense digoxigenin-11-UTP-labeled RNA transcribed from *LjDGD1* and *LjDGD2* cDNA clones. In this case, no significant hybridization signal was visible. The bars represent 100 μm.
showing sequence similarity to Arabidopsis DGDG synthases were induced during nodulation, and the transcripts were abundant in infected cells. Because all of the plants used for these studies were raised under optimal phosphate concentrations, the synthesis and presence of DGDG in the PBM is independent of the availability of external phosphate.

During nodule development, the number of rhizobia in infected cells increases dramatically. This cell division requires membrane biosynthesis not only in the bacteria but also by the plant for the synthesis of PBM to enclose the bacteria in symbiosomes (Fig. 1 and Ref. 49). A large fraction of total cellular membrane biosynthesis not only in the bacteria but also by the plant was induced during nodulation, and the transcripts were showing sequence similarity to Arabidopsis DGDG synthases during this period.

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