Natural Variation in Host-Specific Nodulation of Pea Is Associated with a Haplotype of the SYM37 LysM-Type Receptor-Like Kinase

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Rhizobium leguminosarum bv. *viciae*, which nodulates pea and vetch, makes a mixture of secreted nodulation signals (Nod factors) carrying either a C18:4 or a C18:1 N-linked acyl chain. Mutation of *nodE* blocks the formation of the C18:4 acyl chain, and *nodE* mutants, which produce only C18:1-containing Nod factors, are less efficient at nodulating pea. However, there is significant natural variation in the levels of nodulation of different pea cultivars by a *nodE* mutant of *R. leguminosarum* bv. *viciae*. Using recombinant inbred lines from two pea cultivars, one which nodulated relatively well and one very poorly by the *nodE* mutant, we mapped the *nodE*-dependent nodulation phenotype to a locus on pea linkage group I. This was close to *Sym37* and *PsK1*, predicted to encode LysM-domain Nod-factor receptor-like proteins; the *Sym2* locus that confers Nod-factor-specific nodulation is also in this region. We confirmed the map location using an introgression line carrying this region. Our data indicate that the *nodE*-dependent nodulation is not determined by the *Sym2* locus. We identified several pea lines that are nodulated very poorly by the *R. leguminosarum* bv. *viciae* *nodE* mutant, sequenced the DNA of the predicted LysM-receptor domains of *Sym37* and *PsK1*, and compared the sequences with those derived from pea cultivars that were relatively well nodulated by the *nodE* mutant. This revealed that one haplotype (encoding six conserved polymorphisms) of *Sym37* is associated with very poor nodulation by the *nodE* mutant. There was no such correlation with polymorphisms at the *PsK1* locus. We conclude that the natural variation in *nodE*-dependent nodulation in pea is most probably determined by the *Sym37* haplotype.

The symbiotic interaction between rhizobia and their legume hosts to form nitrogen-fixing nodules shows specificity between the plants and the bacteria because legumes can usually be nodulated by only a limited number of rhizobial strains

(Perret et al. 2000). The range of legumes that can be nodulated by a given rhizobial strain involves signaling specificity mediated by bacterially secreted Nod factors, surface polysaccharides, and secreted proteins (Downie 2010). Nod factors are the major determinants of specificity and are usually made up of a backbone of four or five β -1-4 linked *N*-acetyl glucosamine residues, in which the acetyl group on the terminal non-reducing sugar is replaced by an N-linked acyl chain of 16 to 20 carbon atoms (Perret et al. 2000). *Rhizobium leguminosarum* bv. *viciae* strains, which nodulate pea and vetch, produce a mixture of Nod factors carrying C18:1 or C18:4 acyl chains (Spaink et al. 1991). The synthesis of the C18:4 acyl chain requires the *nodF* and *nodE* gene products (Bloemberg et al. 1995) and, in a *nodE* mutant, a C18:1 acyl chain from normal fatty acid synthesis replaces the C18:4 acyl group. In *R. leguminosarum* bv. *trifolii*, which normally nodulates clover, mutation of *nodE* enabled nodulation of pea, which is not normally a host (Djordjevic et al. 1985). Exchange of the *nodE* genes between *R. leguminosarum* bv. *viciae* and *trifolii* changed their nodulation specificity from clover to pea or vice versa (Spaink et al. 1989; Surin and Downie 1989).

The pea nodulation signaling pathway can be activated with *R. leguminosarum* bv. *viciae* Nod factors carrying either the C18:1 or the C18:4 acyl group but, for infection to occur, a C18:4-substituted Nod factor can be important (Walker and Downie 2000). In *Sinorhizobium meliloti*, the lack of infection by mutants altered in Nod-factor decoration, together with their ability to induce nodule morphogenesis, led to the hypothesis of two types of Nod-factor receptors, one for nodule signaling and another for entry (Ardourel et al. 1994). In *Medicago truncatula*, a host of *S. meliloti*, LYK3, has been proposed as a plant entry receptor that could directly or indirectly be involved in recognition of host-specific Nod factors (Smit et al. 2007).

In addition to the NodF-NodE-determined acyl group, Nod factors can be substituted with various other groups, including acetyl, carbamoyl, methyl, sulfonyl, and several glycosyl groups, all of which are thought to contribute to specificity in signaling between rhizobia and their hosts (Perret et al. 2000). Legumes recognize these Nod factors using transmembrane receptors that have extracellular LysM-domains, which are predicted to bind to the chitin backbone of the Nod factors (Madsen et al. 2003; Radutoiu et al. 2003). Expression of the *Lotus japonicus* receptor genes *NFR1* and *NFR5* in *M. truncatula* allowed the formation of nodules by rhizobial strains that normally nodulate *L. japonicus* but not *M. truncatula* (Radutoiu et al. 2007). Domain-swap experiments between *NFR5* from two *Lotus* spp. with different host-specific nodula-

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tion requirements confirmed the importance of one of the NFR5 LysM domains in recognition of host-specific modifications of Nod factors (Bek et al. 2010).

Natural variation can affect rhizobial recognition by different lines of a single legume species. One example of this in *Pisum sativum* is the resistance of cultivar 'Afghanistan' to nodulation by *R. leguminosarum* bv. *viciae* strains that can nodulate many other cultivars of pea (Lie 1984). Strains of *R. leguminosarum* bv. *viciae* that can nodulate 'Afghanistan' produce a Nod factor that has an O-linked acetyl group on the terminal reducing glucosamine residue. Such strains contain a gene called *nodX* whose product is required for this acetylation (Firmin et al. 1993) and *nodX* is absent from many strains of *R. leguminosarum* bv. *viciae*. 'Afghanistan' pea can also be nodulated if the NodX-determined acetyl group is replaced by fucose (Ovtsyna et al. 1998).

The resistance to nodulation in 'Afghanistan' is recessive and segregates as a single genetic locus (Lie 1984) on pea linkage group I, in a region where there are multiple Nod-factor receptor-like genes (Gualtieri et al. 2002; Zhukov et al. 2008). The locus from 'Afghanistan' is designated *sym^A*, whereas *Sym2^C* has been used to refer to the alleles from cultivated cultivars of pea (Geurts et al. 1997). Some strains of *R. leguminosarum* bv. *viciae* that produce high levels of Nod factors can block nodulation of 'Afghanistan' by other strains and this "competitive nodulation blocking" effect depends on both the *nodE*-determined host-specific Nod factor (Dowling et al. 1989) and the production of high levels of that Nod factor (Hogg et al. 2002). A strain of *R. leguminosarum* bv. *viciae* which carries *nodX* may nodulate 'Afghanistan' poorly if it produces high levels of Nod factor (Hogg et al. 2002).

We were interested in determining whether there were other examples in pea of variation in sensitivity to nodulation signaling by *R. leguminosarum* bv. *viciae*. To this end, we analyzed different pea genotypes for nodulation by a *nodE* mutant of *R. leguminosarum* bv. *viciae* and observed significantly different levels of nodulation. Using a recombinant inbred (RI) population and our introgression line, we were able to show that the major variation in nodulation is associated with *Sym37* on linkage group I.

RESULTS

Quantitative mapping of a locus in pea influencing host-specific nodulation.

In view of the role of the *R. leguminosarum* bv. *viciae* *nodE* gene in determining host-specific nodulation (Spaink et al. 1989; Surin and Downie 1989), we noticed with interest that, whereas nodulation is generally decreased by mutation of *nodE*

Fig. 1. Effect of mutating *nodE* on nodulation of different pea lines. Seedlings of pea lines **A**, JI15 or **B**, JI1194, were inoculated with the wild-type (WT) strain *Rhizobium leguminosarum* bv. *viciae* A34 (closed symbols) or the derivative of it carrying the *nodE*68::Tn5 mutation (open symbols). Data are averages ($\pm$ standard deviation) of data from eight plants.

in *R. leguminosarum* bv. *viciae*, in some cultivars of pea, the reduction consistently appeared to be greater than in others. This suggested that some loci in pea may influence recognition of Nod factors carrying the NodE-determined C18:4 N-linked acyl group. With the aim of mapping this phenotype, we analyzed the effects of a *nodE* mutation on nodulation of different pea lines, which are the parents of RI mapping populations (Ellis et al. 1992, 1998). The *nodE* mutation almost completely blocked nodulation in lines JI15 (Fig. 1A), JI281, and JI399 (data not shown) and reduced nodulation in JI1194 by approximately half (Fig. 1B). An RI population has been produced from the progeny of a cross between JI15 and JI1194; using the RI lines of this cross, nodulation was scored 21 days after inoculation with the *nodE* mutant, when the number of nodules formed is relatively stable under the test conditions used (Fig. 1).

Analysis of the nodulation data revealed a single statistically significant quantitative trait locus (QTL) on linkage group I (Fig. 2) with a log of the difference (LOD) score of 3.57 ($P < 0.01$). There were two other scores with LOD scores of 1.7 and 1.3 on linkage groups III and V, respectively, but these were below the statistically significant ($P < 0.05$) LOD value of 2.9 in these tests. The main QTL, which contributed to 29% of the

Fig. 2. Quantitative trait locus (QTL) mapping of a pea locus affecting nodulation by a *nodE* mutant of *Rhizobium leguminosarum* bv. *viciae*. Recombinant inbred (RI) lines of pea produced from a cross of JI15 $\times$ JI1194 were scored for nodulation 21 days after inoculation with the *nodE* mutant derivative of *R. leguminosarum* bv. *viciae* strain A34. At least 10 plants from each of 49 RI lines were tested and the data were analyzed using MapQTL5.0 (Van Ooijen 2004). Log of the difference (LOD) scores were calculated using data from all linkage groups (LGs) and scores above 2.9 were significant at the 95% confidence level based on a permutation test (10,000 permutations) calculated using all the mapping data for all LGs. The only significant peak ($P < 0.01$) was as marked on LG I, and its location was mapped using all the markers shown segregating in this cross. These markers and a scale bar showing 50 centimorgans are shown on the map of LG I. The only other peaks with a LOD score above 1 were observed on LG III and LG V but are not shown because they were not statistically significant.

Fig. 3. Markers derived from Nod-factor receptor-like genes *Sym37* and *PsK1* on linkage group I. **A**, Intron (open boxes) and exon (black boxes) structure of the *Sym37* and *PsK1* genes derived from previous work (Zhukov et al. 2008) are shown, illustrating the locations of oligonucleotide primers used to amplify regions of *Sym37* (primers a and b) or *PsK1* (primers c and d). DNA products of each gene amplified from J115 and J11194 were sequenced and the nucleotide differences between these two lines are shown below each gene using numbering based on the amplified DNA products. The nucleotides in *italics* show two *MseI* sites in J115, one of which is absent in J11194. **B**, Gel showing the *Sym37* DNA fragments amplified from J115 and J11194. In the absence (–) of digestion, the expected size of 443 nucleotides was obtained and an unexpected fragment of approximately 279 bp was also present; however, this did not interfere with the analysis. After digestion with *MseI* (+), polymorphic fragments of 127 and 289 bp were used to distinguish loci from J115 and J11194, respectively. The *PsK1* loci from J115 and J11194 recombinant inbred lines were distinguished by DNA sequencing using the polymorphisms shown. Mapping of *Sym37* and *PsK1* in the J115 × J11194 mapping population.

total variation in nodulation, mapped at the top of linkage group I, close to markers Tps1/188–, Tps1/199+, and Tps1/204–, which co-segregated in this population. Based on the integrated genetic map (Ellis and Poyser 2002), this location appeared to be close to *Sym2*, *Sym37*, and *PsK1* all of which are very closely linked (Zhukov et al. 2008).

To map these loci in relation to the markers segregating in the J115 × J11194 cross, we generated new genetic markers using the *Sym37* and *PsK1* loci (Gualtieri et al. 2002). *Sym37* and *PsK1* are predicted to encode proteins with structural similarity to putative Nod-factor receptors from *L. japonicus* and *M. truncatula*. Using primers, we amplified and sequenced intron 4 of *Sym37* from J115 and J11194 and identified 10 nucleotide differences, 1 of which corresponded to an *MseI* site difference between J115 and J11194 (Fig. 3A). We mapped this polymorphism in the RI lines, as illustrated with the parental lines in Figure 3B. With *PsK1*, we amplified DNA across introns 6, 7, and 8 of J115 and J11194 and identified nine nucleotide differences (Fig. 3) but, because none could be used to generate a marker that could be cut with a restriction enzyme, we sequenced this region from all the RI lines. There were no plants with recombinations between the *PsK1* and *Sym37*, confirming that they are tightly linked genetically. There was also no recombination between these markers and Tps1/188–, Tps1/199+, and Tps1/204–. This confirmed that the *node*-specific nodulation phenotype is closely linked to *Sym37*, *PsK1*, and, hence, *Sym2*; the location of *Sym37* is shown in Figure 2.

The LOD peak at the markers Tps1/199+, Tps1/204–, and Tps1/188–, among which no recombinations were identified, corresponds to a map distance of approximately 1.5 centimorgans when assessed using JoinMap (Van Ooijen and Voorrips 2001) using the Haldane mapping function. Linkage group I of pea corresponds to *M. truncatula* chromosome 5 (Choi et al. 2004), and pea gene sequences from mapped markers were used to align the pea QTL peak region to the equivalent region in *M. truncatula*. This would be predicted to correspond to approximately 176 kb in *M. truncatula* encompassing approximately 75 genes but, given the nature of QTL mapping data, the region causing the phenotypic differences could be significantly larger. Also, given the large difference in genome sizes of pea and *M. truncatula* (Choi et al. 2004), this estimate of relative gene content in pea must be regarded as an approximation.

An introgression line confirms the map location of the region conferring *node*-dependent nodulation.

Given the linkage between *Sym2* and the J115 locus conferring low nodulation by the *R. leguminosarum* bv. *viciae* *node*

Fig. 4. Effect of *sym2^A* on *nodeE*-dependent nodulation of pea by *Rhizobium leguminosarum* bv. *viciae*. **A**, ‘Afghanistan’ pea inoculated with *R. leguminosarum* bv. *viciae* TOM (filled squares) and the *nodeE* mutant derivative of TOM lacking (open circles) or containing (closed circles) the cloned *nodeE* region on pIM186. **B**, Nodulation of Rondo pea by strain TOM (filled squares) and the *nodeE* mutant derivative of TOM (open circles). **C**, Nodulation tests of variety Rondo carrying the *sym2^A* region introgressed from ‘Afghanistan’ after inoculation with TOM (filled squares), its *nodeE* mutant derivative A804 (open circles), and its *nodeX* mutant derivative (open triangles). Data are averages (± standard deviation) of data from at least 12 plants.

mutant, we decided to score nodulation in pea lines carrying the 'Afghanistan' *sym2^A* locus, which confers resistance to nodulation by rhizobia lacking NodX-acetylated Nod factors. To do this, we made a *nodE* mutant derivative of *R. leguminosarum* bv. *viciae* strain TOM by recombining into TOM the *nodE68::Tn5* mutation described previously (Downie et al. 1985). The TOM *nodE* mutant strain (A804) was almost completely defective for nodulation of 'Afghanistan' pea compared with the high nodulation seen with the control strain (Fig. 4A). This nodulation defect could be complemented by pIM186 (Surin and Downie 1989) carrying *nodE* but not *nodX* (Fig. 4A), confirming that the lack of nodulation by A804 was due to the *nodE* mutation and that the *nodX* gene (required for nodulation of 'Afghanistan') had not been affected by the introduction of the *nodE68::Tn5* mutation.

'Rondo' pea formed nodules when inoculated with the *nodE* mutant A804 (Fig. 4B) and there are enough nodules to determine whether introgression of the *sym2^A* region into Rondo further reduced nodulation by the *nodE* mutant. An introgression line of Rondo carrying *sym2^A* has been generated (Gualtieri et al. 2002; Kozik et al. 1995) and this line showed clear *nodX*-dependent nodulation (Fig. 4C), confirming that this line is homozygous for *sym2^A*. The *nodE* mutant formed almost no nodules with this Rondo-*sym2^A* introgression line, which was nodulated similarly to 'Afghanistan' in that very few nodules were formed by *R. leguminosarum* bv. *viciae* *nodX* or *nodE* mutants. This shows that the *nodE*-dependent nodulation phenotype was co-inherited with *sym2^A*.

Given the co-segregation of the *nodX* and *nodE*-dependent nodulation, we tested nodulation of lines JI15 and JI1194 with strain TOM and its *nodX* and *nodE* mutant derivatives. Neither JI15 nor JI1194 showed *nodX*-dependent nodulation (Fig. 5), showing that they do not carry *sym2^A* and, therefore, the *nodE*-dependent nodulation cannot be determined by the *sym2^A* allele. As expected, mutation of *nodE* in strain TOM greatly reduced nodulation in JI15; the *nodE* mutant nodulated JI1194 at relatively high levels, although with a 2- to 3-day delay compared with the wild-type strain (Fig. 5B).

***nodE*-dependent nodulation is associated with a haplotype of *Sym37*.**

Because the *nodE*-dependent nodulation is co-inherited with but cannot be caused by *sym2^A*, we decided to compare the sequence of parts of *Sym37* and *PsK1* from different lines that are relatively well or very poorly nodulated by *R. leguminosarum* bv. *viciae* *nodE* mutants, to determine whether alleles of either *Sym37* or *PsK1* could cause the host-specific nodulation phenotype. First, we scored nodulation with different pea lines and found five that nodulated relatively well and six that nodu-

lated very poorly 21 days after inoculation with a *nodE* mutant of *R. leguminosarum* bv. *viciae*. In the low-nodulation group were JI15 (2 ± 2 nodules) (Fig. 1A), JI281 (2 ± 2 nodules), JI399 (1 ± 1 nodule), Finale (0 nodules), and 'Afghanistan' (1 ± 0.3 nodules) (Fig. 4A). Pea lines that nodulated relatively well 21 days after inoculation were JI1194 (20 ± 7 nodules) (Fig. 1A), Rondo (39 ± 18 nodules) (Fig. 4B), Frisson (23 ± 5 nodules), Sparkle (38 ± 6 nodules), Sprint-2 (61 ± 11 nodules), and SGE (38 ± 17 nodules).

We sequenced the DNA encoding the predicted LysM domains of *Sym37* and *PsK1* in lines JI15, JI281, JI399, and JI1194 and compared them with the LysM domains previously sequenced in the other pea cultivars (Zhukov et al. 2008). In that work, 10 amino-acid polymorphisms were identified in *Sym37* and 18 were identified in *PsK1*; we identified no additional polymorphisms in JI15, JI281, JI399, or JI1194. The polymorphic sites in *Sym37* and *PsK1* are shown in Figure 6A and B; the five pea lines with very low nodulation are grouped together in the upper half of each figure and those with relatively good nodulation are in the lower groups. It is evident that 6 of the 10 polymorphisms (Fig. 6A, highlighted in bold) in *Sym37* are consistently conserved among the low-nodulation group and are different from conserved residues in the relatively high-nodulation group. This pattern of conserved differences between the low- and relatively high-nodulation groups is not observed with *PsK1*; in fact, there is not one single polymorphism in *PsK1* that is both conserved in the upper group (very poor nodulators) and consistently different in the lower group (relatively good nodulators). Therefore, the six residues of *Sym37* highlighted in Figure 6A constitute a conserved haplotype that is highly associated with *nodE*-dependent host-specific nodulation of pea by *R. leguminosarum* bv. *viciae*.

Loss of function mutations in pea *Sym37* prevented nodulation and the block occurred at the level of infection thread initiation (Zhukov et al. 2008). We tried to determine where the symbiosis was blocked in pea lines JI15 and Finale that nodulated very poorly compared with those that nodulated relatively well (JI1194 and Rondo). Wild-type *R. leguminosarum* bv. *viciae* induced infection threads that could be visualized 7 days after spot inoculation onto lateral roots of JI15, Finale, JI1194, and Rondo; there were also infection foci that had not progressed to infections (Table 1). However, with the *R. leguminosarum* bv. *viciae* *nodE* mutant, no infection threads were observed on any of the four lines, although infection foci were observed (Table 1). Therefore, infection by the *nodE* mutant is significantly reduced in all lines but those nodules that were formed were pink and appeared to be normally infected. Therefore, although no infections were observed in these spot inoculation tests, it seems that some infections can occur but do so relatively infrequently; it may well be that the number of nodules observed corresponds to the total number of successful infections on the root system. However, determining significant differences in successful infection between the very poor and relatively good nodulators would be very difficult, and any

Fig. 5. Nodulation of **A**, JI15 and **B**, JI1194 inoculated with strain TOM (filled squares) and the *nodE* (open circles) and *nodX* (solid diamonds) mutant derivatives of TOM. Data are averages ($\pm$ standard deviation) of data from eight plants.

Table 1. Infection events observed with spot inoculation of different pea cultivars with wild type and the *nodE* mutant of *Rhizobium leguminosarum* bv. *viciae*^a

Pea cultivar	Wild type		<i>nodE</i>	
	Infection threads	Infection foci	Infection threads	Infection foci
JI15	1.75 $\pm$ 0.5	3 $\pm$ 1	0	4.25 $\pm$ 1.5
Finale	2 $\pm$ 0.8	2.5 $\pm$ 0.5	0	5.6 $\pm$ 2.1
JI1194	2.8 $\pm$ 1.5	1.5 $\pm$ 0.6	0	4.8 $\pm$ 2
Rondo	2.25 $\pm$ 1	2 $\pm$ 0.8	0	5.4 $\pm$ 1

^a Data shown are averages $\pm$ standard deviations.

observed differences may not be attributable to the *Sym37* region in view of the fact that these comparisons are being made between nonisogenic pea lines.

DISCUSSION

This work has shown that there is natural diversity among pea cultivars in their ability to be nodulated by strains of *R. leguminosarum* bv. *viciae* that make Nod factors containing a C18:1 rather than the *nodE*-determined C18:4 acyl group. In some cultivars, mutation of *nodE* almost completely abolished nodulation whereas, in others, there was relatively little effect on overall nodulation, with other cultivars showing intermediate effects. We have mapped a pea locus determining a strong *nodE*-dependent nodulation phenotype on linkage group I close to the *Sym2*, *Sym37*, and *PsK1* loci. By comparing the ability of the *R. leguminosarum* bv. *viciae* *nodE* mutant to nodulate several cultivars of pea, we could establish a strong association between very low nodulation and a haplotype of *Sym37* which determines six conserved amino acid polymorphisms. The closely linked *PsK1* locus showed no such association, strongly suggesting that the nodulation phenotype is determined by the *Sym37* haplotype.

Sym37 belongs to the family of receptor kinases that includes *L. japonicus* NFR1 and *M. truncatula* LYK3 (Zhukov et al. 2008). These contain three extracellular LysM domains, which

have been implicated in the binding of Nod factors (Radutoiu et al. 2007), based on the analyses of chimeric genes from *L. filicaulis* and *L. japonicus* which show different specificities for Nod-factor recognition (Radutoiu et al. 2007). This correlates with the predicted role for LYK3 as a candidate entry receptor based on the identified synergistic interactions between appropriately acylated Nod factors and weak alleles of *hcl* mutants of *M. truncatula* (Smit et al. 2007). The six polymorphic residues of *Sym37* that are associated with *nodE*-dependent nodulation are spread across the three LysM domains: glutamine/glutamate43, phenylalanine/serine60, and valine/isoleucine83 are within the first LysM domain; tyrosine/phenylalanine131 and arginine/serine181 are in the second LysM domain; and valine/isoleucine208 is in the third LysM domain. The polymorphic differences involving glutamate and arginine introduce charge differences, while the phenylalanine/serine and arginine/serine polymorphisms will substitute large residues in place of serine. Such substitutions could cause significant structural changes and, therefore, could account for different recognition specificities.

Natural variation in nodulation is probably quite widespread. In soybean, the loci *Rj2*, *Rj3*, *Rj4*, and *Rfg1* have been identified as blocking nodulation in a strain-specific manner (Caldwell 1966; Sadowsky and Cregan 1992; Vest 1970; Vest and Caldwell 1972). *Rj2* and *Rfg1* are allelic variants encoding different forms of a plant disease-resistance protein (Yang et al. 2010),

A Sym37															
J115	Q	S	V	F	V	Y	V	R	N	V					
J1281	Q	S	V	F	V	Y	V	R	N	V					
J1399	Q	S	V	F	V	Y	A	R	K	V					
Finale	Q	S	V	F	V	Y	A	R	N	V					
cv. Afgh.	Q	S	V	F	V	Y	V	R	K	V					
J11194	E	L	V	S	I	F	A	S	K	I					
Rondo	E	S	L	S	I	F	A	S	K	I					
Frisson	E	L	V	S	I	F	A	S	K	I					
Sparkle	E	S	L	S	I	F	A	S	K	I					
Sprint-2	E	S	L	S	I	F	A	S	K	I					
SGE	E	S	L	S	I	F	A	S	K	I					
	43	45	56	60	83	131	141	181	184	208					
B PsK1															
J115	F	A	L	V	PT	KNY	I	D	S	F	T	S	Q	S	I
J1281	F	A	L	V	PT	KNY	I	D	S	F	T	S	Q	S	I
J1399	F	A	L	I	AT	KNY	I	D	S	F	T	T	Q	S	I
Finale	F	A	L	V	PT	KNY	I	D	S	F	T	S	Q	S	I
cv. Afgh.	F	A	L	V	PT	KNY	I	D	S	S	T	T	Q	S	I
J11194	L	A	L	V	PT	KNY	I	N	S	F	I	S	Q	S	I
Rondo	L	A	I	V	SN	TTF	L	D	R	F	T	S	Q	S	I
Sparkle	L	A	I	V	SN	TTF	L	D	R	F	T	S	Q	S	I
Sprint-2	F	V	I	V	PT	KNY	I	N	S	S	T	T	Q	S	V
SGE	F	A	L	I	PT	KNY	L	N	S	S	T	T	Q	S	I
	13	17	31	38	45,46	48-50	55	61	65	81	104	124	157	175	208

Fig. 6. Amino acid sequence polymorphisms of the LysM domains of *Sym37* and *PsK1* from different pea cultivars. Sequences of the predicted LysM domains of **A**, *Sym37* and **B**, *PsK1* from pea lines J115, J1281, J1399, and J11194 were determined and the polymorphisms were aligned with previously identified polymorphisms (Zhukov et al. 2008) in other pea cultivars. Only polymorphic residues are shown, and these are numbered relative to the translation start as described previously (Zhukov et al. 2008). The five pea varieties (J115, J1281, J1399, Finale, and 'Afghanistan') that are nodulated very poorly by the *Rhizobium leguminosarum* bv. *viciae* *nodE* mutant are grouped together in the upper parts of A and B, and the pea varieties (J11194, Rondo, Sparkle, Sprint-2, and SGE) that nodulate relatively well by the *nodE* mutant are in the lower groupings. Residues in bold correspond to those that are consistently different between the lines which nodulate very poorly and those which nodulate relatively well with the *R. leguminosarum* bv. *viciae* *nodE* mutant.

although the effector binding to these leucine-rich-repeat domain proteins remains to be identified. Several mutations affecting the bacterial membrane structure have been shown to overcome the nodulation resistance conferred by the soybean *Rj4* locus (Tsurumaru et al. 2008) but the cause of the strain selectivity is not understood. In *Vicia faba* (Essermonning et al. 1995), two loci conferring strain-specific nodulation were found and one locus was identified in clover (Lewis-Henderson and Djordjevic, 1991). Of these, only the latter has been correlated with Nod-factor decorations. Common bean is preferentially nodulated by different strains of *R. etli* which can be distinguished on the basis of the types of *nodC* genes they contain; this selection is correlated with the induction of a gene encoding a C-subunit of a heterotrimeric nuclear factor, which appears to act at the level of infection and nodule development, possibly by activating the cell cycle in response to Nod factors (Zanetti et al. 2010).

A picture is emerging of a group of predicted Nod-factor receptor-like genes around the syntenic region marked by *Sym2*, *Sym37*, and *PsK1* in pea and the *LYK3/HCL* region of *M. truncatula* (Gualtieri and Bisseling 2002; Gualtieri et al. 2002; Limpens et al. 2003; Smit et al. 2007; Zhukov et al. 2008). Gene duplication and divergence could lead to changes in recognition specificity, leading to selection of different rhizobial strains (Lohmann et al. 2010). With the pea *Sym37* locus, it appears that changes affecting the LysM-like extracellular recognition domains may be able to compensate for the lack of infection that is normally observed with the *R. leguminosarum* bv. *viciae* *nodE* mutant. This *nodE* mutation causes the production of only C18:1-substituted Nod factors rather than a mix of C18:1 and C18:4 Nod factors (Spaink et al. 1991). The role of the C18:1-substituted Nod factors is not clear; they are present at significant levels in very different strains of *R. leguminosarum* bv. *viciae* (Firmin et al. 1993; Spaink et al. 1991). One possibility is that the C18:1 and C18:4 substituted Nod factors could act synergistically; cooperative activity between different Nod factor-like structures has been noted previously in soybean (Minami et al. 1996a,b). If this is the case, then it could be mediated by specialization of different but related Nod-factor receptors, and this could allow different recognition specificities to evolve within one species. Evolutionary changes in recognition between mutualistic partners have been proposed to be a mechanism of maintaining sanctions that select against potential partners that establish less beneficial symbioses (Kiers and Denison 2008).

Two mechanisms could contribute to changes in recognition specificity. One is changes in specificity of individual Nod-factor receptor genes, and that seems to have occurred (Bek et al. 2010; Radutoiu et al. 2007). Therefore, it is reasonable to conclude that the specificity is determined by the *Sym37* haplotype. However, this does not rule out the possibility that there could be different assortments of related gene products in a single recognition complex. Possibly such diverse assortments could affect nodulation efficiency.

MATERIALS AND METHODS

Bacterial strains and growth.

R. leguminosarum bv. *viciae* strain A34 and the derivative of it carrying the *nodE68::Tn5* allele were described previously (Downie et al. 1985). Strain TOM (Winarno and Lie 1979) carrying the *nodE68::Tn5* allele was made by recombination. First, cosmid pIJ1095 (carrying the TOM *nod* gene region) was conjugated into the A34 mutant carrying the *nodE68::Tn5* allele, which was recombined onto pIJ1095 by preparing DNA and selecting for neomycin- and tetracycline-resistant transformants of *Escherichia coli* DH5 α . The resulting plasmid,

pIJ9288, was confirmed to contain the *nodE* allele by digesting the DNA with *EcoRI*; only one band was altered compared with pIJ1095, and the band of 4.3 kb was replaced with a band of 10 kb, corresponding to the expected size after insertion of Tn5. The position of Tn5 was determined by amplifying DNA fragments with the 5' and 3' *nodE*-specific primers 5'-CACCG ATGCTTCCTCTATCTGGAC-3' (nucleotides 21 to 37) and 5'-AACGCAAGGACAGCATTTCAT-3' (nucleotides 1,977 to 1,996), respectively, together with the Tn5 primer 5'-CGTTAC CATGTTAGGAGGTC-3'. DNA sequencing revealed the Tn5 insertion to be 208 nucleotides downstream from the predicted translation start codon. The *nodE* mutation was then recombined from pIJ9288 into a streptomycin-resistant derivative of strain TOM selecting for neomycin-resistant and tetracycline-sensitive recombinants by plasmid incompatibility, as described previously (Downie et al. 1985). Plasmid pIM186 describing the cloned *nodFE* gene region was described previously (Surin and Downie 1989). Strain TOM carrying a *nodX* mutation (*nodX24::Tn3HoHo1*) was described previously (Davis et al. 1988). Bacteria were grown on TY medium (Beringer 1974) and conjugations from *E. coli* to *R. leguminosarum* bv. *viciae* and other microbial methods were as described previously (Downie et al. 1985). Plants were inoculated with approximately 10⁶ freshly grown bacteria.

Pea lines and inoculation.

Most of the pea lines used, including the RI progeny from the cross of JI15 $\times$ JI 1194 (Ellis et al. 1992) and lines SGE (JI3023, Finale (JI2678), Sprint2 (JI2612), Rondo (JI1502), and Frisson (JI2491), were obtained from the *Pisum sativum* germplasm collection curated by M. Ambrose at the John Innes Centre. The line of Rondo carrying the *sym2^A* allele from 'Afghanistan' was line A.5.4.3 (Gualtieri et al. 2002). Pea seedlings were germinated on water agar and grown in flasks as described previously (Beynon et al. 1980). For tests with the RI lines, germination rates were relatively variable and, therefore, plants were inoculated at the same developmental stage, when the stipule on the first true leaf was long enough to come through the foam bung in the 250-ml Erlenmeyer flask. Nodulation was scored either three times per week or 21 days after inoculation, as described in the text. For spot-inoculation experiments, pea plants were grown on agar plates and inoculated with *R. leguminosarum* bv. *viciae* or the *nodE* mutant derivative carrying the *lacZ* plasmid pXLGD4 (Leong et al. 1985). The bacteria were resuspended in water to an optical density of 0.5 and approximately 0.2 μ l were inoculated onto a marked site on lateral roots, generated after excision of the main root on germinated seedlings. Infection events were stained as described previously (Walker and Downie 2000), except that 0.5-cm sections of root around the inoculation site were examined. The numbers of infection events shown are averages and standard deviations determined using at least 20 such root sections per assay.

Genetic mapping, generation of markers, and DNA sequencing.

The JI15 $\times$ JI1194 RI genetic map described previously (Ellis et al. 1992) has had additional dominant markers added: they are Tps1/xx (Ellis et al. 1998) and spmxx (Knox and Ellis 2002), where xx is the marker number followed by either + (a JI15 marker) or - (a JI1194 marker). JoinMap 3.0 (Van Ooijen and Voorrips 2001) and Thread Mapper (Cheema et al. 2010) were used to construct the RI map of JI15 $\times$ JI1194 using 49 individuals and 228 markers. QTL analysis, using interval mapping and the associated permutation testing to determine the LOD score significance threshold, was carried out using MapQTL 5 (Plant Research Intl., Wageningen, The Netherlands).

To generate polymorphic markers for *Sym37*, a 443-bp fragment spanning intron 4 was amplified using oligonucleotides 5'-TATTGCGCTTCTGTTATTTGTTATCT-3' and 5'-ACTACCGGTACCATGCCACTG-3', which were designed based on published pea genome sequence of exons 4 and 5 in GenBank (EU564096). The fragment was amplified from genomic DNA of both JI15 and JI1194 using the following polymerase chain reaction (PCR) conditions: 95°C for 5 min; then, 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; followed by 72°C for 10 min to complete the extension. The resulting products were checked by agarose gel electrophoresis, the fragments of expected size (443 bp) were excised and purified using a Qiagen (West Sussex, U.K.) gel extraction kit, and their sequences were determined with both primers using the Big Dye kit version 3.1 and the 3730XL Sequencer (Life Technologies Ltd., Paisley, U.K.). The sequences were deposited in the EMBL database as FR750007 (JI15) and FR750008 (JI1194). For mapping using the RI lines, genomic DNA from each line was amplified with the same primers and the products were digested with *MseI* (New England Biolabs, Hertfordshire, U.K.) using the conditions and buffer supplied by the manufacturer and analyzed by gel electrophoresis.

To generate polymorphic markers for PsK1, fragments of 656 and 653 bp were amplified from JI15 and JI1194 using primers 5'-GCAAGCATCATCAGAATTTCTG-3' and 5'-ACTTCAATAAGTTTGGTCAAGCC-3', which were designed to span introns 6, 7, and 8 using sequences from exons 6 and 8 in the published pea genomic sequence (EU564091) of PsK1. The PCR conditions and DNA sequence analyses were as described above for *Sym37*, except that the annealing temperature was 55°C rather than 60°C and no gel purification step was needed. The DNA sequences were deposited as FR750009 (JI15) and FR750010 (JI1194). Polymorphic markers for PsK1 were scored by sequencing PCR products generated from the parental and RI lines.

The DNA fragments encoding the predicted extracellular LysM-containing regions of *Sym37* and PsK1 were amplified by PCR and sequenced with the primers TTCATGAACTCAAAAATG and GAAGAAAAACATACATACCTC (for *Sym37*) or TATTGACCAAATATGAACTA and CTATAAAGCATTC AACATGAG (for *PsK1*) using the conditions described above. The first primer of each pair binds to the 5' ends of the genes and the second primer of each pair binds to the 5' end of the first intron of *Sym37* and the second intron of *PsK1*. The DNA sequences were submitted to the GenBank database under accession numbers JF921200 to JF921203 (for *Sym37*) and JF921204 to JF921207 (for *PsK1*).

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