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*, *NFR5*5 and *Medicago* *LYK3*7 encode transmembrane receptor-like serine/threonine kinases with...
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, CASTOR and POLLUX, *M. truncatula* DMI1, DMI2 and DMI3, and *L. japonicus* CCAMK and Nup133 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, Ca\(^{2+}\) influx and Ca\(^{2+}\) spiking. 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 Ca\(^{2+}\) spiking and CCA MK in *Medicago truncatula*.

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 *Ljnsp2-1* was isolated from the EMS-mutagenesis experiments of *L. japonicus* Gifu B-129. The mutant *Ljsym35* was isolated from a population of Gifu derived from a transposon-DNA-tagging trial, as a non-tagged culture mutant, and was kindly provided by Prof. Jens Stougaard (Arrhus University, Denmark).

2.2. Root hair deformation and assays of Ca\(^{2+}\) spiking

Seeds of *L. japonicus* were germinated and grown on BNM agar medium essentially as described previously, 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. Ca\(^{2+}\) spiking was assayed as described previously, 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 *Ljnsp2-1* (Gifu B-129) and the early flowering ecotype Miyakojima MG-20 or *Lotus burttii* B-303. 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 system 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 library were screened with *LjNSP2*-linked...
HEGS/AFLP or SCAR markers as described above. HindIII-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 Pef 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 Ljnsp2-1 mutant was performed as described.26 The plants with transgenic hairy roots were grown in vermiculite pots and inoculated with Mesorhizobium loti. 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 EcoRI, 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'-ACTTCCACCACTCATC-GAC-3' and 5'-ACAAGTGCAAAAGGGATGCAG-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 2x SSC containing 0.5% SDS at room temperature for 10 min, and twice in 0.2x 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 reprobed 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’-ACCGGTGACTAG-TGGAATTGATATAGTTGCATCC-3’ (SacI-site underlined), 5’-CATGTCATGAAATGCACAAATCTGA-TTCTGAGAAC-3’ (BspHI-site underlined)], digested with SacI and BspHI, and cloned into pUC18-CaMV35S-sGFP(s65T)-NOS plasmid29 at the SacI/Ncol 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).29 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 Ljnsp2 mutant

The Ljnsp2-1 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 Ljnsp2-1 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 mycorrhization,1 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 Ljnsp2 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 Ljnsp2-1 (Gifu B-129) was crossed to the early flowering ecotype Miyakojima MG-20. The
LjNSP2 locus was mapped near translocation site of the short arm of chromosome 1. 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−/C0 recessive and Nod+/dominant homozygous F2 plants in the cross of Ljnsp2-1 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 Ljnsp2-1 x Miyakojima MG-20, even with new markers developed from the BAC clones in the contig, we made another cross with L. burttii. 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 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. AtSCSL26 of Arabidopsis was about twice distant from them, and together these proteins made an apparent subfamily of GRAS proteins (Fig. 4). However, genome
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 (LHRII); and a Src-homology 2 (SH2)-like domain (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 Ljnsp2-1 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 I revealed that Ljspm35 carried a mutation allelic to Ljnsp2. DNA hybridizations detected no signal in Ljspm35 (Fig. 6A) and PCR analyses indicated that it has a deletion of >100 kb around the LjNSP2 gene; this allele was renamed Ljnsp2-2.

### Table 1. AFLP markers linked to LjNSP2 locus

| AFLP marker | Primer combination | Approximate length of the AFLP fragment (bp) | Marker type |
|-------------|-------------------|-------------------------------------------|-------------|
| EM117       | E-CGA/M-GTG       | 700                                       | Dominant    |
| EM140       | E-CTT/M-CAA       | 700                                       | Co-dominant |
| EM157       | E-GAC/M-GGC       | 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-AGC/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    |
| EM911       | E-AAG/M-TTC       | 200                                       | Dominant    |
| EM913       | E-AAT/M-CCT       | 120                                       | Dominant    |
| EM116       | E-AGA/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-CTC       | 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-GTGT/M-TCT      | 80                                        | Co-dominant |
| EM802       | E-TGG/M-AAT       | 200                                       | Dominant    |

36 LjNSP2-linked AFLP markers were selected from 3009 F2/F3 plants in the cross of Ljnsp2-1 (Gifu B-129) and Miyakojima MG-20.

AFLP fragments were derived from Miyakojima MG-20.

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 NSP218. 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, NFR4 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 NSP218 and L. japonicus NIN6(Fig. 6A) whose expression increased following inoculation.

3.6. Expression of NIN and LjENOD40-1 genes in Ljnsp2-2 mutant

In the Ljnsp2-2 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 Ljnsp2-2 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
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 Ljnsp2-1-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 Ljnsp2-2 mutants. These constructs complemented the Ljnsp2-2 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 Ljnsp2, nfr1,5 nfr56 and nin.15 One key difference among these mutants is the...
induction of Ca$^{2+}$ 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 Ca$^{2+}$ 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 Ca$^{2+}$ 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 3009 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 8472 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.

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

| PCR marker | Origin | Sequence (5′→3′) | Annealing temperature (℃) | Length (bp)* | Marker type |
|------------|--------|------------------|---------------------------|--------------|-------------|
| SEM140     | EM140  | GAATTCCCTCCCCGGTTCTTC | 65                        | 750          | Dominant (M) |
| SEM299     | EM299  | GAATTCATCCGAGTGGAGAT | 60                        | 250          | Dominant (M, b) |
| SEM341     | EM341  | TTAATTGTCCGATTCCCTGA | 50                        | 500          | Co-dominant (M, b) |
| SEM390     | EM390  | GAATTCGACACAGCTCTGA | 50                        | 230          | Dominant (M) |
| SEM160     | EM160  | GAATTCGAGAACATTGGAGAAG | 60                      | 220          | Dominant (M) |
| SEM190     | EM190  | TTAAACAGACACTTCTCATA | 50                        | 340          | Co-dominant (M) |
| SEM261     | EM261  | TTCTCCGCGGTTCCACCAAT | 68                        | 290          | Dominant (M) |
| SEM115     | EM115  | TTAATTACATTTATGAGTAC | 50                        | 350          | Co-dominant (M, b) |
| SEM163     | EM163  | TTAACTGCGATAGCCACGG | 60                        | 290          | Dominant (M) |
| SEM214     | EM214  | GAATTCGGGGTGTTACACCTC | 60                        | 580          | Dominant (M) |
| SEM892     | EM892  | TTAAATCCCTAATTGGAGAAG | 60                        | 190          | Co-dominant (M) |
| s26d       | 188C5  | TCACTGTTGAGGCTTCTGATT | 68                        | 100          | Co-dominant (M, b) |
| S11        | 188C5  | GCACCGTAGTACCCGTAGTTTCC | 60                      | 520          | Co-dominant (M, b) |
| 183R       | 183A2  | CATCGATGTTGAAGAGAATGAT | 60                        | 580          | Co-dominant (M, b) |

PCR was performed using the indicated primer pairs with the following cycling parameter: 4 min at 94℃; 35 cycles of 30 s at 94℃, 30 s at the temperature indicated, 1 min at 72℃; 10 min at 72℃; and hold at 4℃. PCR fragments were electrophoresed on 13% acrylamide gels with HEGS system.

*PCR fragments are derived from Miyakojima MG-20.

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

Figure 3. Complementation of Ljnsp2-1 mutant. Roots of the Ljnsp2 mutant were transformed by *Agrobacterium rhizogenes* strain LBA1334 carrying appropriate plasmids carrying the 1500 bp LjNSP2 ORF and 486 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, *Leucoglycine esculenta*; NSP, nodulation signaling pathway; SCR, SCARECROW; SCL, SCARECROW-like; SHR, short-root; HAM, hairy meristem; Ls, lateral suppressor; RGA, repressor of ga1-3; PAT, phytocrome 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, D-STAT: Drosophila stat; Q24151, Dd-STAT: Dictyostelium discoideum STAT; Y13097, GAI: Arabidopsis GAI (gibberellin insensitive); At1g14920, SCR: Arabidopsis SCR (SCARECROW); At1g54220, GAI and SCR are the representatives of plant GRAS family proteins. Conserved amino acids are indicated by red boxes. A missense mutation in Ljnsp2-1 is indicated by arrowhead.
and PsNSP2 positions in the nodulation signal transduction. The 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 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. 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. ENOD40 RNAi knock-down lines or the possible co-suppression 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 Ljnsps-1 gene 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. 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 nsps 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

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 Ljnsps-2. Genomic DNA was digested with EcoRI. The LjNSP2 homolog seems to be unique in WT, but cannot be detected in Ljnsps-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 Ljnsps. 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 100). Standard deviations of three independent experiments are indicated by error bars. DPI: days post inoculation.

Figure 8. Nuclear localization of the LjNSP2-GFP. LjNSP2-GFP (A), Ljnsps-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 Ljnsps-1 mutant gene product is significantly suppressed compared with LjNSP2. Scale: 10 μm.

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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 NUP133 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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