The Pea *Sym37* Receptor Kinase Gene Controls Infection-Thread Initiation and Nodule Development

Vladimir Zhukov,¹ Simona Radutoiu,² Lene H. Madsen,² Tamara Rychagova,¹ Evgenia Ovchinnikova,¹ Alex Borisov,¹ Igor Tikhonovich,¹ and Jens Stougaard²

¹Laboratory of Genetics of Plant-Microbe Interactions, All-Russia Research Institute for Agricultural Microbiology, Podbelsky chausse 3, 196608 Saint-Petersburg-Pushkin, Russia; ²Centre for Carbohydrate Recognition and Signalling, Department of Molecular Biology, University of Aarhus, Gustav Wieds vej 10, 8000 Aarhus C, Denmark

Submitted 18 July 2008. Accepted 25 August 2008.

Phenotypic characterization of pea symbiotic mutants has provided a detailed description of the symbiosis with *Rhizobium leguminosarum* bv. *viciae* strains. We show here that two allelic non-nodulating pea mutants, *RisNod4* and *K24*, are affected in the *PsSym37* gene, encoding a LysM receptor kinase similar to *Lotus japonicus* NFR1 and *Medicago truncatula* LYK3. Phenotypic analysis of *RisNod4* and *K24* suggests a role for the *SYM37* in regulation of infection-thread initiation and nodule development from cortical-cell division foci. We show that *RisNod4* plants carrying an L to F substitution in the LysM1 domain display a restrictive symbiotic phenotype comparable to the *PsSym2*^A lines that distinguish ‘European’ and ‘Middle East’ *Rhizobium leguminosarum* bv. *viciae* strains. *RisNod4* mutants develop nodules only in the presence of a ‘Middle East’ *Rhizobium* strain producing *O*-acetylated Nod factors indicating the *SYM37* involvement in Nod-factor recognition. Along with the *PsSym37*, a homologous LysM receptor kinase gene, *PsK1*, was isolated and characterized. We show that *PsK1* and *PsSym37* are genetically linked to each other and to the *PsSym2* locus. Allelic complementation analyses and sequencing of the extracellular regions of *PsSym37* and *PsK1* in several ‘European’ and ‘Afghan’ pea cultivars point towards *PsK1* as possible candidate for the elusive *PsSym2* gene.

Adapting to diverse habitats, living organisms have evolved a wide range of strategies for acquiring their nutrients. A restricted group of plants, most of them belonging to the family *Leguminosae*, evolved the ability to obtain atmospheric dinitrogen in symbiosis with soil bacteria collectively called rhizobia. This symbiosis is based on mutual partner recognition in which bacteria are attracted and stimulated by plant-exuded (iso)flavonoids to synthesize signal molecules known as Nod factors (lipochitin-oligosaccharide molecules). In turn, Nod factors secreted from the bacteria induce development of specialized nitrogen-fixing organs, the nodules, in the legume root (Oldroyd and Downie 2008). Upon Nod-factor recognition and perception, the legume root hairs undergo a series of specific

morphological changes in order to entrap bacteria, form infection threads, and allow successful invasion. Infection threads are plant-derived structures acting as conduits from which bacteria will be endocytosed into nodule cells in which they, contained in symbiosomes, differentiate into nitrogen-fixing bacteroids and provide reduced nitrogen to the plant host under nitrogen-limiting soil conditions.

The relationship between legumes, *Rhizobium* spp. and allied bacteria, is selective. Different rhizobial species show various degrees of host specificity towards their eukaryotic symbiotic partner and vice versa. The host plant has a tight control over the nodule formation and rhizobial infection processes, and mutants as well as natural variants impaired in their ability to bring the symbiotic process to completion were isolated in several legume species (Borisov et al. 2007; Stougaard 2001).

A naturally occurring diversity in strain specificity has been described in *Pisum sativum*, in which two alleles of the *PsSym2* gene differing in their geographic origin determine the outcome of the interaction with *Rhizobium leguminosarum* bv. *viciae* strains. European pea varieties carrying the *PsSym2*^C allele and Afghan varieties carrying the *PsSym2*^A are both able to recognize and form functional nodules with *R. leguminosarum* bv. *viciae* strains from Middle East soils, which produce Nod factors that are *O*-acetylated at the reducing end (Firmin et al. 1993). The rhizobial *nodX* gene encoding the acetyl transferase protein that adds this specific decoration is absent from the genome of most European *R. leguminosarum* bv. *viciae* strains (Kozik et al. 1995; Olsthoorn et al. 2000). As a consequence, these rhizobial strains lack the *O*-acetyl decoration on their Nod factors and induce functional nodules only on the European pea varieties (Kozik et al. 1995). The *PsSym2*^A allele confers a developmentally arrested and defective symbiosis on Afghan peas inoculated with European strains (Hogg et al. 2002). Moreover, it was shown that the type of Nod-factor decoration at the reducing end was not restrictive, since *R. leguminosarum* bv. *viciae* strains producing either fucosylated or acetylated Nod-factor molecules had a similar efficiency in nodulation and nitrogen fixation on Afghan pea hosts (Ovtsyna et al. 1998).

Genetic analyses and phenotypic characterization of plant mutants allowed dissection of the early events that take place in the legume-rhizobium symbiosis (Stougaard 2001). These first molecular studies performed on the model legumes *Lotus japonicus* and *Medicago truncatula* led to identification of genes involved in the early Nod-factor signal perception and transduction pathways (Oldroyd and Downie 2008). LysM receptor kinases were found to be required for Nod-factor perception (Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al.

Corresponding author: Jens Stougaard; Telephone: +8942 5011; E-mail: stougaard@mb.au.dk

Nucleotide sequence data have been deposited in the GenBank database under accession numbers EU564087 to EU564102.

*The e-Xtra logo stands for “electronic extra” and indicates that four supplemental figures and two supplemental tables are published online.

2003, 2007; Smit et al. 2007), while a leucine-rich repeat receptor-like kinase (Stracke et al. 2002) and cation transporters (Imaizumi-Anraku et al. 2005) were found essential for signal perception and transduction. Nuclear pore components are involved in integrating the signal from the cytoplasm into the nucleus (Kanamori et al. 2006; Saito et al. 2007), and a secondary signal in the form of calcium spikes was suggested to be interpreted by a plant protein, CCaMK (Levy et al. 2004; Mitra et al. 2004; Tirichine et al. 2006). Downstream of the signal transduction cascade the putative transcriptional regulators NSP1 (Heckmann et al. 2006), NSP2 (Kalo et al. 2005; Murakami et al. 2006), NIN (Schäuser et al. 1999), and ERN (Middleton et al. 2007) are essential for inducing the specific gene activation required for nodule organogenesis and bacterial infection.

This knowledge of legume-rhizobium symbiosis was mainly accumulated in model legumes, which are of limited importance as agricultural crops. Large genome sizes and reduced availability of molecular tools have hampered molecular genetics studies of the economically important crop legumes. However, the availability of large symbiotic mutant collections, coupled with extensive phenotypic analyses and detailed genomic maps has promoted the use of comparative genomics between legume species (Cronk et al. 2006; Hougaard et al. 2008). Identification of symbiotic genes in agronomically important legume species like pea and soybean (Borisov et al. 2007) was achieved using this approach.

In pea, seven symbiotic genes have been, so far, identified and characterized taking advantage of the 20 symbiotic loci represented by over 70 lines displaying a nodulation deficient phenotype (*Nod*⁻, *Nod*^{+/-}) (Borisov et al. 2007). Among them is *PsSym10* the ortholog of *LjNfr5* (*L. japonicus* Nod-factor receptor5), one of the two genes known to be involved in Nod-factor perception and host determination (Madsen et al. 2003, Radutoiu et al. 2007), while the identity of the *LjNfr1* (*L. japonicus* Nod-factor receptor1) ortholog in pea is unknown.

We present here the isolation of two genes highly similar to the *LjNfr1* and show that non-nodulating *PsSym37* pea mutants are impaired in one of the newly identified receptors. Mutant phenotypic analysis suggests a role for the SYM37 receptor kinase in the Nod-factor perception required to initiate infection-thread growth.

RESULTS

Two closely related LysM receptor kinases are expressed in pea roots.

In order to identify one or more pea genes serving a function similar to the *L. japonicus* *Nfr1* Nod-factor receptor gene, a probe derived from the *LjNfr1* genomic region encoding the extracellular LysM domains was used to screen a pea root cDNA library. The screening resulted in identification of two types of cDNAs, *PsK1* and *PsK10*. Subsequent sequencing of the clones revealed that they correspond to transcriptional products originating from two different but homologous genes. Using cDNA-derived primers the genomic sequences of the two genes in cv. Finale were amplified and sequenced and the exon-intron structures were defined. Alignment of the *PsK10* cDNA with the genomic sequence revealed a gene structure similar to *LjNfr1*, 12 exons and 11 introns (Fig. 1A). In addition, two different versions of the *PsK10* cDNA were identified that match the alternative splicing of exon 4 previously found in *LjNfr1* (Fig. 1A) (Radutoiu et al. 2003). Alignment of the *PsK1* cDNA and the genomic sequence revealed a gene structure containing 11 exons and 10 introns, and evidence for alternative splicing of exon 4 was obtained upon sequencing of *PsK1* transcripts (Fig. 1B). The conceptual proteins encoded

by the *PsK10* and *PsK1* genes are similar and, in cv. Finale, show 86% identity. The kinase domains are 95% identical, while the predicted extracellular regions are quite diverse (75% identity) (Fig. S1). Both have similar levels of identity when compared with *LjNFR1*, 78% for *PsK1* and 76% for *PsK10*, or to *MtLYK3* (LysM domain-containing receptor-like kinase) (84% for both), making them candidates for the *L. japonicus* *Nfr1* ortholog in pea. However, conservation of the gene structure favors *PsK10* as a better candidate. Interestingly, the additional amino acids (GN) that result from the alternative splicing of exon 4 are conserved in pea and *Lotus japonicus*. A gene structure similar to *PsK1* with 11 exons and 10 introns was described for the *MtLyk6* gene (AY372404) (Limpens et al. 2003); however, the level of conservation between *MtLYK6* and *PsK1* (55% identity) is relatively low. When compared with other LysM receptor kinases (Zhang et al. 2007), both *PsK1* and *PsK10* group together with *LjNFR1* and *MtLYK3* (Fig. S2).

PsK1 and *PsK10* are closely linked on pea linkage group I.

The *LjNfr1* locus was positioned on the upper part of linkage group I in *L. japonicus* (Sandal et al. 2002; Radutoiu et al. 2003), and comparative genomics showed this region to be highly syntenic to the lower part of linkage group V in *M. truncatula* and to the upper part of linkage group I in pea (Sandal et

Fig. 1. A, *PsSym37* (*PsK10*) and B, *PsK1* gene structures. The exon (boxes) and intron (line) size in nucleotides are presented in Finale/Rondo background. The numbers in brackets show the alternative splicing forms of exon and intron 4 in *PsSym37*. Mutations in *RisNod4* and *K24* alleles are presented inside the text boxes, and the location of the amplification fragment length polymorphism marker *W62-1* is shown (open rectangle). The predicted conserved LysM domains (dark gray), the transmembrane regions (light gray), and the kinase domains (black) are shown. C, *PsSym37* and *PsK1* locations on linkage group I shown as genetic distances in Kosambi units (cM) to other symbiotic loci (*sym35* and *Enod40*) and to the morphological marker *d* (regulation of axil pigmentation patterning).

al. 2006). In *M. truncatula*, six LysM receptor kinases are located in this region, and mutations in one of them, *MtLyk3*, leads to an altered symbiotic phenotype (Gualtieri et al. 2002). In pea, the *Sym2* locus has been mapped to this syntenic region of the genome (Gualtieri et al. 2002). In addition, segregation analyses of different markers and the symbiotic phenotype revealed that the *Pssym37* mutation is located in the same region (Fig. 1C) (Borisov et al. 2007). Since *PsK1* and *PsK10* are homologs of

LjNfr1 and *MtLyk3*, they emerged as plausible candidates for either the *PsSym37* or *PsSym2* genes, or both. In order to test this hypothesis, *PsK1* and *PsK10* gene-specific cleaved amplified polymorphic sequence (CAPS) or derived (d)CAPS markers were designed and were used for segregation analyses in a *PsSym37* mapping population (Table S1). A total of 235 F_2 plants (59 mutants) were analyzed, and no recombination was detected between the mutant phenotype and the *PsK1* or *PsK10* genes. Moreover, the *PsW62-1*, a restriction fragment length polymorphism marker previously identified to be tightly linked to the *PsSym2* locus was found to be identical to a 304-nucleotide fragment (from 4,179 to 4,482 bp) located in the 3' region of the *PsK10* gene (Fig. 1A and C). Taken together, these results show that i) *PsSym37* and *PsSym2* are tightly linked, ii) *PsK1* and *PsK10* cosegregate, and iii) *PsK1* and *PsK10* are closely linked or identical to the previously identified symbiotic loci, *PsSym37* and *PsSym2*.

***PsK10* is the *PsSym37* gene.**

Two allelic non-nodulating symbiotic mutants obtained from independent chemical mutagenesis programs define the *PsSym37* locus (Borisov et al. 2007; Engvild 1987; Jacobsen 1984; Postma et al. 1988). The *RisNod4* allele was obtained in cv. Finale, while the K24 allele was isolated in cv. Rondo background. In order to investigate whether the two alleles carry a mutation in either of the *PsK1* or *PsK10* genes, the two corresponding genomic regions were amplified from both mutants and wild types and were sequenced. Comparison of the *Pssym37* mutant alleles to the corresponding *PsK10* wild-type gene sequences revealed the presence of single nucleotide substitutions in both *RisNod4* and K24. A transition (C to T) in position 229 (exon 1) of the *PsK10* coding region, leading to an amino acid substitution in the LysM1 domain (L₇₇ to F), was found in the *RisNod4* background, while a C to T transition in exon 11, leading to a premature stop codon (Q₅₃₉ to stop), was identified in the K24 kinase domain (Fig. 1A). No nucleotide difference was detected when sequences of the *PsK1* gene in the *Pssym37* mutant background was compared with the corresponding *PsK1* wild-type sequence. Both the cosegregation results and the molecular characterization of mutant alleles, therefore, support the identification of the *PsK10* gene as the previously genetically defined *PsSym37* locus and describe the mutational changes in the two allelic and non-nodulating *Pssym37* mutants.

Both *PsSym37* mutant alleles are impaired in infection-thread initiation.

Compared with the corresponding wild-type plants, both the *RisNod4* and K24 mutant lines display major nodulation defects and, under controlled glass house conditions, they are essentially unable to form visible nodules (non-nodulating) when inoculated with the European *R. leguminosarum* bv. *viciae* CIAM1026. However, under prolonged cultivation in pots, 80% of the mutant plants formed up to five functional nodules compared with the 20 to 30 nodules formed by wild-type plants. Detailed microscopical analyses showed that mutant and wild-type root hairs respond to the presence of the micro-symbiont in a similar fashion. Upon rhizobial inoculation, root-hair tip regrowth and curling takes place (Fig. 2A to C) and an increased number of the mutant root hairs undergo these typical deformations ($9.9\% \pm 0.7$ in *RisNod4*) when compared with wild-type ($4.5\% \pm 0.5$) (Tsyganov et al. 2002). Methylene blue-stained sections of the roots revealed that epidermal responses are accompanied by cortical changes in both mutants and wild type. Cell divisions are initiated in the inner cortical-cell layers showing that Nod-factor perception takes place (Fig. 2D). In spite of this initial recognition, the number

Fig. 2. **A**, Infection-thread formation in wild-type pea root hair (cv. Finale) in the presence of European strain *Rhizobium leguminosarum* bv. *viciae* VF39gusA. **B**, Root-hair curling and microcolony formation in K24 and **C**, *RisNod4* mutant lines in the presence of *R. leguminosarum* bv. *viciae* VF39gusA. The inset is a closeup of a microcolony entrapped in a *RisNod4* root hair. **D**, Cortical cell divisions (*) induced by European *R. leguminosarum* bv. *viciae* CIAM 1026 in the K24 mutant line. **E**, Number of nodules formed on European, Afghan, and *sym37* mutant lines in the presence of *R. leguminosarum* bv. *viciae* CIAM1026 (gray bars) and strain A1 (white bars). Five to ten plants were analyzed for each genotype-rhizobia combination. Error bars represent the 95% confidence intervals. Scale bars are 0.1 mm in A to C and 0.2 mm in D.

of cortical-cell division foci initiated on the *RisNod4* mutant roots at 15 days postinoculation was 10-fold lower (2.2 ± 1.1) as compared with the wild type (32.2 ± 3.8) and failed to develop into functional nodules (Tsyganov et al. 2002). Microscopical inspection of the mutant and wild-type roots inoculated with *gusA*-labeled European *R. leguminosarum* bv. *viciae* VF39 revealed that bacterial microcolonies are formed and are entrapped into the root-hair curls, but the infection process was highly impaired in the *sym37* mutant background (Fig. 2A to C). No infection threads were observed on the K24 roots (100 cm inspected from 10 plants), while on the *RisNod4*, very few infections were occasionally detected (0.4 ± 0.6 in 100 microscopic fields compared with 9.5 ± 6.6 in parent line Finale). These results show that the *PsSYM37* receptor kinase protein controls the infection process, ensuring a link between epidermal and cortical events upon rhizobial inoculation.

Like *nfr1* mutants in *L. japonicus*, the pea K24 mutant that has the most severe symbiotic phenotype has a normal phenotype in the symbiosis with arbuscular mycorrhiza (Yacobi et al. 1998). Based on these observations, we can conclude that the *PsSym37* gene is most likely important only for the legume-rhizobial symbiosis, supporting the notion that *LjNfr1* and *PsSym37* serve similar functions.

***PsK1* and *PsSym37* have distinct expression patterns.**

Morphological analyses performed on the *Pssym37* mutants showed that the plants were able to initiate the first responses upon rhizobial inoculation. Both root-hair curling, formation of infection pockets, and activation of cortical-cell division were observed. A functional *PsSym37* gene seems required for the progression of the process, indicating that the gene is transcribed in root, nodule primordia, or both. In order to gain more insights into the functions of *PsK1* and *PsSym37*, we monitored their steady-state expression pattern by real-time reverse transcription-polymerase chain reaction (RT-PCR) in different organs of wild-type pea plants. For comparison, we analyzed two other symbiotic genes, *PsSym10* (the *LjNfr5* homolog) and *PsSym35* (*PsNin*), known to be upregulated in pea after inoculation (Borisov et al. 2003; Madsen et al. 2003). All four genes were found to be significantly upregulated in mature nodules as compared with uninoculated roots (Fig. 3A). This suggests a requirement for increased levels of the four transcripts in wild-type functional nodules. Besides nodules, an increased level of *PsK1* transcript was detected in the stems.

In a timecourse experiment, rhizobial inoculation leads to a rapid transcriptional induction of *PsSym37*, *PsSym10*, and *PsSym35*, while the *PsK1* transcript was found to remain at the same level within the first 7 days after inoculation. These results suggest a requirement for higher transcript levels of the three symbiotic genes in the early phases of the symbiotic development (Fig. 3B), and a lower demand for transcriptional activation of the *PsK1* gene that might be required later on in nodulation (Fig. 3A and B).

In *L. japonicus* and *M. truncatula*, activation of the *Nin* gene is a prerequisite for the induction of cortical-cell divisions (Marsh et al. 2007; Tirichine et al. 2007). In order to determine whether the early responses detected in the *Pssym37* mutants and the Afghan cultivar NGB2150 inoculated with the European rhizobial strain are accompanied as well by specific gene activation, we used real-time RT-PCR to monitor the steady-state level of the *PsSym35* transcript in mutants and their corresponding wild types 1 day after rhizobial inoculation. An increase in *PsSym35* transcript was detected in all tested genotypes; however, the levels of induction in the *RisNod4* and K24 mutants were significantly lower than the wild type (Fig. 3C), showing that a full activation of this putative transcriptional regulator gene requires a functional *PsSym37* gene. A similar situation

was observed for the Afghan cultivar when inoculated with the European rhizobial strain, showing that *PsSym35* transcript regulation depends on a compatible legume-rhizobia interaction.

***RisNod4* mutants display a restrictive symbiotic phenotype similar to the Afghan cultivars.**

A symbiotic root-hair phenotype similar to the developmentally arrested phenotype observed for the *RisNod4* and K24 was previously reported for the Afghan pea cultivars in the presence of European *R. leguminosarum* strains (Geurts et al. 1997). Along the same line, our real-time RT-PCR analyses of the *sym37* mutants and Afghan line NGB2150, revealed a compara-

Fig. 3. A, Expression pattern of symbiotic genes *Sym37*, *Sym10*, *Sym35*, and *PsK1* in different organs of cv. Finale and **B**, in roots upon inoculation with European *R. leguminosarum* bv. *viciae* CIAM1026. **C**, *PsSym35* expression levels in uninoculated (u) and inoculated (i) roots of European wild-type cultivars (Finale and Rondo), *sym37* mutants (*RisNod4* and K24), and Afghan cultivar (NGB2150). Results are shown as fold increase compared with uninoculated roots from real-time reverse transcription-polymerase chain reaction analyses. Error bars represent the 95% confidence intervals.

ble impairment in the *PsSym35* transcript regulation upon inoculation with the European rhizobial strain (Fig. 3C). An examination of the *RisNod4* and K24 symbiotic phenotype with different strains of *R. leguminosarum* bv. *viciae* was therefore pertinent. Along with the two mutant lines, eight European and three Afghan pea cultivars were tested with the European (*R. leguminosarum* bv. *viciae* CIAM1026) and Middle East (*R. leguminosarum* bv. *viciae* A1) strains. As expected, the European pea cultivars were able to form functional nodules with both rhizobial strains, while the Afghan cultivars formed nodules only in the presence of the A1 strain (Fig. 2E). The K24 mutant line, which contains a premature stop codon in the *PsSym37* gene, showed the most stringent phenotype, and no nodules were observed after inoculation with either of the two strains. Contrary to expectation, *RisNod4* plants carrying the L to F amino acid change in the LysM1 of *PsSYM37* were able to form a significant number of nodules in the presence of *R. leguminosarum* bv. *viciae* A1, like the Afghan lines carrying the *PsSym2^A* allele.

The elusive *PsSym2* gene.

The *PsSym37* and *PsSym2* loci were located at the same position on the pea linkage map, and the natural variant *PsSym2^A* providing specificity for the Middle East rhizobial strains could, therefore, be an allele of *PsSym37*. To test this hypothesis, the relation between *PsSym37* and *PsSym2* was examined using genetics. Allelism tests between the *PsSym37* K24 mutant line (cv. Rondo background) and *PsSym2^A* introgression lines (cv. Rondo background) were performed. The nodules induced by the European strain *R. leguminosarum* bv. *viciae* CIAM1026 on the roots of *F₁* plants were counted and, in all

17 *F₁* plants scored, the recognition of this rhizobial strain was restored (Fig. S3). These results indicate that *PsSym37* and *PsSym2* are most likely separate genes.

Taking the similarity of the *PsSym2^A* and *RisNod4* phenotypes and the cosegregation of the *PsSym37*, *PsK1*, and *PsSym2* loci into consideration, we decided to take a second approach to further investigate the relation between *PsSym2* and *PsSym37* or *PsK1*. Based on the previous knowledge that Afghan cultivars recognize an *O*-acetylated Nod factor (Firmin et al. 1993) and that LysM domains function as determinants of strain specificity by recognizing the Nod factors (Radutoiu et al. 2007), we sequenced the *PsSym37* gene segment encoding the extracellular region of *PsSym37* in several pea varieties. Amino-acid sequence comparison of seven European and four Afghan cultivars (NGB2150, VIR1887, VIR6559, and CIAM31/6) revealed that one European line and the four Afghan cultivars had an identical primary structure of the SYM37 extracellular domain (Fig. 4A). This result supports the genetic allelism tests and strengthens the interpretation of *PsSym37* and *PsSym2* as two independent genes. On the other hand, the sequence of the *PsK1* extracellular region distinguishes the European and Afghan groups. The *PsK1* gene and amino-acid sequences were unique for the Afghan varieties as compared with the European cultivars included in the analysis (Fig. 4B). Moreover, sequencing of the *PsSym37* and *PsK1* genes in four *PsSym2^A* introgression lines of Rondo (5.4.3, 5.6.9, 15.5, and 33.18) showed no difference in nucleotide sequences compared with the Afghan lines. Altogether, sequencing of four Afghan cultivars and four introgression lines identified the same alleles of the two genes.

Fig. 4. Alignment of the predicted extracellular domains of *PsSYM37* and **B**, *PsK1* in European and Afghan (NGB2150) pea cultivars. The four Afghan cultivars analyzed have an identical sequence; therefore, only the sequence of NGB2150 is presented. Amino-acid differences are highlighted in black and the predicted LysM domains are underlined. The Leucine₇₇ of SYM37 is marked with a box.

Results obtained from the sequencing of *PsSym37* and *PsK1* in these lines and the allelism tests make it unlikely that the *PsSym37* gene determines the Afghan phenotype and that differences in the amino-acid sequences located in the extracellular domain of the *PsSYM37* protein distinguish the *O*-acetylated Nod-factor. Moreover, our analysis of the *PsK1* extracellular domain showed that the Afghan sequence was unique compared with the European lines tested. The *PsSym2* might, therefore, be allelic to *PsK1*, but our data does not provide direct evidence in support of such a conclusion. Both *LjNfr1* and *MtLyk3* belong to a gene family with some of the members closely linked. A similar clustering of the pea genes is not unlikely, and this complicates the final identification of *PsSym2* among alternative candidates.

DISCUSSION

We have characterized two allelic pea mutants, *RisNod4* and *K24*, previously isolated from two independent mutagenesis programs. A combination of cosegregation analysis and genotyping of the two alleles identified *PsSym37* as the likely candidate gene. Complementation of one or both of the mutant alleles with wild-type *PsSym37* could have confirmed this gene identification, but the recalcitrance of pea towards genetic transformation excluded this approach. *PsSym37* has a gene structure similar to *LjNfr1* and *MtLyk3*. Furthermore, we found that *PsSym37* has a conserved alternative splicing of exon four, like *LjNfr1*, showing that this is not limited to legume species with determinant nodules. We show that C to T nucleotide transitions changing the amino-acid composition of *PsSYM37* leads to the non-nodulating phenotypes of the pea *RisNod4* and *K24* mutants. Two types of mutations were induced, a missense mutation in *RisNod4* mutants (*L*₇₇ to F) and a stop codon at position *Q*₅₃₉ in *K24*. The two mutants have a similar symbiotic phenotype in the presence of a European *R. leguminosarum* bv. *viciae* strain; root hairs curl, microcolonies are formed, but the infection process is severely impaired. At the same time, cell divisions initiate in the inner cortex but are stopped in their development towards nodule primordia. However, microscopic analyses of infection-thread formation and analyses of *RisNod4*

mutant symbiotic phenotype in the presence of an Middle East strain of *R. leguminosarum* bv. *viciae* (A1) revealed that differences exist between plants carrying these two alleles, and *K24* mutants show the most severe phenotype. A strong phenotype was previously observed in *Lotus* mutants with stop codons in the kinase-coding domain of the *Nfr1* gene (Radutoiu et al. 2003). In *SYM37*, protein *L*₇₇, which is mutated to F in *RisNod4*, is located within the predicted *LysM1* domain and, although in *NFR1*-like proteins the *LysM1* domain is less conserved compared with *LysM2* and *LysM3*, this particular amino acid is conserved in all analyzed pea cultivars and in *MtLYK3* and *MtLYK4*. In *LjNFR1*, it is replaced by isoleucine (Figs. 4A and 5). An L to F alteration in the predicted Nod factor-binding domain might induce changes in the domain itself or in the overall structure of the extracellular region, which could affect the affinity for particularly decorated ligand molecules. A similar example in which a single amino-acid variation distinguishes between Nod-factor structures was previously described for *LysM2* of *LjNFR5* (Radutoiu et al. 2007). Steric or conformational changes could thus explain the ability of *RisNod4* mutant plants to recognize and form nodules when challenged by the *O*-acetylated Nod-factor molecules produced by the A1 strain of *R. leguminosarum* bv. *viciae*. In the presence of the European type of *R. leguminosarum* bv. *viciae*, lacking this decoration at the reducing end of the Nod factor, both mutants start cortical-cell divisions that subsequently stop developing into nodules. These results, taken together with the fact that European *R. leguminosarum* bv. *viciae* strains induce infection-thread formation in *RisNod4* mutant root hairs, indicate that, in pea, the *SYM37* receptor kinase protein is involved in Nod-factor perception and is required for infection-thread formation and progression of nodule organogenesis.

In our search for *LjNfr1*-like genes in pea, two genes encoding highly similar kinases and quite diverse extracellular domains were identified. The two genes have a slightly different exon/intron structure and show a distinctly different organ expression pattern as well as different induction kinetics during nodulation (Figs. 1 and 3). Based on the results we present here, a role in the rhizobial symbiosis can be assigned only for *PsSym37*. However, there are several clues that point toward

Fig. 5. Alignment of the predicted extracellular domains of *PsSYM37*, *MtLYK3*, and *LjNFR1*. The predicted locations of the *LysM* domains (M1 to M3) are underlined. The Leucine₇₇ is marked with a box, an asterisk (*) indicates identical residues, a colon (:) indicates conserved, and a dot (.) indicates semiconserved substitutions.

a role for *PsK1* during nodulation. The high similarity to PsSYM37, MtLYK3, and LjNFR1, the elevated expression in mature nodules, the genetic linkage to *PsSym2*, and the distinctive extracellular domain of Afghan pea varieties are in favor of such a function.

In *L. japonicus*, mutation in the *LjNfr1* gene leads to a complete loss of susceptibility towards rhizobia and Nod-factor molecules, while in *M. truncatula*, plants with downregulation or mutations of *MtLyk3* and *MtLyk4* genes maintain their initial epidermal and cortical responses. A similar situation is encountered for *PsSym37* mutations and specific *PsSym2* variants in pea. Our analysis of the amino-acid variation of the extracellular domains between European and Afghan pea lines and the complementation analysis does, however, suggest that *PsSym37* and *PsSym2* are separate genes. Based on the results of our mutant analyses and sequencing of *PsSym37* and *PsK1*, a situation in which both receptor kinase proteins are involved in *R. leguminosarum* bv. *viciae* Nod-factor recognition in pea roots can be envisioned. In the absence of a functional PsSYM37, a possible overlapping function of PsK1 or a partially functional receptor (complex) coded by redundant receptor genes could explain the differences in the restrictive symbiotic phenotypes of *LjNfr1* and the more permissive *PsSym37* mutants. In an alternative scenario, PsSYM37 could function as entry receptor in a similar fashion as suggested for the MtLYK3. Detailed biochemical analysis of SYM37 and PsK1 proteins coupled with a more comprehensive analysis of the *PsSym2* locus will lead to a better understanding of the Nod-factor perception process in legumes with indeterminate type of nodules.

MATERIALS AND METHODS

Plant material and bacterial strains.

The allelic RisNod4 and K24 mutants were obtained upon ethylmethane sulphonate treatment in Finale and Rondo background, respectively, and their isolation has been described previously (Engvild 1987; Jacobsen 1984). The RisNod4 and K24 mutant seeds were provided by K. J. Engvild, Agricultural Research Department, Risø National Laboratory, Roskilde, Denmark and E. Jacobsen, Agricultural University, Wageningen, The Netherlands, respectively. The lines NGB2150, VIR1887, VIR6559, and CIAM31/6 carry the Afghan allele of *PsSym2*, and the introgression lines (5.4.3, 5.6.9, 15.5, and 33.18) have the Afghan allele (*PsSym2^A*) in the genotype of cv. Rondo (Kozik et al. 1996). The seeds of the Afghan lines were obtained from the Nordic Gene Bank (NGB2150) and from the N. I. Vavilov collection of Institute of Plant Industry, St. Petersburg, Russia (VIR1887, VIR6559, and CIAM31/6). Seeds of introgression lines were provided by T. Bisseling (Wageningen, The Netherlands). All genotypes were propagated in the Laboratory of Biotechnology, All-Russia Research Institute for Agricultural Microbiology, St. Petersburg, Russia.

Three strains of *R. leguminosarum* bv. *viciae* were used for inoculation of pea plants: the European strains CIAM1026 (Safronova and Novikova 1996) and VF39*gusA* (Voroshilova et al. 2001) and the Afghan strain A1 (Chetkova and Tikhonovich 1986). The rhizobial strains were grown for 2 days in yeast-mannitol broth media supplemented with the appropriate antibiotics (streptomycin at 600 mg/liter for CIAM1026 and chloramphenicol at 40 mg/liter for VF39*gusA*) diluted 1:100 in sterile water before inoculation.

Plant growth conditions and inoculation.

For mapping experiments, F₂ plants were grown in sand in 41 plastic pots, four to five plants per pot, in field conditions or in a glasshouse, with nitrogen-free nutrient solutions. Inoculation with strain *R. leguminosarum* bv. *viciae* CIAM1026 was

performed immediately after planting the seeds as previously described (Borisov et al. 1997).

For other nodulation experiments, plants were grown in HPS2000 growth chambers (Heraeus Vötsch GmbH, Balingen, Germany) (16-h days and 8-h nights, 21 ± 1°C, relative humidity of 75%, photon irradiance of 490 μmol m⁻² s⁻¹). Nitrogen-free nutrient solutions and methods of seed inoculation have been described previously (Borisov et al. 1997). Germinated seeds were planted individually into ceramic pots with tempered sand and were then inoculated. For expression profiling of *Sym37* and *PsK1* genes, the plants were inoculated 7, 2, and 1 day before harvesting and were harvested on day 7 after planting, together with the uninoculated control plants.

Microscopic analyses.

In order to study the plant responses to the presence of rhizobia, root samples were collected 28 days after inoculation. Five plants per variant were analyzed, and 2-cm sections of five lateral roots from each plant were taken. When inoculated with CIAM1026 or A1, root samples were stained with 0.01 methylene blue and were tested for root-hair deformations and curling and for infection-thread growth (at least 20 visual fields at 300× magnification for each sample; approximately 500 visual fields per genotype). Root samples of plants inoculated with strain VF39*gusA* were stained as described previously (Voroshilova et al. 2001) and microscopically inspected for infection-thread formation. To identify nodule primordia development, the inoculated roots were sliced into 70-μm sections using a vibratome VT1000 S (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) and were stained with methylene blue. All samples were examined under a light microscope Opton Axiovert-35 (Opton Feinteknik GmbH, Oberkochen, Germany).

Mapping of the *Sym37* gene.

For initial mapping of the *Sym37* gene, a primary mapping population A was created by crossing the mutant K24 to the wild-type multiply-marked line NGB1238. F₁ plants were grown under greenhouse conditions with full mineral nutrition to obtain F₂ seeds. A total of 120 plants from the F₂ generation (mapping population A) were analyzed for morphological marker segregation, and seeds were collected from individual plants. The symbiotic phenotype (presence or absence of nodules and signs of nitrogen starvation) of the F₃ generation (grown in sand without combined nitrogen) was analyzed in order to identify F₂ *sym37* homozygous mutant plants. Segregation analyses of different morphological markers and the symbiotic phenotype revealed that the *sym37* mutation is linked to the marker *d* (regulation of axil pigmentation patterning) that was previously located on linkage group I (PGene Pisum Gene List database).

An F₄ progeny of mapping population A, carrying the recessive alleles of both the *Sym37* and *D* genes, was involved in a subsequent cross with the wild-type line NGB2715. A total of 235 F₂ individuals derived from this cross, of which 59 are symbiotic mutants (mapping population B), have been further analyzed for a fine mapping of *Sym37* gene. Segregation of the molecular markers derived from *PsSym35* (AJ493065) and *PsENOD40* (X81064) on this mapping population allowed us to locate the *sym37* mutation at 26.8 cM from *PsSym35* and 35.7 cM from *PsEnod40*.

PsK1 and PsK10- derived molecular markers were created using the dCAPS Finder 2.0 (Neff et al. 2002) and were used to analyze the cosegregation of *PsK1*, *PsK10*, and the *sym37* mutant phenotype in the mapping population B.

Computer analysis of the segregation data was performed with the program MapL98 (Y. Ukai, Biometrics Laboratory, Graduate School of Agricultural Life Science, The University of Tokyo). Genetic distances between markers were determined

by converting the frequency of recombination events into Kosambi units (Kosambi 1944).

PsK1 and PsSym37 cDNA isolation.

Pea cDNA clones were isolated by screening one million clones from a pea cDNA library enriched for root hairs (ZapII vector, cv. Finale), using a 1,280-bp fragment of the *Lotus japonicus* *Nfr1* gene covering the LysM-containing extracellular domain as a probe. Primers for PCR amplification of the *Lotus nfr1* probe were: forward, 5'-TAATTATCAGAGTAAGTGTGAC-3' and reverse, 5'-AGTTACCCACCTGTGGTAC-3'.

DNA methods and sequencing strategy.

Pea genomic DNA was isolated using the CTAB method (Rogers and Bendich 1985). *Sym37* and *PsK1* genes have been fully sequenced in Finale, Rondo, RisNod4, K24, and NGB2150 background. Several nucleotide differences were identified in the *Sym37* gene distinguishing the Afghan from the European cultivars. At the amino-acid level, the Afghan cultivar NGB2150 differed from Finale and Rondo by one and seven residues, respectively, and all variations were found in the predicted extracellular domain (Fig. S4). As a consequence, the sequencing of the two genes in the other genotypes (SGE, Frisson, Sparkle, Sprint-2, NGB1238, and NGB2715) was performed only for the genomic sequence corresponding to the predicted extracellular regions. Sequencher and CEQ 8000 software was used for DNA sequence analyses and alignments and ClustalW2 for the predicted amino-acid alignments.

Real-time RT-PCR analyses.

Total RNA from different pea tissues was isolated using Trizol (Sigma-Aldrich Danmark A/S, Brøndby, Denmark) and was treated with DNase (RQ1 DNase, Promega Biotech AB, Nacka, Sweden) in order to remove DNA contaminants. The RNA was subsequently used for cDNA synthesis using the RevertAid first-strand cDNA synthesis kit (FermentasGMBH, Helsingborg, Sweden). Quantitative PCR was performed on a light cycler (Roche Applied Science, Hvidovre, Denmark), using Fast Start DNA master SYBR green kit (Roche) to amplify the target transcripts from 5 µl of diluted cDNA samples. The cDNAs were tested for DNA contamination using primers derived from the *PsSym35* promoter region. Three housekeeping genes (*Actin2*, *GAPDH*, and *PP2A*) were used to determine the relative expression of target genes. The primers used for amplification are presented in Supplementary Table S2. The identity of PCR products was verified by sequencing. Three biological and three technical replicates were performed for each sample and each analyzed transcript. The normalized relative ratios of the target genes and the three independent housekeeping genes have been calculated using the QBASE v.1.3.5 software (Hellemans et al. 2007).

Phylogenetic analyses.

Sequence alignments were performed using ClustalX 2.0.7 with a gap opening of 15 and the Blosom protein weight matrix. The phylogenetic tree was generated using the program Mega 4.1, using the neighbor-joining method and the Poisson correction as a substitution model.

ACKNOWLEDGMENTS

We thank H. Franssen for making the pea cDNA library available, V. A. Voroshilova for performing the allelism tests between *sym2* and *sym37* mutant plants, and L. E. Dvoryaninova for technical assistance. This work was supported by the Danish National Research Foundation and European Union FP6 project FOOD-CT-2004-506223, Russian Foundation for Basic Research 07-04-01558 and 07-04-01171, the Danish Research Agency–Young Scientists grant and state contracts from the Government of Rus-

sian Federation (HIII-5399.2008.4, 02.512.11.2254), Russian Foundation for Basic Research (07-04-89000-NWOC_a), and The Netherlands Organization for Scientific Research (047.018.001). V. Zhukov was supported by a traveling fellowship from Development.

LITERATURE CITED

- Borisov, A. Y., Rozov, S. M., Tsyganov, V. E., Morzhina, E. V., Lebsky, V. K., and Tikhonovich, I. A. 1997. Sequential functioning of *Sym-13* and *Sym-31*, two genes affecting symbiosome development in root nodules of pea (*Pisum sativum* L.). *Mol. Gen. Genet.* 254:592-598.
- Borisov, A. Y., Madsen, L. H., Tsyganov, V. E., Umehara, Y., Voroshilova, V. A., Batagov, A. O., Sandal, N., Mortensen, A., Schausser, L., Ellis, N., Tikhonovich, I. A., and Stougaard, J. 2003. The *Sym35* gene required for root nodule development in pea is an ortholog of *Nin* from *Lotus japonicus*. *Plant Physiol.* 131:1009-1017.
- Borisov, A. Y., Vasil'chikov, A. G., Voroshilova, V. A., Danilova, T. N., Zhernakov, A. I., Zhukov, V. A., Koroleva, T. A., Kuznetsova, E. V., Madsen, L., Mofett, M., Nemankin, T. A., Ovchinnikova, E. S., Pavlova, Z. B., Petrova, N. E., Pinaev, A. G., Radutoiu, S., Rozov, S. M., Rychagova, T. S., Solovov, I. I., Topunov, A. F., Weeden, N. F., Tsyganov, V. E., Shtark, O. Y., Stougaard, J., Naumkina, T. S., and Tikhonovich, I. A. 2007. Regulatory genes of garden pea (*Pisum sativum* L.) controlling the development of nitrogen-fixing nodules and arbuscular mycorrhiza: A review of basic and applied aspects. *Appl. Biochem. Microbiol.* 43:237-243.
- Chetkova, S. A., and Tikhonovich, I. A. 1986. Isolation and study of *Rhizobium leguminosarum* strains effective on peas from Afghanistan. *Microbiology-Russia* 55:143-147.
- Cronk, Q., Ojeda, I., and Pennington, R. T. 2006. Legume comparative genomics: Progress in phylogenetics and phylogenomics. *Curr. Opin. Plant Biol.* 9:99-103.
- Engvild, K. 1987. Nodulation and nitrogen fixation mutants of pea, *Pisum sativum*. *Theor. Appl. Genet.* 74:711-713.
- Firmin, J. L., Wilson, K. E., Carlson, R. W., Davies, A. E., and Downie, J. A. 1993. Resistance to nodulation of cv. Afghanistan peas is overcome by nodX, which mediates an *O*-acetylation of the *Rhizobium leguminosarum* lipo-oligosaccharide nodulation factor. *Mol. Microbiol.* 10:351-360.
- Geurts, R., Heidstra, R., Hadri, A. E., Downie, J. A., Franssen, H., Van Kammen, A., and Bisseling, T. 1997. *Sym2* of pea is involved in a nodulation factor-perception mechanism that controls the infection process in the epidermis. *Plant Physiol.* 115:351-359.
- Gualtieri, G., Kulikova, O., Limpens, E., Kim, D. J., Cook, D. R., Bisseling, T., and Geurts, R. 2002. Microsynteny between pea and *Medicago truncatula* in the *SYM2* region. *Plant Mol. Biol.* 50:225-235.
- Heckmann, A. B., Lombardo, F., Miwa, H., Perry, J. A., Bunnewell, S., Parniske, M., Wang, T. L., and Downie, J. A. 2006. *Lotus japonicus* nodulation requires two GRAS domain regulators, one of which is functionally conserved in a non-legume. *Plant Physiol.* 142:1739-1750.
- Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* 8:R19.
- Hogg, B., Davies, A. E., Wilson, K., Bisseling, T., and Downie, J. A. 2002. Competitive nodulation blocking of cv. Afghanistan pea is related to high levels of nodulation factors made by some strains of *Rhizobium leguminosarum* bv. *viciae*. *Mol. Plant-Microbe Interact.* 15:60-68.
- Hougaard, B. K., Madsen, L. H., Sandal, N., de Carvalho Moretzsohn, M., Fredslund, J., Schausser, L., Nielsen, A. M., Rohde, T., Sato, S., Tabata, S., Bertoli, D. J., and Stougaard, J. 2008. Legume anchor markers link syntenic regions between *Phaseolus vulgaris*, *Lotus japonicus*, *Medicago truncatula* and *arachis*. *Genetics* 179:2299-2312.
- Imaizumi-Anraku, H., Takeda, N., Charpentier, M., Perry, J., Miwa, H., Umehara, Y., Kouchi, H., Murakami, Y., Mulder, L., Vickers, K., Pike, J., Downie, J. A., Wang, T., Sato, S., Asamizu, E., Tabata, S., Yoshikawa, M., Murooka, Y., Wu, G. J., Kawaguchi, M., Kawasaki, S., Parniske, M., and Hayashi, M. 2005. Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. *Nature* 433:527-531.
- Jacobsen, E. 1984. Modification of symbiotic interaction of pea (*Pisum sativum* L.) and *Rhizobium leguminosarum* by induced mutations. *Plant and Soil* 82:427-438.
- Kalo, P., Gleason, C., Edwards, A., Marsh, J., Mitra, R. M., Hirsch, S., Jakab, J., Sims, S., Long, S. R., Rogers, J., Kiss, G. B., Downie, J. A., and Oldroyd, G. E. 2005. Nodulation signaling in legumes requires *NSP2*, a member of the GRAS family of transcriptional regulators. *Science* 308:1786-1789.
- Kanamori, N., Madsen, L. H., Radutoiu, S., Frantescu, M., Quistgaard, E. M. H., Miwa, H., Downie, J. A., James, E. K., Felle, H. H., Haaning, L. L., Jensen, T. H., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and

- Stougaard, J. 2006. A nucleoporin is required for induction of Ca^{2+} spiking in legume nodule development and essential for rhizobial and fungal symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 103:359-364.
- Kosambi, D. D. 1944. The estimation of map distance from recombination values. *Ann. Eugen.* 12:172-175.
- Kozik, A., Heidstra, R., Horvath, B., Kulikova, O., Tikhonovich, I., Ellis, T. H. N., van Kammen, A., Lie, T. A., and Bisseling, T. 1995. Pea lines carrying *sym1* or *sym2* can be nodulated by *Rhizobium* strains containing *nodX*; *sym1* and *sym2* are allelic. *Plant Sci.* 108:41-49.
- Kozik, A., Geurts, R., Heidstra, R., Kulikova, O., Ellis, T. H. N., Bisseling, T., LaRue, T., and Weeden, N. 1996. Detailed map of the *sym2* region of pea linkage group I. In: *Fine mapping of the sym2 locus of pea linkage group I*. Ph.D. thesis. Wageningen University. Wageningen, The Netherlands.
- Lévy, J., Bres, C., Geurts, R., Chalhou, B., Kulikova, O., Duc, G., Journet, E. P., Ané, J. M., Lauber, E., Bisseling, T., Dénarié, J., Rosenberg, C., and Debelle, F. 2004. A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. *Science* 303:1361-1364.
- Limpens, E., Franken, C., Smit, P., Willemse, J., Bisseling, T., and Geurts, R. 2003. LysM domain receptor kinases regulating rhizobial Nod-factor-induced infection. *Science* 302:630-633.
- Madsen, E. B., Madsen, L. H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczygłowski, K., Sato, S., Kaneko, T., Tabata, S., Sandal, N., and Stougaard, J. 2003. A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. *Nature* 425:637-640.
- Marsh, J. F., Rakocevic, A., Mitra, R. M., Brocard, L., Sun, J., Eschstruth, A., Long, S. R., Schultze, M., Ratet, P., and Oldroyd, G. E. 2007. *Medicago truncatula* *NIN* is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. *Plant Physiol.* 144:324-335.
- Middleton, P. H., Jakab, J., Penmetts, R. V., Starker, C. G., Doll, J., Kalo, P., Prabhu, R., Marsh, J. F., Mitra, R. M., Kereszt, A., Dudas, B., VandenBosch, K., Long, S. R., Cook, D. R., Kiss, G. B., and Oldroyd, G. E. 2007. An ERF transcription factor in *Medicago truncatula* that is essential for Nod factor signal transduction. *Plant Cell* 19:1221-1234.
- Mitra, R. M., Gleason, C. A., Edwards, A., Hadfield, J., Downie, J. A., Oldroyd, G. E. D., and Long, S. R. 2004. A Ca^{2+} /calmodulin-dependent protein kinase required for symbiotic nodule development: Gene identification by transcript-based cloning. *Proc. Natl. Acad. Sci. U.S.A.* 101:4701-4705.
- Murakami, Y., Miwa, H., Imaizumi-Anraku, H., Kouchi, H., Downie, J. A., Kawaguchi, M., and Kawasaki, S. 2006. Positional cloning identifies *Lotus japonicus* *NSP2*, a putative transcription factor of the GRAS family, required for *NIN* and *ENOD40* gene expression in nodule initiation. *DNA Res.* 13:255-265.
- Neff, M. M., Turk, E., and Kalishman, M. 2002. Web-based primer design for single nucleotide polymorphism analysis. *Trends Genet.* 18:613-615.
- Oldroyd, G. E. D., and Downie, J. A. 2008. Coordinating nodule with rhizobial infection in legumes. *Annu. Rev. Plant Biol.* 59:519-546.
- Olsthoorn, M. M. A., Stokvis, E., Haverkamp, J., Spaik, H. P., and Thomas-Oates, J. E. 2000. Growth temperature regulation of host-specific modifications of rhizobial lipo-chitin oligosaccharides: The function of *nodX* is temperature regulated. *Mol. Plant-Microbe Interact.* 13:808-820.
- Ovtsyna, A. O., Geurts, R., Bisseling, T., Lugtenberg, B. J. J., Tikhonovich, I. A., and Spaik, H. P. 1998. Restriction of host range by *sym2* allele of Afghan pea is nonspecific for the type of modification at the reducing terminus of nodulation signal. *Mol. Plant-Microbe Interact.* 11:418-422.
- Postma, J. G., Jacobsen, E., and Feenstra, W. J. 1988. Three pea mutants with an altered nodulation studied by genetic analysis and grafting. *J. Plant Physiol.* 132:424-430.
- Radutoiu, S., Madsen, L. H., Madsen, E. B., Felle, H. H., Umehara, Y., Gronlund, M., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and Stougaard, J. 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* 425:585-592.
- Radutoiu, S., Madsen, L. H., Madsen, E. B., Jurkiewicz, A., Fukai, E., Quistgaard, E. M. H., Albrechtsen, A. S., James, E. K., Thirup, S., and Stougaard, J. 2007. LysM domains mediate lipochitin-oligosaccharide recognition and Nfr genes extend the symbiotic host range. *EMBO (Eur. Mol. Biol. Organ.) J.* 26:3923-3935.
- Rogers, S. O., and Bendich, A. J. 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Mol. Biol.* 5:69-76.
- Saito, K., Yoshikawa, M., Yano, K., Miwa, H., Uchida, H., Asamizu, E., Sato, S., Tabata, S., Imaizumi-Anraku, H., Umehara, Y., Kouchi, H., Murooka, Y., Szczygłowski, K., Downie, J. A., Parniske, M., Hayashi, M., and Kawaguchi, M. 2007. NUCLEOPORIN85 is required for calcium spiking, fungal and bacterial symbioses, and seed production in *Lotus japonicus*. *Plant Cell* 19:610-624.
- Safronova, V. I., and Novikova, N. I. 1996. Comparison of two methods for root nodule bacteria preservation: Lyophilization and liquid nitrogen freezing. *J. Microbiol. Methods* 24:231-237.
- Sandal, N., Krusell, L., Radutoiu, S., Olbryt, M., Pedrosa, A., Stracke, S., Sato, S., Kato, T., Tabata, S., Parniske, M., Bachmair, A., Ketelsen, T., and Stougaard, J. 2002. A genetic linkage map of the model legume *Lotus japonicus* and strategies for fast mapping of new loci. *Genetics* 161:1673-1683.
- Sandal, N., Petersen, T. R., Murray, J., Umehara, Y., Karas, B., Yano, K., Kumagai, H., Yoshikawa, M., Saito, K., Hayashi, M., Murakami, Y., Wang, X. W., Hakoyama, T., Imaizumi-Anraku, H., Sato, S., Kato, T., Chen, W. L., Hossain, M. S., Shibata, S., Wang, T. L., Yokota, K., Larsen, K., Kanamori, N., Madsen, E., Radutoiu, S., Madsen, L. H., Radu, T. G., Krusell, L., Ooki, Y., Banba, M., Betti, M., Rispail, N., Skot, L., Tuck, E., Perry, J., Yoshida, S., Vickers, K., Pike, J., Mulder, L., Charpentier, M., Muller, J., Ohtomo, R., Kojima, T., Ando, S., Marquez, A. J., Gresshoff, P. M., Harada, K., Webb, J., Hata, S., Saganuma, N., Kouchi, H., Kawasaki, S., Tabata, S., Parniske, M., Szczygłowski, K., Kawaguchi, M., and Stougaard, J. 2006. Genetics of symbiosis in *Lotus japonicus*: Recombinant inbred lines, comparative genetic maps, and map position of 35 symbiotic loci. *Mol. Plant-Microbe Interact.* 19:80-91.
- Schauser, L., Roussis, A., Stiller, J., and Stougaard, J. 1999. A plant regulator controlling development of symbiotic root nodules. *Nature* 402:191-195.
- Smit, P., Limpens, E., Geurts, R., Fedorova, E., Dolgikh, E., Gough, C., and Bisseling, T. 2007. *Medicago* LYK3, an entry receptor in rhizobial nodulation factor signaling. *Plant Physiol.* 145:183-191.
- Stacey, G., Libault, M., Brechenmacher, L., Wan, J., and May, G. 2006. Genetics and functional genomics of legume nodulation. *Curr. Opin. Plant Biol.* 9:110-121.
- Stougaard, J. 2001. Genetics and genomics of root symbiosis. *Curr. Opin. Plant Biol.* 4:328-335.
- Stracke, S., Kistner, C., Yoshida, S., Mulder, L., Sato, S., Kaneko, T., Tabata, S., Sandal, N., Stougaard, J., Szczygłowski, K., and Parniske, M. 2002. A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature* 417:959-962.
- Tirichine, L., Imaizumi-Anraku, H., Yoshida, S., Murakami, Y., Madsen, L. H., Miwa, H., Nakagawa, T., Sandal, N., Albrechtsen, A. S., Kawaguchi, M., Downie, A., Sato, S., Tabata, S., Kouchi, H., Parniske, M., Kawasaki, S., and Stougaard, J. 2006. Deregulation of a Ca^{2+} /calmodulin-dependent kinase leads to spontaneous nodule development. *Nature* 441:1153-1156.
- Tirichine, L., Sandal, N., Madsen, L. H., Radutoiu, S., Albrechtsen, A. S., Sato, S., Asamizu, E., Tabata, S., and Stougaard, J. 2007. A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis. *Science* 315:104-107.
- Tsyganov, V. E., Voroshilova, V. A., Priefer, U. B., Borisov, A. Y., and Tikhonovich, I. A. 2002. Genetic dissection of the initiation of the infection process and nodule tissue development in the *Rhizobium*-pea (*Pisum sativum* L.) symbiosis. *Annals of Botany* 89:357-366.
- Voroshilova, V. A., Boesten, B., Tsyganov, V. E., Borisov, A. Y., Tikhonovich, I. A., and Priefer, U. B. 2001. Effect of mutations in *Pisum sativum* L. genes (*sym13*, *sym31*, *sym33*, *sym40*) blocking different stages of nodule development on the expression of late symbiotic genes in *Rhizobium leguminosarum* bv. *viciae*. *Mol. Plant-Microbe Interact.* 14:471-476.
- Yacobi, L. M., Voroshilova, V. A., Tsyganov, V. E., Borisov, A. Y., and Tikhonovich, I. A. 1998. Pea mutants K5, K24, FN1 and nod3 induced in cv. Rondo are able to form arbuscular endomycorrhiza. Mutant K24 is not an allele of *sym19*. *Pisum Genetics* 30:30.
- Zhang, X. C., Wu, X., Findley, S., Wan, J., Libault, M., Nguyen, H. T., Cannon, S. B., and Stacey, G. 2007. Molecular evolution of lysin motif-type receptor-like kinases in plants. *Plant Physiol.* 144:623-636.

AUTHOR-RECOMMENDED INTERNET RESOURCES

PGene *Pisum* Gene List database:
data.jic.bbsrc.ac.uk/cgi-bin/pgene/default.asp?ID=234
 Washington University Biology Department dCAPS Finder 2.0:
helix.wustl.edu/dcaps/dcaps.html
 European Bioinformatics Institute ClustalW2 database:
www.ebi.ac.uk/Tools/clustalw2/index.html
 QBASE software: medgen.ugent.be/qbase