

Positional Cloning Identifies *Lotus japonicus* NSP2, A Putative Transcription Factor of the GRAS Family, Required for *NIN* and *ENOD40* Gene Expression in Nodule Initiation

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Abstract

Rhizobia-secreted Nod-factors (NFs) are required for nodulation. In the early developmental process of nodulation, a large number of changes occur in gene expression. *Lotus japonicus nsp2* mutants isolated from Gifu B-129 ecotype have defects in nodule initiation and display non-nodulating phenotype. Here, we describe positional cloning of *LjNSP2* as a component of the nodulation-specific signaling pathway. *LjNSP2* was mapped near the translocation site of chromosome 1 where the recombination is severely suppressed. To circumvent this problem, we introduced *Lotus burttii* as an alternative crossing partner in place of *L. japonicus* Miyakojima. The development of the high-resolution map using a total of 11 481 F2 plants, in combination with newly developed DNA markers and construction of BAC library, enabled us to identify the gene responsible for mutant phenotype. *LjNSP2* encodes a putative transcription factor of the GRAS family that constitutes a subfamily with *Medicago truncatula NSP2*. *LjNSP2* was expressed in roots and early nodules, but strongly suppressed in matured nodules. The expression analysis of *NIN* and *LjENOD40-1* genes in *Ljnsp2* mutants indicates that *LjNSP2* functions upstream of these genes. These results suggest that *LjNSP2* acts as a transcription factor to directly or indirectly switch on the NF-induced genes required for nodule initiation.

Key words: positional cloning; *Lotus japonicus*; transcription factor; nodule initiation

1. Introduction

The legume–rhizobia symbioses lead to the formation of novel organs, termed nodules, which arise from division of cortical cells in the root and the infection of these nodules by rhizobia. Rhizobia within nodule cells differentiate to bacteroids which fix atmospheric nitrogen. Lipochitin oligosaccharides, Nod-Factors (NFs) secreted

by rhizobia are responsible for nodule formation and induce a variety of responses in a host-specific manner, including root hair deformation and cortical cell division, during the early steps of nodulation. Ca²⁺ spiking in root hair cells is one of the most early responses to NFs.¹

In the past few years, the phenotypes of an increasing number of symbiotically defective mutants have been analyzed in the model legumes, *Lotus japonicus* and *Medicago truncatula* to dissect the NF signaling pathway.^{2–4} Several genes required for nodulation have been identified by positional cloning. *L. japonicus NFR1*,⁵ *NFR5*⁶ and *Medicago LYK3*⁷ encode transmembrane receptor-like serine/threonine kinases with

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putative extracellular regions similar to LysM domains, which are thought to be directly involved in perception of NF signal. In subsequent signal transduction, *L. japonicus* SYMRK,⁸ CASTOR and POLLUX,⁹ *M. truncatula* DMI1,¹⁰ DMI2¹¹ and DMI3¹², and *L. japonicus* CCaMK¹³ and Nup133¹⁴ have been identified as components required for the common symbiosis pathway shared between the fungal and bacterial endosymbiotic systems. The *L. japonicus* nin mutant showed normal mycorrhization and early responses following rhizobial inoculation, including root hair deformation,¹⁵ Ca²⁺ influx and Ca²⁺ spiking.¹⁶ NIN was cloned as a putative transcription factor gene and shown to be expressed during both early and late stages of nodule development, suggesting that NIN controls various developmental aspects of nodulation after the perception of rhizobia in root hairs. However, little is known about what signal component relays the signal from the common symbiotic pathway into the nodulation-specific program in *L. japonicus*. Recently, GRAS family genes, *nodulation signaling pathway 1* (*NSP1*) and *NSP2*, have been identified as putative transcription factors functioning downstream of Ca²⁺ spiking and CCaMK in *Medicago truncatula*.^{17,18}

Here, we describe the positional cloning and characterization of nodulation-specific *LjNSP2* gene encoding a plant-specific GRAS protein most similar to *MtNSP2*, which might lead to the induction of expression of genes required for rhizobial infection and early nodule development.

2. Materials and methods

2.1. Plant materials

The mutant carrying *Ljnspp2-1* was isolated from the EMS-mutagenesis experiments of *L. japonicus* Gifu B-129.³ The mutant *Ljsym35* was isolated from a population of Gifu derived from a transposon-/DNA-tagging trial, as a non-tagged culture mutant,¹⁹ and was kindly provided by Prof. Jens Stougaard (Arrhus University, Denmark).

2.2. Root hair deformation and assays of Ca²⁺ spiking

Seeds of *L. japonicus* were germinated and grown on BNM agar medium essentially as described previously,⁹ except that the roots were grown between two filter papers (grade 0860; Schleicher and Schüll, UK), one of which was on the agar surface. Root hair deformation was scored as described previously following 16 h exposure to 10⁻⁸ M NF in 1 ml BNM medium in a chamber on a microscope slide. Images were taken with a digital-camera attached to an inverted microscope. Ca²⁺ spiking was assayed as described previously,⁹ following

the addition of 10⁻⁸ M NF. Representative traces were selected from at least 10 independent cells.

2.3. Genetic mapping population and genomic DNA isolation

Genetic mapping of the *LjNSP2* gene was performed with F2/F3 population derived from cross between *Ljnspp2-1* (Gifu B-129) and the early flowering ecotype Miyakojima MG-20²⁰ or *Lotus burttii* B-303.²¹ For AFLP analysis, genomic DNA was extracted from 0.1 g leaves using a Qiagen DNeasy plant kit according to the manufacture's protocol. For PCR markers analysis, DNA was extracted from one young leaf in 100 µl of PEB (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS), precipitated with isopropanol, washed and dissolved in 100 µl of TE (pH 8.0).

2.4. High efficiency genome scanning (HEGS)/AFLP or PCR-based screening of *LjNSP2*-inked markers

Genomic DNA (100 ng) was digested with *EcoRI* and *MseI*, ligated to *EcoRI*- and *MseI*-adaptors and preamplified by using *EcoRI*- and *MseI*-adaptor primers. Preamplified DNA was prepared at 0.05 mg/µl concentration before amplification with selective primers. A bulked segregant analysis was performed to identify markers linked to *LjNSP2*. Bulks were constructed from preamplified DNAs of 10 recessive (Nod⁻) or dominant (Nod⁺) homozygous F2 plants, 4096 selective primer combinations of *EcoRI*+3/*MseI*+3 were screened to identify markers present only in the bulk of dominant homozygotes.

Subsequently, *LjNSP2*-linked HEGS/AFLP markers were excised directly from polyacrylamide gels and cloned with the TOPO TA Cloning Kit (Invitrogen). The cloned markers were sequenced and primers were designed by using the software Primer 3 (Whitehead Institute, Cambridge, MA). SCAR (sequence characterized amplified region) markers, which revealed polymorphism between *L. japonicus* accession Gifu and Miyakojima MG-20 or *L. burttii*, were analyzed in 2989 or 8472 F2/3 individuals from Miyakojima MG-20 or *L. burttii*, respectively.

To facilitate the efficiency of electrophoresis of AFLP or PCR products, the HEGS system²² was adapted. In this system, a set of electrophoresis apparatus is equipped with two sets of 24.5 × 26.5 cm glass plates, each accommodating a gel with 100 lanes and analysis of 400 samples is practicable in single run. After electrophoresis, the gels were stained with Vistra Green (Amersham Biosciences) and scanned by fluorescent gel scanner (FluorImager 595; Amersham Biosciences).

2.5. BAC contig development

Three-dimensional BAC DNA pools prepared from our BAC library²³ were screened with *LjNSP2*-linked

HEGS/AFLP or SCAR markers as described above. *Hind*III-digested DNA from positive BACs was fractionated on an agarose gel for fingerprinting and determination of overlaps. BAC ends were sequenced using M13 reverse or forward primer and non-repetitive sequences in BAC were used for chromosome walking to screen 3-D BAC pools with PCR or AFLP. The process was repeated as needed to complete the BAC contig. Polymorphic PCR fragments in BAC sequences were analyzed to directly score recombinants in F2/F3 populations.

BAC clone 188C5 containing the *LjNSP2* gene was shotgun-sequenced and then annotated by Rice-GAAS (Rice Genome Automated Annotation System) (<http://ricegaas.dna.affrc.go.jp/>).

2.6. Complementation experiments

For complementation, a 6.9 kb *Pst*I fragment, carrying only the wild-type *LjNSP2* 1500 bp ORF and 4486 and 899 bp of upstream and downstream sequence, respectively, was cloned into the hairy root transformation vector, which was made from pCAMBIA1300 by replacing the hygromycin-resistant gene with *sGFP(S65T)*.²⁴ The resulting *LjNSP2* recombinant plasmid was introduced into *A. rhizogenes* LBA1334²⁵ by electroporation. Hairy root transformation of *Ljnspp2-1* mutant was performed as described.²⁶ The plants with transgenic hairy roots were grown in vermiculite pots and inoculated with *Mesorhizobium loti* TONO. GFP fluorescence and nodule formations were confirmed 4 weeks after inoculation.

2.7. Southern and northern hybridizations

Genomic DNA was extracted from leaves using CTAB method²⁷ from *L. japonicus*, and 2.5 µg DNA were digested by *Eco*RI, electrophoresed on 0.8% agarose gel and blotted to nylon membrane (Biodyne A, Pall).

Twelve-day-old plants were inoculated with *M. loti* TONO. Infected roots at 4 days post inoculation (dpi) and nodules at 8–32 dpi were immediately frozen in liquid nitrogen. Total RNA was isolated from flower, shoot, root and nodule tissue, and 5 µg aliquots were electrophoresed by denaturing agarose gel and blotted as above.

LjNSP2 probe (1322 bp) was amplified from 188C5 BAC DNA with primers 5'-ACTTCCACCACCTCATC-GAC-3' and 5'-ACAAGTCCAAAGGGATGCAG-3', and labeled with ³²P using a random primer labeling kit (Takara). Hybridization was done at 63°C in Church buffer [0.5 M sodium phosphate, pH 7.2, 7% (w/v) SDS, 1 mM EDTA]²⁸ and the filters were washed once in 2× SSC containing 0.5% SDS at room temperature for 10 min, and twice in 0.2× SSC, 0.1% SDS at 63°C for 15 min. The hybridized membranes were then exposed for 3 days to phosphor imaging plates (Fuji, Tokyo, Japan), which were then scanned by a phosphor imaging

scanner (Storm840, Amersham Bioscience). After stripping, the same filters were reprobated with the 400 bp fragment from *L. japonicus* ubiquitin cDNA as a loading control.²⁸

2.8. Transient expression of the *LjNSP2*-GFP fusion protein in onion epidermal cells

The 1.5 kb ORF of *LjNSP2* was amplified from 188C5 BAC DNA using the primers: [5'-ACGCGTCTCGACA-TGGAAATGGATATAGATTGCATCC-3' (*Sa*II-site underlined), 5'-CATGTTCATGAATGCACAATCTGATTCTGAAGAAC-3' (*Bsp*HI-site underlined)], digested with *Sa*II and *Bsp*HI, and cloned into pUC18-CaMV35SΩ-sGFP(s65T)-NOS plasmid²⁹ at the *Sa*II/*Nco*I sites just upstream of GFP gene. Onion epidermal cells were bombarded with DNA-coated particles using a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad).⁹ About 18–24 h after bombardment, the cells were observed using a Bio-Rad Radiance2000 confocal laser scanning microscope.

The sequence data of *LjNSP2* and genomic sequences (B-129 Gifu) of *LjNSP2* have been deposited with the DDBJ data library under accession numbers AB241456 and AB241457, respectively.

3. Results

3.1. Early infection phenotypes of a *Ljnspp2* mutant

The *Ljnspp2-1* mutant of *L. japonicus* grew normally in nitrogen-rich compost, and established a normal symbiosis with the mycorrhizal fungus,³⁰ but did not form nodules under nitrogen limitation when inoculated with *M. loti*. The addition of *M. loti* to *Ljnspp2-1* seedlings induced no phenotypes, such as root hair curling, infection thread formation and cortical cell division, typically seen in the wild-type (data not shown). However, NF did induce swelling and branching in root hair tips, although at a reduced level compared with the wild-type (Fig. 1A and H). Cytological staining of *M. loti* expressing *lacZ* revealed no infection foci and no infection thread formation (data not shown). Intracellular Ca²⁺ spiking, which is induced by NF and has been proposed to be integrated by a Ca²⁺-calmodulin-dependent protein kinase required for activation of early nodulation gene expression and mycorrhization¹, was indistinguishable in the mutant from the wild-type (Fig. 1I). The observed induction of Ca²⁺ spiking and normal mycorrhization of the mutant indicates that the mutation in *Ljnspp2* affects a nodulation-specific signaling component that is downstream of the common pathway for mycorrhizal and rhizobial symbioses.

3.2. High-resolution genetic mapping of *LjNSP2*

The mutant allele *Ljnspp2-1* (Gifu B-129) was crossed to the early flowering ecotype Miyakojima MG-20. The

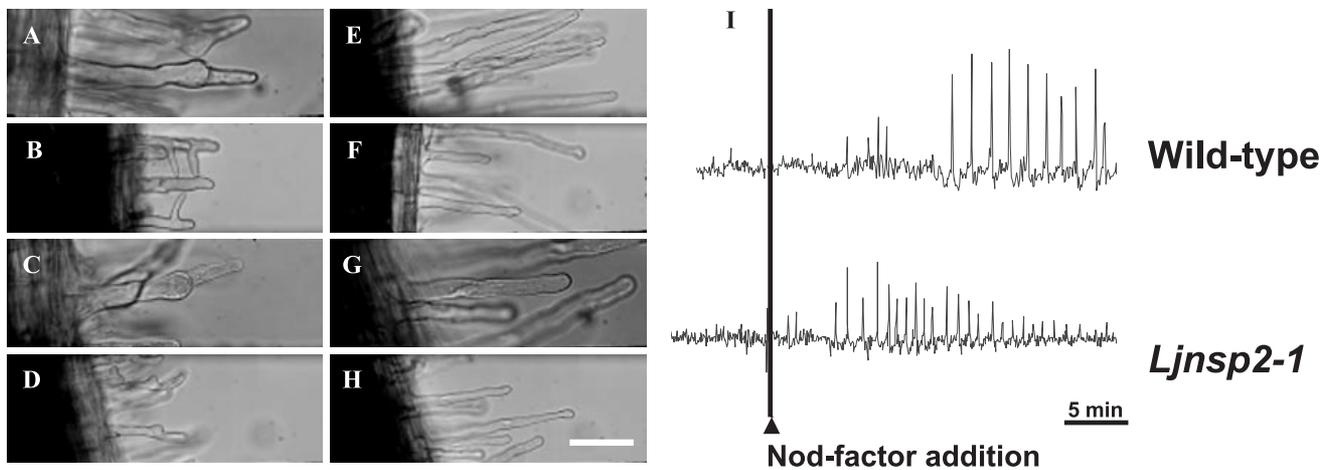


Figure 1. *L. japonicus* root hair responses induced by NF. (A–D) Root hair deformation induced with 10^{-8} M NF. Slight swelling and branching were observed also in *Ljnspp2-1* mutant (C and D), although to a lesser extent than the wild-type (Gifu B-129; A and B). (E–H) Non-treated root hairs look similar between the mutant (G and H) and wild-type (E and F). Scale bar: 50 μm . (I) Analysis of Ca^{2+} spiking in root hairs. Each trace is from a single root hair using seedlings of wild-type (Gifu B-129), or the *Ljnspp2-1* mutant. After about 20 min from injection of the Ca^{2+} -sensitive dye Oregon green-dextran to the root hairs, NF was added to the medium at 10^{-8} M. Both wild-type (upper) and the mutant (lower) responded positively for the addition of NF in 10–20 min and similar effects were observed with at least 10 independent seedlings.

LjNSP2 locus was mapped near translocation site of the short arm of chromosome 1.³¹ Since the recombination is significantly suppressed in the chromosomal segment, total of 4096 *EcoRI*/*MseI* primer combinations were examined for bulked segregant analysis on bulks 10 Nod^- recessive and Nod^+ dominant homozygous F2 plants in the cross of *Ljnspp2-1* and Miyakojima MG-20. *LjNSP2*-linked AFLP markers from this screen were further analyzed in additional 2989 F2 plants with HEGS/AFLP system. Nineteen markers are located on the southern side of *LjNSP2* locus at distances of 0.07 cM and seven markers on the northern side of *LjNSP2* locus at distances of 0.45 cM, respectively, while nine markers cosegregated with *LjNSP2* locus (Table 1, Fig. 2A). The screened markers were converted into the 11 polymorphic SCAR markers (Table 2).

3.3. Construction of a 2 Mb physical BAC contig spanning *LjNSP2*

At the first step, all SCAR markers were used to screen 3-D BAC DNA pools prepared from our *L. japonicus* Gifu BAC library. The contigs containing these SCAR markers were extended by the chromosome walking with the screening of 3-D BAC DNA pool using PCR primer combinations based on end sequences of BAC clones. In several cases, however, we could not obtain non-repetitive PCR fragments from BAC end sequences and screen BAC library. As an alternative strategy, AFLP fragments from BAC with *EcoRI*+1 selective and *MseI*+1 selective primers were searched for non-repetitive sequences of BAC inner. Subsequently, the corresponding *EcoRI*+3 selective and *MseI*+3 selective primers were used to screen 3-D BAC DNA

pools with AFLP system. Finally, we constructed a ~ 2 Mb physical BAC contig that spans the *LjNSP2* locus (Fig. 2B).

3.4. The narrowing of *LjNSP2* genomic region using *L. burttii* as an alternative crossing partner and identification of *LjNSP2* gene

As no more recombination was found from the population of this cross of *Ljnspp2-1* \times Miyakojima MG-20, even with new markers developed from the BAC clones in the contig, we made another cross with *L. burttii*.²¹ Among 8472 F2 progenies from this cross, six recombination events were found between the flanking markers S26d and 183R derived from the BAC clone 188C5; this located the mutation within a 130 kb region (Fig. 2B). Among the 5 ORFs (Fig. 2C) predicted from the sequence in this region (excluding transposable elements), only one was identified as having a mutation in the mutant. A 6.9 kb fragment including this ORF complemented the mutant for nodulation in hairy roots transformed by the *Agrobacterium rhizogenes* carrying the cloned region (Fig. 3). No nodules were formed using empty vector, confirming that mutation of this gene caused the mutant phenotype.

The ORF in this region corresponding to *LjNSP2* encodes a protein belonging to the plant GRAS family of putative transcription factors^{32–34} and analysis of the major plant GRAS family protein sequences indicated that the closest in sequence to *LjNSP2* were the *M. truncatula* and *Pisum sativum* NSP2 proteins.¹⁸ *AtSCL26* of *Arabidopsis* was about twice distant from them, and together these proteins made an apparent subfamily of GRAS proteins (Fig. 4). However, genome

Table 1. AFLP markers linked to *LjNSP2* locus

AFLP marker	Primer combination	Approximate length of the AFLP fragment (bp) ^a	Marker type
EM117	E-CGA/M-GTG	700	Dominant
EM140	E-CTT/M-CAA	700	Co-dominant
EM157	E-GAC/M-GCC	130	Dominant
EM177	E-GCA/M-CAA	550	Dominant
EM205	E-GGC/M-TGG	1500	Dominant
EM242	E-TAG/M-CTC	130	Dominant
EM262	E-TCT/M-ACT	500	Co-dominant
EM265	E-TCT/M-GGC	250	Co-dominant
EM299	E-TTA/M-TGT	250	Dominant
EM341	E-AAT/M-GTT	500	Co-dominant
EM367	E-ACG/M-GGA	400	Dominant
EM390	E-AGC/M-AAA	1000	Co-dominant
EM480	E-CCA/M-GCA	190	Dominant
EM527	E-CGT/M-CTC	70	Dominant
EM582	E-GAC/M-TGG	800	Dominant
EM603	E-GCA/M-TTA	800	Dominant
EM713	E-TAC/M-AAA	400	Dominant
EM784	E-TCT/M-TTC	100	Dominant
EM807	E-TGG/M-CGA	90	Dominant
EM011	E-AAG/M-TTC	200	Dominant
EM013	E-AAT/M-CCT	120	Dominant
EM116	E-CGA/M-GGA	500	Dominant
EM160	E-GAG/M-AAT	250	Co-dominant
EM190	E-GCT/M-CAA	350	Co-dominant
EM261	E-TCG/M-TTG	250	Co-dominant
EM283	E-TGG/M-CTG	500	Dominant
EM350	E-ACA/M-TCA	250	Dominant
EM466	E-CAT/M-ACT	800	Dominant
EM130	E-CGT/M-TGG	600	Dominant
EM686	E-GTC/M-GAT	600	Dominant
EM163	E-GAG/M-GTC	300	Co-dominant
EM115	E-CGA/M-AGT	400	Co-dominant
EM214	E-GGG/M-TTA	850	Dominant
EM231	E-GTT/M-TCT	80	Dominant
EM802	E-TGG/M-AAT	200	Dominant

36 *LjNSP2*-linked AFLP markers were selected from 3009 F2/F3 plants in the cross of *Ljnspp2-1* (Gifu B-129) and Miyakojima MG-20.

^aAFLP fragments were derived from Miyakojima MG-20.

sequences around the *LjNSP2* and *M. truncatula NSP2* revealed no clear co-linearity based on the available data.

The *LjNSP2* gene consists of a 1500 bp exon with no introns, encoding a predicted 499 amino acids protein of 55 kDa, containing the following GRAS family-specific domains: homopolymeric stretches (HPS) of polyE and polyT; first leucine heptad repeat (LHRI); a VHIID

DNA-binding sequence, second leucine heptad repeat (LHR2); and a *Src*-homology 2 (SH2)-like domain³⁵ (Fig. 5A). About 15% of the N-terminal region is divergent among most GRAS family members but that of *LjNSP2* showed strong homology with the *M. truncatula* and *Pisum sativum* NSP2 proteins¹⁸ suggesting functional equivalence and that they are probably orthologs. Furthermore, *LjNSP2* has a well-conserved SH2-like domain among GRAS proteins (Fig. 5B). The *Ljnspp2-1* mutation causes a substitution of a conserved valine (V) to glutamate (E) in this SH2-like domain (Fig. 5C).

Complementation tests with the various *L. japonicus* nodulation mutants carrying mutations mapped to linkage group 1 revealed that *Ljsym35* carried a mutation allelic to *Ljnspp2*. DNA hybridizations detected no signal in *Ljsym35* (Fig. 6A) and PCR analyses indicated that it has a deletion of >100 kb around the *LjNSP2* gene; this allele was renamed *Ljnspp2-2*.

3.5. Expression of *LjNSP2* during nodulation and in different organs

RNA hybridization showed that *LjNSP2* expression was detectable in roots but not in shoots and flowers (Fig. 6B), in contrast to the ubiquitously expressed *Medicago NSP2*.¹⁸ *LjNSP2* was also expressed in infected roots at 4 dpi and early nodules but strongly suppressed in matured nodules (Fig. 6C). These expression patterns of *LjNSP2* are similar to that of the putative Nod factor receptor kinase genes, *NFR1*⁵ and *NFR5*,⁶ and the signal transduction components *CASTOR*, although the decrease in expression of the latter was slight.⁹ This contrasts with the expression patterns of the genes encoding the nodulation signaling pathway components *M. truncatula NSP1*,¹⁷ *M. truncatula NSP2*¹⁸ and *L. japonicus NIN*¹⁵ (Fig. 6A) whose expression increased following inoculation.

3.6. Expression of *NIN* and *LjENOD40-1* genes in *Ljnspp2-2* mutant

In the *Ljnspp2-2* null mutant, RT-PCR revealed that even before inoculation with *M. loti* the expression levels of early nodulation genes, *NIN* and *LjENOD40-1*, were both <40% of the wild-type (Fig. 7A and B). Upon inoculation of the wild-type with *M. loti*, *NIN* and *LjENOD40-1* increased by up to 65- or 3.5-fold, respectively. In contrast, the levels of *NIN* and *LjENOD40-1* transcripts in the *Ljnspp2-2* mutant remained low after inoculation reaching only about 5–20% of wild-type levels (Fig. 7A and B). This demonstrates that *LjNSP2* function is required, directly or indirectly, for either the expression and/or the induction of these early nodulins.

3.7. Nuclear localization of *LjNSP2* in onion cells

Although most GRAS family proteins have a putative nuclear localization sequence (NLS)³⁴, PSORT II

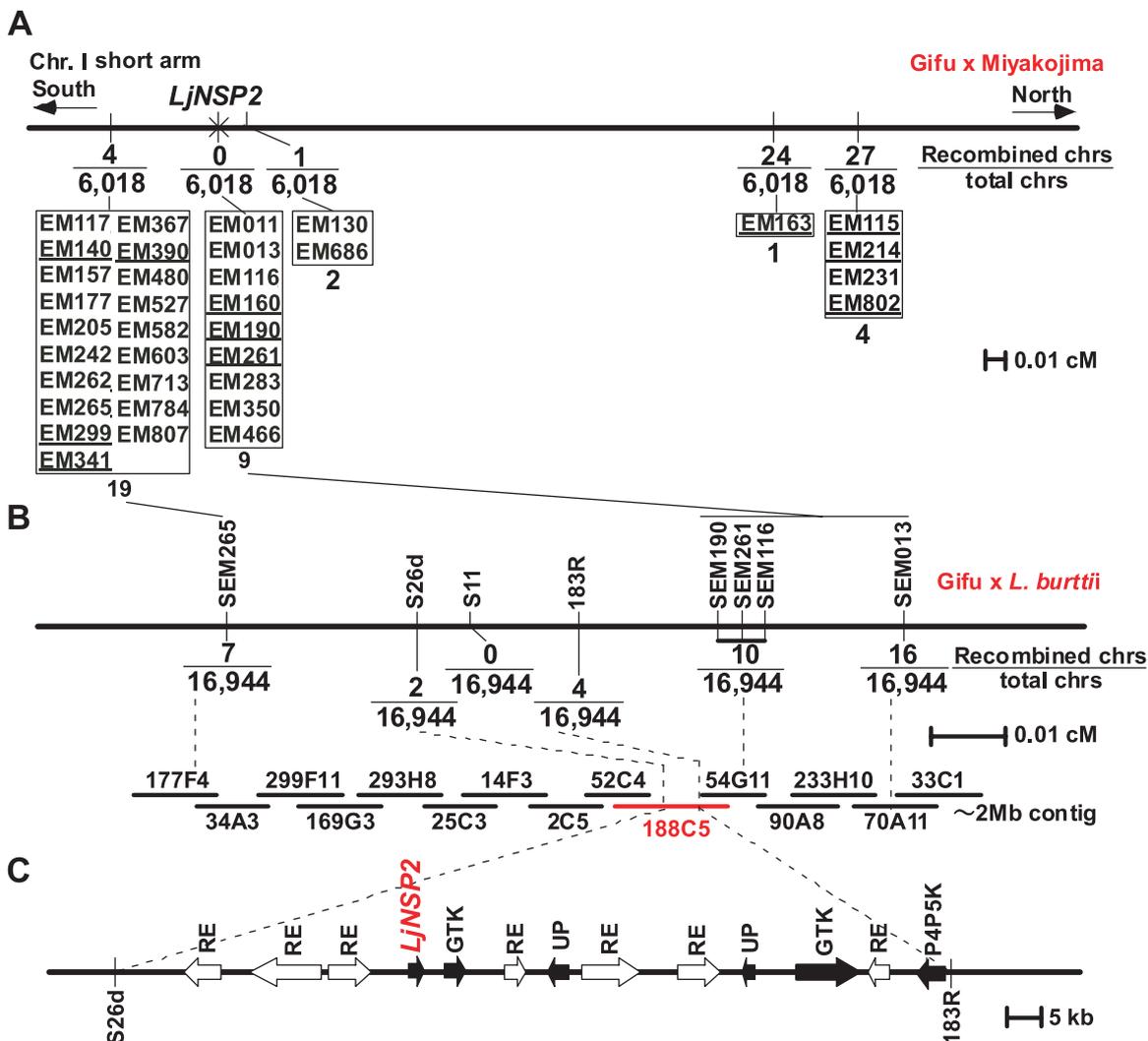


Figure 2. Positional cloning of the *LjNSP2* gene (A) Genetic map of *LjNSP2* region. This map is based on the analysis of 3 009 F₂ plant of a cross between *Ljnspp2-1* and Miyakojima MG-20. These HEGS/AFLP makers were selected from 4 096 of AFLP primer combinations with HEGS system. The markers converted into polymorphic SCAR markers are underlined. (B) About 2 Mb of BAC contig around *LjNSP2* genomic region. The numbers below the markers indicates recombination events detected in 16 944 chromosomes from 8 472 siblings of the heterozygotes from crosses of *Ljnspp2-1* × *L. burttii*²¹. Only overlapping BAC clones spanning *LjNSP2* are displayed in this figure. Finally, *LjNSP2* region was delimited within a 130 kb region on a 211 kb BAC clone (188C5). (C) Putative genes in *LjNSP2* region. ORFs of putative genes (closed arrows) and retro-elements (RE; open arrows) are marked in this 130 kb region. Predicted genes are GTK, glutamine transferase K; P4P5K, phosphatidylinositol-4-phosphate-5-kinase; UP, unknown proteins. There were no introns in the 1 500 bp of the predicted *LjNSP2* ORF.

analysis did not identify an NLS-like sequence in *LjNSP2*. However, *LjNSP2*-GFP fusion delivered into onion (*Allium cepa*) epidermal cells by particle bombardment revealed it to be exclusively localized in nuclei, although not in nucleoli (Fig. 8A). The mutation in the SH-2-like domain reduced nuclear localization of *Ljnspp2-1*-GFP and fluorescence in the cytoplasm became noticeable in at least 30 GFP-expressed cells (Fig. 8B), but not as strong as seen with GFP alone (Fig. 8C). These observations are consistent with *LjNSP2* acting as a transcription factor, and the SH2-like domain facilitating its nuclear localization.

The above *LjNSP2*-GFP fusion under the control of the CaMV 35S promoter or the *LjNSP2* promoter

containing a 5.1 kb fragment of the 5' flanking sequence was constructed and introduced into *Ljnspp2-2* mutants. These constructs complemented the *Ljnspp2-2* mutant phenotype, indicating that this fusion protein retained the *LjNSP2* activity for nodulation. However, the transgenic roots showed no detectable GFP fluorescence (data not shown).

4. Discussion

So far the only described nodulation-specific mutants of *L. japonicus*, which completely lack nodules but have normal mycorrhization, are *Ljnspp2*, *nfr1*,⁵ *nfr5*⁶ and *nin*.¹⁵ One key difference among these mutants is the

Table 2. PCR-based markers linked to *LjNSP2* locus

PCR marker	Origin	Sequence (5'→3')	Annealing temperature (°C)	Length (bp) ^a	Marker type
SEM140	EM140	GAATTCCTTCCCGTTCTTC TTAACAACAGCAACTTCACT	65	750	Dominant (M) ^b
SEM299	EM299	GAATTCCTTACCGAGTGAGAT TTAATGTTTCTGACAAAGGC	60	250	Dominant (M, b)
SEM341	EM341	GAATTC AATGATTGTTGGGA TTAAGTTGTTTCAATTGATA	50	500	Co-dominant (M, b)
SEM390	EM390	GAATTCAGCCACAGCCTCTG AACCACAAGTCAAATGCAAC	50	230	Dominant (M)
SEM160	EM160	GAATTCGAGAATTGGAGAAG TTAAAATCAAACCCCTAACAA	60	220	Dominant (M)
SEM190	EM190	GAATTCGCTTGCAAGTAGTG TTAACAAGAGTCTCATAATC	50	340	Co-dominant (M)
SEM261	EM261	TTCTCGGCGGTTCCACCAAT GCTTGTTTGAAAGTGGCTTT	68	290	Dominant (M) Co-dominant (b)
SEM115	EM115	CCGATTACATTTATGAGTAC TTAAAGTCGATAGACCGACG	50	350	Co-dominant (M, b)
SEM163	EM163	GAATTCGAGATCCAGAATCT TTAAGTCTGAACCATTGCTA	60	290	Dominant (M)
SEM214	EM214	GAATTCGGGGTGTACACCTC TTAATTACCCCTCGTTCTC	65	580	Dominant (M)
SEM802	EM802	GAATTCGGAAGCACGTTGA TTAAAATCATCATCTGAGAG	60	190	Co-dominant (M)
s26d	188C5	TCATGTTTAGCGCTCTGATT GCACCGTAGTACCGTCAGTTTCC	60	100	Co-dominant (M, b)
S11	188C5	TTGCAGGTTTTTCAGGAACATT GAAGTTCATTGTAACCTCAAACG	60	520	Co-dominant (M, b)
183R	183A2	CATCGTATGTTGAAAAGAAGAATGAT TCTTCAGTTCTTCCCTTATAGCC	60	580	Co-dominant (M, b)

PCR was performed using the indicated primer pairs with the following cycling parameter: 4 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at the temperature indicated, 1 min at 72°C; 10 min at 72°C; and hold at 4°C.

PCR fragments were electrophoresed on 13% acrylamide gels with HEGS system.

^aPCR fragments are derived from Miyakojima MG-20.

^bM and b indicates the polymorphism of Miyakojima MG-20 and *L. burttii*, respectively, compared with Gifu B-129.

induction of Ca²⁺ spiking, which might be required for the early common response in activation of the symbioses with both rhizobia and mycorrhizal fungi. The *LjnsP2* mutants are normal for Ca²⁺ spiking but the *nfr1* and *nfr5* mutants are blocked. Recently, *NFR1* and *NFR5* were shown to encode LysM-receptor-like-kinases, that were predicted to function in NF perception.^{5,6} NIN functions downstream of *LjNSP2* as indicated in this study. It is possible that *LjNSP2* is earliest known protein executing nodulation-specific gene expression from Ca²⁺ spiking induced through *NFR1* and *NFR5* in *L. japonicus*.

We have established a ~2 Mb physical BAC contig that spans the *LjNSP2* locus (Fig. 2B). However, *LjNSP2* region was only closed to the minimum of 14 BAC clones

even in the population of 3 009 F2/F3 plants of the cross with Miyakojima MG-20. This corresponds to 24 Mb/cM, ~78-fold greater than the average physical to genetic distance found in our high-density map of the *L. japonicus* (Wang et al., manuscript in preparation). This indicates that recombination in the chromosomal segment near the translocation site is highly suppressed in the cross combination of Gifu B-129 × Miyakojima MG-20. Therefore, we changed the crossing partner from Miyakojima MG-20 to *L. burttii*.²¹ As a result, *LjNSP2* region narrowed up to 130 kb in one BAC clone (188C5) using the population of 8 472 F2 plants of the cross with *L. burttii*, indicating that *L. burttii* is significantly useful as alternative crossing partner of Gifu especially near the translocation site of chromosome 1.

LjNSP2 encodes a GRAS family protein of a putative transcription factor. The phylogenetic analysis revealed that *LjNSP2* is most closely related to *M. truncatula* NSP2 and *P. sativum* NSP2.¹⁸ The comparison of the

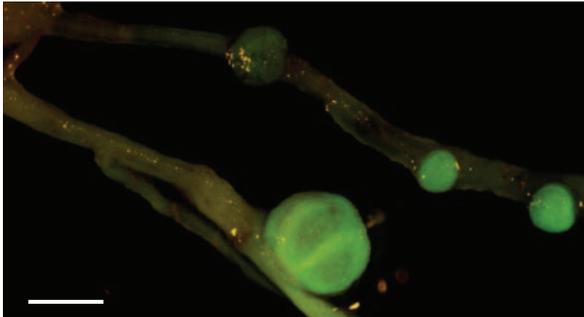


Figure 3. Complementation of *Ljnspp2-1* mutant. Roots of the *Ljnspp2* mutant were transformed by *Agrobacterium rhizogenes* strain LBA1334 carrying appropriate plasmids carrying the 1500 bp *LjNSP2* ORF and 4486 and 899 bp of upstream and downstream sequences and a transformation indicator 35S-*GFP*. On inoculation with *Mesorhizobium loti*, the GFP-marked transformants formed nodules complementing the mutant phenotype. Scale bar: 1 mm.

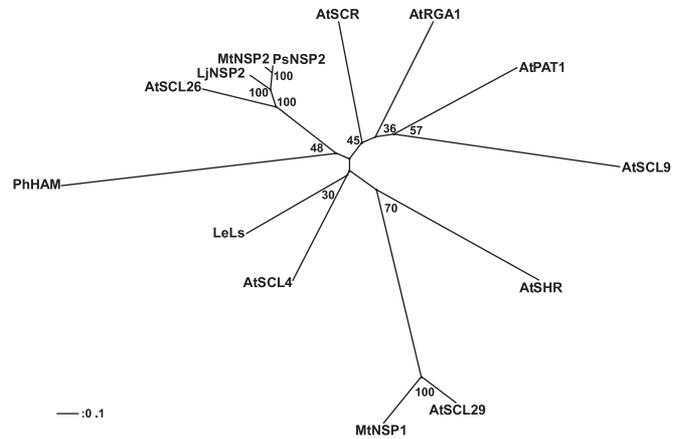


Figure 4. Phylogenetic tree of plant GRAS family proteins. Members of the family of plant GRAS proteins aligned using Clustal W are shown as a neighbor-joining dendrogram with 1000 bootstrap replicates. At, *Arabidopsis thaliana*; Mt, *Medicago truncatula*; Ph, *Petunia hybrida*; Ps, *Pisum sativum*; Le, *Lycopersicon esculentum*; NSP, nodulation signaling pathway; SCR, SCARECROW; SCL, SCARECROW-like; SHR, short-root; HAM, hairy meristem; Ls, lateral suppressor; RGA, repressor of *gal-3*; PAT, phytochrome A signal transduction.

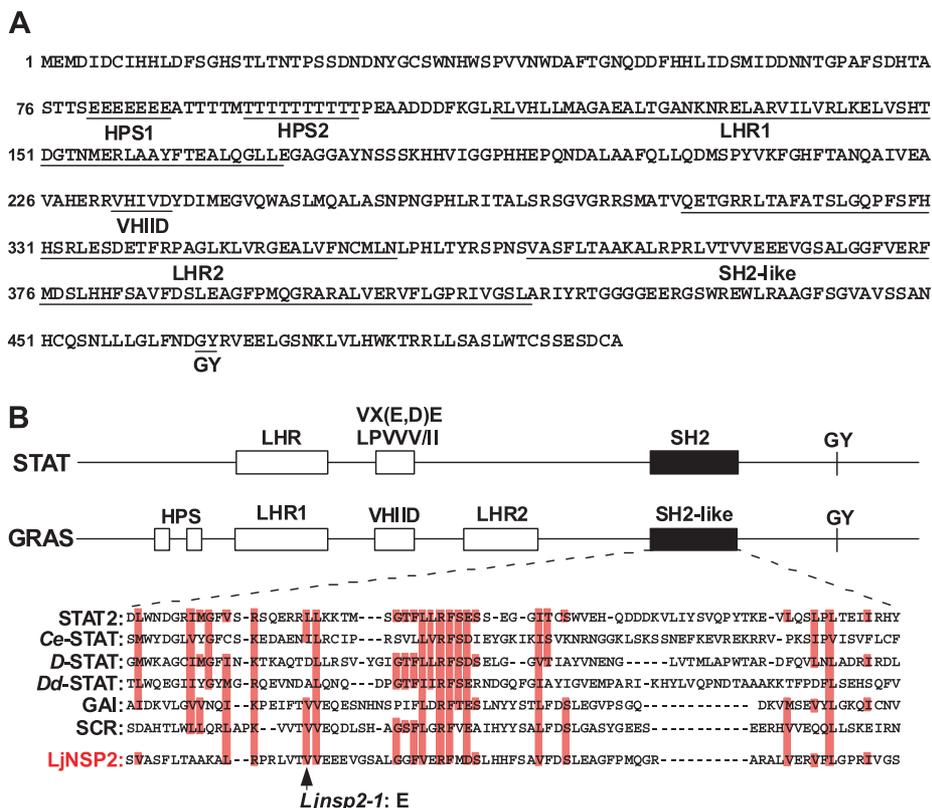


Figure 5. Domain structure of the *LjNSP2* protein. (A) Amino acid sequence of 499 residues and predicted functional domains of *LjNSP2*. HPS; homopolymeric stretches characteristic for GRAS protein near to the N-terminal, LHR (leucine heptad repeat) 1 and 2; putative leucine zipper, VHIID; putative DNA-binding sites, SH2-like region, GY; GY, Y of which is phosphorylated in STATs, is conserved about 100 residues downstream from SH2-like domain. (B) Comparison of GRAS and STAT family proteins with *LjNSP2* in their SH2-like domains. STAT2: human STAT2; P52630, *Ce-STAT*: *Caenorhabditis elegans* STAT; Z70754, *Dd-STAT*: *Drosophila* stat; Q24151, *Dd-STAT*: *Dictyostelium discoideum* STAT; Y13097, GAI: *Arabidopsis* GAI (gibberellin insensitive); At1g14920, SCR: *Arabidopsis* SCR (SCARECROW); At3g54220. GAI and SCR are the representatives of plant GRAS family proteins. Conserved amino acids are indicated by red boxes. A missense mutation in *Ljnspp2-1* is indicated by arrowhead.

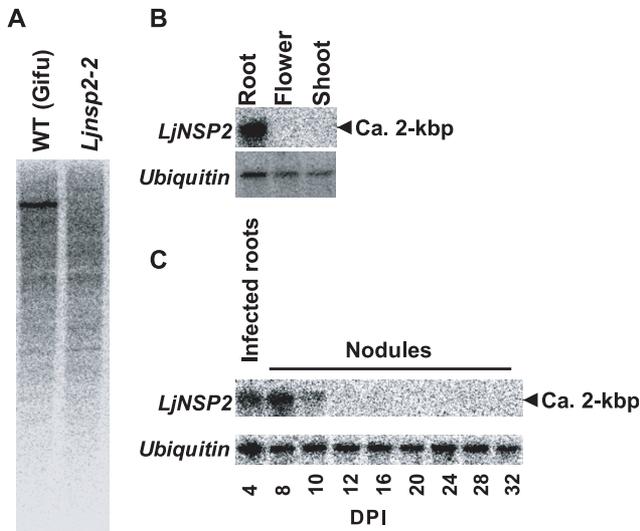


Figure 6. DNA, RNA hybridization and RT-PCR analyses of *LjNSP2*. (A) Genomic hybridization with the full-length *LjNSP2* ORF probe to the wild-type (WT; Gifu B-129) and a null deletion mutant *Ljnspp2-2*. Genomic DNA was digested with *EcoRI*. The *LjNSP2* homolog seems to be unique in WT, but cannot be detected in *Ljnspp2-2*. (B and C) Northern analysis of *LjNSP2* expression in various organs (B), and infected roots at 4 dpi of *M. loti* TONO and nodules at 8–32 dpi of *M. loti* TONO, respectively (C). DPI: days post inoculation.

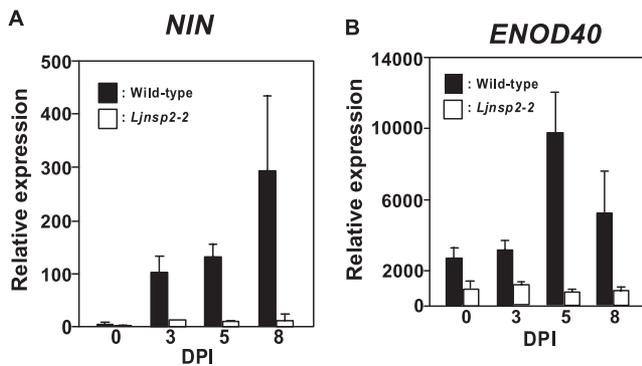


Figure 7. RT-PCR analyses of expression of *LjNSP2* and the early nodulins. (A and B) RT-PCR analysis of the change of expression of *NIN* (A) and *LjENOD40-1* (B) after *M. loti* inoculation, in wild-type and *Ljnspp2*. Suppression of *LjNSP2* expression after inoculation was confirmed, and almost complete loss of induction in *NIN* and *LjENOD40-1* were apparent. *Ubiquitin* is as a loading control. Relative expression levels were normalized against the amount of *ubiquitin* (set as 10^5). Standard deviations of three independent experiments are indicated by error bars. DPI: days post inoculation.

mutant phenotypes suggests that the *LjNSP2*, *MtNSP2* and *PsNSP2* genes function at similar or parallel positions in the nodulation signal transduction. However, the expression pattern is different between *LjNSP2* and *M. truncatula NSP2*. *LjNSP2* is predominantly expressed in roots and its expression decreases in developed nodules. The expression of *M. truncatula NSP2* is observed in shoots as well as roots and is induced

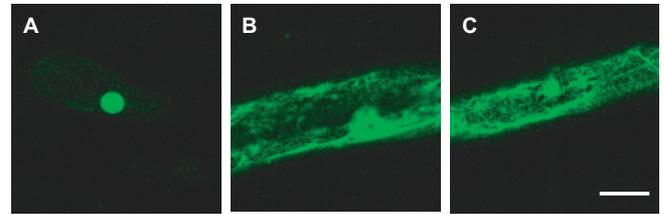


Figure 8. Nuclear localization of the *LjNSP2*-GFP. *LjNSP2*-GFP (A), *Ljnspp2-1*-GFP (B) fusion genes and control GFP gene (C), delivered by particle bombardment, were transiently expressed in onion epidermal cells and observed with a laser confocal microscope 24 h after bombardment. The nuclear localization of the *Ljnspp2-1* mutant gene product is significantly suppressed compared with *LjNSP2*. Scale: 10 μ m.

after rhizobial inoculation. These results suggest that, in contrast with a possible role for *M. truncatula NSP2* in another organ development in addition to nodulation, *LjNSP2* seems to be specialized in nodule initiation.

The expression of *NIN* and *ENOD40* is induced rapidly after rhizobial inoculation and NF-treatment. The *NIN* transcripts are detected in different tissues during various nodule stages, such as the dividing cells of the nodule primordia and the nodule vascular bundles. The *Ljnin* mutants are nodulation-minus and blocked in both the infection thread formation and the cortical cell division.¹⁵ *ENOD40* is induced in the root pericycle a few hours after rhizobial inoculation, and subsequently in the dividing cortical cells of the root and nodule primordia.^{36–38} *ENOD40* RNAi knock-down lines³⁹ or the possible co-suppression⁴⁰ suppressed nodule primordium formation, resulting in very poor nodulation. Despite of their function in nodule initiation, however, little is known about what kinds of transcription factors in the root activate *NIN* and *ENOD40* gene expression in response to rhizobia. In this study, we demonstrate that the induction of *NIN* and *LjENOD40-1* gene expression is clearly cancelled by the *Ljnspp2* mutation (Fig. 7). On the basis of these findings, we speculate that *LjNSP2* may function as a transcriptional activator to directly or indirectly switch on the *NIN* and *LjENOD40-1* gene expression in nodule initiation. In order to address this issue, identification of the promoter region and subsequent binding assay using *LjNSP2* protein would be of great importance in future.

Kaló et al.¹⁸ reported that *M. truncatula NSP2*-GFP localizes in the endoplasmic reticulum and nuclear envelope and re-localizes into the nucleus rapidly after NF-treatment. In this case, they made a functional C-terminal GFP fusion under control of the constitutive CaMV 35S promoter that was introduced in *Medicago nsp2* mutant plants. Here, we attempted to detect *LjNSP2*-GFP, -YFP or -DsRED2 fusion in *L. japonicus* hairy roots. These fusions could complement the mutant phenotype but no fluorescence was detected even under the control of CaMV35S promoter. In place of *L. japonicus* we delivered *LjNSP2*-GFP fusion into onion

epidermal cells by particle bombardment. The fusion exclusively localizes in the nucleus but not in the nuclear envelope. Although there is no way to explain the peculiar difference of subcellular localization of NSP2 between *Medicago* and onion, putative nucleoporins such as NUP133¹⁴ and NUP85 (Saito et al. unpublished data) required for rhizobial and arbuscular mycorrhizal symbioses may retain the NSP2 putative transcription factor in the nuclear envelope of *M. truncatula*. Most recently, Heckmann et al. reported that non-nodulating mutant, SL781-3, carrying an allele of *LjNSP2* was found by TILLING.⁴¹

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