Digalactosyldiacylglycerol Synthase Gene *MtDGD1* Plays an Essential Role in Nodule Development and Nitrogen Fixation

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Little is known about the genes participating in digalactosyldiacylglycerol (DGDG) synthesis during nodule symbiosis. Here, we identified full-length *MtDGD1*, a synthase of DGDG, and characterized its effect on symbiotic nitrogen fixation in *Medicago truncatula*. Immunofluorescence and immunoelectron microscopy showed that *MtDGD1* was located on the symbiosome membranes in the infected cells. β -Glucuronidase histochemical staining revealed that *MtDGD1* was highly expressed in the infection zone of young nodules as well as in the whole mature nodules. Compared with the control, *MtDGD1*-RNA interference transgenic plants exhibited significant decreases in nodule number, symbiotic nitrogen fixation activity, and DGDG abundance in the nodules, as well as abnormal nodule and symbiosome development. Overexpression of *MtDGD1* resulted in enhancement of nodule number and nitrogen fixation activity. In response to phosphorus starvation, the *MtDGD1* expression level was substantially upregulated and the abundance of non-phospholipid DGDG was significantly increased in the roots and nodules, accompanied by corresponding decreases in the abundance of phospholipids such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. Overall, our results indicate that *DGD1* contributes to effective nodule organogenesis and nitrogen fixation by affecting the synthesis and content of DGDG during symbiosis.

Keywords: DGDG, *Medicago truncatula*, *MtDGD1*, phospholipids, rhizobium-legume symbiosis, RNAi, symbiotic phenotype

Lipids, including phospholipids and glycolipids, are the main constituents of membranes and play key roles in the biological function processes of membranes. In many higher animals and yeast cells, phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) are the main components of membrane lipids. However, in plant

leaf cells, the contents of glycolipids, particularly galactolipids, are much higher than those of phospholipids (Dörmann and Benning 2002). Galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), the two most common nonphospholipids, account for approximately 80% of the total membrane lipids in plant cells, and are also the main components of chloroplast thylakoid membranes (Dörmann and Benning 2002). It has been reported that MGDG and DGDG are enriched in thylakoids to maintain their stability (Krumova et al. 2010; Shimojima and Ohta 2011), the functional intactness of the complexes of photosystems I and II (Härtel et al. 1997; Reifarh et al. 1997), and the thermosensitivity of the photosynthetic apparatus (Essemine et al. 2011). Interestingly, the nonredundant function of MGDG and DGDG was found in systemic acquired resistance in plants, and DGDG was reported to be essential for pathogen-responsive nitric oxide and salicylic acid accumulation (Gao et al. 2014). MGDG can be converted into DGDG and oligogalactolipids under extreme conditions, and the generated DGDG would accumulate on the extraplastidic membranes to resist stressful environments (Slack et al. 1977). The symbiosis establishment of fungi and bacteria—namely, *Rhizopus microsporus* (Mucoromycotina) and endobacteria in genus *Burkholderia*—was found to be accompanied by specific changes in the fungal lipid profile. Diacylglycerol kinase (DGK) activity is critical for these lipid metabolic changes when DGK inhibition alters the fungal lipid profile, resulting in a shift from the host–bacteria interaction to antagonism (Lastovetsky et al. 2016). All of these studies indicate the importance of host lipid remodeling in the interaction between the eukaryote and bacteria or the environment.

Nodule formation and development involve mutualistic interactions between legume plants and rhizobia and are essential for symbiotic nitrogen fixation (Oldroyd et al. 2011). In response to the symbiotic signal from the roots of plants, the rhizobia invade root hairs through infection threads (IT), and eventually reach cortical cells and enter infected plant cells, where each rhizobium cell is wrapped by a membrane of plant origin and is separated into a compartment (Jones et al. 2007). The membrane is formed by invagination of the plasma membrane of plant cells, in which the rhizobia are deposited in a process resembling endocytosis. These compartments, consisting of one or more bacteroids surrounded by a plant-derived membrane, are called symbiosomes. The membrane of symbiosomes is also called the peribacteroid membrane (PBM), which serves as the boundary of plant cell cytoplasm and bacteroids. The symbiosome responsible for symbiotic nitrogen fixation is a functional unit in nodules. Symbiosome membranes regulate the exchanges of ammonium, nutrients, and signaling molecules between the bacteroids and host plants (Suzaki and Kawaguchi 2014). The function of the symbiosome

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membrane in symbiotic nitrogen fixation depends on its specific composition of proteins and lipids. The symbiosome membrane is both the structural and functional interface between plant cells and bacterial cells.

Notably, DGDG was also detected in the roots, leaves, symbiosomes, and symbiosome membranes of soybean (*Glycine max*) and *Lotus japonicus* but is not present in bacteroids and free-living rhizobial cells, suggesting that DGDG is a type of host plant-synthesized lipid which is transported into the symbiosome. Accumulated DGDG in nodules and symbiosome membranes might help to save phosphorus for effective legume-rhizobium symbiosis (Gaude et al. 2004). In another study, *Bradyrhizobium* and soybean nodules grown in nitrogen-deficient soil showed dramatic increases in the levels of MGDG and DGDG in the leaves (Narasimhan et al. 2013). However, there have been no reports about the variation of DGDG in *Medicago truncatula* in response to environmental stress. Lipids in the host-derived membrane are considered to be synthesized in the endoplasmic reticulum and eventually transported to the PBM through the Golgi apparatus (Brewin 1990). In Chinese milk vetch (*Astragalus sinicus*), a nodule-specific lipid transfer protein, AsE246, which is specifically expressed in nodules, could bind the plant-synthesized membrane lipid DGDG in vivo. Furthermore, the knockout of AsE246 not only reduced the formation of infection threads and nodule primordia but also decreased the content of lipids, particularly that of DGDG in the nodules, suggesting that AsE246 is involved in the transport of lipids into the symbiosome membrane. In addition, it was confirmed that DGDG was colocalized with the bacteroids and was localized on the symbiosome membranes (Lei et al. 2014). All of these studies indicate that symbiotic nitrogen fixation has important effects on membrane lipid remodeling and DGDG biosynthesis plays an important role in nodule development and symbiotic nitrogen fixation. However, these effects have not been fully characterized yet.

The biosynthesis of DGDG in *Arabidopsis thaliana* was reported to be involved in the galactosylation of MGDG by the galactolipid:galactolipid galactosyltransferase (GGGT), and three genes, including digalactosyldiacylglycerol synthase 1 (*DGD1*), *DGD2* and *GGGT*, are known to encode DGDG synthases (Benning and Ohta 2005). The *dgd1* mutant of *A. thaliana* showed a 90% decrease in the content of DGDG and severely suppressed growth. However, there was little difference in phenotype between the *dgd2-1* mutant and the wild type (WT) (Kelly et al. 2003). In addition, *DGD2* was expressed at low levels under normal conditions but strongly induced under phosphorus deprivation conditions (Kobayashi et al. 2009). Reverse genetic analysis showed that *GGGT* is a cold-sensitive gene in *A. thaliana*, indicating that *GGGT* is not essential for the normal growth and development of plants (Moellering et al. 2010). These previous findings suggest that *DGD1* is critical for the biosynthesis of the bulk amount of DGDG in the chloroplasts (Kelly and Dörmann 2002). It is generally known that *DGD1* is mainly responsible for the biosynthesis of DGDG in plant cells under normal conditions.

As described above, a large amount of DGDG needs to be synthesized by the host plants to facilitate rhizobial infection, symbiosome development, and nitrogen fixation; however, the biological function of DGDG in the nodules of legume plants is not fully understood. The symbiosis phenotypes have not been characterized when DGDG synthesis is affected. In particular, little is known about the functions of those genes involved in DGDG biosynthesis in symbiotic interactions between legume plants and rhizobia. In addition, *M. truncatula* forms indeterminate-type nodules and is different from soybean and *L. japonicas*, which form determinate-type nodules. Hence, it is highly essential to clarify the function of DGDG in symbiotic nitrogen fixation in *M. truncatula*. In addition, there have been

few reports about the changes in the biosynthesis and abundance of DGDG in response to low phosphorus stress during legume-rhizobia symbiosis under nitrogen-deficient conditions.

This study identified *MtDGD1*, a synthase of DGDG, and characterized its effect on the symbiotic nitrogen fixation in *M. truncatula* through a series of experiments, including promoter cloning, in situ expression pattern analysis, protein subcellular localization, symbiotic phenotype analysis of transgenic plants with gene silencing or overexpression, and determination of changes in DGDG accumulation in response to low phosphorus stress. Our results are expected to clarify the relationship between the *MtDGD1* gene related to DGDG biosynthesis and DGDG accumulation in nodules as well as the effect of the *MtDGD1* gene on nodulation and nitrogen fixation. Our findings provide novel insights into the membrane lipid remodeling in root nodule development and nitrogen fixation.

RESULTS

Cloning and phylogenetic characterization of *MtDGD1* gene.

We performed a blast analysis based on the query sequences available in a previous publication (Gaude et al. 2004), including AY635907 (Gm-DGD1), AY635908 (Gm-DGD2), Y635909 (Lj-DGD1), and AY635910 (Lj-DGD2). The results showed that, in the *M. truncatula* genome, there were two DGD1—namely, XM_013594759.2 (Medtr7g106250.1) and XM_013612439.2 (Medtr1g057700.1)—and four DGD2—namely, XM_024775447.1 (Medtr1g047640.2), XM_013611780.2 (Medtr1g047640.1), XM_003620822.3 (Medtr6g091670.1), and XM_003623811.3 (Medtr7g076400.1). The transcriptome data (Young et al. 2011) indicated that the transcript levels of XM_013594759.2 and XM_003620822.3 were the highest in the nodule (Supplementary Fig. S1), which were therefore chosen for further analysis and named MtDGD1 and MtDGD2, respectively.

Based on the data from phytozome v11.0, PCR primers were designed to clone the gene *MtDGD1* (accession number XM_013594759.2). The full-length cDNA of *MtDGD1* was obtained, which contained an open reading frame of 2,343 bp that encodes a protein of 780 amino acid (aa) residues with a putative molecular weight of approximately 88 kDa. The full-length cDNA of *MtDGD2* (accession number XM_003620822.3) contained an open reading frame of 1,392 bp that encodes a protein of 464 aa residues with a putative molecular weight of approximately 53 kDa. Conserved domain analysis showed that the proteins MtDGD1 (accession number XP_013450213.1) and MtDGD2 (accession number XP_003620870.1) belonged to the glycosyltransferase GTB-type superfamily (Supplementary Fig. S2). MtDGD1 and MtDGD2 had a glycosyltransferase GTB-type superfamily domain at the positions of 535 to 670 and 1 to 464 aa, respectively, in the C terminal. Amino acid sequence alignment of MtDGD1 and another DGD1 (XP_013467893.1) indicated that MtDGD1 was longer than XP_013467893.1 in the N terminal, and they had a high degree of homology at the C terminal. Amino acid sequence alignment of MtDGD2 and three other DGD2 proteins (XP_024631215.1, XP_013467234.1, and XP_003623859.1) indicated that these four DGD2 proteins had almost identical amino acid sequences. In addition, the amino acid sequence of MtDGD1 was compared with that of DGD1 in other higher plants, including leguminous plants. The results revealed that MtDGD1 had 91, 88, and 87% similarities in amino acid sequence to the DGD1 in *Cicer arietinum*, *L. japonicus*, and soybean (*G. max*), respectively (Supplementary Table S1) but only a 71% similarity to the DGD1 in *Arabidopsis*. In addition, a maximum-likelihood phylogenetic analysis of these DGD1 proteins was performed using MEGA6.0 software. The phylogenetic tree (Fig. 1) indicated that DGD1

Fig. 1. Phylogenetic analysis of the MtDGD1 protein. BLASTP of MtDGD1 and its homologs from phytozome v11.0 and NCBI was performed. Accession numbers of the proteins are indicated in the subsequent parentheses. The maximum-likelihood method was used for the phylogenetic analysis. Evolutionary analyses were performed by MEGA6.0 software. Bootstrap values were from 1,000 replications.

proteins are widely present in higher plants, including leguminous plants.

Expression and localization of *MtDGD1* protein during symbiosis.

To determine the subcellular location of *MtDGD1* under symbiotic conditions, an immunofluorescence analysis of WT *M. truncatula* inoculated with green fluorescent protein (GFP)-labeled *Sinorhizobium meliloti* 1021 was carried out. The anti-*MtDGD1* antibody was used and its specificity was confirmed via Western blotting (Supplementary Fig. S3). The nodules were harvested at 35 days postinoculation (dpi). The localization of *MtDGD1* was observed by a laser confocal microscopy. The observation showed a substantial overlap of CY3-red and GFP-green fluorescence in the nodules and infected cells (Fig. 2C and 2D), and no red fluorescence was found in the negative nodule slides without the addition of *MtDGD1* antibodies (Fig. 2A) or with the addition of preimmune sera (Fig. 2B). These results indicated that *MtDGD1* was colocalized with bacteroids in the infected cells. Considering that DGDG is a plant-derived lipid and accumulates in the PBM (Gaude et al. 2004), it can be hypothesized that *MtDGD1* may be localized on the symbiosome membrane. Immunoelectron microscopy was implemented to determine the subcellular localization of *MtDGD1* during symbiosis. The results showed that *MtDGD1* was located on the symbiosome membrane (Fig. 3C to F), and no immunogold signal was observed in the negative control without the addition of *MtDGD1* antibody (Fig. 3A) or with the addition of preimmune sera (Fig. 3B). All of these results indicated that *MtDGD1* is localized on the symbiosome membrane. In addition, the immune

Fig. 2. Immunolocalization of *MtDGD1* in the nodules of *Medicago truncatula*. Different signal dots indicate green fluorescent protein (GFP)-labeled *Sinorhizobium meliloti* 1021 or protein *MtDGD1*. Secondary antibody is tagged with CY3. **A** and **B**, Negative control (CK-); **A**, without the addition of *MtDGD1* antibody and **B**, with the addition of the preimmune sera. Other treatments were the same as those in the experimental group. No red fluorescence CY3 signal was detected. **C**, Immunolocalization of *MtDGD1* in the root nodules. The signal was only observed in the infected cells. **D**, Immunolocalization of *MtDGD1* in infected cells. The signal was observed to be specifically colocalized with symbiosomes in root nodules. Bars = 250 μ m (**A**, **B**, and **C**) and 10 μ m (**D**).

localization of *MtDGD1* in the roots of *M. truncatula* was examined, and the results showed that *MtDGD1* was located in the root hair cells and the membrane of root cells (Supplementary Fig. S4).

Expression pattern of *MtDGD1* during nodule development.

The gene expression profiles in *M. truncatula* revealed a higher transcript level of *MtDGD1* in nodules than in roots, as well as in the nitrogen-fixation zone (zone III) than in other zones of the whole nodule (Supplementary Fig. S5). To validate this result, real-time PCR was performed to examine the spatial transcription of *MtDGD1* in different tissues inoculated or uninoculated with rhizobia. The transcript levels of *MtDGD1* in the stems, leaves, and roots inoculated with *S. meliloti* 1021 were upregulated compared with those of the control group uninoculated with rhizobia, and the plants watered with nitrogen-free Fahraeus nutrient solution (NFS). The highest transcript level of *MtDGD1* was observed in the leaves, followed by the nodules (Fig. 4A). To further determine whether *MtDGD1* is involved in nodule development or nitrogen fixation, the temporal expression pattern of *MtDGD1* was also examined. The results showed that the transcript levels of *MtDGD1* in the roots and nodules increased gradually at 5 dpi and reached the maximal level at 15 dpi (Fig. 4B). In addition, we analyzed the gene expression pattern of *MtCOPT1* (XM_003608827), the copper transporter 1 of *M. truncatula*,

Fig. 3. Ultrastructural localization of *MtDGD1* on the symbiosome membranes in root nodules. **A**, Negative control without the addition of *MtDGD1* antibody. **B**, Negative control with addition of the preimmune sera as the first antibody. **C**, **D**, **E**, and **F**, Immunogold signals were detected (but not in **A** and **B**). The 10-nm gold particles were present on the symbiosome membranes (immunogold signal is marked as black dots, indicated by black arrowheads). Bars = 300 nm (**A** and **C**), 500 nm (**B** and **D**), and 200 nm (**E** and **F**).

whose expression is known to be specific to nodules (Senovilla et al. 2018). The results showed that *MtCOPT1* was only expressed in root nodules, and reached the highest level at 15 dpi (Fig. 4C). In addition, the transcript levels of housekeeping gene *MtEF1- α* (XM_013595882.2) were also checked. The results showed that the expression of *MtEF1- α* was rather stable, without remarkable changes in the process of nodule development (Fig. 4D). These findings indicated that *MtDGD1* is involved in nodule development or nitrogen fixation.

To further analyze the spatial expression pattern of the *MtDGD1* gene during nodule development, transformation of *M. truncatula* was carried out. *M. truncatula* transfected with a plasmid pDX2181G-mcherry containing a fusion of the *MtDGD1* promoter (a 2.4-kb fragment upstream of the *MtDGD1* ATG codon) served as the treatment group, while *M. truncatula* transfected with an empty plasmid pDX2181G-mcherry was used as the control. The transgenic roots were confirmed and then inoculated with *S. meliloti* 1021. Histochemical β -glucuronidase (GUS) staining was performed to analyze the spatial distribution of the GUS activity (Sessions et al. 1999). As a result, GUS activity was detected in the vascular cylinder and the tip of transgenic roots (Fig. 5B), as

well as in the nodule primordia at 5 dpi (Fig. 5C). It was noteworthy that *MtDGD1* was highly expressed in the infection zone of young nodules (Fig. 5E). In 15-dpi nodules, GUS activity was observed in the cells in the infection zone but not found in the cells in the nitrogen fixation zone (Fig. 5E and F). However, GUS activity was detected in the infection zone as well as in the entire nodule at 21 and 32 dpi (Fig. 5H, I, K, and L). No GUS activity was detected in the control group (Fig. 5A, D, G, and J). Both histochemical GUS and real-time PCR analysis showed the highest expression level of *MtDGD1* in the nodules. These results demonstrated a dynamic expression pattern of *MtDGD1* during nodule development, suggesting that *MtDGD1* affects nodule development and symbiotic nitrogen fixation.

Overexpression of *MtDGD1* in hairy roots increased the nodule number.

Transgenic *M. truncatula* plants overexpressing *MtDGD1* were generated by transformation utilizing *Agrobacterium rhizogenes* MSU440 and confirmed by GUS staining. Nodules were collected at 28 dpi, and the transcript levels of *MtDGD1* were examined by real-time PCR. The transcript level of

Fig. 4. Expression patterns of *MtDGD1* under symbiosis in *Medicago truncatula*. **A**, Tissue-specific transcription of *MtDGD1* in the roots (R), stems (S), leaves (L), and nodules (N) at 21 days postinoculation (dpi). Noninoculated plants watered with nitrogen-free Fahraeus nutrient solution served as the control. **B**, Transcription of *MtDGD1* in the roots and nodules at 5, 9, 15, 21, and 32 dpi. **C**, Transcription of *MtCOPT1* in the roots and nodules at 5, 9, 15, 21, and 32 dpi. **D**, Transcription of *MtEF1- α* , a housekeeping gene in the roots or nodules at 5, 9, 15, 21, and 32 dpi. All of the transcript levels were determined using *GAPDH* as the internal standard gene. Error bars represent the standard deviations of three independent experiments.

MtDGD1 in the transgenic nodules was 38-fold that in the control nodules transfected with the empty vector pCambia 2301 (Supplementary Fig. S6). Liquid chromatography–mass spectrometry (LC-MS) analysis was used to measure the abundance of DGDG in *MtDGD1*-overexpressing transgenic nodules. The results showed slightly higher abundance of DGDG in transgenic nodules than in the control (Supplementary Table S3). Additionally, symbiotic phenotypes were examined at 28 dpi. The results showed that the nitrogenase activity of the transgenic nodules was slightly higher than that of the control, and the average number of nodules on the

transgenic roots was 23.78 per hairy root ($n = 20$), which was significantly higher than that on the control roots (17.6 per hairy root, $n = 20$) (Table 1).

MtDGD1 RNA interference-impaired nodule development and nitrogen fixation.

To address the functional phenotypes of *MtDGD1* in nodule development, RNA interference (RNAi) was used to suppress the expression of *MtDGD1*. Real-time PCR showed that the RNAi efficiency of *MtDGD1* in transgenic nodules was approximately 70% (Fig. 6D) lower than that in the control nodules

Fig. 5. Temporal and spatial in situ expression of *MtDGD1* during symbiotic conditions. **A, D, G, and J**, Control group with empty plasmid DX2181G-mcherry. **B and C**, Transgenic plants in β -glucuronidase (GUS) staining at 5 days postinoculation (dpi); **E and F**, transgenic plants in GUS staining at 15 dpi; **H and I**, transgenic plants in GUS staining at 21 dpi; **K and L**, transgenic plants in GUS staining at 32 dpi. Bars = 500 μ m (**A, F, K, and L**), 100 μ m (**C, D, G, H, I, and J**), and 200 μ m (**B and E**).

transfected with the empty vector pCAMBIA1301-35S-int-T7. We also checked the transcript level of *MtEF1-α* in the transgenic RNAi nodules. The results indicated that the transcript level of *MtEF1-α* was unchanged (Fig. 6D). In addition, we noted that *MtDGD1*-RNAi did not suppress the transcription of *MtDGD2* in the nodules. Conversely, the transcript level of *MtDGD2* was increased compared with that in the control nodules (Fig. 6D). The control plantlets displayed vigorous growth, with green leaves and red nodules, whereas the RNAi transgenic plantlets were small, with chlorotic leaves and white, small nodules (Fig. 6A to C). Symbiotic phenotypes were further examined and quantified at 28 dpi. The results showed that the fresh weight of the aerial parts of the RNAi transgenic plantlets was significantly lower than that of the control (Table 1). Moreover, the

nodule nitrogenase activity ($9.51 \mu\text{mol g nodule}^{-1} \text{h}^{-1}$, $n = 9$) and number of root nodules (10.4 , $n = 20$) per hairy root on the RNAi hairy roots were significantly decreased compared with those in the control (Table 1).

To further clarify the influence of *MtDGD1* RNAi on nodule formation and development, paraffin sections of *MtDGD1*-RNAi nodules at 28 dpi were observed. Significantly fewer infected cells and rhizobia were observed in *MtDGD1*-RNAi nodules, and most infected cells were localized in the infection zone (Fig. 7B and D). Few infected cells were found in the nitrogen fixation zone of the nodules. The infected cells displayed deformed morphologies with indistinct boundaries compared with the control (Fig. 7A to D). In addition, ultrastructural comparison of the *MtDGD1*-RNAi

Table 1. Symbiotic phenotypes of *MtDGD1*-RNAi and over-expression transgenic plants^a

Group	Nodule nitrogenase activity ($\mu\text{mol g nodule}^{-1} \text{h}^{-1}$)	Shoot fresh weight (g/hairy plant)	Nodule number (per hairy root)
MtDGD1-CK	23.90 ± 4.03	0.35 ± 0.13	17.6 ± 5.36
MtDGD1-RNAi	$9.51 \pm 1.50^*$	$0.06 \pm 0.015^*$	$10.4 \pm 2.8^*$
MtDGD1-OX	25.64 ± 2.76	0.37 ± 0.16	$23.78 \pm 4.8^*$

^a Symbiotic phenotypes (nodule nitrogenase activity, shoot fresh weight, and nodule numbers per hairy root) were examined at 28 days postinoculation. MtDGD1-CK was the transgenic plants transfected with the empty vector. MtDGD1-RNAi and MtDGD1-OX were transgenic plants transfected with the RNA interference and the overexpression vector, respectively. Asterisks (*) indicate significant differences between the two groups at the level of $P < 0.05$. The data represent three independent experiments ($n = 20$).

Fig. 6. Nodulation phenotype and digalactosyldiacylglycerol (DGDG) abundance of nodules in *MtDGD1*-RNA interference (RNAi) transgenic plants. **A**, Appearances of the control (the left) and *MtDGD1*-RNAi hairy root plants (the right); **B**, appearance of root nodules on control hairy roots; **C**, appearance of root nodules on *MtDGD1*-RNAi hairy roots; **D**, relative transcript levels of *MtDGD1*, *MtDGD2*, and *MtEF1-α* in overexpression nodules; **E**, DGDG content in *MtDGD1*-RNAi nodules. Asterisks: ** indicates extremely significant differences at the $P < 0.01$ level (Dunnnett's multiple comparison test) and * indicates significant differences at the $P < 0.05$ level. All of the transcript levels were determined using *GAPDH* as the internal standard gene. Error bars represent the standard deviations of three independent experiments. Bars = 2 cm (A) and 2 mm (B and C).

and control nodules was conducted by transmission electron microscopy (TEM). The results showed that the symbiosomes of the control nodules developed normally, with rounded smooth boundaries and homogeneous cytoplasm (Fig. 7E and G); in contrast, most of the symbiosomes in the *MtDGD1*-RNAi nodule cells were smaller in size and displayed abnormal morphologies. In addition, the symbiosomes in *MtDGD1*-RNAi nodules exhibited enlarged PBM spaces and wrinkled membranes. Most symbiosomes showed severe shrinking and significant increases in electron density, with a quite dark color (Fig. 7F and H). These characteristics might be attributed to the accumulation of precursors for DGDG synthesis in the symbiosomes. Overall, it could be inferred that *MtDGD1* RNAi impaired nodule development and nitrogen fixation. Based on these findings, it can be hypothesized that *MtDGD1* plays an essential role in symbiosome development and nitrogen fixation.

Knockdown of *MtDGD1* affected the abundance of DGDG in the nodules.

LC-MS was performed to measure the abundance of DGDG in *MtDGD1*-RNAi nodules. The results showed that the abundance of DGDG in *MtDGD1*-RNAi nodules was significantly decreased by approximately 50% compared with that of the control, suggesting that *MtDGD1* participates in DGDG synthesis in the nodules of *M. truncatula* (Fig. 6E).

Low phosphorus treatment affected *MtDGD1* expression and lipid abundance in the roots and nodules.

Under phosphorus starvation, the synthesis of phospholipids was decreased while that of galactolipids was increased. This membrane lipid remodeling is a defense mechanism of plants to survive under low phosphorus conditions (Klaus et al. 2002; Shimojima et al. 2015). In this study, we investigated the influence of low phosphorus treatment on the transcription of *MtDGD1* and lipid abundance in the roots and nodules under nonsymbiotic or symbiotic conditions.

Under nonsymbiotic conditions, the *MtDGD1* transcript levels in the uninoculated roots after low-phosphorus (LP) Hoagland's treatment (1 mmol KH_2PO_4 in Hoagland's was replaced by 5 μmol KH_2PO_4) for 21 days were 13-fold higher than those in the control watered with normal Hoagland's (Hoagland and Arnon 1950; Xie et al. 2013). At the same time, the transcription of *MtDGD2* was also induced by low phosphorus treatment (up to 2.7-fold higher than the control) (Fig. 8A). A phosphate transporter gene *MtPT2* (AF000355.1), whose expression was enhanced under low phosphorus conditions in *M. truncatula* (Liu et al. 1998), was used as a positive control in this study. The results indicated that the transcript level of *MtPT2* was significantly increased under low phosphorus conditions (Fig. 8A). The transcript level of *MtEF1- α* was quite stable (Fig. 8A). The abundance of all phospholipids such as PC, PE, and PI decreased, while that of non-phospholipid DGDG significantly increased by threefold compared with that in the control (Fig. 8B). In addition, to examine the effect of low phosphorus treatment on *MtDGD1* expression and lipid level under symbiotic conditions, the plants watered with NFS (Fahraeus 1957) or suffered from LP NFS (LP NFS: Na_2HPO_4 in NFS was replaced by Na_2SO_4 at 0.4 mmol liter^{-1} , and KH_2PO_4 at 0.1 g liter^{-1} was replaced by KH_2PO_4 at 5 μmol liter^{-1} and KCl at 0.7 mmol liter^{-1}) (Wang et al. 2015) were inoculated with *S. meliloti* 1021. The results showed that the transcript levels of *MtDGD1* in roots and nodules were elevated to 17- and 2-fold, respectively, of that in the control at 21 dpi. However, the transcript level of *MtDGD2* displayed no significant changes in both the roots and nodules. The transcript level of the control gene *MtPT2* was 2.7-

and 6-fold that in the control roots and nodules, respectively, while that of the control gene *MtEF1- α* showed little change (Fig. 9A). In addition, the content of DGDG in the LP roots and nodules was increased to three- and twofold, respectively, of that in the control watered with normal NFS. However, the contents of PC, PE, and PI were reduced by 94.8, 59.4, and 44.9% in the LP roots and by 41.2, 58.1, and 36.6% in the nodules, respectively (Fig. 9B). These data showed that the expression of *MtDGD1* and the abundance of DGDG increased correspondingly in the roots and nodules under phosphorus starvation, whereas the phospholipids were reduced.

These results indicated that the membrane lipids in legume host plants are remodeled under nitrogen and phosphorus nutrient deficiency, resulting in the accumulation of DGDG

Fig. 7. Microscopic observations of the development of root nodules and symbiosomes in *MtDGD1*-RNA interference (RNAi) experiment. **A** to **D**, Paraffin sections of nodules and toluidine blue staining. **A**, Longitudinal section of nodule at 28 days postinoculation (dpi) from the control with empty vector; **B**, longitudinal section of a *MtDGD1*-RNAi nodule at 28 dpi; **C**, magnification of nitrogen-fixing zone (fixz) in **A**; and **D**, Magnification of fixz in **B**. Abbreviations: mer = meristem and infz = infection zone. Bars = 500 μm (**A** and **B**) and 100 μm (**C** and **D**). **E** to **H**, Transmission electron microscopy (TEM) images of symbiosomes of nodule. **E**, TEM images of symbiosomes of the control nodule with empty vector at 28 dpi; **F**, TEM images of symbiosomes of *MtDGD1*-RNAi nodule at 28 dpi; **G**, magnification of symbiosomes in **E**; and **H**, magnification of symbiosomes in **F**. Bars = 1 μm (**E** and **F**) and 500 nm (**G** and **H**).

in the roots and nodules to facilitate symbiotic nitrogen fixation.

DISCUSSION

Glycerol glycolipids were first extracted and identified in wheat (Carter et al. 1956), and were reported to be the main components of the photosynthetic membranes in cyanobacteria and higher plants (Kobayashi et al. 2007; Murata and Nishida 1987). As the main lipid of the thylakoid membrane, DGDG is also present on the PBMs of the legume plants *G. max* and *L. japonicas*. This finding indicates that DGDG plays an essential role in photosynthesis and symbiotic nitrogen fixation (Dörmann et al. 1999; Gaude et al. 2004; Sato and Awai 2016).

MtDGD1 was identified as a galactosyltransferase-like protein that could catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules to form glycosidic bonds (Albesa-Jové et al. 2014; Blanco Capurro et al. 2017; Kozmon and Tvaroška 2006). DGDG is a kind of glycoconjugate. It is possible that the biosynthesis of DGDG in *M. truncatula* is similar to that in *Arabidopsis*, in which DGDG is produced by transferring a galactosyl residue

from uridine diphospho-galactose to MGDG (Holzl and Dörmann 2007).

However, the functional phenotype and mechanism of DGDG in nodule development and symbiotic nitrogen fixation still remain elusive. In particular, the genes involved in DGDG synthesis during nodule symbiosis have not been elucidated. In this study, we identified the symbiotic phenotypes of the *MtDGD1* gene which encode a DGDG synthase in *M. truncatula*. Direct experimental evidence was obtained to characterize the function and phenotype of the *MtDGD1* gene during early infection and nodule symbiosis. The results provide novel insights into DGDG synthesis and its significance in nodule development and nitrogen fixation.

Our results revealed that *MtDGD1* plays an important role in the synthesis of DGDG in nodules under symbiotic conditions. The real-time quantitative PCR (qPCR) showed that *MtDGD1* expression was induced by rhizobia, and was significantly upregulated in the stems, leaves, and roots. The spatial

Fig. 8. *MtDGD1* expression and digalactosyldiacylglycerol abundance in roots in response to low phosphorus (LP) under nonsymbiotic conditions. **A**, Transcript levels of *MtDGD1*, *MtDGD2*, *MtPT2*, and *MtEF1-α* in uninoculated roots after LP treatment on day 21. **B**, Abundance of different lipid components in uninoculated roots after LP treatment on day 21. Asterisks: * indicates significant differences between the two groups ($P < 0.05$) and ** indicates extremely significant differences between the two groups ($P < 0.01$). All of the transcript levels were determined using *GAPDH* as the internal standard gene. Error bars represent the standard deviations of three independent experiments.

Fig. 9. *MtDGD1* expression and digalactosyldiacylglycerol (DGDG) abundance in roots and nodules in response to low phosphorus (LP) under symbiotic conditions. **A**, Transcript levels of *MtDGD1*, *MtDGD2*, *MtPT2*, and *MtEF1-α* in infected roots and nodules after LP treatment on day 21 and **B**, abundance of different lipid components in infected roots and nodules after LP treatment on day 21. Asterisks: * indicates significant differences between the two groups ($P < 0.05$) and ** indicates extremely significant differences between the two groups ($P < 0.01$). All of the transcript levels were determined using *GAPDH* as the internal standard gene. NFS = nitrogen-free Fahraeus nutrient solution, LP NFS = nitrogen free solution with low phosphorus, R = infected roots, N = nodules, PI = phosphatidylglycerol, PC = phosphatidylcholine, and PE = phosphatidylethanolamine. Error bars represent the standard deviations of three independent experiments.

expression analysis of *MtDGD1* revealed a dynamic expression pattern of *MtDGD1* during nodule development and, in particular, higher expression of *MtDGD1* was observed in the infection zone of young nodules. These observations suggest that the biosynthesis of DGDG is important for the whole progress of nodule development and nitrogen fixation. In addition, immunofluorescence and immunoelectron microscopy experiments also supported the hypothesis that *MtDGD1* participates in DGDG synthesis. MtDGD1 was observed in the infected cells of nodules and was colocalized with bacteroids, suggesting that *MtDGD1* is localized on the symbiosome membranes, and plays an important role in symbiosome development and lipid synthesis (Kelly and Dörmann 2002). Meanwhile, there was a significant decrease in DGDG content in the nodules after *MtDGD1* silencing. All of these results support the hypothesis that *MtDGD1* is a key gene involved in DGDG synthesis during nodule development. The findings provide new insights into the mechanism of membrane lipid biosynthesis and metabolism during legume–rhizobia symbiotic interaction.

Our results indicated that *MtDGD1* plays an essential role in nodule development and nitrogen fixation by affecting DGDG synthesis. DGDG is one of the main components of the symbiosome membranes and is essential for maintaining the integrity and stability of the symbiosome membrane structures required for effective symbiotic nitrogen fixation. In contrast to the control, *MtDGD1*-RNAi plants showed significant decreases in the abundance of DGDG in the nodules, which severely impaired the nodule development and nitrogen fixation, and ultimately resulted in the formation of fewer and smaller nodules. Some of the nodules were found to be white, with much lower nitrogenase activities and abnormal symbiosome morphologies, leading to nitrogen deficiency in the *MtDGD1*-RNAi plants. As a result, the plant growth was hindered. In addition, overexpression of *MtDGD1* increased the abundance of DGDG in the nodules as well as the number of nodules in transgenic plants but no other differences in symbiotic phenotype were observed between *MtDGD1* overexpressing transgenic plants and the control, suggesting that the increase in DGDG would facilitate nodulation. All the findings of this study are consistent with a previous report on the functional phenotypes of a lipid transfer protein gene *AsE246* in symbiotic nitrogen fixation (Lei et al. 2014). It should be noted that, in a recent study of the function mechanism of another lipid transfer protein gene, *MtN5*, in *M. truncatula*, overexpression of *MtN5* changed the lipid profiles of rhizobia-inoculated *MtN5ox* roots. Several lipids, including some DGDGs, one MGDG, the PE phospholipid, and the two GlcCer, showed higher signals in *MtN5ox* roots than in the WT roots, and the numbers of invaded primordia and mature nodules per plant were significantly increased in comparison with those in WT plants (Santi et al. 2017). All of these findings further confirm the essential role of DGDG in nodule formation and nitrogen fixation. In summary, our results suggest that MtDGD1 is involved in DGDG synthesis in nodules, and that this synthesis is essential for the development of symbiosomes and nitrogen fixation.

Taken together, our results demonstrate that DGDG has important biological functions in the progression of symbiosis and, thus, the possible functional mechanism should be further dissected.

First, the accumulation of the nonphospholipid DGDG under phosphorus starvation indicates that DGDG plays an important role in membrane lipid remodeling under the conditions of nitrogen and phosphorus nutrient deficiency. Because phosphorus is essential to plant growth and development, the effective supply of phosphorus can significantly promote the development of nodules and nitrogen fixation of plants, especially under nitrogen-deficient conditions (Leidi and

Rodríguez-Navarro 2000). Under phosphorus and nitrogen deficiency, DGDG instead of phospholipids can accumulate on the symbiosome membranes for possible membrane remodeling. This process could preserve the phosphorus elements for other important cellular activities in nodule development and nitrogen fixation. For normal and successful nodule development, phosphorus saving is necessary to meet the demand of the synthesis of phosphorus-containing biological macromolecules such as phosphorus-related proteins and nucleic acids during nodule development and nitrogen fixation (Narasimhan et al. 2013). In response to phosphorus stress, the components of the symbiosome membranes will be changed, and the original phospholipids will be degraded or replaced by nonphospholipid DGDG, which is a common phenomenon in plants. In brief, the accumulation of DGDG under symbiosis may also be a unique strategy of leguminous plants to preserve phosphorus for nodule development, particularly under phosphorous starvation.

Second, a previous study has reported a significant correlation between the MGDG and DGDG content in wheat leaves and resistance to wheat leaf spot caused by pathogenic fungi (Kim et al. 2013). Moreover, (Santi et al. 2017) reported that several genes involved in the response to biotic stress were modulated in *MtN5hp* roots, and two proteins (Medtr4g021037 and Medtr8g446950) involved in disease resistance and a cationic peroxidase (Medtr7g107520) were upregulated, suggesting that the content of glycolipids can affect disease resistance. In this study, a dynamic expression pattern was observed for *MtDGD1* in the infection zone of young nodules and in the entire mature nodules, suggesting the importance of DGDG biosynthesis in nodule development and symbiotic nitrogen fixation. Considering that root nodule formation is the outcome of interaction and coordination between symbiosis and defense, DGDG biosynthesis might be a possible strategy for the intracellular symbiosis between plants and rhizobia.

Finally, in the *dgd1* mutant of *Arabidopsis*, some excess MDGs were converted into a large quantity of jasmonic acid (JA), causing visible phenotypes such as extremely short inflorescence stems, lignified phloem cap cells, and ruffled leaves (Lin et al. 2016). This finding suggests that *DGD1* might also indirectly affect the symbiotic phenotypes by increasing JA levels, because JA was reported to be associated with nodule formation and nitrogen fixation (Isayenkov et al. 2005; Kinkema and Gresshoff 2008).

MATERIALS AND METHODS

Plant materials.

The accession Jemalong A17 of *M. truncatula* was used in this study. Seed were scratched lightly with a needle, surface sterilized in 2% hypochlorite (commercial bleach) for 2 to 5 min, then washed several times in sterile water. The seed were then immersed in distilled water for 3 h and placed in a medium with 0.5% sucrose and 1.2% agar for germination (Garcia et al. 2006). Germinated seed were planted in pots filled with sterile sand and watered with NFS. After cotyledon expansion, the plantlets were inoculated with or without *S. meliloti* 1021. All plants were grown in a greenhouse under daily conditions of 16 h of light and 8 h of darkness at 24 and 20°C, respectively.

Bioinformatic and phylogenetic analyses.

Based on the query sequences AY635907 (*Gm-DGD1*), AY635908 (*Gm-DGD2*), Y635909 (*Lj-DGD1*), and AY635910 (*Lj-DGD2*) (Gaude et al. 2004), two DGDG synthase 1 and four DGDG synthase 2 were obtained from the *M. truncatula* genome by blasting on the NCBI Standard Nucleotide BLAST website. The transcript levels of different DGDG synthases in

different tissues of *M. truncatula* were obtained from the transcriptome data (Young et al. 2011). The physical and chemical parameters of MtDGD1 were predicted using the ExPASy website. The sequences of MtDGD1 and MtDGD2 were blasted on the BLAST website, and their similarities to the homologs in higher plants, including other leguminous plants, were detected. Conserved domain analysis was performed on the NCBI conserved domains website. Amino acid sequence alignment was performed by DNAMAN software.

MtDGD1 homologous proteins were downloaded from the genomes of *M. truncatula* (Mt), *L. japonicus*, *G. max*, *Phaseolus vulgaris*, *Arabidopsis thaliana*, *Oryza sativa*, and *Nicotiana benthamiana* from phytozome v11.0 and the NCBI database. All alignments were performed by using ClustalW method. The maximum-likelihood method was used for the phylogenetic analysis. The phylogenetic tree was constructed with MEGA6.0 software. Bootstrap values were from 1,000 replications.

Cloning of full-length *MtDGD1* cDNA and plasmid construction.

Nodules were harvested at 40 dpi. Total RNA was extracted with the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The primers of full-length *MtDGD1* cDNA were 5' GAGCTCATGGCTACACAGCATCCACCTTC 3' and 5' CTGCAGCACAGTACAACCTACAACCTGAAC 3'. To construct the overexpression plasmid, the open reading frame of *MtDGD1* was amplified by reverse-transcription PCR using the abovementioned primers containing a *SacI* site and a *SalI* site at each 5' end of the primer. The amplification product was digested with *SacI*-*SalI* and ligated into the pCambia 2301 vector (Gene ID: AF234316). To generate the RNAi, a 287-bp fragment with a short part of the coding region of *MtDGD1* in the 3' untranslated region was amplified by reverse-transcription PCR using the following primers: 5' GAGCTC CACTATTGTCTCACAGGGAACG 3' containing a *Bam*HI or *Pst*I site at the 5' end and 5' GGATCCAACAAGCAGAAA GCCGAACCTAC 3' containing a *Sac*I or *Xba*I site at the 5' end. The amplification products were digested with *Bam*HI-*Sac*I or with *Pst*I-*Xba*I and ligated into the pCambia1301-35S-int-T7 plasmid vector (Chen et al. 2012). To generate the promoter-GUS vector, the promoter of *MtDGD1* was amplified with the following primers: 5' GCAGGTCGACGGATCCCCGGGGTT AACCTATTAACCTTGTCAAATG 3' containing 15 bp of pDX2181G-mCherry vector at a *Sma*I site upstream and 5' ATAAGGGACTGACCACCCGGGGCTTTTGTCTATATATCA ACAATC 3' containing 15 bp of pDX2181G-mCherry vector at a *Sma*I site downstream at each 5' end. The amplification product and pDX2181G-mCherry vector, a pDX2181 derivative (Ye et al. 2012), were linked with recombinase. The constructs were transferred into *Agrobacterium rhizogenes* MSU 440 by electroporation.

Antibody preparation.

The *MtDGD1* coding sequence (accession number XM_013594759) was amplified from complementary DNA. The upstream primer was 5' CATATGGCTACACAGCATCCACCTT 3' and the downstream primer was 5' GCGGCCGCACCAGCCA TAGATAGGACTTTC 3'. Considering that MtDGD1 and MtDGD2 had a high homology in the C-terminal sequence, the coding sequence of MtDGD1 was double digested into an N-terminal truncated fragment (576 bp, encoding 189 aa) with *Nde*I and *Hind*III. This truncated fragment was distinguished from MtDGD2 and then cloned into pET-28a(+). The fragment sequence was confirmed by sequencing. The recombinant vector pET-28a-*MtDGD1* was transformed into the host strain *Escherichia coli* BL21 DE3. The MtDGD1-truncated protein was purified from the *E. coli* lysate utilizing an HIS cartridge (Bio-Rad).

Purified MtDGD1 protein was used for immunization in New Zealand white rabbits to obtain the anti-MtDGD1 antibody.

RNA extraction and real-time PCR.

To study the expression patterns of *MtDGD1* during nodule development, leaves, stems, roots, and nodules were harvested at 21 dpi with or without *S. meliloti* 1021 inoculation. The leaves and nodules inoculated with *S. meliloti* 1021 were harvested at different times (days 5, 9, 15, 21 and 32). The LP stress experiment was performed as described previously (Wang et al. 2015). When the first cotyledon was fully extended, under nonsymbiotic conditions, the seedling was treated with Hoagland's or LP Hoagland's, with 1 mmol KH₂PO₄ in Hoagland's being replaced by 5 μmol KH₂PO₄; while, under the symbiotic conditions, the seedling was treated with NFS (Fahraeus 1957) or LP NFS, with Na₂HPO₄ in NFS being replaced by 0.4 mmol Na₂SO₄ and KH₂PO₄ at 0.1 g liter⁻¹ being replaced by 5 μmol KH₂PO₄ and 0.7 mmol KCl. The roots or nodules of *M. truncatula* were harvested on day 21.

Total RNA was extracted from each tissue sample using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Genomic DNA was eliminated by DNAaseI (Fermentas). The purity and concentration of each RNA sample were measured by a Nano Drop 2000 spectrophotometer (Thermo Scientific). After adjusting the amount of RNA, the cDNA was synthesized by RevertAid rReverse transcription (Fermentas) using oligo(dT)18 primers. The fluorescence qPCR was performed using the Faststart Universal SYBR Green Master Kits (Roche), and qPCRs were monitored and analyzed using the Applied Biosystems (ABI) StepOne. The transcript levels of *MtDGD1*, *MtDGD2*, *MtPT2*, *MtGAPDH*, *MtEF1-α*, and *MtCOPT1* were assayed by fluorescence qPCR. *MtGAPDH* served as an internal standard gene, and housekeeping gene *MtEF1-α* served as the other reference gene. All of the real-time PCR primers were listed in the Supplementary Table S2. The data were analyzed using the 2^{-ΔΔCT} method. Each experiment was repeated at least three times.

Immunofluorescence experiment.

Nodules were hand sectioned using a double-edged razor blade. Nodule sections or roots were fixed in 1% (wt/vol) freshly depolymerized paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4 for 30 min at 4°C. Nodule sections were blocked in 3% (wt/vol) bovine serum albumin (BSA) and further incubated with the primary antibody overnight at 4°C in PBS containing 0.3% (vol/vol) Triton X-100. The secondary antibody (goat antirabbit) conjugated with CY3, a red fluorescence dye, was used. The control experiment was performed without adding primary antibodies or with the addition of the preimmune sera. Nodule sections containing GFP-labeled *S. meliloti* 1021 were examined by a confocal microscopy. Antibody dilution was prepared as follows: anti-MtDGD1 antibody at 1:100 and goat antirabbit (CY3) at 1:1,000.

Microscopic analysis.

Microscopic analysis was performed as previously described (Li et al. 2008). The 28-dpi nodules were analyzed. For light microscopy, nodules were cut longitudinally, fixed in formalin-aceto-alcohol buffer, washed, dehydrated, and embedded in paraffin, and the slides were stained with toluidine blue, then observed with an Olympus light microscope. For electron microscopy analysis, nodules were fixed in 2.5% glutaraldehyde for 4 h, then post fixed in 1% osmium tetroxide for 3 h, washed, dehydrated through an ethanol series, and embedded in London resin white. Ultrathin sections were examined by TEM (H-7650; Hitachi).

Immunoelectron microscopy analysis.

Immunoelectron microscopy analysis was performed as reported previously (Limpens et al. 2009). The nodules were fixed by 4% paraformaldehyde in 2.5% glutaraldehyde, and postfixed with 1% osmium tetroxide for 3 h. After washing and dehydrating through an ethanol series, the tissues were embedded in LR White and polymerized at 60°C. Then, the nodules were cut into ultrathin sections (60 nm) by a Leica EM UC7 ultramicrotome. Nickel grids with sections (60 nm) were blocked with 2% (wt/vol) BSA in PBS and incubated with the primary antibody overnight at 4°C in PBS according to the dilutions described above. Goat antirabbit antibody coupled with 10 nm gold (1:1,000 dilution) was used as the secondary antibody and incubated for 2 h. The sections were examined by TEM (H-7650; Hitachi).

Plant transformation.

A. rhizogenes MSU440 cells containing the binary vectors were used to induce hairy root formation on *M. truncatula* seedlings, as described previously (Chabaud et al. 2006; Limpens et al. 2004). Briefly, radicles approximately 3 mm in length were cut from the root tip with a sterile scalpel when the seedlings had a radicle length of approximately 1 cm. Then, the sectioned surface was coated with *A. rhizogenes* by light scraping. The seedlings were transferred onto agar with modified Fahraeus medium and cocultivated with *A. rhizogenes* MSU440 for 5 days in a growth chamber. The plants were transferred onto hairy root emergence medium plates containing timentin at 300 µg/ml and grown for 10 more days until the development of hairy roots from the hypocotyls. To select the transgenic hairy roots, root tips of 2 to 3 mm were excised for GUS staining overnight at 37°C in a solution containing 100 mM sodium phosphate (pH 7.0), 0.1% Triton X-100, 0.1% N-lauroylsarcosine, 10 mM EDTA, 1 mM K₃Fe(CN)₆, 1 mM K₄Fe(CN)₆, and 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide cyclohexylammonium salt at 0.5 mg/ml. The remaining portion of the hairy root was labeled. If the hairy root tip was GUS negative, the whole hairy root would be discarded; if it was GUS positive, the remaining portion of the hairy root would be retained. One to three transgenic hairy roots were preserved for each seedling. Plants harboring transgenic hairy roots were transferred to pots filled with vermiculite and sand (1:1) and grown in a chamber with a cycle of 16 h (day) and 8 h (night) at 24 and 20°C, respectively. After 5 to 7 days, the plants were inoculated with *S. meliloti* 1021. Symbiotic phenotypes were examined 4 weeks after inoculation.

Nitrogenase activity measurement.

Nitrogenase activity was measured with the method of the acetylene reduction activity (Wych and Rains 1978). Nine hairy root lines from each sample were analyzed. The hypogeal parts, including the nodules and roots, were incubated for 2 h at 28°C in 30-ml glass bottles containing 2 ml of C₂H₂. The amount of ethylene was measured using an East & West Analytical Instrument GC 4000A gas chromatograph.

Lipid extraction and quantification.

To determine the lipid composition of the roots and nodules, plant tissues at different stages were harvested (100 mg) for lipid extraction, and were homogenized in liquid nitrogen (Bligh and Dyer 1959). Samples were resuspended in 50 ml of chloroform and 100 ml of methanol. Subsequently, they were sonicated three to five times. Afterward, 50 ml of chloroform and 50 ml of distilled and deionized water were added to the mixture. Then, the solution was transferred to a separatory funnel and allowed to stand for approximately 30 min to achieve complete clarification and separation. Finally, lipids extracted from the lower organic phase were

dried by rotary evaporation, then dissolved in methanol (Lei et al. 2014).

The lipid extracts were introduced into a quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent 6540 Accurate Mass Q-TOF LC-MS unit) operating in the positive mode. Lipids were supplied to the mass spectrometer in methanol. Q-TOF LC-MS was performed in an Agilent 6540 UHD accurate-mass Q-TOF LC-MS system with a Zorbax Eclipse Plus C18 column (2.1 × 100 mm, 3.5 µm). The sample of 1 µl was loaded at a flow rate of 0.3 ml min⁻¹ in solvent A (5% methanol and 10 mM NH₄AcOA) and solvent B (methanol). Solvent B was 25% (vol/vol) at the start, 100% (vol/vol) at 5 min, 100% (vol/vol) at 10 min, and 25% (vol/vol) again at 15 min. The acquisition range was 400 to 1,100 m/z; nebulizer pressure, 40 pounds per square inch gauge; drying gas, N₂ at 350°C and 9 liters min⁻¹; electrospray ionization voltage of capillary, 4,000 V; fragment of 214v; skimmer, 65 V; and octopole radio frequency programming mode voltage, 750 V. MGDG and DGDG were analyzed as [M+NH₄]⁺ and [M+Na]⁺ ions, respectively (Wu and Xue 2010).

Statistical analysis.

Statistical significance was analyzed using independent-sample *t* test and single-factor analysis of variance method by IBM SPSS Statistics, version 20. Three biological replicates were carried out for each experiment. The bars represent the standard deviations of three independent experiments in the figure legends. *P* values <0.05 or <0.01 (Dunnett's multiple comparison test) were considered statistically significant.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Phytozome v11.0:
https://phytozome.jgi.doe.gov/pz/portal.html#!search?show=BLAST&method=Org_Mtruncatula
Gene expression profiles in *Medicago truncatula*:
<https://iant.toulouse.inra.fr/symbimics/>
NCBI Standard Nucleotide BLAST website:
https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome
ExpASY website: <https://web.expasy.org/protparam/>
NCBI BLAST website: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>
NCBI conserved domains website:
<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?RID=U1GG5DSH015&mode=all>
DNAMAN software:
<https://dnaman-32-en-001.software.informer.com/>

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