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The Integral Membrane Protein SEN1 is Required for Symbiotic Nitrogen Fixation in *Lotus japonicus* Nodules

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Legume plants establish a symbiotic association with bacteria called rhizobia, resulting in the formation of nitrogen-fixing root nodules. A *Lotus japonicus* symbiotic mutant, *sen1*, forms nodules that are infected by rhizobia but that do not fix nitrogen. Here, we report molecular identification of the causal gene, *SEN1*, by map-based cloning. The *SEN1* gene encodes an integral membrane protein homologous to *Glycine max* nodulin-21, and also to CCC1, a vacuolar iron/manganese transporter of *Saccharomyces cerevisiae*, and VIT1, a vacuolar iron transporter of *Arabidopsis thaliana*. Expression of the *SEN1* gene was detected exclusively in nodule-infected cells and increased during nodule development. *Nif* gene expression as well as the presence of nitrogenase proteins was detected in rhizobia from *sen1* nodules, although the levels of expression were low compared with those from wild-type nodules. Microscopic observations revealed that symbiosome and/or bacteroid differentiation are impaired in the *sen1* nodules even at a very early stage of nodule development. Phylogenetic analysis indicated that SEN1 belongs to a protein clade specific to legumes. These results indicate that SEN1 is essential for nitrogen fixation activity and symbiosome/bacteroid differentiation in legume nodules.

Keywords: Bacteroid differentiation • Legume–*Rhizobium* symbiosis • *Lotus japonicus* • Nitrogen fixation • Nodule • Symbiosome.

Abbreviations: dpi, days post-inoculation; EMS, ethylmethane sulfonate; EST, expressed sequence tag; GFP, green fluorescent protein; GUS, β-glucuronidase; ORF, open reading flame; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR; SM, symbiosome membrane; TAC, transformation-competent artificial chromosome.

The nucleotide sequence of *SEN1* has been submitted to the DNA Data Bank of Japan under the accession number AB573230.

Introduction

Biological reduction or fixation of atmospheric dinitrogen serves as a primary source of nitrogen for living organisms. Dinitrogen is reduced to ammonia by diazotrophic bacteria that have the nitrogenase enzyme system. In particular, a group of soil bacteria collectively termed rhizobia performs highly efficient nitrogen fixation in symbiotic associations with legume host plants and, thus, has critical importance in agriculture. Legume plants establish symbiosis with rhizobia and form a unique organ, the root nodule, in which the endosymbiotic rhizobia fix atmospheric dinitrogen by utilizing plant photosynthates and export ammonia to the host.

The symbiotic association between legumes and rhizobia is regulated by coordinated and complex interactions between

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the symbiotic partners. Flavonoids released from plant roots activate nodulation (*Nod*) genes in rhizobia that are required for synthesis and excretion of lipochitin oligosaccharide signal molecules (Nod factors). Nod factors elicit both rhizobial infection and nodule organogenesis in the host legume plants, and their diverse modifications determine the strict specificity between rhizobia and host legume species (Radutoiu et al. 2007, for a review, see Dénarié et al. 1996). In many legumes, rhizobia invade the host roots through infection threads in root hair cells, and concomitantly cell division is induced in the root cortex to form nodule primordia. During nodule development, rhizobia are released into nodule cells enclosed in a plant-derived membrane called the symbiosome membrane (SM) and then differentiate into a symbiosis-specific form, the bacteroid, which develops nitrogen fixation activity. Molecular genetics approaches using two model legumes, *Lotus japonicus* and *Medicago truncatula*, have identified a number of the host legume genes involved in Nod factor perception and immediate downstream signaling pathways and others involved in rhizobial infection and nodule organogenesis (Oldroyd and Downie 2008, Hayashi et al. 2010, Kouchi et al. 2010, Madsen et al. 2010). In contrast to the recent rapid progress in understanding early symbiotic signaling cascades, little is known about the host legume genes involved in the regulation of rhizobial nitrogen fixation in mature nodules.

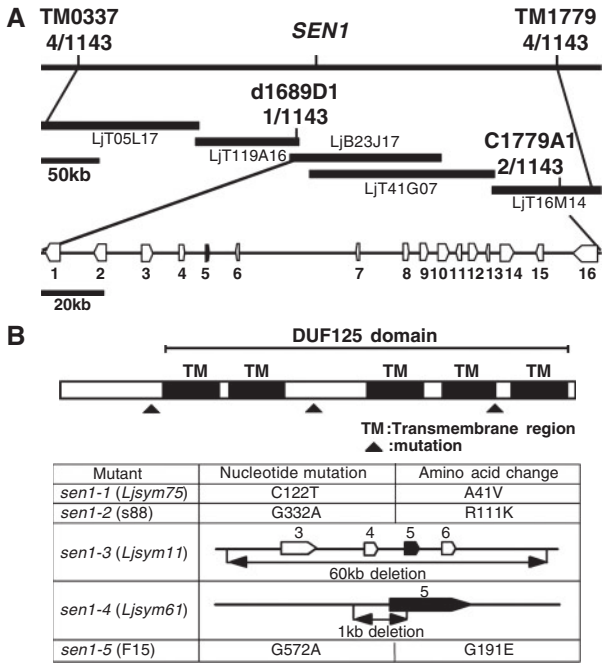
The host-determined Fix$^-$ mutants, which form morphologically normal nodules with endosymbiotic bacteria but are defective in nitrogen fixation activity, are key tools for identification of plant genes required for the establishment and maintenance of symbiotic nitrogen fixation. From *L. japonicus*, three genes, *SST1*, *IGN1* and *FEN1*, have been isolated so far. SST1 is located in the SM and is a nodule-specific sulfate transporter (Krusell et al. 2005). IGN1 is a novel plasma membrane protein with ankyrin repeats that is proposed to play a role in bacteroid differentiation and/or its maintenance (Kumagai et al. 2007). FEN1 is a homocitrate synthase specific to nodule-infected cells, and has been shown to supply homocitrate to bacteroids to support synthesis of iron molybdenum cofactor (FeMo-co) in the nitrogenase complex (Hakoyama et al. 2009). From *M. truncatula*, *DNF1* was recently shown to encode a component of the signal peptidase complex involved in a nodule-specific secretory pathway required for terminal differentiation of bacteroids (Wang et al. 2010).

In this study, we focused on a *L. japonicus* Fix$^-$ mutant, *sen1*. The *sen1* mutant forms nodules in which rhizobia are endocytosed normally but nitrogen fixation activity is completely absent (Kawaguchi et al. 2002, Suganuma et al. 2003). Thus, the *sen1* mutant is distinct from other *L. japonicus* Fix$^-$ mutants such as *sst1*, *fen1*, *ign1* and *Ljsym105* (Hossain et al. 2006); all these mutant nodules exhibit low levels of nitrogen fixation activity. To advance our understanding of the legume genetic program(s) involved in regulation of rhizobial nitrogen fixation, we present here the molecular identification of the *SEN1* gene.

Results

Map-based cloning of the *SEN1* gene

The *sen1-1* (*Ljsym75*) and *sen1-2* (s88) mutants were produced from ethylmethane sulfonate (EMS) mutagenesis of *L. japonicus* ecotype Gifu B-129 (Kawaguchi et al. 2002). *sen1-3* (*Ljsym11*) and *sen1-4* (*Ljsym61*) derived from Gifu were isolated from a progeny of descendants after T-DNA transformation (Schauser et al. 1998), and *sen1-5* (F15) was isolated from EMS mutagenesis of *L. japonicus* ecotype Miyakojima MG-20 in this study. Linkage analysis of 1,143 homozygous F_2 mutant plants generated by crossing the *sen1-1* mutant with wild-type Miyakojima positioned the *SEN1* gene between the molecular markers, TM0337 and TM1779, on the upper part of the chromosome 4 (**Fig. 1A**). A series of transformation-competent artificial chromosome (TAC) clones from this region was obtained and new markers were generated based on the draft sequences. Finally, we delimited the *SEN1* locus within a region of approximately 200 kb length, which was covered by three TAC clones. Fifteen genes (open reading flames; ORFs) were predicted in this region, and we found a single nucleotide mutation that causes an amino acid substitution in the *sen1-1* mutant by direct sequencing of these genes. In the other four *sen1* alleles,



Mutant	Nucleotide mutation	Amino acid change
sen1-1 (*Ljsym75*)	C122T	A41V
sen1-2 (s88)	G332A	R111K
sen1-3 (*Ljsym11*)	60kb deletion	
sen1-4 (*Ljsym61*)	1kb deletion	
sen1-5 (F15)	G572A	G191E

Fig. 1 Map-based cloning of the *SEN1* gene. (A) Genetic map of the *SEN1* locus and physical map of the TAC clones between the closest linked markers. The position of microsatellite markers and of sequence-derived markers and the fraction of recombinant plants found in the mapping population are indicated. In the region delimited by the closest recombination events, 15 genes are predicted by computational analysis. (B) Schematic domain structure of the SEN1 protein (the fifth ORF in A) and the positions of the mutation (arrowheads) found in five mutants of the *sen1* alleles.

mutations were found in the same gene (**Fig. 1B**). *sen1-2* and *sen1-5* both harbored single nucleotide mutations leading to amino acid substitutions. *sen1-3* had a large deletion covering the entire coding region of the *SEN1* gene, and *sen1-4* had a small deletion which includes a part of the *SEN1* coding region. Despite the differences in the mutations/deletions in the alleles, all mutants exhibited essentially the same symbiotic phenotype. They formed smaller white nodules and showed symptoms of severe nitrogen deficiency under symbiotic conditions (**Fig. 2A**; see also Schauser et al. 1998, Kawaguchi et al. 2002, Suganuma et al. 2003).

Transformation of the entire *SEN1* gene including its promoter and terminator regions into *sen1-1* using the *Agrobacterium rhizogenes*-mediated hairy root transformation procedure fully complemented the *sen1-1* mutant phenotype (**Fig. 2**). Nodules on transformed *sen1-1* roots developed similarly to wild-type plants, which were clearly distinguishable from the mutant nodules in color and size. They were red in color, showing the abundant accumulation of leghemoglobin, and were larger than the mutant nodules. Shoot growth of *sen1-1* with the transformed roots was comparable with that of the wild-type plants (**Fig. 2A**). Nitrogen fixation (acetylene reduction) activity of the transformed nodules also recovered to a level comparable with the wild-type nodules (**Fig. 2B**). Although the transformed nodules of *sen1-1* exhibited higher nitrogen fixation activity than the nodules on either the non-transformed or transformed roots of the wild-type plants, the differences were not statistically significant. Based on these results, together with direct sequencing of different *sen1* alleles, we concluded that defects in this gene are responsible for the *sen1* mutant phenotype.

The *SEN1* gene is expressed exclusively in nodule-infected cells

Since there was no expressed sequence tag (EST) clone corresponding to the *SEN1* gene in the *L. japonicus* EST database (http://est.kazusa.or.jp/en/plant/lotus/EST/index.html), we amplified a nearly full-length cDNA by reverse transcription–PCR (RT–PCR) of RNA prepared from nodules. By comparing the cDNA and genome sequences, we found that the *SEN1* gene has no introns. Northern blot analysis showed that expression of the *SEN1* gene is regulated in a nodule-specific manner. Transcripts of *SEN1* were detected exclusively in nodules and their level increased during nodule development (**Fig. 3A**). On the basis of sequence analysis, two more clones homologous to the *SEN1* gene, MWM137c08 and MWM075h10, were found in the *L. japonicus* EST database. The expression patterns of *MWM137c08* and *MWM075h10* were quite different from that of *SEN1*. Both *MWM137c08* and *MWM075h10* were expressed in nodules and roots, but their transcript levels were lower than that of the *SEN1* gene, and not enhanced during nodule development (**Fig. 3A**). Nodule-specific expression of the *SEN1* gene was also confirmed by the *SEN1* promoter–*GUS* (β-glucuronidase) fusion analysis (**Fig. 3B, C**), which indicated

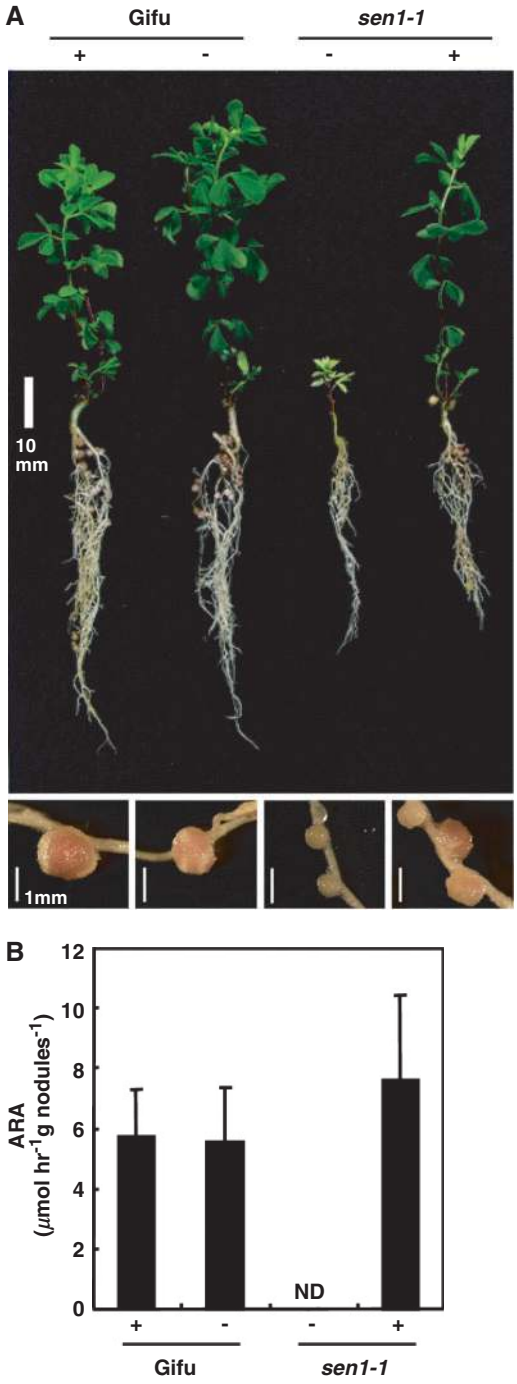


Fig. 2 Complementation of the *Lotus japonicus sen1* mutant by introduction of the wild-type *SEN1* gene. (A) Growth and nodule appearance of the plants transformed with the native promoter-driven full-length cDNA for the *SEN1* gene (+) or empty vector (−) by means of *Agrobacterium rhizogenes*-mediated hairy root transformation, at 28 d after *Mesorhizobium loti* inoculation. (B) Acetylene reduction activity (ARA) of the transformed nodules. Nodulated transformed roots were incubated with acetylene gas in a closed vial and production of ethylene was determined. The data are the means of six independent transgenic roots and the vertical bars represent standard errors. ND, not detected.

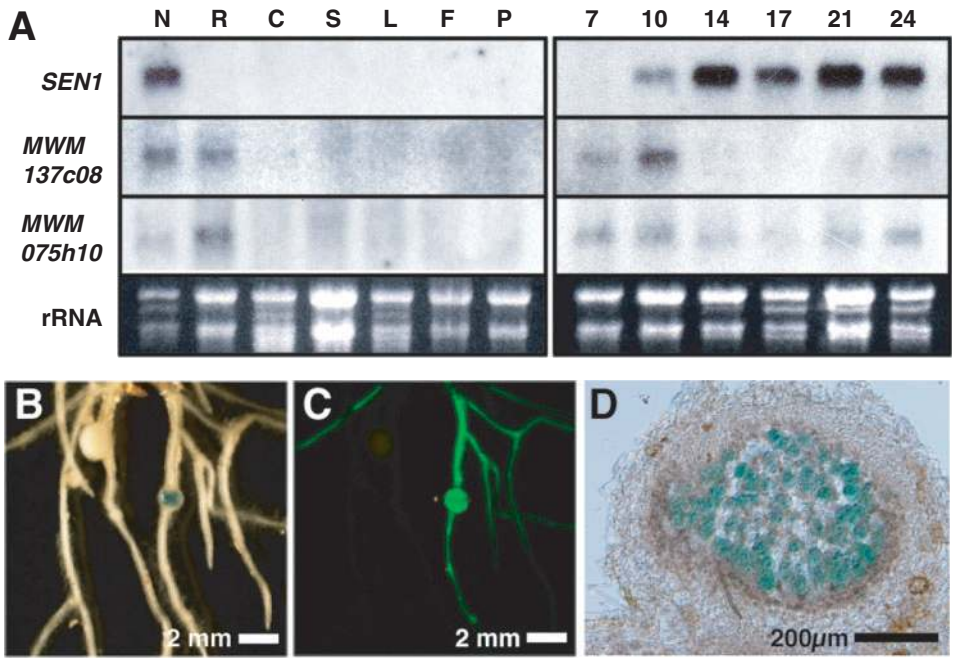


Fig. 3 Expression of *SEN1* and its homologous genes in *Lotus japonicus*. (A) Northern blot analysis of *SEN1* mRNA and the homologous mRNAs *MWM137c08* and *MWM075h10*. Total RNA was isolated from various organs (left) and from the roots and nodules (right) of the wild-type plants. In the left panel, nodules (N), roots (R) and cotyledons (C) were harvested from 3-week-old plants, and stems (S), leaves (L), flowers (F) and pods (P) were obtained from 3-month-old plants. In the right panel, RNAs were prepared from 7-day-old uninoculated roots (7), nodulated roots 10 dpi (10) and nodules (14, 17, 21 and 24 dpi). Ethidium bromide-stained rRNAs are also shown. (B, C and D) Spatial expression of the *SEN1* gene in nodules by *SEN1* promoter–*GUS* fusion. GUS activity shown with blue color was detected only in the nodules on transformed roots (B). Transformed roots were distinguished by fluorescence of GFP as a transformation marker (C). In the transformed nodule, blue areas representing activities of GUS were detected in the infected cells (D).

that GUS activity was detected only in the central zone of nodules formed on transformed hairy roots. Observation at higher magnification demonstrated that expression of the *SEN1* gene was limited to the infected cells of the nodules (**Fig. 3D**).

SEN1 encodes an integral membrane protein

The predicted polypeptide for *SEN1* is 226 amino acids in length with a molecular mass of 23.8 kDa. The SOSUI domain prediction program (Hirokawa et al. 1998) suggested that SEN1 is an integral membrane protein with five transmembrane regions (**Fig. 1B**). Determination of the nucleotide sequences of two EST clones, MWM137c08 and MWM075h10, showed that their deduced amino acid sequences are 57 and 61% identical to SEN1, respectively. SEN1 is homologous to the *Glycine max* nodule-specific polypeptide nodulin-21 with 66% amino acid identity. Nodulin-21 is characterized as a methionine-rich protein, which is specific to root nodules, though its function is unknown (Delauney et al. 1990). In *M. truncatula*, a nodulin cDNA clone termed MtN21 (or nodulin 21) has been reported (Gamas et al. 1996, Ranocha et al. 2010). However, it is noted that MtN21 belongs to a protein family distinct from that of *G. max* nodulin-21. Since the published sequence of *G. max* nodulin-21 was 206 amino acids in length and appeared to be shorter than SEN1 in the C-terminal region, we cloned a cDNA for nodulin-21 from nodule RNA of *G. max* cv. Akisengoku by RT–PCR. It appeared to encode a polypeptide of 236 amino acids, and thus we used this sequence for comparison. The SEN1 protein also shows low but significant homology to CCC1 and VIT1 (approximately 23% overall identity and 61% similarity) which was demonstrated to function as a vacuolar cation transporter in *Saccharomyces cerevisiae* (Fu et al. 1994, Lapinskas et al. 1996, Li et al. 2001) and in *Arabidopsis thaliana* (Kim et al. 2006), respectively. Furthermore, the predicted domain structures of SEN1, NODULIN-21, CCC1 and VIT1 are very similar; they have five membrane-spanning domains, relatively long amino acid stretches between the second and third transmembrane domains, and very short C-terminal sequences following the last transmembrane domain (**Supplementary Fig. S1**).

Glycine max nodulin-21-like proteins contain the DUF125 domain of unknown function, as do other nodulin-21-like proteins of bacteria, fungi, yeasts and plants such as *A. thaliana* and *Oryza sativa* (http://www.ebi.ac.uk/interpro). However, genomic Southern hybridization did not detect DNA fragments homologous to the *SEN1* gene in *A. thaliana* and *O. sativa*, although hybridizing bands were found in multiple legume species (**Fig. 4**). When the amino acid sequences of SEN1 and *G. max*

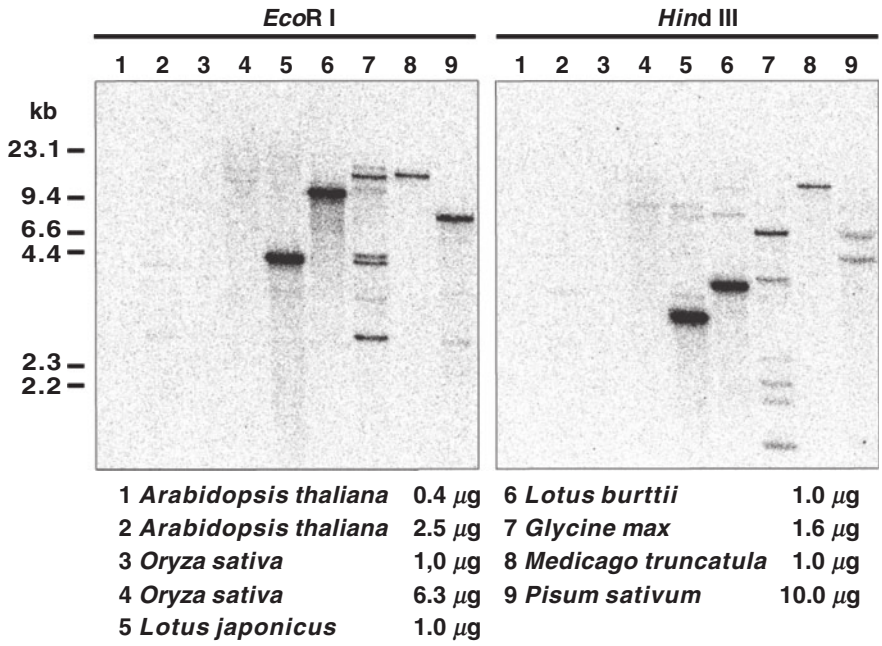


Fig. 4 Detection of the *SEN1* gene in various plant species. Genomic DNAs were isolated from the leaves, digested with *Eco*RI or *Hin*dIII and subjected to Southern blot hybridization with ^{32}P-labeled full-length *SEN1* cDNA as a probe.

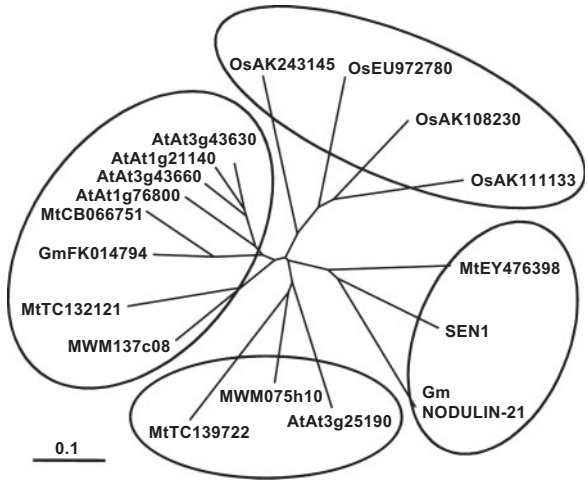


Fig. 5 A phylogenetic tree of SEN1 and the *G. max* nodulin-21-like proteins from various plant species. Amino acid sequences from *Lotus japonicus* (Lj), *Glycine max* (Gm), *Medicago truncatula* (Mt), *Arabidopsis thaliana* (At) and *Oryza sativa* (Os) followed by each accession number were compared by CLUSTALW and the tree was depicted by the TreeView program. Accession numbers: *SEN1* (AB573230), *MWM137c08* (AV766881), *MWM075h10* (AV427050) and *GmNODULIN-21* (X16488).

nodulin-21-like proteins from *L. japonicus*, *G. max*, *M. truncatula*, *A. thaliana* and *O. sativa* were compared, they were categorized into four distinct phylogenetic clades, i.e. the SEN1 type, the MWM137c08 type, the MWM075h10 type and the monocotyledonous (*O. sativa*) type (**Fig. 5**). In this phylogenetic tree, the SEN1 clade consists of genes from only legumes represented by *L. japonicus*, *G. max* and *M. truncatula*. Thus, the SEN1 clade appears to be specific to legumes.

The SEN1 protein was predicted to be localized at the plasma membrane by the PLOC program (Park and Kanehisa 2003) or at the nuclear membrane by the ESL pred program (Bhasin and Raghava 2004), respectively. In contrast, no membrane-targeting signal was predicted with confidence by the WoLF PSORT (Horton et al. 2007), TargetP (Emanuelsson et al. 2007) and predotar (Small et al. 2004) programs. To determine the subcellular location of the SEN1 protein, we introduced *SEN1* fused with a green fluorescent protein (GFP) gene driven by the *SEN1* gene's own promoter into the *sen1-1* mutant, and observed GFP fluorescence in the complemented nodules formed on the transformed hairy roots. However, the GFP signal was too weak to determine the target membrane of the SEN1 protein.

As described above, SEN1 has similarity to the CCC1 and VIT1 proteins of *S. cerevisiae* and *A. thaliana*, respectively. *CCC1* encodes a transmembrane protein and was found to cross-complement the Ca^{2+}-sensitive mutant of *S. cerevisiae* (Fu et al. 1994). Furthermore, CCC1 has been shown to be an iron (Fe)/manganese (Mn) transporter that mediates the accumulation of these metals in the vacuole (Lapinskas et al. 1996, Li et al. 2001). Arabidopsis VIT1, which is an ortholog of CCC1, has been demonstrated to be a vacuolar iron uptake transporter (Kim et al. 2006). Based on these results, we postulate that SEN1 is involved in transport of Fe and/or some other metal ions across the SM from the host cell cytosol to bacteroids. Fe is an essential component of the nitrogenase complex as well as electron transport proteins required for bacteroid respiration and nitrogen fixation, so that lack of Fe could account for the

metabolic defects of *sen1* nodules. To test whether SEN1 can transport Fe, we introduced the *SEN1* gene into *S. cerevisiae*, and determined the concentrations of Fe in the transformed *S. cerevisiae*. However, no significant differences in concentrations of Fe were detected between wild-type and transformed *S. cerevisiae* (**Supplementary Fig. S2**). In addition, attempts to detect differences in the content of Fe between bacteroids of *sen1* nodules and wild-type nodules were unsuccessful (**Supplementary Fig. S2**).

Further characterization of *sen1* mutant phenotypes

We previously described the *sen1-1* (*Ljsym75*) symbiotic phenotypes with regard to bacteroid differentiation and expression of nitrogenase proteins, and reported that in *sen1-1* nodules nitrogenase proteins were not detectable (Suganuma et al. 2003). However, since proteome analysis of the bacteroids prepared from the *sen1-1* nodules indicated the presence of small amounts of nitrogenase proteins (N. Suganuma, M. Nomura and S. Tajima, unpublished data), we re-examined the expression of the *NifH* gene and the presence of nitrogenase proteins in bacteroids isolated from the *sen1-1* nodules. Expression of the *NifH* gene in *sen1-1* bacteroids was detected by quantitative RT–PCR analysis, although the level of expression was significantly lower than that in the wild type (**Fig. 6A**). Furthermore, trace amounts of nitrogenase proteins, both component-I and component-II, were detected in the mutant by immunoblotting in combination with a chemoluminescent detection system that has much higher sensitivity than the chromogenic detection system used previously (**Fig. 6B**). Nevertheless, the nitrogenase activity of *sen1* nodules was undetectable (**Fig. 2B**; see also Suganuma et al. 2003), indicating that SEN1 is essential for nitrogenase activity but not nitrogenase biosynthesis per se.

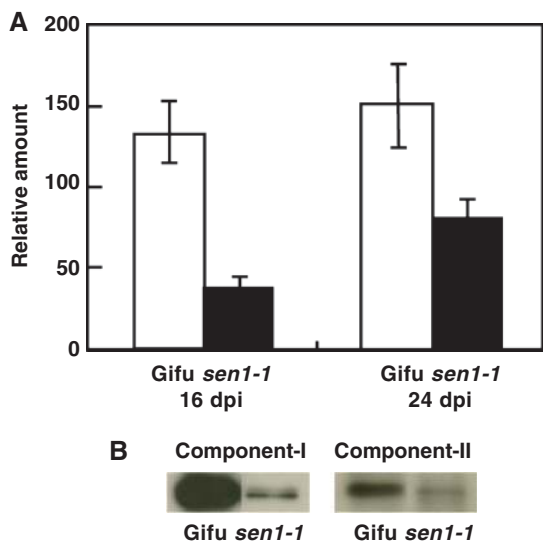


Fig. 6 Expression of *NifH* and nitrogenase proteins in nodules formed on the wild-type plant and the *sen1* mutant. (A) Expression of the *NifH* gene. Bacteroids were isolated from nodules at 16 and 24 dpi, and total RNAs were prepared. Expression of *NifH* was evaluated by quantitative RT–PCR with *sigA* as an internal standard. (B) Immunological detection of nitrogenase component-I and -II proteins. Bacteroids were isolated from nodules of the wild-type Gifu plants and the *sen1-1* mutants at 16 dpi. Aliquots of protein were fractionated by SDS–PAGE, blotted onto a membrane, and Western blot analysis was carried out with antisera raised against nitrogenase component-I and -II from pea nodule bacteroids.

We further examined the structure of *sen1-1* nodules at early stages of endosymbiosis by light and electron microscopy, because our previous observations were made at late stages (3–8 weeks post-inoculation) of nodule development (Kawaguchi et al. 2002, Suganuma et al. 2003). In *sen1* nodules at very early stages of nodule development (8 days post-inoculation; dpi), infected cells were highly vacuolated with a large number of small vacuoles, whereas the wild-type nodules did not appear to have vacuoles at this stage, as observed in the nodules at 21 dpi (Kawaguchi et al. 2002) (**Fig. 7A, B**). At the ultrastructural level, irregularly shaped, enlarged symbiosomes were observed in the *sen1* nodules at 8 dpi (**Fig. 7E, H**). The enlargement of symbiosomes was more drastic at 10 dpi (**Fig. 7F, I**), the bacteroids were smaller in size compared with the wild type (**Fig. 7D, G**) and there was less electron-dense material inside the bacteroids of mutant nodules. These symptoms were not observed in ineffective nodules formed by an *NifH*-defective (Δ*NifH*) mutant strain of *M. loti* (**Fig. 7C**; see also Kumagai et al. 2007).

Discussion

Lotus japonicus symbiotic mutants have been grouped into three categories, i.e. Nod$^-$ mutants in which both nodulation and rhizobial infection do not occur, Hist$^-$ mutants which have defects in infection thread formation and produce rudimentary nodules (mostly small bumps) with no endosymbiotic bacteria inside, and Fix$^-$ mutants which form nodules filled with endosymbionts but have defects in nitrogen-fixing activity (Kawaguchi et al. 2002). In this paper, we focused on characterization of one of the Fix$^-$ mutants to shed light on the function and regulation of symbiotic nitrogen fixation rather than on the bacterial infection process or nodule development.

The *sen1* mutant has a unique characteristic among the Fix$^-$ mutants isolated so far, because it completely lacks nitrogen fixation activity throughout nodule development (Suganuma et al. 2003). This is in contrast to other Fix$^-$ mutants from *L. japonicus*, such as *sst1* (Krusell et al. 2005), *fen1* (Hakoyama et al. 2009), *ign1* (Kumagai et al. 2007), *sym105* (Hossain et al. 2006), and *sym102*, *sym104* and *sym43* (Sandal et al. 2006), all of which show low nitrogen fixation activity. The molecular identification of the *SEN1* gene presented here provides insights into the essential role of SEN1 protein for nodule function.

SEN1 encodes an integral membrane protein that shows high homology to *G. max* nodulin-21, which represents an

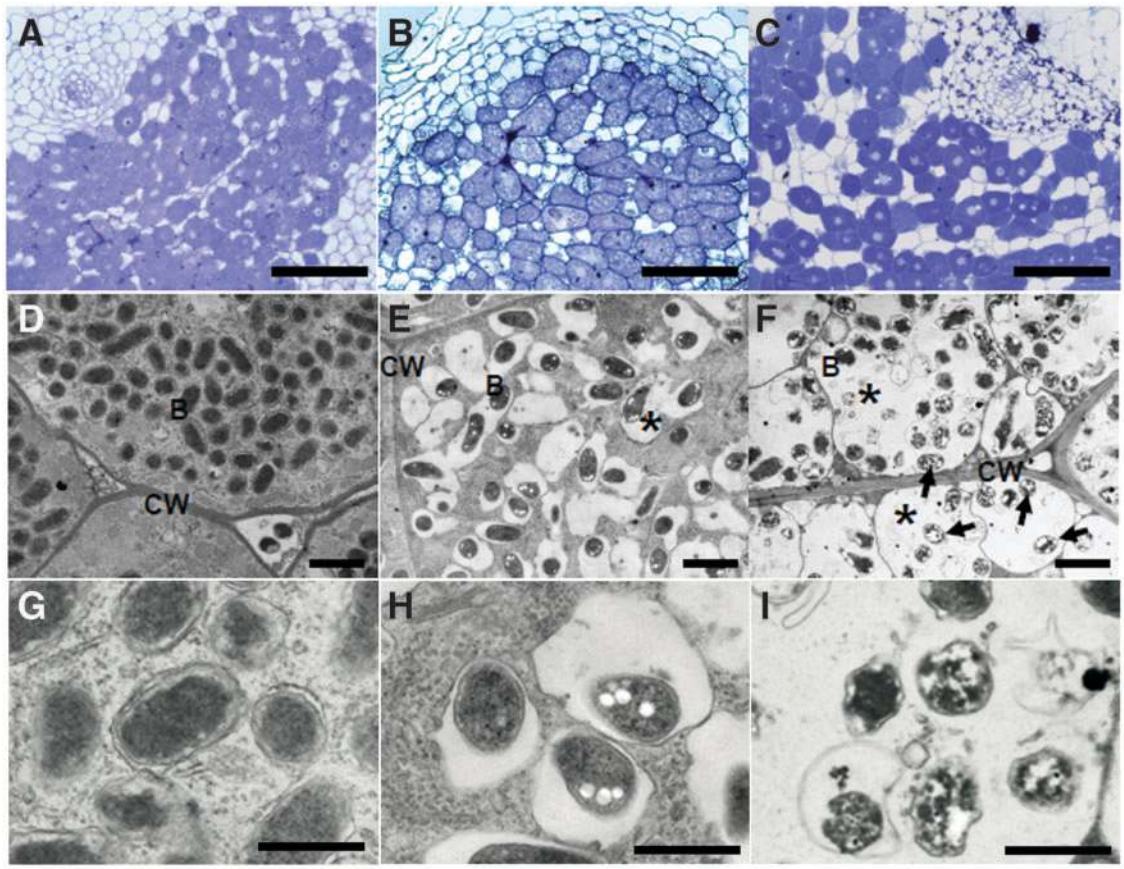


Fig. 7 Nodule and infected cell structures of the *sen1* mutant at early stages of nodule development. Light micrographs of nodules 8 dpi are shown for wild-type Gifu plants (A), the *sen1-1* mutant (B) and nodules formed by inoculation of the Δ*NifH Mesorhizobium loti* strain (C). Electron micrographs of infected cells are shown for an 8 dpi wild-type nodule (D), and 8 dpi (E) and 10 dpi *sen1-1* (F) nodules. Asterisks indicate irregularly shaped and/or enlarged symbiosomes, and arrows indicate deteriorated bacteroids (E, F). Magnifications of symbiosomes are also shown for the 8 dpi wild-type nodule (G), and 8 dpi (H) and 10 dpi *sen1-1* (I) nodules. Bars = 100 μm (A, B, C), 2 μm (D, E, F) and 1 μm (G, H, I). B, bacteroid; CW, cell wall.

abundant nodulin in soybean nodules (Delauney et al. 1990). The *G. max* nodulin-21-like gene family is widely spread in the plant kingdom, including dicots and monocots (**Fig. 5**), although none of them has been assigned a specific biological function. Among the proteins of the *G. max* nodulin-21 family, SEN1 belongs to a legume-specific clade that is comprised of SEN1, Gm nodulin-21 and MtEY476398. As shown in **Fig. 3**, two genes, *MWM137c08* and *MWM075h10*, which have high similarity to SEN1, were expressed constitutively in both roots and nodules, suggesting that these genes are the paralogous housekeeping genes. *SEN1*, corresponding to ORF No. 5 in **Fig. 1**, and *MWM075h10*, corresponding to ORF No. 6, are adjacent on *Lotus* chromosome 4. Therefore, it is likely that *SEN1* was recruited from the housekeeping gene(s) by tandem duplication during evolution of nitrogen-fixing symbiosis in legumes.

In the present study, we could not demonstrate the target membrane of the SEN1 protein by GFP fusion experiment. However, expression analyses showed that *SEN1* expression is restricted in nodule-infected cells (**Fig. 3**). Therefore, it is possible that SEN1 is an integral SM protein. The SM serves as the physical and functional interface between the host plant cell cytoplasm and symbiotic rhizobia, and it controls transport of nutrients between the two (Udvardi and Day 1997). In fact, the bacteria are completely dependent on the plant for reduced carbon and all other nutrients, including metal ions required for metabolism and cofactor synthesis. Therefore, the SM must contain a diverse array of transporters, many of which may be nodule specific given the unique nature of this membrane and the legume–*Rhizobium* symbiosis. Indeed, a number of proteins with putative transport functions have been identified by proteome analyses of the SM prepared from nodules of various legume species (Saalbach et al. 2002, Wienkoop and Saalbach 2003, Catalano et al. 2004). The predicted SEN1 protein is homologous to *G. max* nodulin-21, and also to CCC1, a vacuolar Fe/Mn transporter of *S. cerevisiae*, and to VIT1, a vacuolar iron transporter of *A. thaliana*. From these results, we hypothesize that SEN1 is involved in transport of Fe across the SM, though we have no direct evidence about the transport function of SEN1 at present. Further investigations to demonstrate

the subcellular localization of the SEN1 protein and its transport function are currently under way in our laboratory.

The *sen1* mutant completely lacks nitrogen fixation activity (**Fig. 2B**; Suganuma et al. 2003) despite the presence of low levels of nitrogenase proteins (**Fig. 6**). At the same time, microscopic observations of nodule and infected cell structures strongly suggest that SEN1 is also essential for symbiosome and/or bacteroid differentiation, as suggested by our previous report (Suganuma et al. 2003). Even at a very early stage of nodule development, abnormal and incomplete differentiation of symbiosome and bacteroids was observed in the *sen1* nodules (**Fig. 7B, E, F**). This might be due to incorporation of bacteroids and/or degrading symbiosomes into lytic vacuoles as suggested for *sst1* (Krusell et al. 2005) and *sym105* mutants (Hossain et al. 2006). Interestingly, the infected cells of nodules formed by inoculation with an *NifH*-defective *M. loti* mutant strain did not show such severe deterioration of symbiosomes and bacteroids inside, at least at the same developmental stages (**Fig. 7C**; Kumagai et al. 2007), even though they completely lack nitrogenase activity. Therefore, it is evident that abnormal and incomplete differentiation of symbiosomes and bacteroids in the *sen1* nodules is not simply due to the absence of nitrogen fixation activity. Rather, such extremely rapid deterioration of symbiosomes and bacteroids suggests that SEN1 is essential for symbiosome organogenesis and/or bacteroid differentiation. This is not inconsistent with the hypothesis that SEN1 is involved in Fe transport to bacteroids inside the symbiosomes. Fe is crucial to many proteins, including bacteroid-specific cytochrome oxidases (Nellen-Anthamatten et al. 1998, Sciotti et al. 2003), which enable respiration under low oxygen concentrations in nodules, and thus probably precede the induction of nitrogenase activity. In addition, heme proteins are also involved in redox sensing that is required for bacteroid differentiation (Dixon and Kahn 2004). An alternative possibility is that SEN1 is more directly involved in symbiosome and/or bacteroid differentiation like, for example, DNF1, a component of the signal peptidase complex in *M. truncatula* (Van de Velde et al. 2010, Wang et al. 2010).

In the symbiotic association of plant and arbuscular mycorrhizal fungi, it has been shown that arbuscule development and the ability to import phosphate to the host plant are impaired in a *M. truncatula str* mutant defective in a half-ABC transporter that is located in the peri-arbuscular membrane (Zhang et al. 2010). Although its precise function is unknown, the transporter is predicted to export a signal molecule or a nutrient from the host cortical cell to the peri-arbuscular apoplastic space. In legumes, the initial stages of rhizobial and mycorrhizal symbiosis are controlled by a common symbiosis signaling pathway (Parniske 2008). However, endosymbiotic rhizobia and mycorrhizal fungi finally display disparate functions. In addition, SEN1 belongs to a family distinct from ABC transporters, and thus each function is likely to be different. Nevertheless, it is intriguing to note that transporters encoded in the host plant genome may regulate the developmental programs of intracellular symbiotic organelles and their functions in a different type of plant and microbe symbiotic association.

In this study, SEN1 was identified by map-based cloning of an *L. japonicus* Fix$^-$ mutant *sen1*. By re-examination of the symbiotic phenotypes of the *sen1* mutant nodules, it appears that SEN1 is required for nitrogenase activity and is also involved in symbiosome and/or bacteroid differentiation. The predicted structure of the SEN1 protein suggested that it has a transport function. Although the subcellular localization and exact biochemical function of the SEN1 protein remains to be elucidated, its discovery will provide further insight into legume and *Rhizobium* symbiosis.

Materials and Methods

Plant and bacterial materials

Seeds of the parent *L. japonicus* ecotype Gifu B-129 and the Fix$^-$ mutant *sen1-1* (*Ljsym75*), *sen1-2* (s88), *sen1-3* (*Ljsym11*) and *sen1-4* (*Ljsym61*) derived from it (Schauser et al. 1998, Kawaguchi et al. 2002) and *sen1-5* (F15) derived from ecotype Miyakojima MG-20 were surface-sterilized and sown in sterilized vermiculite with *M. loti* MAFF303099 or an *NifH*-defective mutant of MAFF303099. The plants were grown with 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 by Imaizumi-Anraku et al. (1997).

Map-based cloning

Mapping was performed as described previously (Suganuma et al. 2003). Total DNA was extracted from leaves of F_2 homozygous plants generated by crossing with *L. japonicus* ecotype Miyakojima MG-20, and PCR was carried out with SSR (simple sequence repeat) and dCAPS (derived cleaved amplified polymorphic sequence) 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 Vistra Green (GE Healthcare).

cDNA cloning

The *SEN1* cDNA was prepared from RNA isolated from wild-type Gifu nodules using a SMART RACE cDNA amplification kit (TAKARA). 5′ and 3′ RACE (rapid amplification of cDNA ends) were performed with the 5′ RACE-specific primer 5′-GTTAATTGCTCTCATTAGACAAGTGAC-3′ and the 3′ RACE-specific primer 5′-AGTCTTGACTCTTTGTTCTCTACC TC-3′ in combination with the universal primer supplied in the kit. Based on the sequence of the 5′ RACE product, the 5′Sen1 primer 5′-TCCACCCTCTTCATCTCTCAAGTC-3′ was generated, and the full-length *SEN1* cDNA was amplified from the 3′ RACE cDNA pool with 5′Sen1 and the universal primers.

Complementation test

The *SEN1* promoter and the *SEN1* terminator fragments were amplified from genomic DNA isolated from wild-type Gifu leaves with the pSen1F primer 5′-TGGGTACCACAACTTCAA GGACAAATTGACT-3′, the pSen1R primer 5′-CGGATCCTTGG TGCGAACAAGCAAAAAAGTAG-3′, the tSen1F primer 5′-GA ACTGCAGAATCCTCTTGGCCCTGGTTTTTAGT-3′ and the tSen1R primer 5′-TCCAAGCTTTTCCGGTTCGAGTGAGCCTC AGGA-3′. The coding region of the *SEN1* gene was amplified from full-length cDNA with the Sen1ORFF primer 5′-GGATCC ATGGCTGCTGGTGCACCCAA-3′ and the Sen1ORFR primer 5′-CTGCAGTTATATTTCCAACCCACCAG-3′. These three amplified fragments were inserted together into cDNA, and amplified fragments were inserted together into pC1300GFP. The pC1300GFP was made from pCAMBIA1300 (CAMBIA), where the hygromycin resistance gene was replaced with the sGFP gene (Niwa et al. 1999, Kumagai and Kouchi 2003). The resultant construct was transformed into *A. rhizogenes* LBA1334.

Hairy root transformation of the *sen1-1* mutant and wild-type Gifu using *A. rhizogenes* LBA1334 was performed as described by Kumagai and Kouchi (2003). In brief, the seedlings (5–6 d old) were placed in *A. rhizogenes* suspension and then cut at the base of the hypocotyl. The seedlings were transferred onto agar plates containing half-strength Jensen +N medium (Diaz et al. 1989), placed vertically in a growth cabinet and grown for 5 d for co-cultivation. Then the plants were transferred onto HRE agar medium containing 150 μg ml^{-1} cefotaxime and grown for 10 d. The plants with transgenic hairy roots were transferred to vermiculite pots supplied with half-strength B & D medium and grown in a growth cabinet (16 h day and 8 h night, 24°C). Four days after the transfer, the plants were inoculated with *M. loti* strain Tono (Kawaguchi et al. 2002). Twenty-four days after the inoculation with *M. loti*, non-transformed nodules that showed no GFP fluorescence were detached from roots and then the acetylene reduction activity of the remaining transformed nodules was measured.

Acetylene reduction assay

Acetylene reduction activity was assayed in a closed system with nodulated roots detached from freshly harvested intact plants (Suganuma et al. 2003). Nodulated roots were placed in 35 ml vials and incubated at 25°C. After 30 min, the amount of ethylene produced was determined by gas chromatography.

Northern and Southern blot analyses

Northern and Southern blot analyses were carried out as described (Suganuma et al. 2004). Total RNA or genomic DNA digested with *Eco*RI or *Hin*dIII was separated by electrophoresis and transferred to a Hybond-XL membrane (GE Healthcare). The membranes were hybridized with DNA probes labeled with [^{32}P]dCTP. Hybridization was carried out in 0.5 M sodium phosphate buffer (pH 7.2) that contained 1 mM EDTA and 7% SDS at 65°C for 16 h. After incubation, the filters were washed with 2 × SSC (0.6 M NaCl and 0.06 M sodium citrate) containing 1% SDS at room temperature and twice with 0.1 × SSC containing 0.1% SDS at 65°C.

Promoter–GUS analysis

The amplified *SEN1* promoter fragment was inserted into pC1301GFP, resulting in construction of the *SEN1* promoter–*GUS* fusion gene. pC1301GFP was made from pCAMBIA1301 (CAMBIA) by replacement of its hygromycin resistance gene with the sGFP gene. The wild-type Gifu was transformed with *A. rhizogenes* LBA1334 carrying the *SEN1* promoter–*GUS* constructs. Whole roots were immersed in a staining solution (2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 100 mM sodium phosphate, pH 7.0) and incubated at 37°C for 16 h in the dark. To observe GUS activity inside the nodules, transformed nodules were excised from transgenic hairy roots and embedded in 5% agar. The embedded nodules were sectioned at 60 μm thickness using a microslicer (VT1200S, Leica). The sections placed on a glass slide were covered by a staining solution and incubated at 37°C for 16 h in the dark. The stained materials were observed with a light microscope.

Phylogenetic analysis

Amino acid sequences of Nodulin-like proteins were aligned using the CLUSTALW program from the GenomeNet server (http://www.genome.jp). The phylogenetic tree was drawn using the program TreeView32 (Page 1996).

Fluorescence microscopy

The coding region of the *SEN1* gene was amplified from the *SEN1* cDNA with the Sen1F primer 5′-TGTACAAGATGGCTGC TGGTGCACCCAA-3′ and the Sen1R primer 5′-CTGCAGTTAT ATTTCCAACCCACCAG-3′, and the amplified fragment was fused to the *sGFP* (S65T) cDNA in-frame at the *Bsr*GI site (Niwa et al. 1999). The *GFP–SEN1* fusion fragment and *SEN1* promoter and terminator fragments described above were inserted together into pCAMBIA1300 (CAMBIA), and the resulting plasmid was introduced into the *sen1-1* mutant by the hairy root transformation system. Fluorescence of GFP was observed using TCS SP5 confocal laser scanning microscopy (Leica).

Determination of iron (Fe) concentration

The coding region for *SEN1* was amplified from the *SEN1* cDNA with the Sen1YESF primer 5′-GGGGTACCATGGCTGCTGGTG CACCCA-3′ and the Sen1YESR primer 5′-CGGGATCCCTATAT TTCCAACCCACCAGC-3′. The mutated *SEN1* gene containing a single nucleotide mutation (*SEN1m*, corresponding to mutation in *sen1-1*) was amplified from a mixture of two overlapping DNA fragments, which were amplified by the Sen1YESF primer and the Sen1m internal reverse primer 5′-AGCACGAAGCCAT TGAACCCTCTGCCAGTA-3′, and the Sen1m internal forward primer 5′-AGGGTTCAATGGCTTCGTGCTGCGGTGTTA-3′ and the Sen1YESR primer. The amplified PCR fragments were

ligated into the pYES2 (Invitrogen) yeast expression vector containing the *GAL1* promoter. The production of recombinant proteins was induced by incubation at 25°C with addition of galactose. The *S. cerevisiae* cells were collected by centrifugation and were dried. Nodules harvested from the wild-type Gifu plants and the *sen1-1* mutants at 24 dpi with *M. loti* were homogenized in 50 mM Tris–HCl (pH 7.5) and 0.15 M NaCl, and separated into a host plant fraction and a bacteroid fraction by centrifugation. Dried *S. cerevisiae* cells were digested using a microwave, and the host plant and bacteroid fractions were digested with nitric acid and hydrogen peroxide. Concentrations of Fe was determined by inductively coupled plasma atomic emission spectoscopy.

Quantitative RT–PCR analysis

Nodules were harvested from the wild-type Gifu plants and the *sen1-1* mutants at 16 and 24 dpi with *M. loti*, and bacteroids were prepared from each nodule by centrifugation as described previously (Suganuma et al. 2003). Total RNA was isolated from the bacteroids by the method of Sung et al. (2003). In brief, cells were suspended in extraction buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.2% Triton-X 100), and boiled at 100°C for 10 min. Boiled samples were extracted by chloroform–methanol, and finally total RNA was precipitated by ethanol. The RNA was further treated with DNase (RQ1, Promega) and reverse-transcribed by Superscript II (Invitrogen) with the NifH primer 5′-AAGCTCGGCAAGCATTTGCT-3′ and the sigA primer, 5′-CCTTTTCCTTCTCCAGCTCT-3′, respectively. The resultant cDNA was used as template for real-time PCR with a Lightcycler model 350 S (Roche Diagnostics) according to the standard procedures described in the manufacturer's manual. Expression of the *NifH* gene was examined with the NifHF primer 5′-CTCAATTCCAAGCTCATCCA-3′ and the NifHR primer 5′-TCTTCATGATGCCGAAGTCG-3′, and *sigA* gene expression as an internal standard was examined with the sigAF primer 5′-GCCCTCTGCTCGACCTTTCC-3′ and the sigAR primer 5′-AGCATCGCCATCGTGTCCTC-3′ (Ott et al. 2005).

Immunoblotting analysis

Bacteroids were isolated from nodules of wild-type Gifu plants and the *sen1-1* mutants at 16 dpi with *M. loti*. The bacteroids were suspended in SDS–PAGE sample buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 50 mM dithiothreitol, 0.1% bromophenol blue) and fractionated by SDS–PAGE with 10% (w/v) polyacrylamide gel. After electrophoresis, the proteins were blotted onto an Immobilon-P filter (Millipore) and reacted with antisera against nitrogenase components-I and -II from pea bacteroids provided by Dr. T. Bisseling. Immnunoreactive protein was visualized by using the ECL plus Western blotting detection system (GE Healthcare).

Light and electron microscopy

Nodule samples were cut into small pieces (1 mm thick) and fixed in 2.5% glutaraldehyde and 4.0% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 3 h at room temperature and then overnight at 4°C. After washing in the same buffer, the samples were post-fixed in 0.5% osmium tetraoxide in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature, dehydrated through a graded ethanol and then an acetone series, and embedded in an epoxy resin (Quetol-812, Nisshin EM). Thin and ultrathin sections were made using an ultramicrotome (UltraCut-R, Leica) equipped with a glass knife and a diamond knife (Diatome, JEOL), respectively. Thin sections were observed by light microscopy after staining with 0.002% toluidine blue. Ultrathin sections were post-stained with uranium acetate and lead citrate, and were observed by electron microscopy (H-7100, Hitachi).

Supplementary data

Supplementary data are available at PCP online.

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