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Research

A MYB coiled-coil transcription factor interacts with NSP2 and is involved in nodulation in *Lotus japonicus*

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Summary

- Transcription factor complex formation is a central step in regulating gene expression. In this report, a novel MYB coiled-coil transcription factor referred to as IPN2, for Interacting Protein of NSP2, is described.
- The interaction between IPN2 and NSP2 was examined by protein pull-down assays and bimolecular fluorescence complementation (BiFC). Subcellular localization of proteins, gene expression and gene function were assessed in transgenic hairy roots expressing tagged recombinant proteins, promoter-reporter and RNA interference (RNAi) constructs, respectively.
- The GRAS domain of NSP2 and the coiled-coil domain of IPN2 were found to be responsible for the interaction between the two proteins. IPN2 had strong transcription activation activity, bound directly to the *NIN* gene promoter, and was localized to the nuclei of *Lotus japonicus* root cells. The expression of *IPN2* was elevated during nodule development, coinciding with increased *NSP2* gene expression during nodule organogenesis. RNAi-mediated knockdown expression of *IPN2* did not affect arbuscular mycorrhizal development, but had deleterious effects on rhizobial infection and nodule formation in *L. japonicus*.
- These results demonstrate an important role of *IPN2* in nodule organogenesis and place a new MYB transcription factor in the Nod signaling pathway.

Introduction

The unique and complex interactions between legume roots and rhizobia led to the development of a special organ, the root nodule. Genetic and genomic studies in the model legumes *Lotus japonicus* and *Medicago truncatula* have identified a set of genes essential for the perception and signal transduction of rhizobial Nod factors (Oldroyd, 2013). NFR1 and NFR5 of *L. japonicus* (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003) and LYK3 and NFP of *M. truncatula* (Amor *et al.*, 2003; Smit *et al.*, 2007) are receptor-like kinases that have been shown to serve as the receptors of Nod factors. These receptor-like kinases contain chitin-binding LysM motifs that have been shown to directly bind the chitin-like Nod factors (Broghammer *et al.*, 2012).

Recent studies have shown that several downstream components of the Nod factor signal transduction pathway also function in the signaling pathway that leads to the establishment of arbuscular mycorrhizal (AM) symbiosis. These protein components form a 'common symbiosis pathway' (CSP) that is responsible for relaying the symbiotic signals coming from either the nitrogen-fixing rhizobia or the nutrient-beneficial AM fungi (Kouchi *et al.*, 2010). Two GRAS family transcriptional regulators, nodulation signaling pathway 1 (NSP1) and NSP2, have been shown to participate in the nitrogen fixation symbiosis pathway downstream of CCaMK (Kaló *et al.*, 2005; Smit *et al.*, 2005; Heckmann *et al.*, 2006). Interestingly, NSP2 and NSP1 have recently been shown to promote root colonization by mycorrhizal fungi (Maillet *et al.*, 2011; Delaux *et al.*, 2013), implicating a role of NSP2 and NSP1 in the AM symbiosis. Thus, NSP2 and NSP1 do not function exclusively in the nodulation signaling pathway.

GRAS proteins are a family of key plant-specific transcription regulators, and play diverse roles in root and shoot development, and transduction of the gibberellic acid (GA) signal, and regulation of the signaling pathway downstream of phytochrome A (Bolle, 2004). One of the most prominent characters for GRAS domain proteins is their ability to move intercellularly through plasmodesmata (Cui *et al.*, 2007). There are at least 33 and 60 GRAS genes that have been identified in *Arabidopsis* and rice, respectively (Tian *et al.*, 2004). Putative orthologs of NSP1 and NSP2 can be found in most of the nonlegume plant species (Kaló *et al.*, 2005; Smit *et al.*, 2005; Heckmann *et al.*, 2006). Rice NSP1 and NSP2, for example, are able to fully rescue the nodule symbiosis-defective phenotypes of *L. japonicus nsp1* and *nsp2* mutants (Yokota *et al.*, 2010). In addition, NSP1 and NSP2 are found to be indispensable for strigolactone (SL) biosynthesis in *M. truncatula* and rice (Liu *et al.*, 2011).

NIN (nodule inception) is another transcriptional regulator expressed specifically during root nodule symbiosis. NIN protein shows similarity to Notch and sterol responsive element binding protein (SREBP) transcription factors in animals. The predicted DNA-binding/dimerization domain of NIN proteins contains a consensus motif that has been implicated in nitrogen-controlled development in plants (Schauser *et al.*, 1999; Marsh *et al.*, 2007). The *nin* mutants exhibit unusually excessive root hair curling, abortive infection, and no cortical cell division (CCD; Schauser *et al.*, 1999). *NIN* expression is strongly induced upon inoculation of rhizobia or application of Nod factors, and this induction requires NSP1 and NSP2 (Murakami *et al.*, 2006; Hirsch *et al.*, 2009). *NIN* is also upregulated by cytokinins and may mediate the crosstalk between Nod-factor and cytokinin signals (Tirichine *et al.*, 2007; Heckmann *et al.*, 2011).

In addition to NSP1, NSP2 and NIN, other transcriptional regulators required for nodule symbiosis have recently been identified. The *M. truncatula ERN1* (*ERF required for nodulation*) gene encodes a transcription factor in the APETALA 2/Ethylene Response Element (AP2/ERF) family that acts downstream of CCaMK-dependent Nod factor signaling (Middleton *et al.*, 2007). Three closely related AP2/ERF transcription factors (Mt ERN1, 2 and 3) have been shown to bind to the Nod factors responsive regulatory unit (the NF box) present in the promoter of Mt *ENOD11* (Andriankaja *et al.*, 2007). Another symbiosis mutant *astray* in *L. japonicus* contains a mutation in Lj Bzf, which is closely related to the *Arabidopsis* HY5, a transcription factor having a basic leucine zipper in the C-terminus and a RING-finger motif in the N-terminus (Nishimura *et al.*, 2002). Moreover, reverse-genetic approaches have led to functional characterization of additional transcriptional regulators, such as Ms ZPT2-1 (Frugier *et al.*, 2000), Mt HAP2-1 (Combier *et al.*, 2006), Lj ERF1 (Asamizu *et al.*, 2008) and Lj SIP1 (Zhu *et al.*, 2008).

In order to better understand how legume root cells respond to rhizobial Nod factors, it is crucial to identify the transcription regulatory networks involved in nodule symbiosis and to characterize their target promoters. Mt NSP1 and Mt NSP2 have recently been shown to form a transcription factor complex and bind to the promoters of early nodulin genes, such as Mt *ENOD11*, Mt *NIN* and Mt *ERN* (Hirsch *et al.*, 2009). In this study, we used the C-terminal GRAS domain of Lj NSP2 as bait in yeast two-hybrid screens and identified a novel MYB family transcription factor, designated as IPN2 for Interacting Protein of NSP2, in *L. japonicus*. The IPN2 protein shows strong transcription activation activity and is able to directly bind to the *Lj NIN* promoter in yeast cells and *in vitro*. We also explored the potential role of IPN2 in root nodule symbiosis.

Materials and Methods

Plant materials and growth conditions

Seeds of *Lotus japonicus* (Regel) K. Larsen MG-20 were surface-sterilized in 75% ethanol for 2 min and then in 8% sodium hypochlorite for 10 min and washed six times with sterile water. The seeds were germinated for 48 h at 22°C on sterile 0.8% water agar plates in Petri dishes in dark. Seedlings were planted in pots on sterile vermiculite and sand (1 : 1) supplemented with nitrogen-free Fahraeus medium and grown on in a growth chamber maintained at 22°C with a 16 : 8 h day : night cycle. Five-day-old seedlings were inoculated with *c.* 10^7 CFU ml^{-1} of *Mesorhizobium loti* MAFF303099. Wild-type *Nicotiana benthamiana* plants were grown in a growth chamber at 25°C with a 16-h light : 8-h dark cycle for *c.* 1–1.5 months before infiltration with *Agrobacterium tumefaciens*.

Yeast two-hybrid library screening

A cDNA fragment encoding the NSP2 region (105–499 amino acids), which lacks the auto-activation domain, was fused in-frame with the GAL4 DNA-binding domain in the bait vector pGBKT7. Bait constructs were transformed into yeast strain Y187 by the lithium acetate method (Gietz *et al.*, 1995). Screening of interaction clones was carried out as described elsewhere (Zhu *et al.*, 2008; Kang *et al.*, 2011).

β-Galactosidase assay

Yeast cells grown in liquid selection media were pelleted and assayed for β-galactosidase activity as described previously (Zhu *et al.*, 2008; Kang *et al.*, 2011).

In vitro protein–protein interaction

In order to assay the interaction between NSP2 and IPN2, GST-tagged NSP2 or GST alone were bound to glutathione Sepharose 4B columns (Sigma). The beads were incubated with 2 μg of purified His-IPN2, His-IPN2 (1–105), or His-IPN2 (1–178) protein in 1 ml of interaction buffer (20 mM Tris-HCl, 100 mM KCl, 2 mM MgCl, 5% glycerol, pH 8.0). The reaction was incubated for 1 h on ice with gentle shaking, and the interaction was assayed by immunoblotting with the anti-His-tag antibody as described previously (Zhu *et al.*, 2008; Kang *et al.*, 2011).

Subcellular localization of IPN2 in hairy root cells

The full-length cDNA of *IPN2* without stop codon was PCR amplified using the primers 5′-CATGCC ATGGTA GAGAGA ATGTTC CCTCCC-3′ and 5′-GACTAG TGCCAA ATGGTG AGCTTC TACCTT GAGCCA TAG-3′. The amplified PCR fragment was cloned into the *Nco*I/*Spe*I site of pCAMBIA1302 vector (CAMBIA, Canberra, ACT, Australia). For selection of transformed hairy roots of *L. japonicus*, the hygromycin resistance gene in pCAMBIA1302 was replaced by the GUS gene. For hairy root transformation, the construct was transferred into *Agrobacterium rhizogenes* LBA1334 by electroporation. Transgenic hairy roots selected by GUS staining were examined for the subcellular localization of GFP fusion proteins using confocal laser-scanning microscope Zeiss LSM510 with excitation and emission wavelengths of 488 and 550 nm, respectively.

BiFC analysis

The full-length cDNAs of *NSP2* and *IPN2* were amplified by PCR and cloned into the *Bam*HI/*Sma*I and *Sal*I/*Sma*I site of pSCYCE-R vector (Waadt *et al.*, 2008), generating CFPC-NSP2 and CFPC-IPN2 fusion constructs. The full-length cDNAs of *NSP2* and *IPN2* without the stop codon were cloned into the *Bam*HI/*Sma*I site and the *Sal*I/*Sma*I site of pSCYNE to obtain NSP2-CFPN and IPN2-CFPN, respectively. The constructs were transformed into *A. tumefaciens* strain GV3101/PMP90 by electroporation. Transient expression of proteins in *N. benthamiana* leaves by *A. tumefaciens* infiltration was conducted as described in Kang *et al.* (2011). Cyan fluorescence of fusion proteins was assayed 2–3 d after infiltration using the Zeiss LSM510 with excitation/emission wavelengths of 405 and 477 nm, respectively.

Yeast one-hybrid assay

The *IPN2* cDNA was fused to the Gal4 activation domain in pGADT7-Rec2 (Clontech, Mountain View, CA, USA), generating pGADT7-IPN2. The promoter regions of *NIN* (−500 to −1), *CBP1* (−462 to +1) and *CCaMK* (−315 to +1) were amplified by PCR using genomic DNA as template. The promoter fragments of the *NIN*, *CBP1* and *CCaMK* genes were inserted into the *Eco*RI-*Mlu*I site of pHIS2 (Clontech), producing *NINpro:HIS3*, *CBP1pro:HIS3* or *CCaMKpro:HIS3* fusion, respectively, which would express the HIS3 reporter under the control of the corresponding promoter. The plasmid was transformed into yeast Y187 cells harboring pGADT7-IPN2. The DNA-binding activity of IPN2 was determined by the expression of the HIS3 reporter for growth on the SD/-Trp-Leu-His plates in the presence of 20- or 40-mM 3-AT.

Gel mobility shift assay

Recombinant GST-IPN2 protein was produced in *Escherichia coli* strains BL21-Codon Plus (DE3)-RIL (Stratagene, La Jolla, CA, USA) and purified using glutathione sepharose 4B columns (GE Healthcare). The amount of purified proteins was estimated by the Bradford method using a protein assay kit (Fermentas). Oligonucleotide probes were labeled with [γ-^{32}P]ATP using T4 nucleotide kinase (TaKaRa). Electrophoretic mobility shift assays (EMSA) were performed as described previously (Zhu *et al.*, 2008).

Quantitative real-time reverse transcription PCR (qRT-PCR) analysis

The expression profile of the *IPN2* gene was analyzed by quantitative RT-PCR using RNA samples isolated from leaves, shoots and roots of *L. japonicus* MG-20 following inoculation with *M. loti* MAFF303099. Total RNA was isolated using TRIZOL reagent (Invitrogen) and treated with DNase I (Promega) to eliminate genomic DNA contamination. The amount of total RNA was normalized by measuring the RNA concentration at 260 nm, and 1 μg of total RNA was added to individual tubes to synthesize first-strand cDNA using Oligo $(dT)_{18}$ primer. Real-time RT-PCR reactions were performed as described previously (Ke *et al.*, 2012). *IPN2* transcripts were amplified using primers 5′-ATCCGC ATTTGT TCTTGA ATC-3′ and 5′-AGTTGA GGGCTT CTTGGG AG-3′.

IPN2 promoter-GUS construction

For the promoter-GUS fusion, a 3-kb promoter with the 5′ untranslated region (UTR) was amplified using primers 5′-AA CTGC AGGTTG TATATG ATTGAG AAC-3′, 5′- GGAATT CCATTC TCTCCA TCTCTC ACC-3′. The PCR fragment was cloned into the *Pst*I/*Eco*RI site of pCAMBIA1391Z vector (CAMBIA). The construct was transferred into *A. rhizogenes* LBA1334 by electroporation.

Knockdown expression of the IPN2 gene by RNAi

A 201-bp fragment of the 5′-UTR with a short coding region of *IPN2* was amplified by PCR using two primer pairs: 5′-TC CCCC GGGTCT CTTCTT CAAAAC GCA-3′ and 5′- ATCG GA TCCACC TGAGTC TCCTTG AAC-3′; and 5′-AACTGC AGTCTC TTCTTC AAAACG CA-3′ and 5′-AACTGC AGAC CT GAGTCT CCTTGA AC-3′. For IPN2 RNAi-2 construction, a 250-bp fragment of the 3′-UTR was amplified using 5′-A ACTGC AGCCCG GGATGA CTCAAA GCCTGA AGAG-3′ and 5′- ACGCGT CGACGG ATCCAG ATTCCC AACCAA AGAC-3′. The cDNA products were digested with *Sma*I-*Bam*HI and with *Pst*I-*Sal*I, and ligated into pCAMBIA1301-35S-int-T7 vector, in which the sense and antisense *IPN2* DNA sequence were located in tandem separated by the *Arabidopsis actin-11* intron. This intron-hairpin RNA (ihpRNA) construct was placed behind the dual CaMV 35S promoter. The RNAi binary vector was transferred into *A. rhizogenes* LBA1334 by electroporation. Plants harbouring transformed hairy roots were transferred to pots filled with vermiculite and sand (1 : 1) with half-strength Broughton & Dilworth (B&D) medium and grown in a chamber in 16 : 8 h day : night cycle at 22°C. After 5–7 d. Plants were inoculated with *M. loti* MAFF303099 and grown with the above medium.

Results

Identification of IPN2 as an interacting protein of NSP2

Both NSP1 and NSP2 contain the conserved C-terminal GRAS domain, but have a variable N-terminal region. Fusion of NSP1 or NSP2 to the Gal4 DNA binding domain is known to lead to auto-activation of the Gal4-mediated yeast two-hybrid system (Hirsch *et al.*, 2009). Truncated NSP1 and NSP2 lacking the N-terminal region did not exhibit auto-activation in the yeast two-hybrid system (Supporting Information Fig. S1b), suggesting that auto-activation is caused by the expression of the N-terminal region. In an attempt to identify NSP1 and NSP2-binding proteins, we used the GRAS domains (Fig. S1a) as bait to screen a *L. japonicus* root cDNA library (Zhu *et al.*, 2008). Positive

interaction clones from the primary screens were tested on stringent selective medium (SD-Trp-Leu-His-Ade). Screening with the GRAS domain of Lj NSP1 did not identify any interacting clone. Screening with the GRAS domain of Lj NSP2 resulted in isolation of two independent positive clones (Fig. S1d). Sequencing analysis of the plasmids revealed that the two clones represent the same gene encoding a novel protein, designated IPN2 for Interacting Protein of NSP2.

IPN2, a MYB coiled-coil transcription factor of the GARP subfamily

The full-length Lj *IPN2* cDNA (GenBank acc. HQ343457) contains an open reading frame of 1074 nucleotides encoding a peptide of 358 amino acid residues (Fig. S2b), with a predicted isoelectric point of 8.3 and molecular mass of 40.2 kD. A BLAST search for Lj *IPN2* sequences in the National Center for Biotechnology Information (NCBI) high-throughput genomic sequence database identified a perfect match with a Transformation-competent Artificial Chromosome (TAC) clone (GenBank accession no. AP010214). Comparison between the genomic DNA and the cDNA sequence revealed that Lj *IPN2* is composed of six exons and five introns. The Lj IPN2 protein contains a single MYB-like DNA-binding domain (1R) and a predicted coiled-coil dimerization domain (Fig. 1a). MYB family transcription factors contain one to three copies (termed R1 to 3) of a highly characteristic DNA-binding domain. These are typically slightly over 50 amino acids in length. Each domain contains three helices and folds into a helix-turn-helix motif (Fig. 1b) with three highly conserved Trp (W) residues regularly spaced by 18 or 19 amino acids, and the motif plays a critical role in stabilizing the DNA binding domain (Ogata *et al.*, 1992). An alignment of the Lj IPN2 1R region, other plant MYB-like DNA-binding domains, and the R1, R2 and R3 repeats of human c-Myb (Majello *et al.*, 1986) showed that the IPN2 1R region contains only one conserved Trp (W) at the first helix. The tther two positions expected to have 'W' residues are replaced with Pro (P) and Leu (L), respectively (Fig. 1b). Based on these substitutions, the Lj IPN2 1R region can be classified into the GARP subfamily, which is more distantly related to the MYB superfamily (Riechmann *et al.*, 2000).

Other members in the GARP family include maize GOLDEN2 (G2; Hall *et al.*, 1998), *Arabidopsis* ARRs (Hosoda *et al.*, 2002), *Chlamydomonas reinhardtii* PSR1 (Wykoff *et al.*, 1999), and

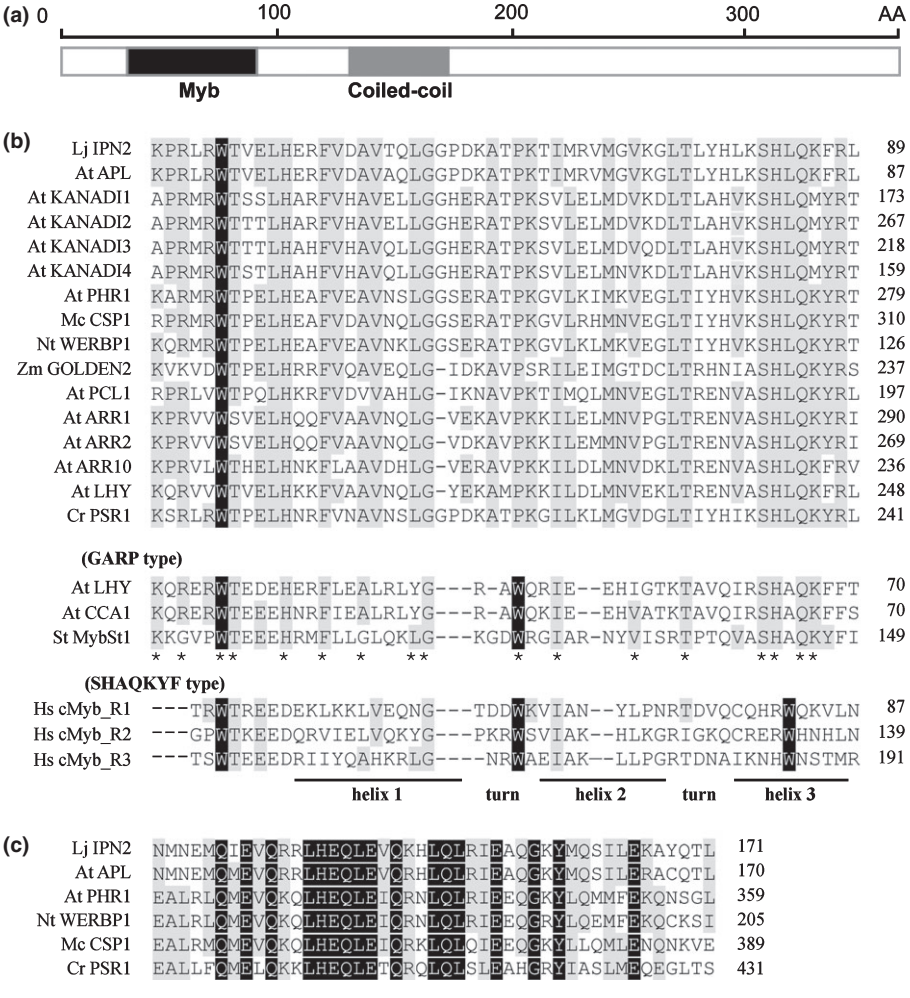

Fig. 1 IPN2 from *Lotus japonicus* belongs to the GARP subfamily of Myb-related proteins. (a) Schematic illustration of functional domains of IPN2 protein. The single repeat Myb-like DNA binding domain is indicated by a solid box and the predicted coiled-coil domain is shown in a shaded box. (b) Alignment of the Myb-like domain of Lj IPN2 and other Myb-related proteins from various organisms including *Arabidopsis thaliana* (At APL, KANADI1-4, PHR1, PCL1, ARR1, ARR2, ARR10, ARR12), *Mesembryanthemum crystallinum* (Mc_CSP1), *Nicotiana tabacum* (Nt_WERBP1), *Zea mays* (Zm_GOLDEN2), *Chlamydomonas reinhardtii* (Cr_PSR1), *Solanum tuberosum* (St_MybSt1) and *Homo sapiens* (Hs_c-Myb R1-R3). Numbers on the right indicate the amino acid positions of the proteins. The three regularly spaced Trp (W) residues are highlighted in black. Gray letters are residues that share > 75% identity. (c) Alignment of the predicted coiled-coil conserved domain of IPN2 (amino acids 127–171) with the corresponding regions of other GARP proteins. Identical residues are in black and conserved residues are in gray.

Arabidopsis PHR1 (Rubio *et al.*, 2001). A GARP domain has also been found in the identified KANADI and APL proteins in *Arabidopsis*, having regulatory roles in the establishment of organ polarity and vascular identity, respectively (Kerstetter *et al.*, 2001; Bonke *et al.*, 2003). The GARP domains are similar to the MYB-like domain of the single repeat MYB proteins CCA1 (Wang *et al.*, 1997), LHY (Schaffer *et al.*, 1998) and MybSt1 (Baranowskij *et al.*, 1994), which, however, have two conserved 'W' residues at the first and second helices, but have the third 'W' residue replaced with Ala (A) in the context of 'SHAQKY/F' (Fig. 1b). These proteins have also been referred to as the 'SHAQKY/F' subfamily (Fukuzawa *et al.*, 2006). Besides the GARP domain, Lj IPN2 and some of the GARP family proteins also contain a predicted coiled-coil domain with the consensus sequence 'LHEQLE' (Figs 1c, S2a).

Identification of domains required for interaction between NSP2 and IPN2

Lj NSP2 contains two leucine heptad repeats (LHR1 and LHR2), a VHIID motif and a Src-homology 2 (SH2)-like domain (Murakami *et al.*, 2006). To determine which domain of NSP2 was responsible for its interaction with IPN2, we expressed the full-length NSP2 and a series of truncated proteins of NSP2 fused to the Gal4 DNA binding domain (BD). These constructs were tested for interaction with IPN2 fused to the Gal4 activation domain (AD; Fig. 2a). The strength of interaction was measured using β-Galactosidase activity with ONPG as substrate. Most of the N- and C-terminal deletions of NSP2 failed to interact with IPN2. Only the full-length NSP2 and a truncated section (105–499 aa) containing all four domains (LHR1, VHIID, LHR2 and

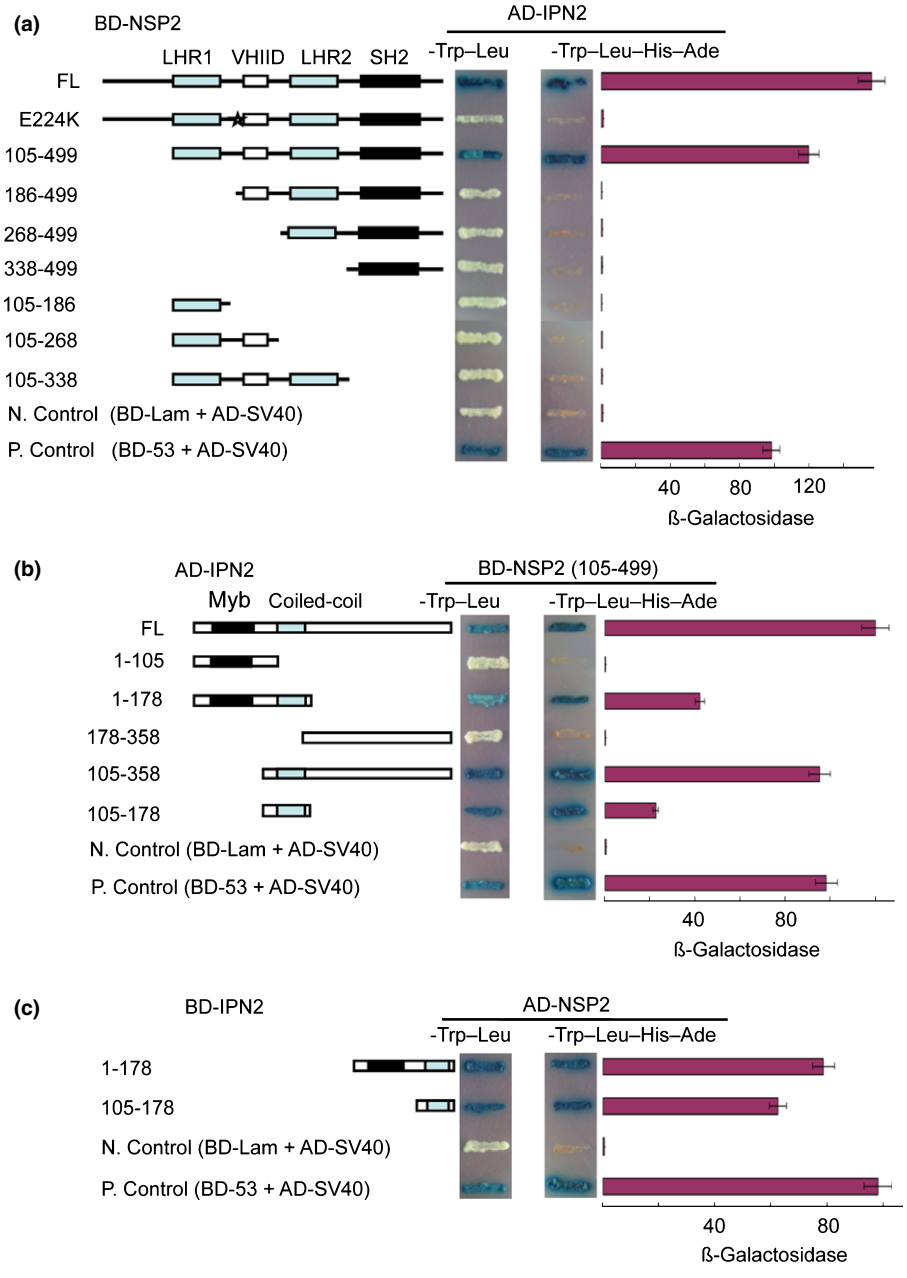

Fig. 2 IPN2 from *Lotus japonicus* interacts with NSP2 in yeast. (a) Dissection of functional domains of NSP2 required for interaction with IPN2. The C-terminal GRAS domain of NSP2 (105–499) was used in the initial isolation of IPN2 from the two-hybrid library screen. SD/-Leu-Trp medium was used for mating selection and SD/-Leu-Trp-His-Ade medium for selection of interacting clones. The interaction strength was measured through the β-Galactosidase activity either on plates containing X-Gal (80 μg ml^{-1}) or by quantification with ONPG as substrate. In the experiment, 1 unit of β-galactosidase was defined as the amount of enzyme that hydrolyzes 1 μmol of ONPG to *o*-nitrophenol and *D*-galactose per min per cell (Miller unit). The combination of BD-53/AD-SV40 was used as a positive control and BD-Lam/AD-SV40 as a negative control (Clontech). BD: Gal4 DNA binding domain; AD: Gal4 activation domain. (b) Dissection of functional domains of IPN2 required for interaction with NSP2. The coiled-coil domain of IPN2 was necessary and sufficient for interaction with NSP2. (c) Exchanging the BD and AD fusion of NSP2 and IPN2 in (b) confirmed the interaction of the coiled-coil domain of IPN2 with NSP2.

SH2-like) were able to interact with IPN2 (Fig. 2a), suggesting that more than one domain of NSP2 is required for this interaction. In contrast, the LHR1 domain of Mt NSP2 alone has been shown to be necessary and sufficient for the interaction with Mt NSP1 (Hirsch *et al.*, 2009). Interestingly, a site mutant (E224K) of Lj NSP2, which is equivalent to the E232K mutant of Mt NSP2, abolished the interaction with Lj IPN2 (Fig. 2a). The Mt NSP2 (E232K) mutant was originally identified as *nsp2-3* mutant in *M. truncatula*, a weak *nsp2* mutant allele showing limited nodule formation in contrast to the null allele *nsp2-2* producing no nodule at all (Kaló *et al.*, 2005). This mutation also abolishes auto-activation in the yeast two-hybrid system, but does not affect the interaction with Mt NSP1 (Hirsch *et al.*, 2009).

The coiled-coil conformation has been considered as a potential homodimerization or heterodimerization motif (Lupas *et al.*, 1991). To determine whether the coiled-coil domain of Lj IPN2 was responsible for the interaction with Lj NSP2, several deletion mutants of Lj IPN2 fused to Gal4 AD were tested for interaction with the Lj NSP2 (105–499) fused to Gal4 BD (Fig. 2b). We found that only the deletion mutants containing the coiled-coil domain interacted with Lj NSP2. On the other hand, the truncated Lj IPN2 (1–178) and Lj IPN2 (105–178) interacted with the full-length Lj NSP2 fused to Gal4 AD (Fig. 2c). These results indicate that the coiled-coil domain of Lj IPN2 is necessary and sufficient for interaction with Lj NSP2.

Interaction of NSP2 and IPN2 *in vitro* and in planta

In order to validate the interaction between Lj NSP2 and Lj IPN2, we performed *in vitro* protein pull-down and bimolecular fluorescence complementation (BiFC) assays. The full-length Lj NSP2 was expressed as glutathione S-transferase (GST) fusion protein and was bound to the glutathione Sepharose 4B columns. Lj IPN2, or its deletion mutants Lj IPN2 (1–105) and Lj IPN2 (1–178) fused with the ×6 His tag, was mixed with Ni-affinity beads, which were then washed repeatedly with buffer to remove nonspecifically-bound proteins. Proteins retained on the beads were eluted in SDS sample buffer and resolved on SDS-PAGE. The presence of Lj IPN2 on the beads was detected by immunoblotting with anti His-tag antibody. As shown in Fig. 3(a), both the Lj IPN2 and Lj IPN2 (1–178) proteins were retained on the GST-NSP2 beads, confirming a direct interaction between Lj NSP2 and Lj IPN2.

For BiFC assay, we constructed plasmids that would express a fusion protein of the C-terminal split cyan fluorescent protein (SCFP; Waadt *et al.*, 2008) with NSP2 (SCFPC-NSP2) and a fusion protein of IPN2 with the N-terminal SCFP (IPN2-SCFPN). The constructs were transiently co-expressed in *N. benthamiana* epidermis cells. Using a confocal laser scanning microscope, we observed strong cyan fluorescence signals in the nucleus (Fig. 3b), suggesting that NSP2 and IPN2 interact with each other in plant cells. The interaction was further confirmed by switching the tags between NSP2 and IPN2 (Fig. 3b).

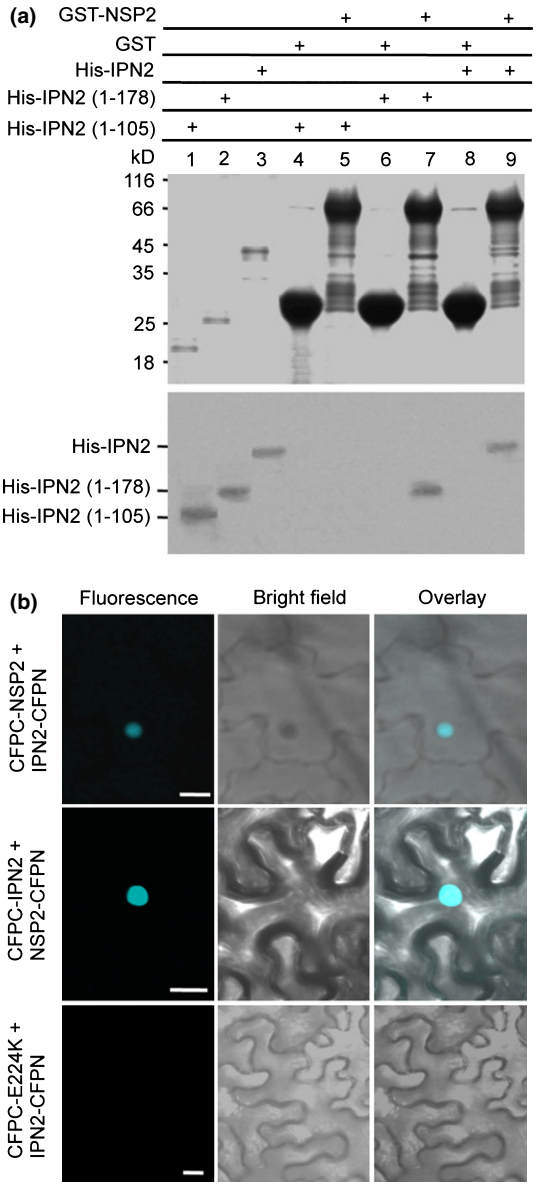

Fig. 3 Interaction of *Lotus japonicus* IPN2 and NSP2 *in vitro* and in plant nuclei. (a) *In vitro* protein pull-down assay for testing the interaction between NSP2 and IPN2. His-tagged IPN2 (1–105), IPN2 (1–178) and IPN2 were incubated with immobilized GST-NSP2 fusion protein or GST control. After washing, proteins retained on the affinity beads were separated on SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R250 (top). An identical gel was used for immunoblot with anti His-tag antibody (bottom). (b) Interaction of NSP2 and IPN2 in planta. *N. benthamiana* leaves cotransformed with CFPC-NSP2 and IPN2-CFPN were observed for protein-protein interactions using a confocal microscope. The split CFP tags were switched between NSP2 and IPN2, and the resulting constructs were used to co-transform *N. benthamiana* leaves. The combination of IPN2 and the point mutant E224K of NSP2 was used as a negative control. Fluorescence and bright-field images of the same leaf cells were superimposed to produce the overlay images. Bars, 20 μm.

DNA binding and transcription activation of IPN2

In *M. truncatula*, NSP1 and NSP2 form a complex that binds directly to the Mt *NIN* (*Nodule Inception*) promoter (Hirsch

et al., 2009). The expression of the *NIN* gene is known to be induced rapidly by rhizobial infection or treatment with Nod factors (Schauser *et al.*, 1999; Murakami *et al.*, 2006), and the regulatory *cis*-DNA sequences required for this induction are conserved within a 500-bp promoter region upstream of the transcript start of Lj *NIN* (Borisov *et al.*, 2003). As an interacting partner of Lj NSP2, Lj IPN2 may play a role in the regulation of Lj *NIN* promoter in concert with Lj NSP1 and Lj NSP2. To test whether Lj IPN2 could bind to the Lj *NIN* promoter, we carried out the yeast one-hybrid system assays. The 500-bp (−500 to −1) Lj *NIN* promoter was inserted in front of the *HIS3* reporter, generating a construct of *NIN*pro:HIS3 fusion. The 462-bp (−462 to +1) Lj *CBP1* (Webb *et al.*, 2000) and the 315-bp (−315 to +1) Lj *CCaMK* promoter were used as controls. Each of these constructs was cotransformed with pGAD-IPN2 into yeast Y187 cells, where Lj IPN2 was expressed as a fusion protein with Gal4 AD. If Lj IPN2 could bind to the promoter, the Gal4 AD would drive the expression of the *HIS3* reporter, and the yeast cells would grow on SD/-Trp-Leu-His plates supplemented with 3-amino-1, 2, 4-triazole (3-AT). Our results showed that the cotransformants of pGAD-IPN2 and *NIN*pro:HIS3 were able to grow on SD/-Trp-Leu-His plates, whereas the cotransformants of pGAD-IPN2 with *CBP1*pro:HIS3 or with *CCaMK*pro:HIS3 could not (Fig. 4a). Thus, Lj IPN2 can specifically bind to the Lj *NIN* promoter but not the Lj *CBP1* and Lj *CCaMK* promoters in yeast cells.

We performed EMSA in order to test whether Lj IPN2 binds to the Lj *NIN* promoter *in vitro*. Purified GST-IPN2 was tested for co-migration with a radio-labeled 500-bp *NIN* promoter fragment, the same promoter used in the yeast one-hybrid assay. We found a clear band shift when the *NIN* promoter was incubated with IPN2, but not with the controls (Fig. 4b). Moreover, the binding of IPN2 to the *NIN* promoter could be outcompeted by the addition of unlabeled *NIN* promoter (Fig. 4b).

We further examined whether Lj IPN2 contains a transcription activation domain (AD). The full-length Lj IPN2 or its deletion fragments were fused to the C-terminus of the DNA binding domain (BD) of Gal4. The constructs were transferred into yeast strain Y187, which contained the *lacZ* reporter gene under the Gal4-responsive promoter (Clontech). If Lj IPN2 contained an AD, the BD-IPN2 fusion protein would bind to the Gal4-responsive promoter and drive the expression of the *lacZ* reporter. The strength of transcription activation was quantified through the β-galactosidase activity with ONPG as substrate. We also compared the activation activity of BD-IPN2 with BD-NSP1 and BD-NSP2. As shown in Fig. 4(c), Lj IPN2 had much higher transcription activation activity than Lj NSP1 and Lj NSP2. The N-terminal region of Lj IPN2 (1–178) did not have transcription activation activity. The transcriptional activation of Lj NSP2 was abolished by the point mutation of Lj NSP2 (E224K), in the same way as the Mt NSP2 (E232K) mutant (Hirsch *et al.*, 2009).

Temporal and spatial expression of *IPN2* gene during nodulation

Gene expression patterns – that is the *IPN2* mRNA transcript levels at different tissues and nodulation stages – were measured

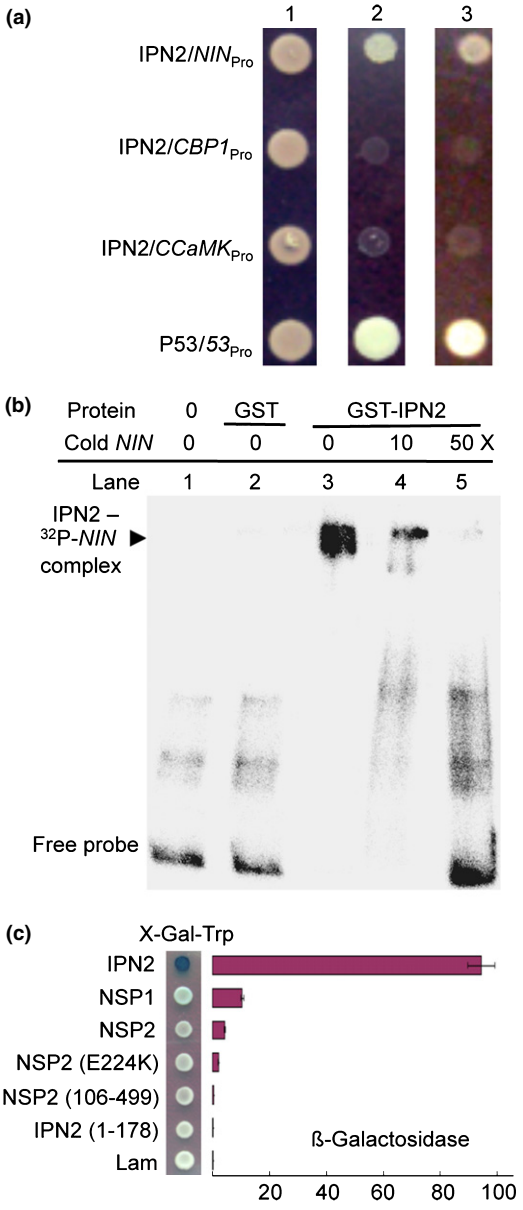

Fig. 4 DNA binding and transcription activation of *Lotus japonicus* IPN2. (a) Yeast one-hybrid assay showing the binding of IPN2 to the *NIN* promoter. IPN2 was expressed as a fusion protein with the Gal4 activation domain in yeast Y187 cells harboring the reporter construct *NIN*pro:HIS3, *CBP1*pro:HIS3, or *CCaMK*pro:HIS3, which express the HIS3 reporter gene under the promoter of the *NIN*, *CBP1*, or *CCaMK* gene. The promoter regions of *NIN* (−500 to −1), *CBP1* (−462 to +1), and *CCaMK* (−315 to +1) were used. Yeast cells were examined for growth on selection medium of SD/-Trp-Leu (control) and SD/-Trp-Leu-His plates in the presence of 20 or 40 mM 3-AT. Yeast cells harboring p53-HIS2 and pGADT7-53 were used as a positive control. (b) EMSA of IPN2 with labeled *NIN* promoter. The *NIN* promoter (−500 to −1) was labeled with ^{32}P, and unlabeled *NIN* fragment was used as a competitor DNA. Arrow indicates a shifted band, which was absent in the control and became weaker in the presence of competitor DNA at 10- and 50-fold excess (×c10, ×c50). (c) Transcription activation activity of IPN2. Plasmids expressing a fusion protein of the Gal4 DNA-binding domain with IPN2 or its deletion fragments were expressed in yeast Y187 cells harboring the *lacZ* reporter gene. Yeast cells were streaked on SD/-Trp plate containing X-Gal (80 μg ml^{-1}) or cultured in SD/-Trp broth for quantification of β-galactosidase activity with ONPG as substrate. Lam (Clontech) was used as a negative control.

by quantitative RT-PCR (qRT-PCR). Roots were harvested at different time points after inoculation with *M. loti* MAFF303099, and noninoculated roots used as control. We observed a slightly elevated level of Lj *IPN2* gene expression 4 and 6 d post inoculation (DPI). The expression level dropped down to the basal level 12 d after inoculation (Fig. 5a). We next examined the expression of Lj *IPN2* in different organs of *L. japonicus* plants. The expression of Lj *IPN2* was detected ubiquitously in leaves, stems, nodules and roots (Fig. 5b). It is likely that Lj *IPN2* may have other roles in the development of *L. japonicus* plants in addition to nodulation.

In order to analyze the spatial expression of Lj *IPN2* gene, we fused a 3-kb genomic DNA segment upstream of the coding region of Lj *IPN2* to the GUS reporter gene. This *IPN2*pro:GUS construct was subsequently transformed into *L. japonicus* by *A. rhizogenes* and monitored for GUS activity in roots and nodules. In noninoculated roots, *IPN2*pro:GUS was mainly expressed in the root tip and vascular bundles (Fig. 6a). After inoculation with *M. loti*, the GUS reporter activity was observed at the nodule primordial initiation sites (arrow in Fig. 6b), nodule primordia (Fig. 6c), and young developing nodules (Fig. 6d). The activity was downregulated drastically in mature nodules (Fig. 6e). We also examined the spatial expression pattern of *NSP2*, the interaction partner of *IPN2*, in order to assess if the expression patterns of the two genes overlap. Transgenic roots

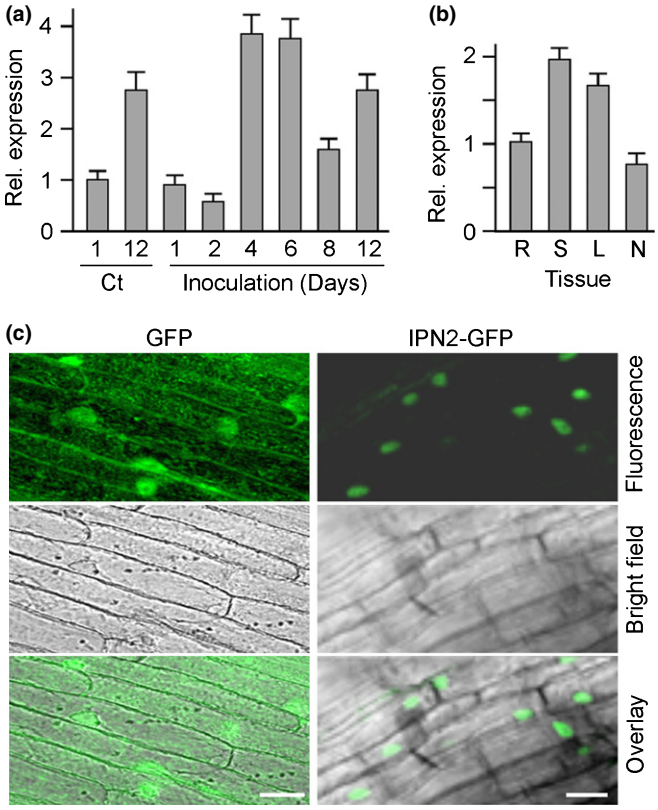

Fig. 5 Gene expression and subcellular localization of *Lotus japonicus* IPN2. (a) Quantification of *IPN2* gene expression in *Rhizobium*-infected roots by quantitative RT-PCR. Relative levels of *IPN2* gene expression (Rel. expression) were calculated using the expression of *Ubiquitin* (AW720576) as an internal control. RNA samples were taken from roots inoculated with *M. loti* over a period of 1–12 d post inoculation. Noninoculated roots (1 and 12 d) served as controls (Ct). (b) *IPN2* expression in various organs. *Rhizobium*-inoculated roots, stems, leaves and nodules were harvested 20 d after inoculation. Error bars indicate ± SD. (c) Subcellular localization of IPN2. Fusion protein of IPN-GFP was expressed in transgenic hairy roots, and observed using a confocal microscope. Green fluorescent and bright-field images were superimposed to produce the overlay images. Note that IPN2-GFP was localized only in the nucleus whereas control GFP was distributed both in the cytoplasmic and nuclear compartments. Bars, 20 μm.

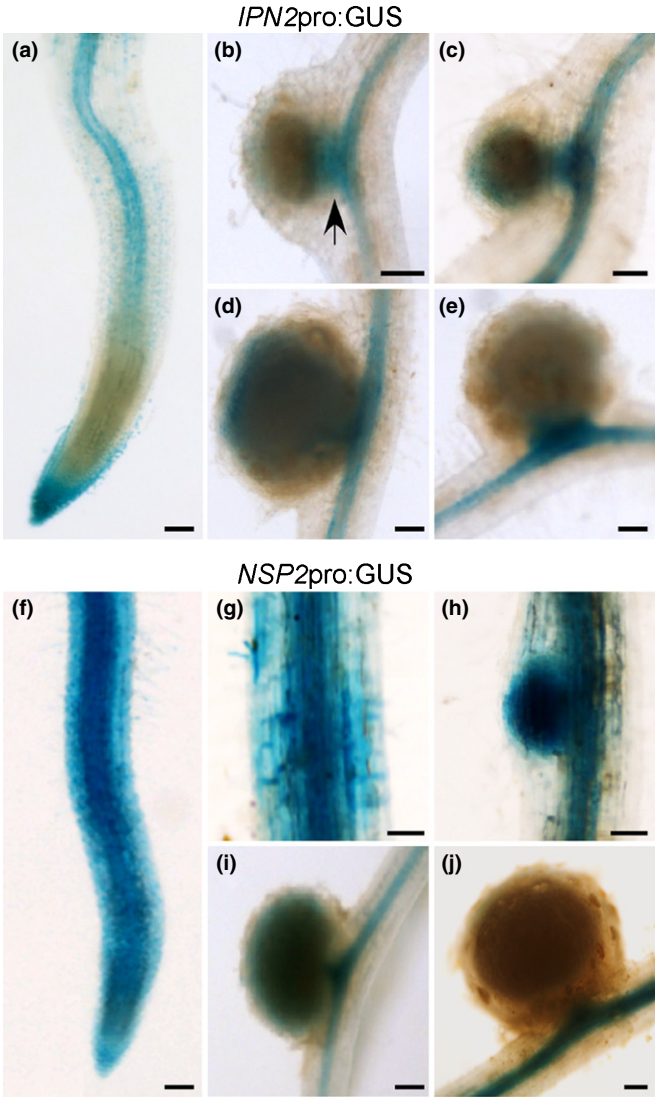

Fig. 6 Analysis of *IPN2*pro:GUS and *NSP2*pro:GUS expression in roots and nodules of *Lotus japonicus*. Stereomicroscopical images of *L. japonicus* transgenic hairy roots expressing *IPN2*pro:GUS (a–e) or *NSP2*pro:GUS (f–j) after GUS staining. In noninoculated roots, the *IPN2* promoter was active in the root tip and vascular bundle (a), whereas the *NSP2* promoter was active all along the root, including root hairs, epidermis, cortex and vascular bundle (f). After rhizobial inoculation, *IPN2* promoter activity was observed at the nodule primordia initiation sites (b), nodule primordia (c) and young developing nodule (d) but not in mature nodules (e). Following inoculation, *NSP2* promoter activity was noticeably stronger in the root hair and epidermal cells in close contact to rhizobial infections (g) and nodule primordia (h). During further development of the nodules, *NSP2* promoter activity became weaker (i) and finally disappeared in fully matured nodules (j). Bars, 100 μm.

containing *NSP2*pro:GUS construct were generated by *A. rhizogenes* transformation. In noninoculated roots, the *NSP2* promoter was active all along the root, including root hairs, epidermis and cortex (Fig. 6f). Following inoculation, *NSP2* promoter activity was noticeably high in the root hairs and epidermal cells in close contact with rhizobial infections (Fig. 6g) and nodule primordia (Fig. 6h). As nodules continued to develop and mature, *NSP2* promoter activity started to drop (Fig. 6i) and eventually disappeared in fully matured nodules (Fig. 6j). Thus, the gene expression patterns of *IPN2* and *NSP2* show partially overlapping, for example, at the nodule primordia and young developing nodules during nodulation.

Subcellular localization of IPN2 protein

The DNA binding activity and the presence of a putative nuclear localization signal (NLS; Fig. S2b) motif of Lj IPN2 implies a possibility of subcellular localization of this protein in the nucleus of living cells. For this, we expressed IPN2-GFP fusion protein in transgenic hairy roots of *L. japonicus*. Confocal laser-scanning microscopy revealed that IPN2-GFP was mainly concentrated in the nucleus of root cells, whereas the GFP control was present both in the cytoplasmic and nuclear compartments (Fig. 5c). The nuclear localization of IPN2-GFP remained unchanged after inoculation with *M. loti*.

Impairment of nodulation by *IPN2* RNAi

In order to elucidate the function of Lj *IPN2* in the nodulation process, we generated transgenic hairy roots that had downregulated expression of *IPN2* by RNAi. Transgenic hairy roots expressing the empty vector served as a control. Two *IPN2*-specific RNAi constructs, with *IPN2* RNAi-1 targeting the 201-bp 5′ untranslated region (5′-UTR) and RNAi-2 targeting the 250-bp 3′-UTR of the *IPN2* cDNA, were expressed in transgenic hairy roots of *L. japonicus*. The levels of the *IPN2* transcripts in transgenic hairy roots were measured by quantitative RT-PCR. The data showed that the *IPN2* transcripts were reduced to 51% in *IPN2* RNAi-1 and 32% in RNAi-2 as compared with the control hairy roots generated using the empty vector (Fig. 7a).

As root growth defects may influence nodule formation, we examined the growth phenotype of *IPN2* RNAi roots under non-nodulating conditions. We did not observe any obvious nonsymbiotic or developmental defects in *IPN2* RNAi roots (Fig. S3a), and the primary root length was comparable between the *IPN2* RNAi and control hairy roots (Fig. S3b).

Transgenic *IPN2* RNAi hairy roots were inoculated with *M. loti* MAFF303099 and grown for 3 wk in a nitrogen-free environment. Nodulation phenotypes were examined. The average number of nodules formed on control hairy roots was 7.8, while that on RNAi-1 and RNAi-2 hairy roots was reduced to 3.5 and 2.7, respectively (Fig. 7b). This reduction in nodule number by RNAi expression was statistically significant ($P < 0.01$, $n = 55$ and $P < 0.01$, $n = 61$). These results indicate that downregulation of *IPN2* gene expression by RNAi has a negative effect on nodulation.

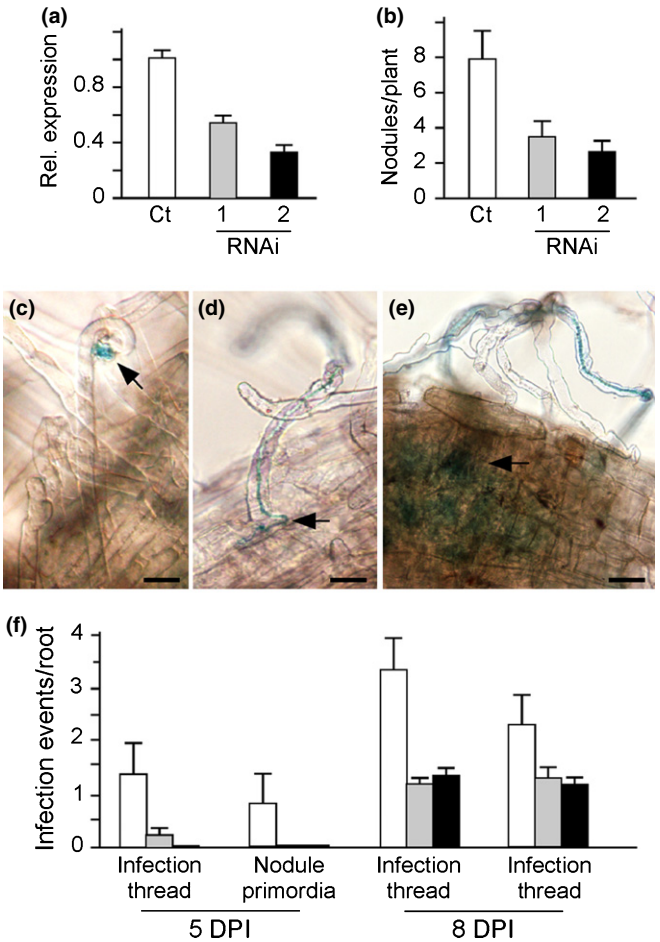

Fig. 7 Rhizobial infection and nodulation phenotype of *Lotus japonicus IPN2*-RNAi hairy roots. (a) Quantitative RT-PCR analysis of *IPN2* expression levels in control hairy roots expressing the empty vector and representatives of hairy roots expressing the *IPN2*-RNAi constructs (RNAi-1 and RNAi-2). Error bars indicate ± SD for six independent transformants per line and three technical replicates. (b) Numbers of nodules in transgenic hairy roots vector expressing the empty vector (control) and two RNAi constructs were determined 3 wk after inoculation with *M. loti* MAFF303099. (c–e) Infection threads (ITs) formation in *IPN2*-RNAi hairy roots. ITs were visualized after staining with X-Gal in hairy roots 8 d post inoculation (DPI) with *M. loti* expressing a *lacZ* construct. The images show the key steps of *Rhizobium* infection in hairy roots, including curled root hairs with entrapped bacterial colony (c), IT growth (d), and release of rhizobia in developing nodule cells (e). Bars, 25 μm. (f) Frequencies of infection events per root of control hairy roots, *IPN2* RNAi-1 and RNAi-2 hairy roots at 5 and 8 DPI, respectively. The data are presented as 15 individual transgenic plants for each construct, and randomly scored in four roots between 4 and 6 cm per plant. Error bars represent ± SD.

Decreases in rhizobial infection and nodule organogenesis by *IPN2* RNAi

In order to determine how *IPN2* RNAi expression affects nodule formation, we analyzed the infection process of RNAi hairy roots inoculated with *lacZ*-labelled *M. loti* cells (Kang *et al.*, 2011). The presence of infection threads (ITs) was visualized under a microscope. At 5 d post inoculation (DPI), neither ITs nor nodule primordia were found in the *IPN2* RNAi-2 roots. No nodule primordia and only a few ITs could be detected in the RNAi-1

roots. By contrast, both ITs and nodule primordia could be readily observed in the control hairy roots expressing the empty vector. At 8 DPI, we could observe the initiation of ITs from the curled root hair tips, IT growth through root hairs to the root epidermis, and even the initiation of the nodule primordia could be observed in *IPN2* RNAi roots (Fig. 7c–e). The average numbers of ITs and nodule primordia were significantly reduced in *IPN2* RNAi roots as compared to those in the control hairy roots (Fig. 7f). Thus, both rhizobial infection and nodule organogenesis appear to be impaired by the knockdown expression of *IPN2* in RNAi hairy roots.

We also analyzed the expression levels of early nodule development marker genes *NIN* and *ENOD40-1* in *IPN2* RNAi hairy roots. Analysis of gene expression by qRT-PCR revealed that both *NIN* and *ENOD40-1* transcripts decreased markedly in *IPN2* RNAi-2 hairy roots as compared to those in the control (Fig. 8b,c). These results suggest that *IPN2* may serve as a transcriptional regulator of *NIN* and other nodulation-related genes.

It has recently been reported that NSP2 promotes root colonization by mycorrhizal fungi (Maillet *et al.*, 2011). We examined the mycorrhization phenotype of *IPN2* RNAi roots. In *IPN2* RNAi-1 and RNAi-2 roots 3 wk post inoculation with *Glomus intraradices*, the AM fungi penetrated into the outer cell layers, colonized the root cortex, and formed arbuscules and vesicles. No observable difference was found in hyphal, arbuscular and vesicular colonization between *IPN2* RNAi roots and vector control (Fig. S4). These observations suggested that RNAi knockdown of *IPN2* expression in hairy roots has no obvious effect on AM fungal colonization.

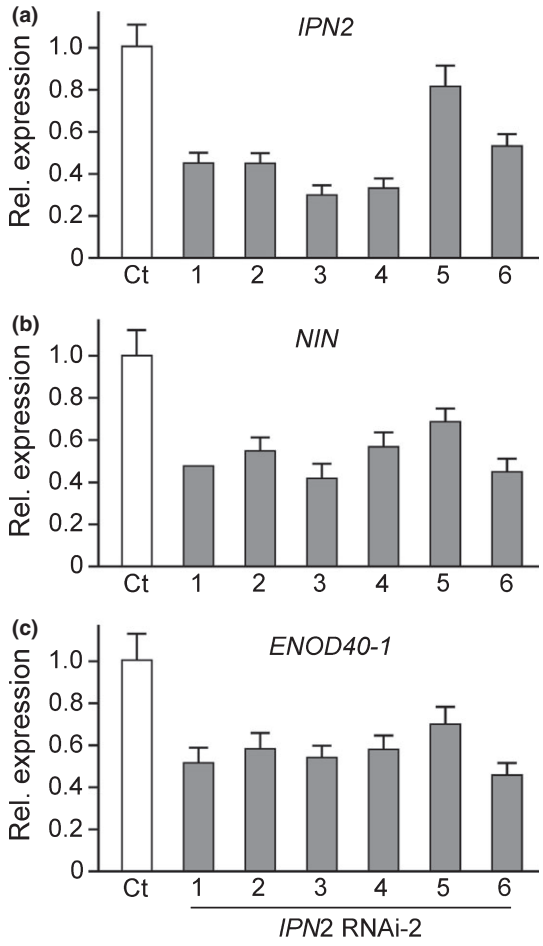

Fig. 8 Suppression of marker genes in *Lotus japonicus IPN2* RNAi-2 hairy roots. Total RNA was isolated from the control hairy roots (Ct) expressing the empty vector and six representative plants expressing *IPN2* RNAi-2. Quantitative RT-PCR analysis was performed to assess the expression levels of *IPN2* (a), *NIN* (b) and *ENOD40-1* (c). Error bars represent ± SD.

Discussion

Formation of a transcription factor complex is a key step in order to bind the promoter DNA and regulate gene expression. Two GRAS family transcription factors, SCARECROW (SCR) and SHORTROOT (SHR), interact with each other and control root radial patterning (Cui *et al.*, 2007). The *Solanum tuberosum* GRAS protein RGA interacts with the PIF4 basic helix-loop-helix (bHLH) transcription factor, and functions to integrate the light and GA signals (de Lucas *et al.*, 2008).

In *M. truncatula*, GRAS family transcription factors Mt NSP1 and Mt NSP2 form a protein complex and induce specific gene expression changes essential to the root nodule symbiosis (Hirsch *et al.*, 2009). In this study, we identified a novel MYB transcription regulator, Lj IPN2, which interacts with Lj NSP2. In *M. truncatula*, the LHR1 domain of Mt NSP2 is necessary and sufficient for interaction with Mt NSP1, whereas the whole GRAS domain of Mt NSP1 (156–554 aa) is required for interaction with Mt NSP2 (Hirsch *et al.*, 2009). In contrast, the whole GRAS domain of Lj NSP2 (105–499 aa) is required for interaction with Lj IPN2, whereas the coiled-coil domain of Lj IPN2 is necessary and sufficient for interaction with Lj NSP2 (Fig. 2).

Both Lj *NSP1* and Lj *NSP2* are expressed mainly in roots, with very low to no expression of Lj *NSP2* in shoots (Heckmann *et al.*, 2006; Murakami *et al.*, 2006). Our data on gene expression revealed that the expression levels of Lj *IPN2* in stems and leaves were higher than in roots (Fig. 5b). This expression pattern of Lj *IPN2* is similar to that observed in Mt *NSP2*, which is ubiquitously expressed in shoots and roots (Kaló *et al.*, 2005). It is possible that Lj *IPN2* has additional functions in shoots in addition to nodulation. Increased expression of Lj *IPN2* was observed in roots 4 and 6 d after inoculation (Fig. 5a), which is similar to that of Lj *NSP2*, with the difference that the upregulation of Lj *IPN2* might be somewhat earlier compared with Lj *NSP2* (Heckmann *et al.*, 2006). This is also similar to what was observed for Mt *NSP2* expression in *M. truncatula* (Kaló *et al.*, 2005). These suggest that the expression of *IPN2* and *NSP2* in roots is induced after rhizobial inoculation, but we could not exclude the possibility that the increased expression of Lj *IPN2* was developmentally regulated during the development of roots and nodules.

In order to identify the cell types in which the interaction of NSP2 and IPN2 might take place, we compared the spatial expression pattern of corresponding genes at tissue and cellular levels by means of promoter:GUS fusion. Following inoculation, *NSP2* promoter activity was noticeably induced in root hairs and

epidermal cells in close contact with rhizobial infections and nodule primordia (Fig. 6g,h). This spatial expression pattern fits well with the proposed function of *NSP2* in rhizobial infection and nodule organogenesis. Unlike *NSP2*, however, *IPN2* promoter activity was only observed at the nodule primordia initiation sites and nodule primordia (Fig. 6b,c), but not in the root hairs and epidermal cells before and after inoculation. This expression pattern is surprising because both rhizobial infection and nodule organogenesis appear to be impaired in *IPN2* RNAi hairy roots. It is possible that the decreased rhizobial infection is a secondary effect of the impaired nodule organogenesis, because cortical cell division and primordium formation are necessary to achieve rhizobial infection. The expression of both *NSP2* and *IPN2* was observed in nodule primordia and developing nodules, but was suppressed in mature nodules (Fig. 6). These overlapping expression patterns of *NSP2* and *IPN2* may implicate potential cell types and developmental stages at which NSP2 and IPN2 interact with each other.

Medicago truncatula NSP2 has been localized to the nuclear envelope and endoplasmic reticulum. The protein is re-localized to the nucleus rapidly upon Nod factor treatment (Kaló *et al.*, 2005). We attempted to detect the subcellular localization of Lj NSP2 in *L. japonicus* hairy roots by NSP2-GFP or NSP2-DsRED fusion under the control of the 35S promoter or Lj *NSP2* native promoter. However, no fluorescence was detected in cells expressing these constructs before and after rhizobial inoculation. Murakami *et al.* (2006) also reported that no fluorescence could be detected in *L. japonicus* hairy roots for Lj NSP2 with C-terminal GFP, YFP or DsRED fusion. However, IPN2-GFP under the 35S promoter showed strong nuclear localization in *L. japonicus* hairy roots (Fig. 5c), whereas no fluorescence could be detected under the control of the *IPN2* native promoter. This may be due to the low expression level of the *IPN2* promoter. The reason why no fluorescence could be detected for Lj NSP2 in *L. japonicus* hairy roots remains unclear, but our BiFC data clearly showed that Lj NSP2 interacts with Lj IPN2 in the nucleus of *N. benthamiana* leaf epidermal cells (Fig. 3b). Accordingly, Mt NSP2 interacts with Mt NSP1 in the nucleus of *N. benthamiana* using BiFC assay (Hirsch *et al.*, 2009). Altogether, these data strongly suggest that NSP2 and IPN2 may meet with each other in the nuclei of plant cells.

Our biochemical data demonstrate that IPN2 is a transcription factor that exhibits strong transcriptional activation activity and binds to the *NIN* promoter *in vitro* and in yeast (Fig. 4). Consistent with this, the expression of *NIN* decreased markedly in *IPN2* RNAi hairy roots (Fig. 8a,b). It is likely that IPN2 also binds to the *NIN* promoter *in vivo* to regulate *NIN* gene expression. The expression of *NIN* has been shown to be suppressed in Lj *NSP2* mutant (Murakami *et al.*, 2006), but no evidence for direct binding of NSP2 to the promoter DNA has been presented so far. In *M. truncatula*, Mt NSP1 binds directly to the *NIN* promoter *in vitro*, and this association *in vivo* requires Mt NSP2 (Hirsch *et al.*, 2009). This raises the possibility that NSP1, NSP2 and IPN2 form a complex on the *NIN* promoter. It is possible that NSP1 and IPN2 bind directly to different *cis*-elements of the *NIN* promoter, while NSP2 serves as a skeleton protein linking NSP1 with IPN2. This hypothesis warrants further experimental investigation.

Having an N-terminal MYB-like DNA binding domain, IPN2 belongs to the large MYB protein family. The MYB protein family is universal in the plant kingdom, and MYB domain proteins are classified based on the numbers of MYB-DNA binding domain repeats. Because of the presence of only one MYB domain, Lj IPN2 is classified as a MYB-related protein. The functions of MYB domain family of proteins may vary drastically according to the number of repeats. MYB proteins with two MYB repeats (R2R3) represent the biggest family in plants. To date, over 100 members have been isolated in *Arabidopsis* and they play various roles in the regulation of secondary metabolism, tissue morphogenesis, phloem formation and cell cycle progression (Riechmann *et al.*, 2000). In summary, plant Myb-related transcription factors are likely to be involved in diverse biological processes including cell proliferation, differentiation and organ development.

Protein domain analysis revealed that the Myb-like DNA binding domain of IPN2 is closely related to the B motif or GARP motif (Hosoda *et al.*, 2002). The B motif is originally identified as a signature of the type-B ARRs involved in the His-Asp phosphor-relay system in *Arabidopsis* (Imamura *et al.*, 1999). The B motif is a representative of the GARP family plant transcription factors, including maize G2 and *C. reinhardtii* PSR1. G2 controls the differentiation of a photosynthetic cell type of the maize leaves (Hall *et al.*, 1998), whereas PSR1 is a regulator of phosphorus metabolism (Wykoff *et al.*, 1999). *Arabidopsis* PHR1, a homolog of PSR1, functions in phosphate starvation signaling (Rubio *et al.*, 2001). The identified KANADI protein, possessing a GARP motif, has been implicated in the regulation of organ polarity in *Arabidopsis* (Kerstetter *et al.*, 2001). Overall, GARP proteins appear to be involved in plant-specific processes. Although their amino acid sequences are distantly related to those of the authentic mammalian Myb repeats, little is known about the structure and function of the GARP motif.

In summary, we have described the identification of IPN2 as an interacting partner of NSP2 in *L. japonicus*. IPN2 is a MYB coiled-coil type transcription factor belonging to the GARP protein family, while NSP2 is a GRAS family transcription regulator. The two proteins may form a transcription factor complex to promote the expression of a specific set of genes essential for nodule development. This work has advanced the understanding of the transcriptional regulatory networks essential to root nodule development.

Acknowledgements

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Screens of NSP2-interaction proteins.

Fig. S2 Structural features of the IPN2 protein.

Fig. S3 Growth phenotypes of *IPN2*-RNAi roots under nonsymbiotic conditions.

Fig. S4 Mycorrhization phenotypes of *IPN2* RNAi roots.

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