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The SNARE Protein SYP71 Expressed in Vascular Tissues is Involved in Symbiotic Nitrogen Fixation in *Lotus japonicus* Nodules

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SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) proteins are crucial for signal transduction and development in plants. Here, we investigate a *Lotus japonicus* symbiotic mutant defective in one of the SNARE proteins. When in symbiosis with rhizobia, the growth of the mutant was retarded compared with that of the wild-type plant. Although the mutant formed nodules, these exhibited lower nitrogen fixation activity than wild-type. The rhizobia were able to invade nodule cells, but enlarged symbiosomes were observed in the infected cells. The causal gene, designated *LjSYP71*, was identified by map-based cloning and shown to encode a Qc-SNARE protein homologous to *Arabidopsis thaliana* SYP71. *LjSYP71* was expressed ubiquitously in shoot, roots, and nodules, and transcripts were detected in the vascular tissues. In the mutant, no other visible defects in plant morphology were observed. Furthermore, in the presence of combined nitrogen, the mutant plant grew almost as well as the wild-type. These results suggest that the vascular tissues expressing *LjSYP71* play a pivotal role in symbiotic nitrogen fixation in *L. japonicus* nodules.
INTRODUCTION

Intracellular membrane fusion in eukaryotic cells involves membrane-associated SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) proteins that contain a conserved coiled-coil domain and are anchored to the membrane by either a C-terminal transmembrane domain or a post-translational addition of lipids (Fukuda et al., 2000; McNew et al., 2000; Parlati et al., 2000). Transport vesicles carrying cargo fuse with the target endomembrane or the plasma membrane and their contents are delivered to different organelles or secreted to the extracellular space, respectively. A SNARE protein localized on the donor vesicle forms a tetrameric bundle of coiled helices with complementary SNARE proteins in the target membrane, which drives the fusion of transport vesicles with the proper target membrane.

These SNARE proteins are divided into v-SNAREs and t-SNAREs depending on their localization on the trafficking transport vesicle or the target membrane, respectively. However, this nomenclature does not apply to homotypic membrane fusion. Thus the SNARE proteins have been reclassified as R-SNAREs and Q-SNAREs based on the conserved arginine or glutamine residue in the center of the SNARE motif (Fasshauer et al., 1998). Furthermore, Q-SNAREs are subdivided into three classes designated Qa-, Qb- and Qc-SNARE depending on their SNARE motif domains. These Q-SNAREs create a three-helix bundle complex (t-SNARE) that interacts with R-SNARE (v-SNARE) in membrane fusion (Bassham and Blatt, 2008).

Syntaxins are one of the components of the t-SNARE complex. In the Arabidopsis thaliana genome, 24 genes were annotated to encode members of the syntaxin family, and were designated syntaxin of plants (SYP) (Sanderfoot et al., 2000). Based on sequence homology,
these 24 genes are primarily divided into eight groups named SYP1 to SYP8, each of which contains one to nine members. Further sequence analysis revealed that the SYP1 to SYP4 groups belong to the Qa-SNAREs subfamily while the SYP5 to SYP8 groups belong to the Qc-SNAREs subfamily (Uemura et al., 2004). All groups play essential roles in various aspects of signaling and development in plants by cooperating with other SNARE proteins (Lipka et al., 2007; Pfeffer, 2007; Bassham and Blatt, 2008).

In legumes, rhizobia generally attach to host plant root hairs, invade the root cortical cells through an infection thread, and inhabit the specialized organ formed on the host plant root, the root nodule. Rhizobia then differentiate into bacteroids that are enclosed by a plant-derived symbiosome membrane, and which reduce atmospheric dinitrogen to ammonia in the nodule cells. Recently, syntaxin SYP132 of the model legume *Medicago truncatula* was shown to be localized not only to the plasma membrane surrounding the infection thread but also abundantly to the symbiosome membrane, suggesting that MtSYP132 is involved in symbiosome formation (Catalano et al., 2007; Limpens et al., 2009). Intriguingly, this contrasts with the plasma membrane syntaxin SYP132 of *Nicotiana benthamiana* which is thought to be involved in plant resistance against pathogenic bacteria (Kalde et al., 2007). In another model legume, *Lotus japonicus*, syntaxin SYP32-1 was shown to be required for normal differentiation of nodule tissues (Mai et al., 2006). These results indicate that SNARE proteins are likely to be crucial for the maintenance of legume-*Rhizobium* symbiosis. However, with the exception of MtSYP132 and LjSYP32-1, little evidence is available regarding the contributions of SNARE proteins to the symbiotic association.

We have been studying *L. japonicus* Fix− mutants that exhibit lower or no nitrogen fixation activity in order to identify plant genes required for the establishment and maintenance of
symbiotic nitrogen fixation. In this study, we demonstrate that one of these mutants is defective in a Qc-SNARE protein homologous to *A. thaliana* SYP71, indicating that *LjSYP71* is required for effective symbiotic nitrogen fixation in *L. japonicus* nodules.

**RESULTS**

**Isolation of a Fix\(^-\) mutant *Ljsyp71***

The *Ljsyp71-1* and *Ljsyp71-2* mutants were produced from ethylmethane sulfonate (EMS) and carbon-ion beam mutagenesis of *L. japonicus* ecotype Miyakojima MG-20, respectively. Both mutants formed nodules but their growth was retarded compared to that of the wild-type plant under symbiotic conditions (Fig. 1); we categorized these two mutants as Fix\(^-\) mutants. Each mutant was crossed with *L. japonicus* ecotype Gifu B-129 for genetic mapping, and linkage analysis with published DNA markers (Sato et al., 2008) was performed using each F1 progeny. The two loci appeared to be located almost in the same region, so we carried out reciprocal crosses, which confirmed that the two mutants were allelic. Hereafter, we used *Ljsyp71-1* for analyses. The F1 plants obtained by back-crossing *Ljsyp71-1* to the parental line Miyakojima displayed a wild-type phenotype and the resulting F2 progenies segregated at 135:46 for the wild type and mutant phenotypes. The observed segregation ratio fitted the expected value of 3:1 (\(\chi^2 = 0.03\)), indicating that the phenotype of the *Ljsyp71-1* mutant is regulated in a monogenic recessive manner.

**Plant growth, nodulation and nitrogen fixation activity in the *Ljsyp71* mutant**
During plant development, growth of the *Ljsyp71* mutant plant was always retarded under symbiotic conditions with the compatible rhizobium *Mesorhizobium loti* (Fig. 2A). The *Ljsyp71* mutant formed smaller and pale white nodules (Fig. 1), of a similar number to that on the wild-type plant throughout plant development (Fig. 2B). However, the growth of *Ljsyp71* nodules was arrested after 4 weeks whereas wild-type plant nodules continued to increase in size (Fig. 2C). The nitrogen fixation activity of *Ljsyp71* nodules was delayed compared to that of wild-type nodules, and the activity level remained lower (Fig. 2D). At 4 weeks, the activity of *Ljsyp71* nodules was approximately 14% that of wild-type nodules (Fig. 2E). After potassium nitrate was supplied to the *Ljsyp71* mutant, plant growth almost recovered to wild-type plant levels (Fig. 3). In addition, under both symbiotic and non-symbiotic conditions, no visible morphological changes in vegetative or reproductive organs of the *Ljsyp71* plant were observed, apart from the nodulation defects.

**Cellular structure of *Ljsyp71* mutant nodules**

The cellular structure of *Ljsyp71* mutant nodules was examined by light and transmission electron microscopy. Twenty-four days after inoculation, toluidine blue-stained infected cells were observed in both *Ljsyp71* and wild-type nodules (Fig. 4A and 4B, respectively). However, some of the infected cells in the *Ljsyp71* nodules stained less densely, and a large vacuole observed in the infected cells of wild-type nodules was absent from most of the infected cells of *Ljsyp71* nodules. The infected cells of wild-type nodules were packed with endosymbionts enclosed by a symbiosome membrane (Fig. 4C). The symbiosomes of *Ljsyp71* nodules were
generally enlarged and, consequently, the symbiosome space between the symbiosome membrane and the bacteroids was increased compared to that of wild-type nodules (Fig. 4C and 4D).

**Map-based cloning of the LjSYP71 gene**

*Ljsyp71-1* was crossed with ecotype Gifu B-129 and linkage analysis was performed using 972 homozygous F2 mutant individuals to enable map-based cloning of the responsible *LjSYP71* gene. This gene was primarily delimited between the simple sequence repeat (SSR) markers TM0696 and TM1077 on the upper part of chromosome 5 (Supplemental Fig. S1A). At the time of this experiment, the genome sequence of this region had not been fully covered, but comparison of the *L. japonicus* genome (Sato et al., 2008) with that of *Glycine max* (Schmutz et al., 2010) predicted that a transformation-competent artificial chromosome (TAC) clone, LjT03L03, would be positioned between the two markers. Direct sequencing of six genes predicted on the TAC clone revealed a single nucleotide mutation at the third exon/intron boundary that caused an error in RNA splicing in one of these genes in the *Ljsyp71-1* mutant (Supplemental Fig. S1B). In the *LjSYP71-2* mutant, it appeared to carry a non-autonomous DNA transposon composed of approximately 500 base pairs inserted in the fifth exon of the same gene (Supplemental Fig. S1B).

To confirm that the gene in question was responsible for the *Ljsyp71* mutant phenotype, we introduced the wild-type complementary DNA (cDNA), prepared from the EST clone as described below, driven by the *L. japonicus* ubiquitin promoter into the *Ljsyp71-1* mutant using *Agrobacterium rhizogenes*-mediated hairy root transformation. Introduction of the gene restored
shoot growth in the transformed \textit{Lj}sp71-1 mutant (Fig. 5A). Nitrogen fixation (acetylene reduction) activity of the transformed nodules was also recovered to a level comparable to that of wild-type nodules (Fig. 5B). From these results, we concluded that this gene was indeed mutated in the mutant.

\textbf{Structure of the LjSYP71 protein}

The EST clone MR020b12, corresponding to the \textit{Lj}SYP71 gene, was found in the \textit{Lj}aponicus EST database (http://est.kazusa.or.jp/en/plant/lotus/EST/index.html). The clone contained a nearly full-length cDNA that encoded a polypeptide composed of 265 amino acids with a molecular mass of 29.8 kDa. \textit{Lj}SYP71 appeared to be homologous to syntaxin SYP71 (At3g09740) of \textit{A. thaliana} (Fig. 6). The \textit{At}SYP7 family, unique to plants, has three members, SYP71, SYP72 and SYP73, with largely unknown functions (Sanderfooot et al., 2000). Sequence analysis of the 54 \textit{A. thaliana} SNARE genes revealed that the \textit{At}SYP7 family belongs to Qc-SNAREs (Uemura et al., 2004). In the deduced \textit{Lj}SYP71 amino acid sequence (Supplemental Fig. S2), the SOSUI domain prediction program (Hirokawa et al., 1998) predicted a trans-membrane domain in the C-terminal region. In addition, the MOTIF program (http://www.genome.jp/tools/motif) detected a SNARE coiled-coil motif centered on a glutamine (Q) residue adjacent to the C-terminal trans-membrane domain. These structural features matched those of Q-SNARE proteins (Fasshauer et al., 1998; Sanderfooot et al., 2000). Consequently, we designated the gene \textit{Lj}SYP71. Determination of the nucleotide sequence of the EST clone MPD004c03 showed that the deduced amino acid sequence is 86\% identical to \textit{Lj}SYP71 (Supplemental Fig. S3). Sequence comparison revealed that the MPD004c03 also
belongs to the AtSYP71 protein family (Fig. 6).

**Expression of the *LjSYP71* gene**

*LjSYP71* gene expression was analyzed by quantitative real-time PCR. RNA transcripts were detected in all vegetative tissues examined, including stems, leaves, roots and nodules (Fig. 7) as well as in reproductive tissues such as flowers and pods (Supplemental Fig. S3). The highest expression was observed in young roots, though the difference in levels of expression was no more than twice as high as that in other tissues. After inoculation with *M. loti*, expression levels decreased in roots; furthermore, the level of expression in nodules was usually maintained during their development. These results are consistent with those obtained from expression analysis using macro-array ([http://est.kazusa.or.jp/en/plant/lotus/EST/cDNA.html](http://est.kazusa.or.jp/en/plant/lotus/EST/cDNA.html); Kouchi et al., 2004). In situ-hybridization further revealed that the *LjSYP71* gene was expressed in vascular tissues in both roots and nodules (Fig. 8A and 8B). This was also confirmed by promoter-β-glucuronidase (GUS) fusion experiments. The development of a blue color was observed in the vascular tissues of the hairy roots transformed with the *LjSYP71* promoter-GUS fusion gene as well as in the vascular tissues of the nodules borne on these hairy roots (Fig. 8C and 8E). By contrast, GUS activities were undetectable in the hairy roots transformed with an empty vector and in the nodules formed on these hairy roots (Fig. 8D and 8F).

**DISCUSSION**

While studying the Fix− mutants of *L. japonicus*, four genes were identified: *SST1*, *IGN1*,
FEN1 and SEN1 (Krusell et al., 2005; Kumagai et al., 2007; Hakoyama et al., 2009; Hakoyama et al., 2012). Two further genes, encoding the EFD transcription factor and DNF1, were shown to be essential for symbiotic nitrogen fixation during the investigation of the M. truncatula Fix− mutants (Vernié et al., 2008; Wang et al., 2010). All of these genes regulate rhizobial symbiotic nitrogen fixation in various aspects of the developmental process. With the exception of IGN1, all genes are expressed in a nodule-specific manner, and their products function in nodule-infected cells. IGN1, by contrast, is expressed constitutively in all organs, although it is uncertain whether its product plays an important role in nodule-infected cells.

In the present study, we identified another L. japonicus Fix− mutant and showed that the mutated gene encodes a Qc-SNARE protein homologous to A. thaliana SYP71. In symbiotic association with M. loti, the Ljsyp71 mutant plant exhibits symptoms of nitrogen deficiency because of the lower nitrogen-fixing activity of the nodules. Inside the Ljsyp71 nodules, enlarged symbiosomes are observed in infected cells like other Fix− mutants (Suganuma et al., 2003; Krusell et al., 2005; Hossain et al., 2006; Kumagai et al., 2007). However, in contrast to LjSST1, LjFEN1, LjSEN1, MtEFD and MtDNF1, but similar to LjIGN1, LjSYP71 is expressed in all vegetative tissues. In addition, no obvious morphological defects of the Ljsyp71 mutant were observed under both symbiotic and non-symbiotic conditions, similar to the Ljign1 mutant. This provides further evidence that regulation of rhizobial symbiotic nitrogen fixation requires both nodule-specific genes and genes expressed throughout the plant.

Members of the SYP7 family, incuding LjSYP71, have not been identified in yeast or mammalian SNAREs (Sanderfoot et al., 2000), so are likely to play specific roles in plant development, one of which may be related to their expression in vascular tissues. In A. thaliana, SYP71 is predominantly localized to the plasma membrane where it is thought to function in the
secretion process (Alexandersson et al., 2004; Marmagne et al., 2004; Tyrrell et al., 2007; Suwastika et al., 2008). The discovery of the LjSYP71-defective Fix− mutant suggests the existence of a systemic regulation of the nitrogen-fixing activity mediated by vascular tissues. A substance in the shoot that affects nitrogen-fixing activity might be secreted via vesicle trafficking into the phloem and transported to infected nodule cells. Alternatively, a substance produced from nitrogen fixation in the nodules might be exported to the plant shoot through the xylem. The nitrogen-fixing activity of the nodules could be impaired by an interruption in the translocation of either substance, which is caused by the LjSYP71 mutation. Guinel (2009) focused on the legume nodule cortex surrounding the central infected zone and highlighted the importance of vascular tissues in the development and regulation of nodules in the legume-Rhizobium symbiosis. Our results present further evidence for the pivotal role played by the vasculature in symbiotic nitrogen fixation.

In A. thaliana, AtSYP71 is expressed in vegetative tissues and its expression is detected in the vascular tissues of the roots (Suwastika et al., 2008). This pattern is similar to that of LjSYP71 in L. japonicus. However, it should be noted that no homozygous T-DNA insertion mutant of A. thaliana SYP71 was isolated from progenies of the SYP71/syp71 heterozygote, suggesting that AtSYP71 is essential for the development of A. thaliana (Suwastika et al., 2008). By contrast, LjSYP71 does not appear to be essential for the development of the L. japonicus plant because under non-symbiotic conditions but supplied with combined nitrogen, the Ljsyp71 mutant grows similarly to the wild-type plant. The cDNA clone, MPD004c03, from the L. japonicus EST database was shown to be homologous to LjSYP71, and the predicted protein belongs to the AtSYP71 family. A previous cDNA array experiment (Kouchi et al., 2004) found that MPD004c03 is also expressed in shoot, roots and nodules. It is likely that LjSYP71 evolved to
fulfill an important role in symbiotic nitrogen fixation and that the paralogous gene expressed in parallel with *LjSYP71* is required for the correct development of *L. japonicus*. In nodules, the paralogous gene may have a partially overlapping function, resulting in basal levels of nitrogen fixation.

Some SYP proteins have already been linked to nodulation. In *M. truncatula*, SYP132 of the Qa-SNARE protein family is located on symbiosome membranes, and is thought to be involved in symbiosome formation (Catalano et al., 2007; Limpens et al., 2009). Another member of the Qa-SNARE protein family, SYP32-1, is involved in symbiotic nitrogen fixation as well as nodule formation of *L. japonicus* (Mai et al., 2006). However, *LjSYP32-1*-suppressed transformants display significantly retarded plant growth even when the nutrient medium contains nitrogen. *LjSYP32-1* is expressed not only in the vascular tissues of roots and nodules but also in the inner cortical cell layer surrounding the infected zone of nodules and in the meristematic area of developing lateral roots. By contrast, the expression of *LjSYP71* is detected in the vascular tissues of roots and nodules, but not in the infected cells of nodules. Thus, it is suggested that the role of *LjSYP71* in symbiotic nitrogen fixation differs from those of MtSYP132 and LjSYP32-1.

In conclusion, this study showed that *LjSYP71*, a component of the SNARE complex that is expressed in vascular tissues, plays an essential role in symbiotic nitrogen fixation in *L. japonicus* nodules. Further analysis of the role of *LjSYP71* will provide novel insights into legume and *Rhizobium* symbiosis.

MATERIALS AND METHODS
Plant and Bacterial Materials

Seeds of *L. japonicus* ecotype Miyakojima MG-20 were used as wild type. The *Ljsyp71-1* mutant was obtained by EMS mutagenesis. Approximately 5,000 seeds were immersed in 0.4% EMS overnight and allowed to germinate. M1 plants were grown in a green-house and M2 seeds collected. A screen for Fix− mutants that form nodules but exhibit retarded plant growth was performed in approximately 25,000 M2 plants. A single individual of the M3 self-progeny from the M2 candidate mutant was backcrossed with the parent MG-20. The *Ljsyp71-2* mutant was produced by carbon-ion beams as described previously (Tanaka et al., 1997; Magori et al., 2009). Seeds were surface-sterilized and sown in sterilized vermiculite with *Mesorhizobium loti* MAFF303099, which had been cultured on yeast-mannitol-agar plates for 7 days. The plants were grown in a nitrogen-free nutrient solution in the greenhouse under natural daylight or in a controlled chamber on a 16-h day/8-h night cycle at 26°C as described (Imaizumi-Anraku et al., 1997). Under non-symbiotic conditions, potassium nitrate (10 mM) was added to the nutrient solution.

Acetylene Reduction Assay

The nitrogenase activity of mutant nodules was measured by an acetylene reduction assay, in a closed system with nodulated roots detached from freshly harvested intact plants. Nodulated roots were placed in 20-mL vials and incubated at 25°C (Suganuma et al., 2003). After 30 min, the amount of ethylene produced was determined by gas chromatography equipped with a flame ionization detector and a column of Porapak N (Waters, Milford, MA).
**Light and Electron Microscopy**

The cellular structure of the *Ljsyp71-1* nodules was observed by light and transmission electron microscopy, and observations were carried out essentially as described previously (Suganuma et al., 2003). Nodules on root segments were fixed in FAA containing 5% (v/v) formaldehyde, 5% (v/v) acetic acid, and 63% (v/v) ethanol. After dehydration, the samples were embedded in Paraplast Plus. Serial microtome sections (10 µm) were stained with 1% (w/v) toluidine blue in 0.5% (w/v) sodium tetraborate (pH 9.0). For transmission electron microscopy, nodules were fixed in 2% glutaraldehyde and post-fixed in 2% osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.2). After being dehydrated, the samples were embedded in an epoxy resin (Quetol-812, Nisshin EM, Tokyo, Japan). Ultrathin sections were stained with uranium acetate and lead citrate, and were observed under electron microscopy.

**Map-based Cloning**

The causal gene of the *Ljsyp71* mutants was identified by map-based cloning as described previously (Suganuma et al., 2003). Total DNA was extracted using the DNeasy Plant Mini kit (QIAGEN, Hilden, Germany) from leaves of F₂ homozygous mutant plants generated by crossing the mutant with *L. japonicus* ecotype Gifu B-128, and PCR was carried out with SSR markers (Sato et al., 2008). For fine mapping, additional PCR markers were developed on the basis of sequence differences between the two parents. The PCR product was resolved on a non-denaturing 15% polyacrylamide gel and stained with SYBR Green I (TaKaRa, Shiga,
Sequence Analysis

EST clones, MR020b12 and MPD004c03 (Asamizu et al., 2000; Sato et al., 2001; Asamizu et al., 2004), were obtained from The National BioResource Project (L. japonicus and G. max) Office, Department of Agriculture, Miyazaki University, Japan. Plasmides containing of each cDNA clone were isolated and the nucleotide sequence of each clone was determined.

Complementation Test

Wild-type cDNA was introduced into the Ljsyp71-1 mutant: the coding region of the LjSYP71 gene was amplified by PCR from MR020b12 cDNA with the F04-ORF forward:

5'-CACCATGAGCGTCATCGACCTTCT-3' and reverse primers: '5-CTGCAGACGCAAGACTGCTT-3'. The amplified fragment was cloned into pENTR/D-TOPO using the TOPO cloning system (Invitrogen, Carlsbad, CA). The fragment placed behind the L. japonicus ubiquitin promoter on pENTR/D-TOPO was further transferred into pUB-GW-GFP (Maekawa et al., 2008) using the LR clonase II enzyme mix (Invitrogen). The resulting plasmid was transformed into Agrobacterium rhizogenes LBA1334 by the freeze-thaw method. The LjSYP71 gene was transformed into both the Ljsyp71-1 mutant plant and wild type Miyakojima using the A. rhizogenes-mediated hairy root transformation system (Diaz et al., 2005). Plants with transgenic hairy roots were transferred to vermiculite pots supplied with 1/2 strength B & D medium and grown in a growth cabinet (16 h day and 8 h
night, 26 °C). Four days after the transfer, plants were inoculated with *M. loti* strain MAFF303099. Twenty-eight days after the inoculation with *M. loti*, non-transformed nodules with no GFP fluorescence were detached from the roots, and the acetylene reduction activity of the remaining transformed nodules was measured.

**Quantitative Real-time PCR**

Expression of *LjSYP71* was analyzed by quantitative real-time PCR. *L. japonicus* Miyakojima seeds were sown on 0.9% water-agar plates and 6 days later the seedlings were transferred to vermiculite pots. These plants were inoculated with *M. loti* strain MAFF303099 4 days after transfer. Some roots were harvested at 0, 4, and 8 days after inoculation (DAI), and some leaves and stems at 8 DAI. Nodules were collected from roots at 12, 17, and 22 days DAI. Total RNAs were isolated from leaves, stems, roots and nodules by the RNeasy Plant Mini Kit (QIAGEN). Total RNAs were reverse-transcribed after removal of genomic DNA using a QuantiTect reverse transcription kit (QIAGEN) according to the manufacturer’s instructions. Aliquots of the resulting cDNA (equivalent to approximately 1 ng RNA) were subjected to real-time PCR analysis using a Light Cycler real-time PCR System (Roche Diagnostics, Basel, Switzerland) with the F04RT forward primer: 5’-TGAGATGGACACTAAGGTGGACCA-3’ and reverse primer: 5’- ATTCGAACACATCCGTGGTCTTTG-3’. *L. japonicus* ubiquitin was used as an internal standard with the forward primer: 5’-TTACCTTTGCTCGTCTTG-3’ and reverse primer: 5’-AACAAACAGCACACACAGCCAATCC-3’ as described by Flemetakis et al. (2000). Independently, flowers and pods were collected from the wild-type Miyakojima mature plants, and total RNAs were isolated from flowers using a Sepasol-RNA I Super G
(Nacalai Tesque, Kyoto, Japan) and from pods by a Plant Total RNA Extraction Miniprep System (VIOGENE, Sunnyvale, CA), respectively. After treatment with DNase I (QIAGEN), RNAs were reverse-transcribed using a ReverTra Ace qPCR RT Kit (TOYOBO, Tokyo, Japan). Quantitative real-time PCR was performed using an iQ SYBR Green Super mix (Bio-Rad, Hercules, CA).

**In situ Hybridization**

Spatial expression of *LjSYP71* in nodules was determined by in situ-hybridization, which was carried out using the method described by Kouchi and Hata (1993). Nodules were fixed in 4% (w/v) paraformaldehyde and 0.25% (w/v) glutaradehyde in 0.1 M sodium phosphate buffer (pH 7.4). The sections were hybridized with RNA probes prepared from linearized plasmids with digoxigenin-UTP (Roche Diagnostics) containing the entire coding region of the *LjSYP71* gene. The hybridization signals were detected using anti-digoxigenin-alkaline phosphatase conjugate with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate toluindinium salt (Roche Diagnostics).

**Promoter GUS Analysis**

Spatial expression patterns of *LjSYP71* in roots and nodules were also studied by promoter GUS analysis. The *LjSYP71* promoter and terminator fragments were amplified by PCR from genomic DNA with proF04-F primer: 5’-GGTACCCGTACCACTAACCACATCAG-3’, proF04-R primer: 5’-GGATCCCGGTGTGGTGGGAGTCTAAG-3’, terF04-F primer:
5'-TCTAGAATAGATGCACAAGACCACGG-3' and terF04-R primer: 5'-GTCTGACCAAGGAACACCAGTAACAC-3', and inserted into pC1300GFP. A Gateway vector conversion cassette (Invitrogen) was inserted between the promoter and terminator fragments. The gusA gene on plasmid pENTR–gusA (Invitrogen) was transferred to the cassette using LR clonase II (Invitrogen) to construct the *LjSYP71* promoter–gusA–*LjSYP71* terminator fusion gene. The resulting plasmid was transformed into *A. rhizogenes* LBA1334, and the wild type Miyakojima was transformed with the *A. rhizogenes*. Transgenic hairy root segments were prefixed in ice-cold 90% acetone, and immersed in a staining solution (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% TritonX-100 and 50 mM sodium phosphate buffer, pH 7.0). Nodules on transgenic hairy roots were sliced longitudinally with a double-edged razor blade and immersed in the staining solution. After incubation for 2 h at 37 °C in the dark, the stained materials were observed with a light microscope.

**Phylogenetic Analysis**

Amino acid sequences of *A. thaliana* syntaxins and *LjSYP71* were aligned using the CLUSTALW program (http://www.ddbj.nig.ac.jp). The phylogenetic tree was drawn using the program TreeView32 (Page, 1996).

Sequence data from this article can be found in the GenBank/EMBL/DDBJ data libraries under accession number AB704757.
Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Map-based cloning of the *LjSYP71* gene.

**Supplemental Figure S2.** The predicted amino acid sequence of *LjSYP71*.

**Supplemental Figure S3.** Alignment of *LjSYP71*, MPD004c03 and *AtSYP71*.

**Supplemental Figure S4.** Expression of the *LjSYP71* in flowers and pods.

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LITERATURE CITED

Alexandersson E, Saalbach G, Larsson C, Kjellbom P (2004) Arabidopsis plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. Plant Cell Physiol 45: 1543–1556

Asamizu E, Nakamura Y, Sato S, Tabata S (2000) Generation of 7137 non-redundant expressed sequence tags from a legume, Lotus japonicus. DNA Res 7: 127–130

Asamizu E, Nakamura Y, Sato S, Tabata S (2004) Characteristics of the Lotus japonicus gene repertoire deduced from large-scale sequence tag (EST) analysis. Plant Mol Biol 54: 405–414

Bassham DC, Blatt MR (2008) SNAREs: cogs and coordinators in signaling and development. Plant Physiol 147: 1504–1515

Catalano CM, Czymmek KJ, Gann JG, Sherrier DJ (2007) Medicago truncatula syntaxin SYP132 defines the symbiosome membrane and infection droplet membrane in root nodules. Planta 225: 541–550

Diaz CL, Grønlund M, Schlaman HRM, Spank HP (2005) Induction of hairy roots for symbiotic gene expression. In AJ Marquez, ed, Lotus japonicus Handbook. Springer, The Netherlands, pp 261–277

Fasshauer D, Sutton RB, Brunger AT, Jahn R (1998) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc Natl Acad Sci USA 95: 15781–15786

Flemetakis E, Kavroulakis N, Quaedvlieg NE, Spank HP, Dimou M, Roussis A, Katinakis P (2000) Lotus japonicus contains two distinct ENOD40 genes that are expressed in
symbiotic, nonsymbiotic, and embryonic tissues. Mol Plant Microbe Interact 13: 987–994

Fukuda R, McNew JA, Weber T, Parlati F, Engel T, Nickel W, Rothman JE, Söllner TH (2000) Functional architecture of an intracellular membrane t-SNARE. Nature 407: 198–202

Guinel FC (2009) Getting around the legume nodule: II. Molecular biology of its peripheral zone and approaches to study its vasculature. Botany 87: 1139–1166

Hakoyama T, Niimi K, Watanabe H, Tabata R, Matsubara J, Sato S, Nakamura S, Tabata S, Jichun L, Matsumoto T, et al (2009) Host plant genome overcomes the lack of a bacterial gene for symbiotic nitrogen fixation. Nature 462: 514–517

Hakoyama T, Niimi K, Yamamoto T, Isobe S, Sato S, Nakamura Y, Tabata S, Kumagai H, Umehara Y, Brossuleit K, et al (2012) The integral membrane protein SEN1 is required for symbiotic nitrogen fixation in \textit{Lotus japonicus} nodules. Plant Cell Physiol 53: 225–236

Hirokawa T, Boon-Chieng S, Mitaku S (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics 14: 378–379

Hossain MS, Umehara Y, Kouchi H (2006) A novel Fix$^{-}$ symbiotic mutant of \textit{Lotus japonicus}, \textit{Ljsym105}, shows impaired development and premature deterioration of nodule infected cells and symbiosomes. Mol Plant Microbe Interact 19: 780–788

Imaizumi-Anraku H, Kawaguchi M, Koiwa H, Akao S, Syono K (1997) Two ineffective-nodulating mutants of \textit{Lotus japonicus} – Different phenotypes caused by the blockage of endocytotic bacterial release and nodule maturation –. Plant Cell Physiol 38: 871–881

Kalde M, Nühse TS, Findlay K, Peck SC (2007) The syntaxin SYP132 contributes to plant resistance against bacteria and secretion of pathogenesis-related protein 1. Proc Natl Acad Sci USA 104: 11850–11855
Kouchi H, Hata S (1993) Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. Mol Gen Genet 238: 106–119

Kouchi H, Shimomura K, Hata S, Hirota A, Wu GJ, Kumagai H, Tajima S, Suganuma N, Suzuki A, Aoki T, et al (2004) Large-scale analysis of gene expression profiles during early stages of root nodule formation in a model legume, *Lotus japonicus*. DNA Res 11: 263–274

Krusell L, Krause K, Ott T, Desbrosses G, Krämer U, Sato S, Nakamura Y, Tabata S, James EK, Sandal N, et al (2005) The sulfate transporter SST1 is crucial for symbiotic nitrogen fixation in *Lotus japonicus* root nodules. Plant Cell 17: 1625–1636

Kumagai H, Hakoyama T, Umehara Y, Sato S, Kaneko T, Tabata S, Kouchi H (2007) A novel ankyrin-repeat membrane protein, IGN1, is required for persistence of nitrogen-fixing symbiosis in root nodules of *Lotus japonicus*. Plant Physiol 143: 1293–1305

Limpens E, Ivanov S, van Esse W, Voets G, Fedorova E, Bisseling T (2009) *Medicago* N2-fixing symbiosomes acquire the endocytic identity marker Rab7 but delay the acquisition of vacuolar identity. Plant Cell 21: 2811–2828

Lipka V, Kwon C, Panstruga R (2007) SNARE-Ware: the role of SNARE-domain proteins in plant biology. Annu Rev Cell Dev Biol 23: 147–174

Maekawa T, Kusakabe M, Shimoda Y, Sato S, Tabata S, Murooka Y, Hayashi M (2008) Polyubiquitin promoter-based binary vectors for overexpression and gene silencing in *Lotus japonicus*. Mol Plant Microbe Interact 21: 375–382

Magori S, Oka-Kira E, Shibata S, Umehara Y, Kouchi H, Hase Y, Tanaka A, Sato S, Tabata S, Kawaguchi M (2009) *TOO MUCH LOVE*, a root regulator associated with the long-distance control of nodulation in *Lotus japonicus*. Mol Plant Microbe Interact 22:
Mai HT, Nomura M, Takegawa K, Asamizu E, Sato S, Kato T, Tabata, S, Tajima S (2006) Identification of a Sed5-like SNARE gene LjSYP32-1 that contributes to nodule tissue formation of Lotus japonicus. Plant Cell Physiol 47: 829–838

Marmagne A, Rouet MA, Ferro M, Rolland N, Alcon C, Joyard J, Garin J, Barbier-Brygoo H, Ephritikhine G (2004) Identification of new intrinsic proteins in Arabidopsis plasma membrane proteome. Mol Cell Proteomics 3: 675–691

McNew JA, Parlati F, Fukuda R, Johnston J, Paz K, Paumet F, Söllner TH, Rothman JH (2000) Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. Nature 407: 153–159

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12: 357–358

Parlati F, McNew JA, Fukuda R, Miller R, Söllner TH, Rothman JH (2000) Topological restriction of SNARE-dependent membrane fusion. Nature 407: 194–198

Pfeffer SR (2007) Unsolved mysteries in membrane traffic. Annu Rev Biochem 76: 629–645

Sanderfoot AA, Assaad FF, Raikhel NV (2000) The Arabidopsis genome. An abundance of soluble N-ethylmaleimide-sensitive factor adaptor protein receptors. Plant Physiol 124: 1558–1569

Sato S, Kaneko T, Nakamura Y, Asamizu E, Kato T, Tabato S (2001) Structural analysis of Lotus japonicus genome. I. Sequence features and mapping of fifty-six TAC clones which cover the 5.4 Mb regions of the genome. DNA Res 8: 311–318

Sato S, Nakamura Y, Kaneko T, Asamizu E, Kato T, Nakao M, Sasamoto S, Watanabe A, Ono A, Kawashima K, et al. (2008) Genome structure of the legume, Lotus japonicus. DNA Res
Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J, et al (2010) Genome sequence of the palaeopolyploid soybean. Nature 463: 178–183

Suganuma N, Nakamura Y, Yamamoto M, Ohta T, Koiwa H, Akao S, Kawaguchi M (2003) The Lotus japonicus Sen1 gene controls rhizobial differentiation into nitrogen-fixing bacteroids in nodules. Mol Gen Genomics 269: 312–320

Suwastika IN, Uemura T, Shiina T, Sato MH, Takeyasu K (2008) SYP71, a plant-specific Qc-SNARE protein, reveals dual localization to the plasma membrane and the endoplasmic reticulum in Arabidopsis. Cell Struct Funct 33: 185–192

Tanaka A, Shikazono N, Yokota Y, Watanabe H, Tano S (1997) Effects of heavy ions on the germination and survival of Arabidopsis thaliana. Int J Radiat Biol 72: 121–127

Tyrrell M, Campanoni P, Sutter JU, Pratelli R, Paneque M, Sokolovski S, Blatt M (2007) Selective targeting of plasma membrane and tonoplast traffic by inhibitory (dominant-negative) SNARE fragments. Plant J 51: 1099–1115

Uemura T, Ueda T, Ohniwa RL, Nakano A, Takeyasu K, Sato MH (2004) Systematic analysis of SNARE molecules in Arabidopsis: dissection of the post-golgi network in plant cells. Cell Struct Funct 29: 49–65

Vernié T, Moreau S, de Billy F, Plet J, Combier JP, Rogers C, Oldroyd G, Frugier F, Niebel A, Gamas P (2008) EFD is an EFR transcription factor involved in the control of nodule number and differentiation in Medicago truncatula. Plant Cell 20: 2696–2713

Wang D, Griffitts J, Starker C, Fedorova E, Limpens E, Ivanov S, Bisseling T, Long S (2010) A nodule-specific protein secretory pathway required for nitrogen-fixing symbiosis. Science
Figure legends

**Figure 1.** Plants and nodules of the wild-type (Wt) Miyakojima (left) and the *Ljsyp71*-1 mutant (right) grown under symbiotic conditions.

**Figure 2.** Growth, nodulation, and Acetylene Reduction Activity (ARA) of the wild-type Miyakojima (open circle) and the *Ljsyp71* mutant (closed circle) during plant development. Plant fresh weight (A), nodule number (B), nodule fresh weight (C), and ARA (D, E) of the wild-type and *Ljsyp71*-1 mutant are shown. ARA is expressed as the basis of plant (D) or g fresh weight of nodules (E). All values are means of three determinations and vertical bars indicate standard errors.

**Figure 3.** Effect of potassium nitrate on growth of the wild-type (Wt) Miyakojima and the *Ljsyp71* mutant. The wild-type and the *Ljsyp71*-1 mutant were grown either with *Mesorhizobium loti* (*M. loti*) or supplemented with 10 mM potassium nitrate (KNO3). After 28 days, plants were harvested. All values are means of six determinations and vertical bars indicate standard errors. An asterisk indicates a statistically significant difference (Student’s *t*-test, *P*<0.05).

**Figure 4.** Nodules and infected cell structures of the wild-type Miyakojima and the *Ljsyp71* mutant. Light micrographs of nodules 28 days after inoculation are shown for the wild-type (A) and *Ljsyp71*-1 mutant (B). Transmission electron micrographs of infected cells are shown for the wild-type nodule (C) and *Ljsyp71*-1 mutant (D) nodules 24 days after inoculation.
Arrowheads indicate enlarged symbiosomes.

**Figure 5.** Complementation of the *Ljsyp71* mutant by introduction of the wild-type *LjSYP71* gene. A, Growth of the wild-type Miyakojima (Wt) and the *Ljsyp71-1* mutants transformed with the *Lotus japonicus* ubiquitin promoter-driven full-length cDNA for the *LjSYP71* gene (+) or empty vector (-) by *Agrobacterium rhizogenes*-mediated hairy root transformation, 28 days after inoculation with *Mesorhizobium loti*. B, Acetylene reduction activity (ARA) of the transformed nodules on the wild type (Wt) and the *Ljsyp71-1* mutant with the *LjSYP71* gene (+) or empty vector (-). Data are the means obtained from six independent transgenic roots and vertical bars represent standard errors.

**Figure 6.** Phylogenic analysis of SYP proteins from *Arabidopsis thaliana* (At), and *LjSYP71* and the paralogous protein (MPD004c03) from *Lotus japonicus*. Amino acid sequences were compared by CLUSTALW and the tree was depicted by TreeView program. AGI identifications for AtSYP; AtSYP111 (At1g08350), AtSYP112 (At2g18260), AtSYP121 (At3g11820), AtSYP122 (At3g52400), AtSYP123 (At4g03330), AtSYP124 (At1g55410), AtSYP125 (At1g10980), AtSYP131 (At3g03800), AtSYP132 (At5g08080), AtSYP21 (At5g16830), AtSYP22 (At5g46860), AtSYP23 (At4g17730), AtSYP31 (At5g05760), AtSYP32 (At3g24350), AtSYP41 (At5g26980), AtSYP42 (At4g02195), AtSYP43 (At3g05710), AtSYP51 (At1g15930), AtSYP52 (At1g73260), AtSYP61 (At1g27550), AtSYP71 (At3g09740), AtSYP72 (At3g45280), AtSYP73 (At3g61450), and AtSYP81 (At1g47920).

**Figure 7.** Relative expression of the *LjSYP71* gene in stems, leaves, roots and nodules. The
wild-type Miyakojima seeds were germinated and the seedlings were transferred to vermiculite 6 days after sowing. After transfer, *Mesorhizobium loti* was inoculated 4 days later. Stems and leaves were harvested at 8 days after inoculation. Roots were harvested 0, 4, and 8 days after inoculation. Nodules were collected from the roots 12, 17, and 22 days after inoculation. The housekeeping gene ubiquitin was used to assess the relative expression of the *LjSYP71* gene. All values are means of three biological independent determinations and vertical bars indicate standard errors.

**Figure 8.** Spatial expression of the *LjSYP71* gene in roots and nodules of the wild-type Miyakojima. A–B, In situ-localization of *LjSYP71* mRNA in roots and nodules 28 days after inoculation. Longitudinal sections were hybridized with digoxigenin-labeled antisense (A) or sense (B) probes. Hybridization signals are visible as purple areas (arrow heads). C–F, Promoter GUS expression patterns of *LjSYP71*. Transgenic hairy roots or sections of nodules containing the *LjSYP71* promoter–GUS fusion (C, E) and empty vector (D, F) 28 days after inoculation were examined with a histochemical GUS assay. Positive signals are visible as blue color development.
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