and carries a carbamoyl group. The reducing terminal residue is substituted with 4-O-acetylfucose. This is consistent with published *M. loti* NF structure for R7A[24]. A derivative in which the terminal residue is substituted with fucose was identified as a minor constituent. At set times after inoculation, root tips (~3 mm) were discarded and susceptible zones (3–4 cm) of 14 roots each were collected in liquid nitrogen and used for RNA preparation. For root hair observations, seedlings were inoculated with *M. loti* four days after germination. To visualize infection threads, roots were inoculated with a *M. loti* R7A derivative carrying pXLGD4 constitutively expressing a *lacZ* reporter gene[11]. Entire root systems of at least 10 seedlings were inspected per allele, 14 and 21 d post inoculation, after staining for β-galactosidase activity.

RT–PCR

To compare cDNA concentrations in different samples, fragments of a *L. japonicus* polyubiquitin cDNA (GenBank accession number AW719307) were PCR-amplified (small fragment 227 bp) with primers fwd P40079 CTACAACATTCAGAAGGAGTCCA and rev P40080 CACAGGCCAGAAGAGGCCACAACA (22 cycles: 94 °C for 15 s, 58 °C for 30 s, 72 °C for 50 s). Amplification was exponential up to 25 cycles. A 257-bp fragment of a leghaemoglobin cDNA (AB042718), was PCR-amplified using primers fwd P40183 GTTCTACACCGTTATATTGGAGATAG and rev P41008 GCAGGGCTTCTTTAACCACCACG (both spanning exon/intron borders) (40 cycles: 94 °C for 15 s, 59 °C for 30 s, 72 °C for 40 s). For (RT)-PCR of *Lotus SYMRK* (Fig. 3e) primers fwd P60243 TGCAGTGAGATCATCCAGGCTC and rev P60244 TCTAAGTTGGTCATCTCAGCAATGCTGG were used. (35 cycles: 94 °C for 15 s, 65 °C for 30 s, 72 °C for 60 s). PCR fragments were analysed by agarose gel electrophoresis and sequencing.

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Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to M.P. (e-mail: martin.parniske@bbsrc.ac.uk). Sequences are deposited at GenBank with the following accession numbers. *Lotus SYMRK*, AF492655; pea *SYM19*, AF491997; *M. truncatula SYMRK*, AF491998. TAC clones: *Lotus* T11E23 (containing the *Lotus SYMRK* gene), AP004579; *Lotus* T01N11, AP004576; *Lotus* T10N22, AP004577; *Lotus* T17H01, AP004578.

A receptor kinase gene regulating symbiotic nodule development

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Leguminous plants are able to establish a nitrogen-fixing symbiosis with soil bacteria generally known as rhizobia. Metabolites exuded by the plant root activate the production of a rhizobial signal molecule, the Nod factor, which is essential for symbiotic nodule development[1,2]. This lipo-chitooligosaccharide signal is active at femtomolar concentrations, and its structure is correlated with host specificity of symbiosis[3], suggesting the involvement of a cognate perception system in the plant host. Here we describe the cloning of a gene from *Medicago sativa* that is essential for Nod-factor perception in alfalfa, and by genetic analogy, in the related legumes *Medicago truncatula* and *Pisum sativum*. The identified 'nodulation receptor kinase', NORK, is predicted to function in the Nod-factor perception/transduction system (the NORK system) that initiates a signal cascade leading to nodulation. The family of 'NORK extracellular-sequence-like' (*NSL*) genes is broadly distributed in the plant kingdom, although their biological function has not been previously ascribed. We suggest that during the evolution of symbiosis an ancestral NSL system was co-opted for transduction of an external ligand, the rhizobial Nod factor, leading to development of the symbiotic root nodule.

The tetraploid alfalfa non-nodulation mutant MN-1008 (ref. 4) is known to lack all symbiotic responses upon inoculation with compatible *Sinorhizobium meliloti* or treatment with the corresponding cognate Nod factor[5–7]. But spontaneous nodulation in the absence of *Sinorhizobium*, a Nod-factor-independent phenotype reported so far only on alfalfa[8], could be detected in the mutant[9]. Consequently, the perception of the extrinsic signal was abolished in MN-1008, but the capacity for nodule initiation and development, as an internal developmental programme of the plant, remained intact. Like several other non-nodulation mutants, MN-1008 is also resistant to colonization by vesicular-arbuscular mycorrhizal fungi (the Myc$^-$ phenotype)[10]. This genetic intersection between mycorrhizal and rhizobial associations has attracted significant interest because it suggests that symbiotic nitrogen fixation may be derived, in part, from the more ancient and widely distributed mycorrhizal symbiosis[11]. Thus, determining the molecular basis of mutations

such as in MN-1008 is central to understanding two of the most important symbiotic associations of plants, and their genetic overlap in legumes.

Map-based cloning of the genetic determinant responsible for the non-nodulation trait of *M. sativa* MN-1008 was initiated by mapping the Nod$^-$ phenotype in a tetraploid segregating population of alfalfa[12]. As this study demonstrated a high level of synteny between the *M. sativa* and *M. truncatula* genomes, a bacterial artificial chromosome (BAC) library of *M. truncatula*[13] was used to construct a contig spanning the mutation. Two tightly linked restriction-fragment length polymorphism (RFLP) markers[12], SHMT and CAD, were used to isolate primary BAC clones, and a 600-kilobase (600-kb) contig (the 'Nod contig') was assembled based on a combination of chromosome walking from BAC end sequences and restriction endonuclease fingerprinting of the clones. Genetic markers internal to the Nod contig were used to position the Nod$^-$ mutation within a fine-structure linkage map (Fig. 1a). Genotypes were scored in a segregating tetraploid *M. sativa* F_2 population (NAB population[12]) with the help of recombinant plants. This analysis revealed correspondence between the *M. truncatula* physical map and the genetic map positions in *M. sativa*. Moreover, two recombinant plants, NAB 4156 and 4443, served to delimit the MN-1008 non-nodulation mutation (*nn1*) to a ~160-kb interval (Fig. 1a, b). BAC clones containing the minimum tiling path of the Nod region (part of BAC 67A11, and the entirety of BACs 2D11 and 28I12; Fig. 1b) were subcloned and sequenced (data available at ⟨http://www.szbk.u-szeged.hu/ ~ alfi/⟩). A total of 15 open reading frames were identified and putative functions were assigned based on sequence homologies (see Fig. 1b and Supplementary Information). The predicted gene content of the Nod contig shared limited microsynteny with regions of the *Arabidopsis thaliana* genome (Fig. 1c), but functional information for the syntenic counterparts in *Arabidopsis* did not enhance our ability to identify candidate genes.

The non-nodulation phenotype of MN-1008 could be explained by an alteration in several candidate genes in this region, including genes predicted to code for an ATP-binding cassette (ABC) transporter, a receptor kinase, a MADS-box protein and lectin proteins. We first chose the ABC transporter and receptor kinase, membrane-spanning proteins to analyse for genetic alterations by comparing the sequence of alleles from wild-type nodulating alfalfa and the mutant MN-1008 plant material. To reduce confusion from different F_2 alleles, F_3 individuals that were homozygous throughout the Nod contig region were identified and used for sequencing. Based on the *M. truncatula* BAC sequences, exon-specific primers were designed to amplify both genomic and complementary DNA templates. At least three clones from independent amplifications of overlapping portions of both genes were analysed from the wild-type and mutant backgrounds of *M. sativa*. Based on this analysis, only synonymous changes were detected in the ABC transporter coding genes, whereas an unambiguous mutation, an 'in frame' stop codon, was identified in the receptor kinase gene from the mutant. This mutation was found consistently in independent amplification products from both genomic and cDNA templates of MN-1008 mutant. We have named this receptor kinase gene 'nodulation receptor kinase', *NORK*.

In addition to the analyses of *Medicago* species, we sequenced the candidate *NORK* gene from related legume species *Melilotus alba*, *P. sativum*, *Vicia hirsuta* and *Lotus japonicus*, and in each case the deduced proteins exhibited high overall homology. As shown in Fig.

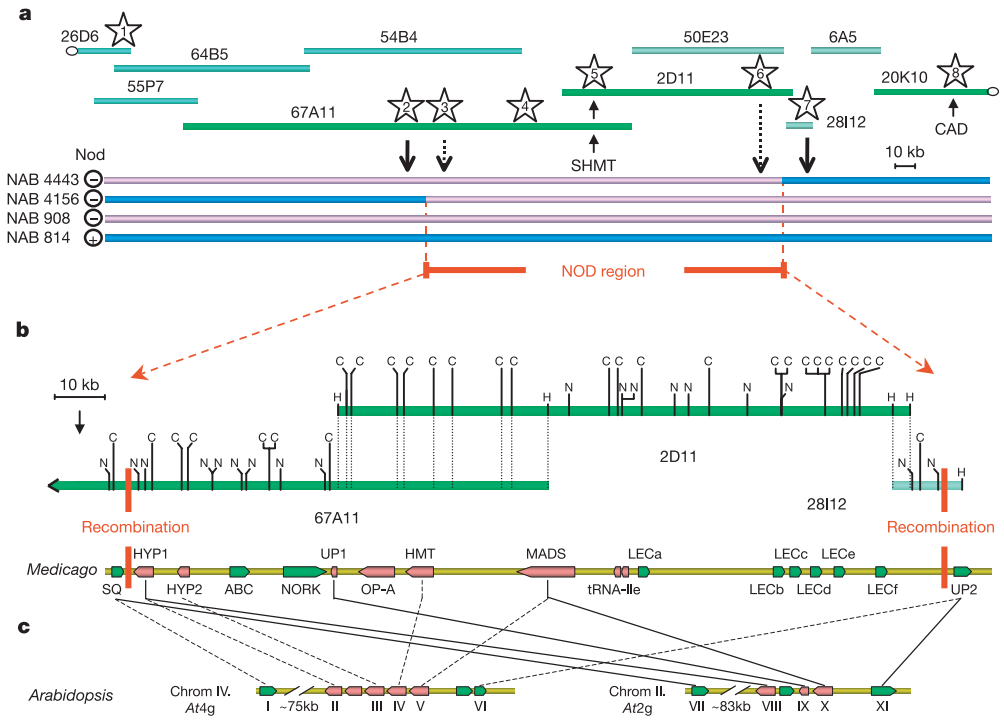

Figure 1 Genetic and physical map around the Nod locus. **a**, Primary BAC clones (67A11, 2D11 and 20K10) are coloured dark green, overlapping BAC clones (26D6, 55P7, 64B5, 54B4, 50E23, 28I12 and 6A5) are coloured light green. Fragments of the BAC clones (indicated as numbers in stars) were used for fine mapping. The Nod$^-$ and one Nod$^+$ genotypes are shown as pink and blue bars, respectively. **b**, Physical map and the gene content of the sequenced region. Restriction cut sites for *Cla*I (C) and *Nhe*I (N), and the *Hin*dIII (H) ends of the overlapping BACs are shown in the upper portion. Orientations of the open reading frames are indicated with green and red colours. **c**, Comparison of gene content with *A. thaliana* chromosomes 2 and 4 showing similarities to the sequenced region of *M. truncatula* I, *At*4g37760; II, *At*4g37900; III, *At*4g37920; IV, *At*4g37930; V, *At*4g37940; VI, *At*4g37960; VII, *At*2g22830; VIII, *At*2g22660; IX, *At*2g22640; X, *At*2g22630; XI, *At*2g22620.

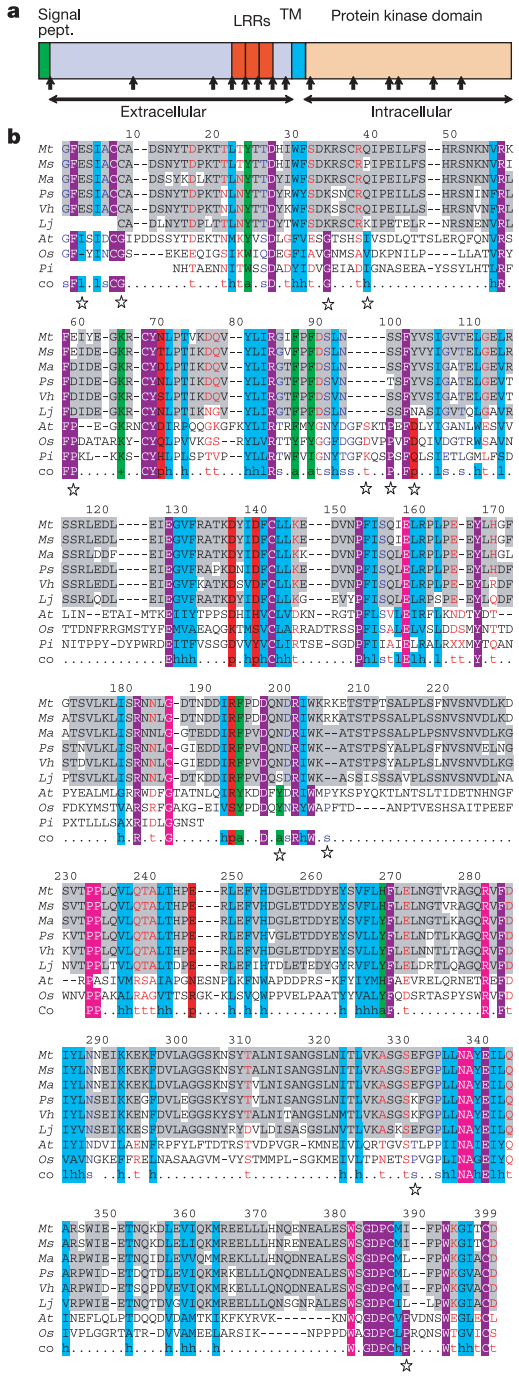

Figure 2 The NORK proteins. **a**, Schematic diagram of the NORK protein. The different domains are predicted on the basis of the deduced AA sequence. Vertical arrows indicate the location of the introns in the genomic DNA sequence. LRR, leucine-rich repeat; TM, transmembrane. **b**, Multiple alignment of the extracellular part of legume NORKs and three representatives of non-legume NSL proteins. *Mt*, *M. truncatula*; *Ms*, *M. sativa*; *Ma*, *Melilotus alba*; *Ps*, *Pisum sativum*; *Vh*, *Vicia hirsuta*; *Lj*, *Lotus japonicus*; *At*, *A. thaliana* LRRPK (accession number X97774); *Os*, *Oryza sativa* (accession number AAK92623); *Pi*, *Pinus taeda* (accession number BE758674); co, consensus. AA residues are coloured according to an 80% consensus (calculation included more NSL sequences, see Supplementary Information): hydrophobic (h: ACFGHIKLMRTVWY) and their aliphatic subset (l: ILV) with turquoise shading; aromatic (a: FHWY) and positive (+: HKR) with green shading; polar (p: CDEHKNQRST) with red shading; turn-like (t: ACDEGHKNQRST) in red; small (s: ACDGNPSTV) in blue. Dark violet background with white letters shows the position of 100% conserved AA, whereas pink background features the 80% consensus AAs. Grey background highlights conservation within legume NORKs, stars indicate the positions where AAs of the legume domain differ from the consensus of other NSL sequences.

2a, the predicted proteins possess an amino-terminal signal peptide motif, a putative extracellular domain containing three leucine-rich repeat (LRR) motifs[14], as well as a transmembrane segment and an intracellular domain with typical serine/threonine protein kinase signatures[15,16]. Several putative phosphorylation sites (see Supplementary Information) suggest a possible role also for (auto)phosphorylation in the protein's function. The predicted LRR motifs may indicate interaction with other protein(s)[17]. Although the N-terminal extracellular domain is well conserved among the legume proteins (Fig. 2b), this stretch, roughly 400 amino acids (AA) long, did not show homology to any known domain.

To further substantiate that in fact the mutation in the *NORK* gene was responsible for the non-nodulation phenotype, a similar sequencing strategy was undertaken on different Nod$^-$ mutants. In *M. truncatula* and *P. sativum*, mutagenesis programmes have yielded non-nodulation mutants with phenotypes and syntenic map positions that are suggestive of orthology with the alfalfa *nn1* gene[18,19]. Each sequenced *NORK* gene from the Nod$^-$ alleles of *M. truncatula* and *P. sativum* contained single base-pair alterations that are predicted to abolish NORK function. The nucleotide changes and their possible effects are shown in Table 1. Among the seven mutant alleles of *NORK*, six resulted in a Nod$^-$/Myc$^-$ phenotype. It is interesting to note that a non-synonymous substitution (see Table 1) in the mutant *M. truncatula* R38 resulted in Nod$^-$/Myc$^+$ phenotype. This suggests a composite role for NORK, branching the signal towards nodule formation and mycorrhiza colonization. The identification of molecular lesions in seven alleles, obtained from independent non-nodulation mutants in three legume species, provides strong circumstantial evidence of a role for NORK in nodulation and mycorrhizal association.

To directly test this hypothesis, we analysed the ability of the wild-type *NORK* gene of *M. truncatula* to complement the non-nodulation mutant, TR25, following transformation by *Agrobacterium rhizogenes*[20]. Roots appearing on the seedlings of the TR25 Nod$^-$ mutants after *Agrobacterium* infection were tested for nodulation by inoculating with *S. meliloti*. Plants transformed by *A. rhizogenes* carrying an empty vector control tested positive for β-glucuronidase (GUS) staining of transgenic roots, but were unable to form nodules. By contrast, TR25 plants infected by *A. rhizogenes* carrying the *NORK* gene on the same plasmid tested positive for GUS staining and developed nodules containing bacteroids (see Supplementary Information). The combination of the positive complementation tests and the genetic evidence provided by sequencing of multiple independent non-nodulation mutants, suggest that NORK is an essential component of the host plant nodulation signalling pathway.

To determine the range of legume and non-legume species in which the NORK protein is conserved, we used the DNA region coding for the postulated extracellular domain of NORK as a hybridization probe on Southern blots of total genomic DNA. Specific hybridizing bands could be detected (Fig. 3) in each of the legume genera tested (*Sesbania*, *Cassia*, *Trifolium*, *Desmodium*, *Vicia*, *Melilotus*, *Vigna*, *Macroptilium*, *Lotus*, *Glycine*, *Pisum*, *Phaseolus*, *Medicago*). Homology in *Cassia emerginata* is particularly interesting because this Caesalpinoid legume species lacks symbiotic nodule formation[21]. Genetic mapping of the *NORK* gene in *Medicago* demonstrated only one segregating locus (data not shown). Similarly, in most other legume species that we tested, only one strong hybridizing band was detected, suggesting the presence of a single copy gene. In a few cases, however, more than one equally hybridizing band could be detected, indicating either a single copy gene with an internal restriction site, or the presence of a small *NORK* gene family. Representative members of non-legume plants (maize, tobacco, wheat, rice and *Arabidopsis*) did not show a specific hybridization signal, indicating that high homology to *NORK* is restricted primarily to legumes.

Despite the absence of high nucleotide similarity outside of

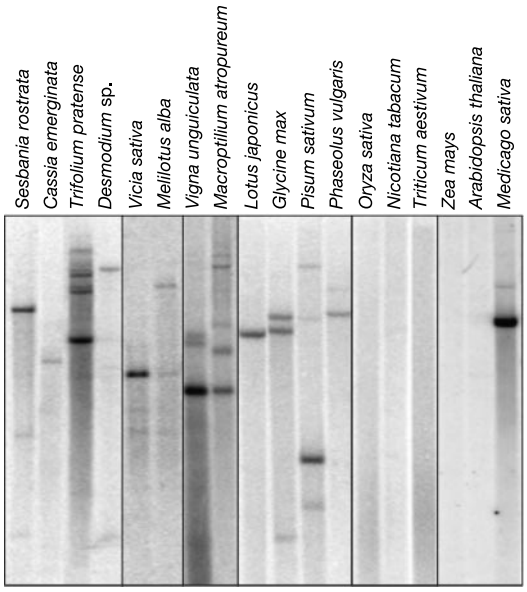

Figure 3 Genomic hybridization with the *NORK* gene to different legume and non-legume plants. Total DNA isolated from young leaf tissue of the plants highlighted were digested by *Eco*RI (except that *Cassia emerginata* DNA was digested by *Hin*dIII), at equivalent amounts. A 1,700-bp PCR fragment containing only the putative extracellular part of the protein was used as the *NORK*-specific probe.

legumes revealed by DNA hybridization, BLASTX searches of the NORK unique extracellular domain identified many homologous sequences from other plant species at the protein level. We refer to these homologues as NSL (NORK extracellular sequence-like) sequences to distinguish them from other non-related receptor kinase homologues. The multiple alignment of these NSL sequences revealed a number of conserved AA residues throughout this domain (Fig. 2b). The presence of about 50 different genomic NSL sequences in the *Arabidopsis* genome indicates the existence of a large gene family containing the NSL motif. Almost all of these *Arabidopsis NSL* genes also have predicted LRR, transmembrane and intracellular kinase domains (NSL-RK). These receptor kinases were recently classified into the LRRI-type category on the basis of their kinase sequences[22]. In addition to genomic sequences, many of the *Arabidopsis* homologues are represented as expressed sequences in GenBank, suggesting that they are likely to code for functional proteins. Besides information on tissue specific expression of two genes[23,24], no mutation/phenotype for any of these NSL sequences has been described. One full-length *Arabidopsis* complementary DNA sequence (AY059761) is predicted to code for a protein containing only the NSL domain. This example suggests that NSL proteins lacking the LRR, transmembrane and intracellular kinase domains may also have biological function.

NSL genes were found (based on either genomic or expressed sequence tag (EST) sequences) not only in other dicotyledonous plants, but also in monocotyledonous plants such as rice, maize and wheat, and a homologue was also identified in Gymnosperms (see Fig. 2b and Supplementary Information). Owing to the limited number of sequences available from these organisms (in some cases only partial cDNA sequences were available), the number of *NSL* genes in these species could not be estimated. On the basis of BLASTX analysis, *NSL* homologues were not identified in animals, lower eukaryotes or prokaryotes, but we can not exclude the possibility that more highly diverged members of the NSL family might also be present in these non-plant genomes.

The mechanism(s) by which bacterial Nod factors are recognized by legume plants has remained obscure. If NORK proteins interact directly with Nod factors, then differences in the AA sequence of NORK extracellular domain from different legumes might reflect specificity for Nod-factor structure. It is also possible that Nod factors are bound by another protein, and that events occurring after ligand binding require the activity of NORK. In fact, inter-

Table 1 Legume *NORK* genes sequenced with description of the mutations

WT NORK sequences	Mutant NORK sequences	Mutant phenotype	Nature of mutation
Medicago sativa	MN-1008 (*nn1*)	Nod$^-$ Myc$^-$	Nonsense mutation, premature translational termination before the kinase active site
Medicago truncatula A17	TR25, TR26 (*dmi2* alleles of *Mt* A17)	Nod$^-$ Myc$^-$	Frame shift due to one basepair deletion, premature translational termination in the extracellular domain
Medicago truncatula A17	P1 (*dmi2* allele of *Mt* A17)	Nod$^-$ Myc$^-$	G to A transition in the splice consensus site of intron 5 caused the loss of exon 6 from the mRNA
Medicago truncatula A17	R38* (*dmi2* allele of *Mt* A17)	Nod$^-$ Myc$^+$	Conserved Gly changed to Glu in the kinase subdomain X which might interact with the substrate[16]
Pisum sativum cv. Frisson	P4 (*sym19* allele of *Ps* cv. Frisson)	Nod$^-$ Myc$^-$	Strictly conserved Gly residue of the ATP-binding domain[16] changed to Glu
Pisum sativum cv. Frisson	P55 (*sym19* allele of *Ps* cv. Frisson)	Nod$^-$ Myc$^-$	Asp of protein kinase subdomain VII[16]—involved in the binding of the ATP-chelating metal—changed to Asn

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action of NORK with other proteins is suggested by the presence of LRR motifs and thus the extracellular domain may be involved in protein–protein interaction. On the basis of pharmacological studies, a G-protein coupled receptor-like protein has been suggested as a candidate for the Nod-factor receptor[25]. Other candidate gene(s) for the Nod-factor receptor(s) include *DMI1* from *M. truncatula*[18,26], *SYM1* or *SYM5* from *L. japonicus*[27], and *SYM8* or *SYM10* from *P. sativum*[28], on the basis of their mutant phenotype. The map-based cloning of these genes (which is in progress) and their analysis will further elucidate the molecular bases of Nod-factor perception. The current study suggests that NORK is an integral component of the signalling pathway, in which the coordinated interaction of the components is needed for Nod-factor signal perception and transduction.

The *NSL* gene family—identified with the help of NORK extracellular domain—is broadly distributed in the plant kingdom. The AA conservation detected in this protein family suggests a similar molecular mechanism in their function. Thus NORK homologues may function in the perception and transduction systems for extracellular ligands in plants. We suggest that during the evolution of symbiosis an ancient NSL system was recruited by an external ligand, the bacterial Nod factors, leading to the developmental regulation of a new plant organ, the symbiotic nodule. □

Methods

Plant materials

M. sativa plants used for studies are individuals of a tetraploid mapping population described earlier[12]. Wild-type and mutant seeds of *M. truncatula* A17 and *P. sativum* cv. Frisson were obtained from D. Cook (*M. truncatula* A17), J. Denarie (*M. truncatula* P1), G. Duc (*M. truncatula* TR25 and TR26, *P. sativum* cv Frisson, mutants P4 and P55) and K. VandenBosch (*M. truncatula* R38). Plants were grown as described previously[12].

Nucleic acid isolation and analysis

Genomic DNA isolation, restriction enzyme digestion, Southern blot, DNA hybridization (RFLP analysis) and polymerase chain reaction (PCR) amplification were carried out as described previously[12]. BAC clone identification by hybridization and BAC DNA isolation were performed according to original descriptions[13]. BAC clones were subcloned after fragmentation by restriction enzyme digestion or random fragmentation by sonication into pUC19. Total RNA from roots was extracted using the High Pure RNA isolation kit (Roche). First strand cDNA was synthesized with the help of the RevertAid H Minus First Strand cDNA Synthesis Kit of MBI Fermentas. PCR amplifications of the overlapping fragments of the *NORK* gene from genomic and cDNA templates were set up in triplicate and each PCR fragment was cloned independently into pGEM-T-Easy vector (Promega). DNA sequencing was carried out on an ABI377 automatic sequencer using fluorescent dye terminators.

A. rhizogenes transformation

The *A. rhizogenes* hairy root transformation system recently developed for *M. truncatula* was used[20]. The wild-type *M. truncatula NORK* gene with its own promoter sequence (more than 700 base pairs (bp) upstream from the proposed translation start site) was cloned on an 8.5-kb *Nhe*I fragment into the modified pPR97 plant transformation vector[29] carrying a constitutively expressed *uid*A gene (clone pBRC1720). *A. rhizogenes* carrying the pBRC1720 clone or empty pPR97 vector (negative control) were used to transform seedlings of the *M. truncatula* TR25 Nod$^-$ mutants. Experiments were repeated twice. In total, 25 GUS-positive roots developed on 10 treated plants, on which 20 nodules appeared that contained bacteroids. In control plants GUS-positive roots also developed but no nodule formation was detected.

Computational analyses

BLAST searches were conducted against the non-redundant and EST databases at NCBI (www.ncbi.nlm.nih.gov/BLAST). Multiple alignment of the NSL sequences was constructed using the ClustalW program[30]. Protein domain predictions were carried out at www.smart.embl-heidelberg.de and www.ch.embnet.org. Consensus sequence was calculated from the multiple alignment at www.bork.embl-heidelberg.de/Alignment/consensus.html.

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Competing interests statement

The authors declare that they have no competing financial interests.

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