FINE MAPPING OF THE *SYM2* LOCUS
OF PEA LINKAGE GROUP I

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Statements

1. The different responses in legume roots to *Rhizobium* Nod factors are induced through more than one recognition mechanism.

   Ardourel et al., 1994, The Plant Cell 6: 1357-1374

2. Chen et al. have not shown that hydrogen peroxide acts as a second messenger in systemic acquired resistance induced by salicylic acid in tobacco plants.

   Z. Chen et al., 1993, Science 262: 1883-1886

3. The contradictory results of Tuerck & Fromm and Grotewold et al. on the elements of the maize A1 promoter required for transactivation by the phlobaphene P regulatory gene can be attributed to the different in vitro systems used by the two groups.

   J.A. Tuerck & M.E. Fromm, 1994, The Plant Cell 6: 1655-1663
   E. Grotewold et al., 1994, Cell 76: 543-553

4. LeGal and Hobbs have wrongly assumed that rhizobia induce the formation of nodule primordia in incompatible interactions with *sym2* containing pea plants.

   M.F. LeGal and S.L.A. Hobbs, 1989, Can. J. Bot. 67: 2435-2443

5. The apparent discrepancy in results of Reuber & Ausubel and Ritter & Dangl on the interference between the RPS2- and RPM1- mediated resistance responses stimulates new lines of thought in the plant-pathogen interaction field.

   T.L. Reuber & F.M. Ausubel, 1996, The Plant Cell 8: 241-249
   C. Ritter & J.L. Dangl, 1996, The Plant Cell 8: 251-257

6. Whereas positively acting intermediates are essential to drive signal transduction, negative elements are responsible for ensuring a response that is quantitatively appropriate, correctly timed and highly coordinated with other activities of the cell.

   C. Bowler and Nam-Hai Chua, 1994, The Plant Cell 6: 1529-1541

7. What Bateson and Pellew have said in 1915; "The main problem remains unsolved and the work is still in progress, but the facts already established are so unusual that it seems desirable to make them generally known." still applies to the science of genetics.

   Bateson and Pellew, 1915, Journal of Genetics vol. V: 13-36

8. "Seek not what is too difficult for you, nor investigate what is beyond your power. Reflect upon what has been assigned to you, for you do not need what is hidden. Do not meddle in what is beyond your tasks, for matters too great for human understanding have been shown you. For their hasty judgment has led many astray, and wrong opinion has caused their thoughts to slip."

   The Apocrypha; Sirach, 3: 21-24

Statements from the thesis entitled:
"Fine mapping of the sym2 locus of pea linkage group I"
Alexander Kozik, Wageningen, 7 June 1996.

CONTENTS

The symbiotic interaction between *Rhizobium* bacteria and leguminous plants results in the formation of root nodules which are specific, nitrogen-fixing organs that supply the plant with ammonia required for its growth. The formation of a nitrogen-fixing root nodule involves a complex series of steps requiring the expression of genes in both the rhizobial symbiont and the host plant. The necessary genes of rhizobia for nodulation, the *nod* genes, and for nitrogen fixation, the *fix* and *nif* genes, are well studied, nearly all of them having been cloned and characterized. In contrast the symbiosis genes of the legumes are not well understood. A number of plant genes which are specifically expressed in nodules or display enhanced expression in nodules, the so-called nodulin genes, have been identified in pea, soybean, alfalfa and other legumes by mRNA analysis and cDNA cloning. The time of expression of several nodulins has been analyzed in a number of cases and the nodulin genes which are expressed shortly after infection and in the first steps of nodule formation are referred to as early nodulins (ENODs).

In addition, a large series of naturally occurring and induced plant mutants, the *sym* mutants, which have an altered symbiosis, have been described but the *sym* genes have not been characterized and the functions of the proteins encoded by these genes are not known. Since it seems a reasonable assumption that there will be a limited number of genes involved in nodule formation and metabolism it might well be that some of the *sym* mutants represent defects in nodulin-coding or controlling sequences. The study presented in this thesis is centred on one of the *sym* genes, *sym2*, as we suspected that the *sym2* gene has a role in the first interaction between the *Rhizobium* bacteria and the legume host plant. A typical characteristic of the *Rhizobium* legume symbiosis is the host specific nature. Most *Rhizobium* species can nodulate only plants of a specific plant genus. In the recently past years it has been demonstrated that in the *Rhizobium* bacteria the *nod* genes are responsible for the strong specificity in the bacteria-plant interaction. The *nod* genes are involved in the synthesis of specific lipo-oligosaccharides called Nod factors, that can induce the early responses in host plants leading to root nodule formation. Nod factors with a very specific structure are required to induce these responses and they are active at very low concentrations. Therefore it is probable that, as a first step in inducing the reactions leading to nodule formation, the Nod factors are recognized by a special receptor in the host plant. There are now several reasons to presume that the plant *sym2* gene might encode the receptor for the specific recognition of Nod factors. The aim of the study presented in this thesis is a further characterization of the interaction between *sym2* containing pea lines and different strains of *Rhizobium leguminosarium* bv. *viciae* in an attempt to find further support for this hypothesis. At the same time, we have started the detailed genetic mapping of *sym2* on the pea genome, in preparation for eventually cloning and molecular characterization of

sym2.

In chapter 1 a general introduction summarizes the history of the discovery of the *sym2* gene and presents the arguments for the hypothesis that *sym2* might code for a receptor of rhizobial Nod factors. In chapter 2 a further genetic characterization of *sym2* is given. Chapter 3 describes how a novel early nodulin gene of *PsENOD7* was characterized and mapped near the *sym2* locus. In chapter 4 a detailed map of the *sym2* locus linkage group I is presented including some new molecular markers tightly linked to the *sym2* locus. In chapter 5 it is demonstrated how the *Rhizobium, nodO* gene determines whether *sym2* is a dominant or a recessive allele. The thesis ends with some concluding remarks on the nature of *sym2* and the impact of the research described in the thesis for the cloning of *sym2*.

CHAPTER 1

GENERAL
INTRODUCTION

Introduction

The interaction between leguminous plants and *Rhizobium* bacteria results in the formation of root nodules in which the bacteria are hosted intracellularly and are able to fix (reduce) atmospheric nitrogen into ammonia. This process is known as symbiotic nitrogen fixation.

The formation of root nodules involves several steps. It starts with colonization of legume roots by *Rhizobium* bacteria, which induces deformation and curling of root hairs. Bacteria entrapped in the curls of the hairs effect a local hydrolysis of the cell wall and at these sites formation of an infection thread is initiated that grows towards the base of the root hair containing epidermal cell. Concomitantly, cortical cells are activated and start to divide, leading to the formation of nodule primordia. The infection threads grow towards these primordia and, while bacteria are released into the cytoplasm, a nodule meristem is formed and the primordia develop into highly differentiated nodules.

A typical characteristic of the legume-*Rhizobium* symbiosis is its host-specific nature. For example, *Rhizobium leguminosarum* bv *viciae* (*Rlv*) can nodulate pea and vetch, but is unable to nodulate alfalfa, whereas *Rhizobium meliloti* can nodulate alfalfa but not pea and vetch. The host specificity is determined by a mutual recognition of signal molecules produced by plant and bacteria. Specific flavonoid compounds secreted by the plant host activate the expression of rhizobial *nod* genes. The signalling molecules then produced by rhizobia are lipo-chito-oligosaccharides named LCO or Nod factors [Lerouge et al., 1990; Spaink et al., 1991]. The Nod factors of most rhizobia have been characterised and they all appear to consist of an acylated chitin oligomer that can contain species-specific modifications which determine the host specificity of the *Rhizobium*-legume interaction. The rhizobial *nod* genes encode enzymes that are involved in the process of chitin synthesis, fatty acid biosynthesis, and chitin modification. Through their essential role in the biosynthesis of the Nod factors, the *nod* genes are major mediators of the host specificity of nodulation.

Nod factors induce reactions in the root that correspond to the early steps of nodulation, such as root hair deformation and cortical cell divisions and they are active at very low concentrations (10^{-12}M) [Spaink et al., 1991; Denarie and Cullimore, 1993]. Furthermore, only molecules with a specific structure are active in a certain host. Therefore it has been postulated that Nod factors are recognized by a host receptor [Denarie and Cullimore, 1993; Ardourel et al, 1994].

At present, one of the major research interests in the *Rhizobium*-legume

studies is to explain the mechanism of Nod factor perception. Nod factor binding proteins may in principal be identified by a biochemical or by a genetic approach. Recently Bono et. al. [1995] reported on the isolation of a Nod factor binding protein, but the protein concerned did not have the expected specificity and also the binding constant was rather low. Therefore it seems unlikely that this binding protein is a real Nod factor receptor.

A genetic approach on the other hand was hampered by lack of genetically well characterised plant material. In this thesis I shall describe the characterisation of the *sym2* allele of Afghanistan pea that encodes a putative Nod factor receptor. Hopefully the genetic characterisation of this locus as presented in this thesis paves the way for the cloning of this gene. In the following I will first describe, how *sym2* has been discovered, what is known about the *sym2* phenotype at present and why it most likely is a putative receptor for Nod factor.

Pea genetics

Pisum sativum (pea) is a self-pollinating, diploid plant, containing seven chromosome pairs [Cannon, 1903], and therefore it is a very suitable plant for genetic studies. Actually Mendel discovered the basic laws of heredety (Mendel's laws) more than hundred years ago using pea [Mendel, 1866]. The first linkage between morphological markers in pea was observed by De Vilmorin and Bateson [1912]. Later mapping studies revealed seven linkage groups corresponding to the seven different chromosomes [Lamprecht, 1948]. Since the cytogenetics of pea, at present, is rather poorly developed and the frequency of translocations is relatively high, the correlation between linkage groups and chromosomes is still ambiguous. A more detailed genetic map was constructed by Lamprecht [Lamprecht, 1974] and was further improved by Blixt [Blixt, 1974 and 1977]. This map, based on many crosses between different pea lines, contains the relative positions of morphological markers only, since a molecular-genetic analysis was not available at that time. More recently, some molecular markers were added to the classical map [Weeden and Wolko, 1990; Weeden et al., 1993] and an RFLP map has been constructed by Ellis [Ellis et al., 1992, 1993]. The latter map derived from the single cross (JI281 x JI399), gives the precise position of about one hundred cDNA markers, but contains only very few morphological markers.

Plant genes involved in symbiosis

Using direct genetic analysis of plant mutants, it has been found that legume nodule formation requires the expression of quite a series of different genes in the host plant. These genes are in general named symbiotic (*sym*) genes. Furthermore, plant genes that are specifically expressed during nodulation, the nodulin genes [Van Kammen 1984], have been identified by molecular methods. In most cases the precise functions of nodulins are not known neither to what extent they are essential for normal nodule development [Franssen, 1992a].

Mutagenesis has especially been successful with pea and many pea mutants were obtained which are disturbed in nodule development or functioning. By chemical mutagenesis and X-ray irradiation about 30 *sym* genes of pea were identified [Duc and Messager, 1989; Kneen et al, 1994]. In addition to the induced mutants, natural variants have been found which has led to the identification of the *sym1*, *sym2*, *sym3*, *sym4*, *sym6* and *sym22* genes [Lie, 1984b; LaRue and Weeden, 1992].

The different *sym* mutants (*sym, nod, brz*) are listed in Table I. Mutations resulting in an increase of the number of nodules are named *nod* mutations. Some symbiotic mutations have a pleotropic effect and are named after a visible trait (i.g. *brz-* small bronze spots on the pea leaves). For the *sym, brz* and *nod* mutants it is known that they are not allelic to each other. For the other mutants it has not been tested whether they are allelic to the previously described *sym/nod* genes. Unfortunately, the phenotype of only a few mutants has carefully been characterised. Among the best studied mutants are P6, P53-P57, DK24 [Sagan et al., 1994]. Of these mutants it is known that they are not allelic to each other, but it is not determined whether they are allelic to the previously described *sym/nod* genes.

Mutations in the *sym* genes have sofar resulted in the following phenotypes;
1. no or a few nodules (Nod⁻/few nodules)
2. dramatic increased nodule number (Nod⁺⁺⁺)
3. the formation of nodules in which nitrogen fixation does not take place (Fix⁻).

Mutations in *sym* genes that are most likely involved in perception (or transduction) of Nod factors will result in no or a few nodules, and null mutants (complete loss of function), most likely, will have lost the ability to deform (Had) and to curl (Hac) root hairs, to form infection threads (Inf) and to induce the formation of nodule primordia (Noi).

At present it can not be concluded which *sym* gene(s) code(s) for a Nod factor

receptor. In most cases only a single mutant of a *sym* gene has been isolated and so it is not known whether this mutation has caused a partial or a complete loss of function (null allele). Hence also *sym* genes, of which a mutant has been isolated that still produces some (a few) nodules, can encode a component of the Nod factor perception or transduction machinery. To determine whether a gene is involved in Nod factor perception or signal transduction, it is essential to obtain insight in the Nod factor activated signal transduction pathways. This knowledge can subsequently be used to determine whether a *sym* mutant is disturbed in a step of Nod factor perception or transduction. For example, it has been shown that like *Rhizobium, E.coli* strains producing cytokinin induce cortical cell division in alfalfa roots [Cooper and Long, 1994]. Assuming that Nod factors activate a signal transduction pathway that results in an increased cytokinin level, it should be possible to induce cortical cell divisions with cytokinin in a Nod⁻ mutant if the mutated gene encodes a protein functioning in Nod factor perception or signal transduction upstream of cytokinin. Thus, further studies elucidating the signal transduction pathways involved in nodulation as well as the obtaining of more alleles of a *sym* gene are required to determine which *sym* gene is involved in perception or transduction of Nod factors.

Of the characterised pea *sym* genes only one gene, namely *sym2* has been shown to play a role in the interaction with Nod factor. Pea lines containing *sym2* can be nodulated only by *Rlv* strains carrying an additional nodulation gene, *nodX*. Since these rhizobia secrete two additional Nod factors, it has been postulated that *sym2* is a Nod factor receptor [Heidstra et al., 1993]. In the following part of this introduction I will give an overview of our knowledge on *sym2* and *nodX*.

Discovery of *sym2*

Cultivated pea lines (*Pisum sativum* L.) have very little genetic variation with regard to the symbiotic response to *Rhizobium leguminosarum* bv *viciae*, while a large genetic variation occurs in wild and primitive pea plants [Lie, 1987].Wild and primitive forms of peas can be found in a region with the Middle East as its centre, including areas of Central Asia, the Mediterranean and Ethiopia. Because these gene centres are very rich in genetic variations they are important as a genetic source for plant breeding [Vavilov, 1951].

Table I. Genetic loci and mutants of pea involved in symbiosis

Symbiotic locus or mutant	Linkage group	Phenotype comments	Name and number of pea variety	Used *Rlv* strains	Reference
sym1 (wt)	I	*sym1* is *sym2*	Iran	PRE, RF2 248, TOM	Lie, 1971a,b 1984b Kozik et al., 1995
sym2 (wt)	I	Nod$^-$ (Hac$^+$, Inf$^-$, Noi$^+$) *nodX* depended nodulation	Afghanistan	PRE, RF2 248, TOM	Firmin et al., 1993; Kozik et al., 1995
sym3 (wt)	nd	Fix$^-$	Afghanistan	commerc. inoculum	Holl, 1975
sym4 (wt)	nd	strain-specific nodulation Nod$^-$ with *Rlv* 310a	*P. humile* (JI261)	PF2, PRE, 310a	Lie, 1984b
sym5 (EMS) (γ-rad.)	I	Nod$^-$ (Hac$^+$, Inf$^+$, Noi$^-$) temperature-sensitive nodulation ethylene- mutant	Sparkle E2, E77, E143; Sparkle R88	128C53	Kneen and LaRue, 1984b Weeden et al., 1990
sym6 (wt)	nd	Fix$^-$ (strain-specific) cv. Afghanistan/*Rlv* strain F13	Afghanistan	F13	Lie and Timmermans, 1983; Lie et al., 1987
sym7 (EMS)	III	Nod$^-$	Sparkle E69	128C53	Kneen and LaRue, 1988 Kneen et al., 1994
sym8 (γ-rad.)	VI	Nod$^-$ (Hac$^-$, Inf$^-$)	Sparkle R25	128C53	Markwei and LaRue, 1992; Kneen et al., 1994
sym9 (γ-rad.)	nd	Nod$^-$ (Had$^+$, Hac$^-$, Inf$^-$)	Sparkle R72	128C53	Markwei and LaRue, 1992; Kneen et al., 1994
sym10 (neutron)	I	Nod$^-$	Sparkle N15	128C53	Kneen and LaRue, 1988 Kneen et al., 1994
sym11 (neutron)	VII	Nod$^-$	Sparkle N24	128C53	Kneen and LaRue, 1988 Kneen et al., 1994
sym12 (EMS)	nd	few nodules (Hac$^+$, Inf$^+$)	Rondo K5	PF2, PRE	Jacobsen, 1984 Postma et al., 1988a
sym13 (EMS)	VII	Fix$^-$	Sparkle E135F	128C53	Kneen et al., 1990b
sym14 (EMS)	II	Nod$^-$	Sparkle E135F	128C53	Kneen et al., 1990b
sym15 (EMS)	VII	few nodules	Sparkle E151	128C53	Kneen and LaRue, 1988 Kneen et al., 1994
sym16 (γ-rad.)	V	few nodules	Sparkle R50	128C53	Kneen and LaRue, 1988 Kneen et al., 1994
sym17 (γ-rad.)	nd	few nodules	Sparkle R82	128C53	Kneen and LaRue, 1988 Kneen et al., 1994
sym18 (EMS)	I	strain-specific nodulation	Sparkle E54	128C53	Weeden et al., 1990
sym19 (EMS) (NMU)	I	Nod$^-$ (Hac$^+$, Inf$^-$)	Rondo K24 Sparkle NEU5	PF2, PRE 128C53	Postma et al., 1988a Weeden et al., 1990
sym20 (γ-rad.)	nd	Nod$^-$	Sparkle R80	128C53	Kneen and LaRue, 1988 LaRue and Weeden, 1992
sym21 (EMS)	nd	few nodules	Sparkle E132	128C53	Kneen and LaRue, 1988 LaRue and Weeden, 1992
sym22 (wt)	II	few nodules	*P. humile* JI1794	128C53	LaRue and Weeden, 1992
*sym23** (EMS)	nd	Fix$^-$	Frisson P59	1007	Duc and Messager, 1989 Sagan et al., 1993
*sym24** (EMS)	nd	Fix$^-$	Frisson P60	1007	Duc and Messager, 1989 Sagan et al., 1993

Symbiotic locus or mutant	Linkage group	Phenotype comments	Name and number of pea variety	Used *Rlv* strains	Reference
*sym25** (EMS)	nd	Fix⁻	Frisson P61	1007	Duc and Messager, 1989 Sagan et al., 1993
*sym26** (EMS)	nd	Fix⁻	Frisson P63	1007	Duc and Messager, 1989 Sagan et al., 1993
*sym27** (EMS)	nd	Fix⁻	Frisson P12	1007	Duc and Messager, 1989 Sagan et al., 1993
*sym28** (EMS)	nd	Nod⁺⁺⁺ (hypernodulation)	Frisson 190F	1007	Duc and Messager, 1989 LaRue and Weeden, 1992
brz (EMS)	IV	few nodules	Sparkle E107	128C53	Kneen et al., 1990a
nod3 (EMS)	I	Nod⁺⁺⁺ (hypernodulation)	Rondo-nod3	PF2 128C53	Jacobsen and Feenstra, 1984; Temnykh et al., 1995
P1 (EMS)	nd	Nod⁻	Frisson P1	1007	Duc and Messager, 1989
P4 (EMS)	nd	Nod⁻	Frisson P4	1007	Duc and Messager 1989 Sagan et al., 1994
P6 (EMS)	nd	Nod⁻ (Had⁺, Hac⁻, Inf⁻, Noi⁻)	Frisson P6	1007	Duc and Messager, 1989 Sagan et al., 1994
P53 (EMS)	nd	Nod⁻ (Had⁻, Hac⁻, Inf⁻, Noi⁻)	Frisson P53	1007	Duc and Messager, 1989 Sagan et al., 1994
P54 (EMS)	nd	Nod⁻ (Had⁻, Hac⁻, Inf⁻, Noi⁻)	Frisson P54	1007	Duc and Messager, 1989 Sagan et al., 1994
P55 (EMS)	nd	Nod⁻ (Had⁺, Hac⁻, Inf⁻, Noi⁻)	Frisson P55	1007	Duc and Messager, 1989 Sagan et al., 1994
P56 (EMS)	nd	Nod⁻ (Had⁻, Hac⁻, Inf⁻, Noi⁻)	Frisson P56	1007	Duc and Messager, 1989 Sagan et al., 1994
P57 (EMS)	nd	few nodules (Hac⁺, Inf⁺, Noi⁺)	Frisson P57	1007	Duc and Messager, 1989 Sagan et al., 1994
DK24 (EMS)	nd	few nodules (Hac⁺, Inf⁺, Noi⁺)	Finale DK24	1007	Engvild, 1987 Sagan et al., 1994
FN1 (EMS)	nd	Fix⁻	Rondo FN1	PRE	Postma et al., 1990
Sprint-2-fix⁻ (EMS)	III	Fix⁻	Sprint-2-fix⁻	commerc. inoculum	Borisov et al., 1993 Rozov et al., 1993
k301 (EMS)	nd	Nod⁺⁺⁺ (hypernodulation)	Ramonskii77 k301	commerc. inoculum	Sidorova and Uzhintseva, 1992
k287 (EMS)	nd	Fix⁻	Ramonskii77 k287	commerc. inoculum	Sidorova and Uzhintseva, 1992
k1005m (EMS)	nd	Nod⁻	Ramonskii77 k1005m	commerc. inoculum	Sidorova and Uzhintseva, 1992
KN7 (EMS)	nd	Nod⁻ (Hac⁺)	nod3, KN7	PRE	Postma et al., 1988b

Abbreviation:
wt - wild type   EMS - ethylmethane sulphonate   NMU - nitrosomethylurea
neutron - fast neutron bombarding   γ-rad. - γ-radiation   * - provisional symbols
Nod⁻ - non nodulating phenotype   Nod⁺⁺⁺- hypernodulating phenotype
Fix⁻ - non nitrogen fixing nodules   Inf⁺ - developed infection threads
Had - root hair deformation   Hac - root hair curling   Noi - formation of nodule primordia
numeration of *nod* mutants started from *nod3*, it is unclear whether *nod1* and *nod2* exist [Gelin and Blixt, 1964; LaRue and Weeden, 1992]

Symbiotic properties of peas from Afghanistan were first studied by Govorov, who observed that certain peas from Afghanistan need *Rhizobium* strains from Afghanistan to form nodules [Govorov, 1928, 1937]. Lie reinitiated the studies on peas from Afghanistan and other countries of the gene centres in a search of naturally occurring variations in pea genes involved in symbiosis. He found several pea lines which upon inoculation with European *Rhizobium* strains are defective either in nodule formation or nitrogen fixation [Lie et al., 1976; Lie, 1978, 1981; Lie et al., 1982; Lie and Timmermans, 1983; Lie, 1984a]. One of the Afghanistan peas failed to form nodules with European *Rlv* strains [Lie, 1984b]. The disability of Afghanistan pea to form nodules upon inoculation with European *Rlv* strains was named nodulation resistance. This phenotype was assigned to the *sym2* locus.

Pisum sativum cv. Afghanistan, which will be named Afghanistan pea in the following part of this thesis, forms nodules with *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) strains from the Middle East (Afghanistan, Turkey, Israel), e.g. strain TOM [Winarno and Lie, 1979], but does not upon inoculation with European *Rlv* strains [Lie, 1978]. At the other hand strain TOM can nodulate European peas as well as Afghanistan pea.

Characterisation of *sym2*

Single genetic locus

By genetic analysis of the nodulation resistance of Afghanistan pea it was shown that the Nod⁻ phenotype of *sym2* upon inoculation with European *Rlv* strains is controlled by a single genetic locus. This conclusion was reached in independent experiments by several groups [Holl, 1975; Lie, 1984b; Kneen and LaRue, 1984a; Young 1985]. In all cases the genetic analyses were complicated by the occurrence of intermediate nodulating plants in the F2 segregating populations. For this reason it was suggested that the genetic background of Afghanistan pea affectes the *sym2* phenotype in segregating F2 individuals [Kneen and LaRue, 1984a; Young, 1985].

Is *sym2* a dominant or a recessive gene?

Studies on the recessive/dominant nature of *sym2* have given contradictory results. By using *Rlv* strain PF2 or a commercial inoculum it was shown that *sym2* is a recessive gene, whereas upon inoculation with *Rlv* strain PRE it was reported to be a dominant gene. However, it could not be excluded that the dominant resistance to

Rlv strain PRE is determined by another genetic locus than the recessive resistance to the other strains [Lie, 1984b].

Using molecular markers Kozik et al. [1995] confirmed that the *sym2* locus is responsible for the Nod⁻ phenotype in the interaction with *Rlv* strains PF2, 248 or PRE, and the dominant or recessive phenotype of *sym2* is determined by the *Rlv* strain. In the interaction with *Rlv* strain PRE *sym2* behaves as a dominant trait, whereas upon inoculation with *Rlv* strains PF2 and 248 *sym2* has a recessive nature [Kozik et al, 1995].

sym2 alleles

In search of additional naturally occurring pea genes conferring nodulation resistance, more than three hundred pea lines were tested for their nodulation ability upon inoculation with European *Rlv* strains [Young et al., 1982; Kneen and LaRue, 1984a; Lie, 1984b]. This resulted in thirty different Nod⁻ peas, most of which originated from Afghanistan. Out of the forty tested pea lines from Afghanistan, twenty four had a Nod⁻ phenotype. Pea lines from other areas were found to be Nod⁻ with a remarkable lower frequency, among thirty seven tested pea lines from Turkey only one was Nod⁻ upon inoculation with European *Rlv* strains [Young et al., 1982]. Ten of the thirty Nod⁻ pea lines described above, were characterised in more detail. In all cases the Nod⁻ phenotype was conferred by alleles of *sym2* [Young, 1985; Kneen and LaRue, 1984a]. Recently it was shown that the *sym1* gene of Iran pea is also a allele of *sym2* [Kozik et al., 1995]. Therefore in all pea lines for which the unability to form nodules upon inoculation with European *Rlv* strains has been characterised, the Nod⁻ phenotype is conferred by an allele of *sym2* . The distribution over the Middle East of pea lines, for which the *sym2* alleles were identified, is shown in Figure 1.

Mapping of the sym2 locus

The first genetic linkage analysis of *sym2* with pea morphological markers was made by Young in 1985. It was shown that *sym2* is located on linkage group I at a distance of 18 cM from *d*, a classical genetic (morphological) marker of pea controlling axillary pigmentation [Young, 1985].

The position of *sym2* on the genetic pea map was determined more precisely by Weeden, who showed that *sym2* is closely linked to the leghemoglobin (*Lb*) cluster on linkage group I near *d* and glutamine synthetase (*Gs*) [Weeden et al., 1990].
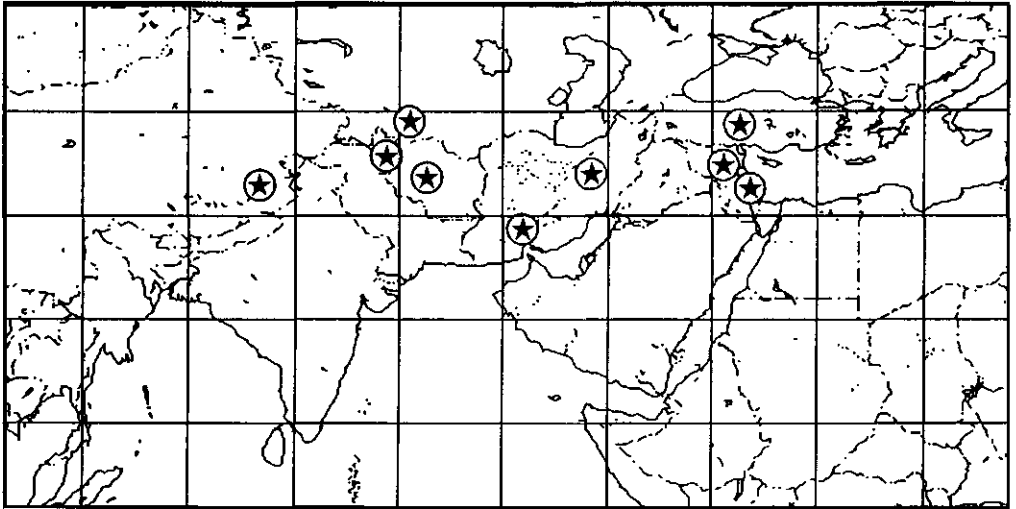
Figure 1. Distribution of the sym2 (asterisk) alleles.

Ellis et al. [1992, 1993] constructed an RFLP map with about one hundred molecular markers (cDNAs) and Kozik et al. [1995] showed that *sym2* is flanked by the markers cDNA44 and cDNA267 and maps close to the *Lb* cluster on linkage group I. Three genetic maps of pea linkage group I constructed by Blixt, Weeden and Ellis are present in Figure 2. Maps constructed by Blixt and Weeden are integrated maps, whereas the RFLP map constructed by Ellis is based on the single cross JI281xJI399.

Rhizobial *nod* gene(s) required to overcome *sym2* nodulation resistance

It was shown by Young that the capability of *Rlv* strain TOM to nodulate *sym2* containing pea lines has to be attributed to the symbiotic plasmid pRL5JI present in this *Rlv* strain [Young et al., 1982]. A comparision with the *nod* region of the well characterised *Rlv* symbiotic plasmid pRL1JI revealed that plasmid pRL5JI contains an additional *nod* gene, named *nodX* [Firmin et al., 1993]. *nodX* mutants have lost the

Figure 2 (see next page). Pea genetic maps of linkage group I constructed by Blixt, Weeden and Ellis. The position of the identical genetic loci on different maps are connected by dashed lines.

S.Blixt 1977   N.F.Weeden 1993   T.H.N.Ellis 1993

a
vi1
chi 3
chi4
chi 22
au
li
lf
miv
vac 1
chi 17
pra
y
rup
sru
d
Pur
Him
re
t
foe
fom
nap
di
cot
mie
lac
am
oli
cor
ins
af
par
sal
o
sb
red
i

C52b
crd
Aat-p
(Est-3)
(Mdh)
Lg-J (Lg-2)
P 114
a   (chi4)
au   (chi3)
lf   (Orc)

y (sym5)
sym 2
sym18
sym19
Lb
d   (rup, sru)
Idh
Pur   (Him, re)
Alat-c
t
(fom, nap
di, cot
mie,lac
oli, cor)
(par, sal, sb)
o
am-1
ms-7
C49c
af   aero
(sym10)
(ms-11)
i
Nco5

cDNA 186
cDNA 267
cDNA 40/3
cDNA 44
cDNA 164
Lb / 1
Gs-10/1
cDNA 40/8
cDNA 125/08
cDNA 150/1
cDNA 206
af
cDNA 76
i

a - blocks all anthocyanin synthesis
Aat-p - plastid aspartate aminotransferase
aero - separation of epidermal layer of leaf
    from subjacent cell layers
af - leaflets transformed into tendrils (afila)
Alat-c - cytosolic alanine aminotransferase
am - flowers white
au - seedlings reddish-yellow; lethal
C49c - RFLP marker
C52b - RFLP marker
chi - light green to yellowish green plant
cor - hilum region colored ochroceous
cot - reduces internode length
crd - waved leaflets and stipules toothed at base
d - absence of maculum ring
di - seeds with depressions in testa and cotyledons
Est-3 - esterase-3

foe - seeds with irregular, small depressions
fom - decreases foliage width
Gs - glutamine synthase
Him - hilum size one half of seed diameter
i - green cotyledons
Idh - isocitrate dehydrogenase
ins - leaflet apex deeply incised
lac - leaflets funnel-shaped; tendrils overdeveloped
Lb - leghemoglobin
lf - early flowering
Lg-J (Lg-2) - legumin, seed protein
Mdh - malate dehydrogenase
mie - decreases internode number
miv - affects distance between seeds
ms-11 - male sterility from breakdown of microsporogenesis
nap - petals of keel broader, winglike
Nco5 - RFLP marker
o - yellowish green foliage color
oli - seedcoat grayish olive
orc - cotyledons orange
P114 - RFLP marker
par - reduces seed weight
pra - 4 -11 sterile nodes to first flower
Pur - pods purple
re - anthers shortened; flowers budlike
red - leaflets very narrow
rup - pods with minute spots of anthocyanin
sal - seedcoat salmon colored
sb - leaves crimpled, rigid, with whitish wax
sru - pod with anthocyanin stripe along upper suture
t - thickens stem
vac 1 - foliage with sectors of different color and structure
y - reduces width of leaflets

ability to nodulate *sym2* containing peas, whereas introduction of *nodX* in *Rlv* strains is sufficient to overcome the nodulation resistance of *sym2* containing peas [Davis et al., 1988, Firmin et al., 1993]. *nodX*, most likely, encodes an *O*-acetyltransferase, since *Rlv* strains containing *nodX* produce pentameric Nod factors with an *O*-acetyl substitution on the reducing terminal sugar residue. The structure of the Nod factor, produced by the *Rlv* strain TOM, modified by NodX, is shown in Figure 3. Since *nodX* is required to overcome *sym2* nodulation resistance, it seems probable, that Nod factors modified by NodX interact with Sym2, and that Sym2 is a Nod*Rlv*-V(Ac,Ac,$C_{18:4}$ or $C_{18:1}$) factor receptor. Strikingly, *Rlv* strains carrying *nodX* maintain the ability to nodulate pea lines lacking *sym2* (Figure 4). This is most likely due to the fact that the *nodX* containing *Rlv* strains produce, in addition to NodX modified factors, also factors lacking the NodX specific modifications.
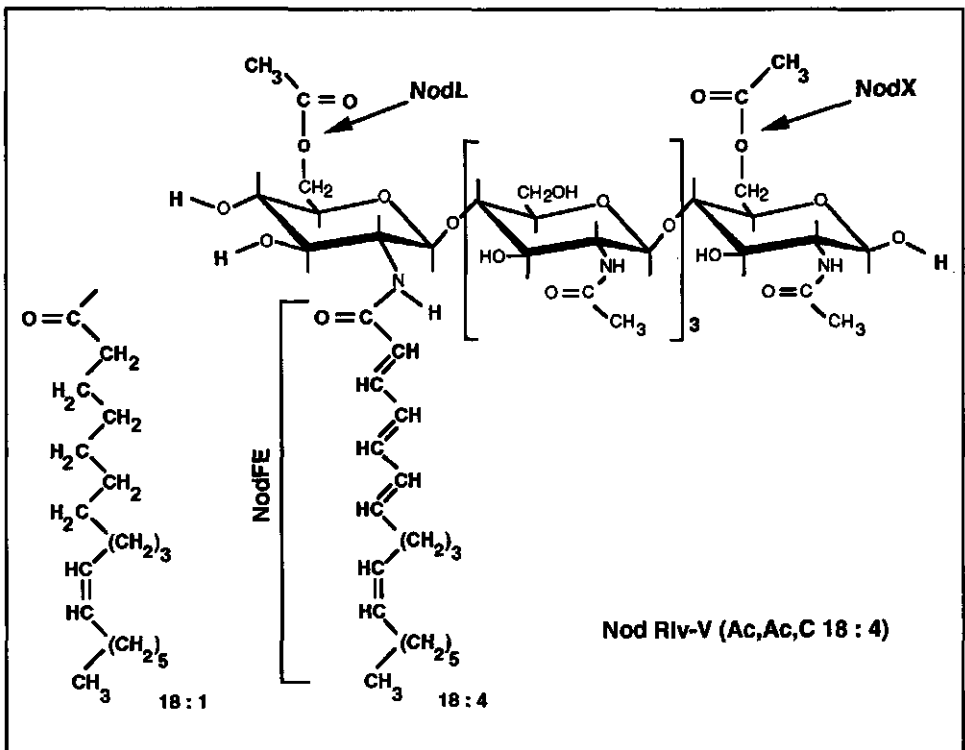

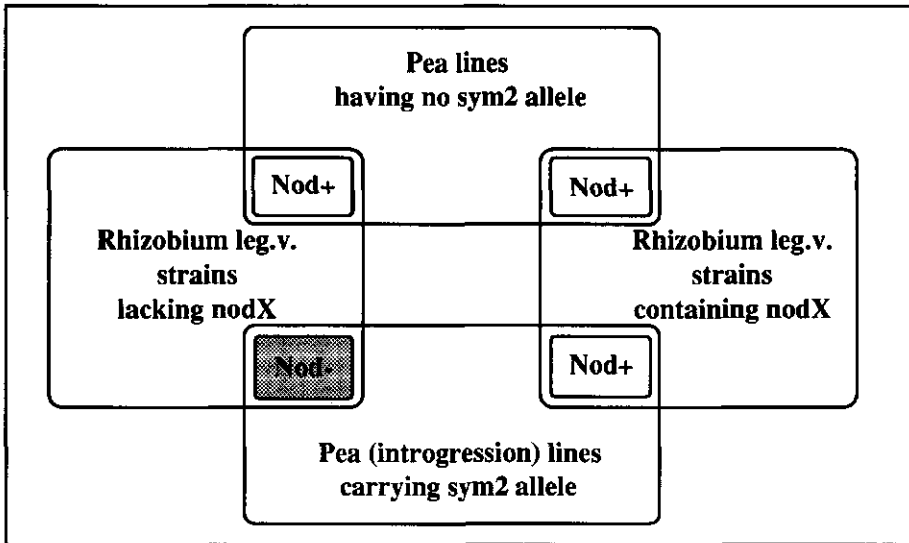Figure 3. Structure of the RlvNod factor modified by NodX.

Figure 4. Rlv strains containing nodX can nodulate pea lines carrying the sym2 allele as well as pea lines having no sym2. Incompatible interaction (Nod⁻) takes place only between pea lines carrying sym2 allele and Rlv strains lacking nodX.

Is Sym2 a receptor for Nod factors?

Nod factors have the ability to induce various responses in the epidermis and cortex of the roots of leguminous plants. It has been possible, using different *Rhizobium nod*-mutants, producing Nod factors with different structures, as well as using purified Nod factors, to determine the structural features of Nod factors that are essential for the induction of a certain response. Such studies on Nod factor structural-functional relationship has provided some insight in the receptors involved in Nod factor perception.

It was shown by Ardourel et al. [1994] that a *Rhizobium meliloti nodF/nodL* mutant induced root hair deformation on its host *Medicago sativa*, but infection threads did not develop. This mutant produces Nod factors with an $C_{18:1}$ fatty acid moiety and without an O-acetyl group at the non-reducing terminal sugar. At the other hand, wild type *Rhizobium meliloti*, producing O-acetylated Nod factors with a $C_{16:2}$ fatty acid moiety, can induce both root hair deformation and the formation of infection threads. Since root hair deformation is induced by the *nodF/nodL* mutant while infection thread formation is blocked, it was proposed by Ardourel et al. [1994]

that there are two different Nod factor receptors present in the root epidermis; a "signalling" receptor and an "entry" receptor. The differences in responses are a result of the existence of more than one type of Nod factor recognition mechanism at the root hair surface what is, most likely, due to presence of different types of receptors. According to this model (Figure 5) the "signalling" receptor is activated by Nod factors, even if the non-reducing end of the Nod factor lacks the O-acetyl group and has a $C_{18:1}$ fatty acid moiety instead of a $C_{16:2}$. The "entry" receptor is activated only by the O-acetylated Nod factors with $C_{16:2}$ fatty acid moiety and, this receptor is involved in the induction of infection thread formation.
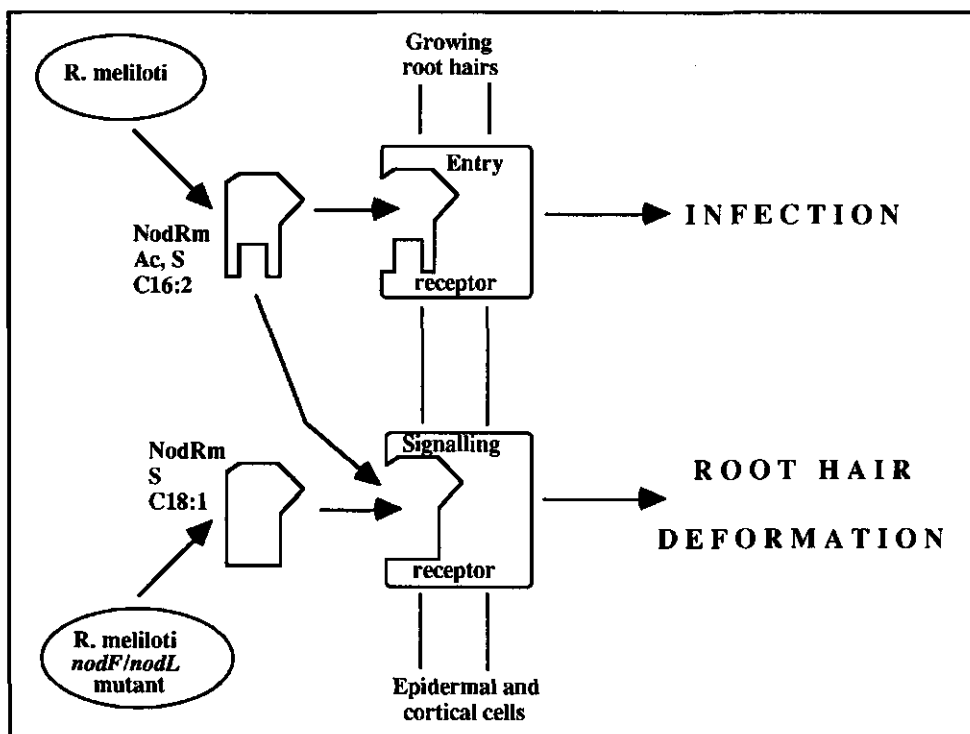
Figure 5. Two types of receptors for Nod factor (modified from Ardourel et al., 1994).
For detailed description of the model; see text.

Concerning the *sym2* phenotype we have to mention the following observations. Although *Rlv* strains lacking *nodX* are unable to form nodules on *sym2* containing pea lines, these bacteria are able to induce several responses in the

incompatible interaction. *Rlv* strains with or without *nodX*, induce on pea lines, containing *sym2*, root hair deformation, and also the early nodulin gene *PsENOD12* is induced in the epidermis to a similar level. However, the number of infection threads on *sym2* containing peas was severely reduced upon inoculation with *Rlv* strains lacking *nodX*. Moreover, the few infections that took place were aborted in the epidermis [Lie, 1984; Le Gal and Hobbs, 1989; Geurts, Hadri and Heidstra, unpublished]. In this respect, there is a striking similarity between the responses induced by the *Rhizobium meliloti nodF/nodL* mutant on alfalfa and those induced by *Rlv* strains lacking *nodX* on *sym2* containing peas. So, it is possible that *sym2* encodes a Nod factor receptor with similar characteristics as the proposed "entry" receptor. In this thesis the *sym2* locus of Afghanistan pea is characterised in more detail.

CHAPTER 2

PEA LINES CARRYING *SYM1* OR *SYM2* CAN BE NODULATED BY *RHIZOBIUM* STRAINS CONTAINING *NODX*; *SYM1* AND *SYM2* ARE ALLELIC

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ABSTRACT

In wild pea varieties two genes, *sym1* and *sym2*, have been identified that cause resistance to European *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) strains. The *sym2* gene has previously been studied in some detail and it was shown that the additional nodulation gene *nodX* is sufficient to overcome the *sym2* controlled nodulation resistance. Here we characterize the *sym1* gene. We show that the resistance conferred by *sym1* can be overcome by the introduction of *nodX* in European *Rlv* strains, indicating that *sym1* just as *sym2* is involved in Nod factor recognition. Both *sym1* and *sym2* display a recessive or dominant nature depending on the *Rlv* strain used for inoculation. Furthermore, introgression lines containing either *sym1* or *sym2* are able to form nodules with *Rlv* strain 248 at 26°C, but not at 18°C, indicating that both *sym1* and *sym2* have a temperature sensitive nature. *sym2* was mapped on the pea RFLP map. We found that *sym1* maps in the same region of linkage group I as *sym2*. By crossing *sym1* and *sym2* containing introgression lines we demonstrate that *sym1* and *sym2* are allelic.

Key words: sym1, sym2, RFLP map, *Rhizobium leguminosarum* bv.*viciae*, *nodX* .

INTRODUCTION

In *Pisum sativum* about 20 different genes have been described that are essential for *Rhizobium leguminosarum* bv *viciae* (*Rlv*) induced nodulation. These are the so-called *sym* genes. In general, mutations in the *sym* genes affect the interaction with all *Rlv* strains, but a few of the *sym* genes, e.g. *sym1*, *sym2* and *sym18* cause strain specific nodulation behaviour [Lie, 1978 and 1984b; Weeden et al., 1990]. *sym1* and *sym2* were first described in the early seventies as naturally occuring nodulation resistance genes of the primitive pea cultivars (cv.) Iran and Afghanistan, respectively [Lie, 1971a, 1971b, 1978; and 1984b]. Both genes are responsible for resistance to nodulation by European *Rlv* strains, but certain *Rlv* strains from the Middle East, like strain TOM, can nodulate Iran and/or Afghanistan peas [Lie, 1978 and 1984b].

After the discovery of *sym1* and *sym2*, several other wild pea cultivars from different geographic regions were examined for the occurrence of resistance genes to European *Rlv* strains. It was shown that such resistance genes occurred in the Middle East, i.e. Turkey, Israel, Irak, Iran, Afghanistan, but also in Tibet. Nine resistant pea lines, originating from these area, were studied in more detail and in all cases the resistance was conferred by alleles of *sym2* [Young et al, 1982; Young and Matthews 1982; Young, 1985; Kneen and LaRue, 1984a]. Thus, two resistance genes have been found in pea, of which *sym2* is widely spread, whereas *sym1* has only been detected in cv. Iran.

Both *sym1* and *sym2* behave in crosses as single (Mendelian) genes. Furthermore, *sym1* was shown to have a dominant character, whereas *sym2* was shown to be a recessive gene [Lie, 1984b; Kneen et al., 1984]. *sym2* was mapped on linkage group I, near the leghemoglobin (Lb) locus, and is located in a cluster with several other *sym* genes and nodule specific genes [Young, 1985; Weeden et al., 1990]. The map position of *sym1* has not been described.

An interesting characteristic unique to the *sym1* carrying Iran pea, is the temperature sensitive nodulation phenotype; Iran pea is unable to form nodules with the European *Rlv* strain PRE at 18°C, but it does at 25°C [Lie, 1971a and 1971b].

Rlv strain TOM has the ability to nodulate Afghanistan peas as well as European pea cultivars. This extended host range of strain TOM is due to an additional nodulation (*nod*) gene. The *nod* genes of rhizobia are involved in the synthesis of specific lipooligosaccharides, named Nod factors. The common *nodABC* genes are shared by all rhizobia and are involved in the synthesis of the core structure of Nod factors [Spaink, 1992; Denarie and Cullimore, 1993], which consists of a tetra- or pentamer of N-acetyl glucosamine with an acyl chain substitution at the non-reducing sugar moiety. In addition, each *Rhizobium* species has several *nod* genes that are involved in the introduction of substitutions at different positions in the Nod factors. Such substitutions have been shown to determine host specificity of *Rhizobium*-legume interactions [Spaink, 1992; Denarie and Cullimore, 1993]. The extended host range of *Rlv* strain TOM is conferred by *nodX*, a gene that has not been found in European *Rlv* strains [Gotz et al., 1985; Davis et al., 1988]. Recently, it was shown that NodX is an acetyl transferase that specifically acetylates the reducing sugar moiety at the C-6 position of the pentameric Nod factor [Firmin et al., 1993].

Nod factors have the ability to trigger early steps in nodulation, like root hair deformation and induction of nodule primordia [Spaink et al., 1991]. In certain legume species, even genuine nodule structures are formed after treatment with

purified Nod factors [Truchet et al., 1991; Stokkermans and Peters, 1994]. Hence, in understanding how *Rhizobium* elicits nodule formation, it will be of major importance to resolve the mode of action of Nod factors.

Pea lines containing *sym2* can be nodulated by *Rlv* strains harbouring *nodX*, whereas strains lacking *nodX* are in general unable to nodulate such peas. Therefore it seems that *sym2* is somehow involved in recognition of Nod factors and the characterisation of this gene might provide insight in the mechanism by which Nod factors are recognised by the host root. Since also *sym1* displays strain specific nodulation, it might be an additional gene involved in Nod factor recognition. With this idea in mind, we studied whether a specific *nod* gene could extend the host range of *Rlv* strains to *sym1* containing pea plants, and we examined the relation between *sym1* and *sym2*.

MATERIALS AND METHODS

Plant material

The pea lines we used in this study are described in Table 1. *sym1* introgression lines were obtained by backcrossing F1 plants of the cross Rondo x Iran three times with Rondo. After selfing, plants morphologically resembling Rondo, but with a Nod⁻ phenotype upon inoculation with *Rlv* strain 248, were selected. Two lines carrying *sym1* (I1.3.10 and I6.2.2) that had been selfed at least four times were used for further investigations. In a similar way two Rondo lines with an introgressed *sym2* region of Afghanistan pea were obtained (A5.4.3 and A5.6.9).

Table 1. Pea Lines

Pea lines	Relevant characteristics	Reference
Parental pea lines		
Afghanistan	*sym2*	[Lie, 1978]
Iran	*sym1*	[Lie, 1971b]
Rondo		[Lie, 1984b]
NGB1238 (=JI 73)		
Introgression lines		
L-4	*sym2*	this study
A5.4.3	*sym2*, 3 backcrosses	this study
A5.6.9	*sym2*, 3 backcrosses	this study
I1.3.10	*sym1*, 3 backcrosses	this study
I6.2.2	*sym1*, 3 backcrosses	this study

Line L-4 was obtained from the cross NGB1238 x Afghanistan. An F1 plant was selfed and among the F2 population a plant morphologically resembling NGB1238, but with a Nod⁻ phenotype upon inoculation with *Rlv* strain 248, was selected and named L-4.

Nodulation assays were performed in modified Leonard jars according to Lie [Lie et al., 1988].

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are described in Table 2.

To determine whether *nodX* is sufficient for nodulation of *sym1* or *sym2* containing plants we decided to clone *nodX* into an expression vector using PCR. Two oligo's spanning the coding region were designed for the *nodX* gene of *Rlv* strain TOM [Davis et al., 1988] (5'-TTCGGCGGCGGCTAATGAGA-3', 220 bp upstream of the translation start and 5'-GGGCTGTGGTGTCTGGGATG-3', 60 bp downstream of the translation stop). Total DNA was isolated from strain TOM essentially as described by Krol [Krol et al., 1980]. DNA (50 ng) was amplified during 25 cycles (94°C, 1 min; 60°C, 1 min; 72°C, 3 min) in a total volume of 50 ml amplification buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3) supplemented with 1 mM dNTPs, 2 mM $MgCl_2$, 50 ng of each oligo and 1 U *AmpliTaq* (Perkin Elmer, Cetus). The PCR product was cloned into Bluescript digested with *Sma*I and the insert was then recloned in the right orientation into the expression vector pMP1070 behind the "nod box". The resulting plasmid was named pMW1071.

Table 2. Strains and plasmids

Strains/Plasmids	Relevant characteristics	Reference
TOM	*Rlv* strain containing pRL5JI Nod⁺ on Afghanistan and Iran pea	[Brewin et al., 1980] [Winarno and Lie, 1979]
248	*Rlv* strain containing pRL1JI	[Josey et al., 1979]
PF2		[Lie, 1984b]
PRE		[Lie, 1984b]
248-X	strain 248 containing pMW1071	this study
PRE-X	strain PRE containing pMW1071	this study
PRE-D	strain PRE containing pMP280	this study
pRK2013	helper plasmid	[Ditta et al., 1980]
pMP280	*nodD* (pRL1JI) cloned in pMP92	[Spaink et al., 1987]
pMP1070	incP, expression vector	[Schlaman et al., 1992]
pMW1071	*nodX* cloned in pMP1070	this study

The plasmids pMW1071 and pMP280 were mobilized from *E.coli* to *Rlv* strains using pRK2013 as a helper plasmid. Selection of transconjugants was done on B⁻ medium [Spaink et al., 1991] with the addition of 2 mg tetracycline per liter.

TLC analysis of the Nod factors produced by different *Rlv* strains was performed according to Spaink [Spaink et al., 1992], using C_{18} reversed-phase TLC plates (Sigma) with acetonitrile:water (1:1) as a mobile phase.

RFLP analysis

Weeden showed that *sym2* is located on linkage group I closely linked to the Lb (leghemoglobin) locus [Weeden et al., 1990]. To position *sym2* on the RFLP map of pea we used the RFLP markers cDNA40, cDNA44, cDNA150, cDNA164, cDNA186, cDNA267 [Ellis et al., 1992 and 1993], and GS (glutamine synthetase) [Gebhardt et al., 1986] surrounding Lb. For the RFLP marker Lb, an *Eco*RI - *Hind*III fragment of the 5'-region of the insert of pPsLb101 [Nap, 1988] was used.

Restriction enzyme digestion, gel electrophoresis, Southern blotting and filter hybridization (Hybond-N⁺ membrane, Amersham) were performed under standard conditions [Sambrook et al., 1989]. The RFLP probes were labelled with α-^{32}P dATP using the random priming method [Feinberg and Vogelstein, 1983].

Genomic DNA was isolated from young pea leaves according to Rogers [Rogers and Bendish, 1988] and digested with *Hind*III or *Eco*RI. The markers 40, 150, 186, GS did not give a polymorphism with these restriction enzymes.

Segregating F2 populations were obtained from the crosses L-4 x NGB1238 and A5.6.9 x Rondo consisting of 64 plants and 48 plants, respectively. These 112 F2 plants were used for mapping markers 44, 164, 267 and Lb and the *sym2* locus. Nod⁻ plants (inoculated with strain 248) were scored as homozygous *sym2* and Nod⁺ plants as having either one or no *sym2* allele. Plants were scored as Nod⁺ if 20-30 nodules (L-4 x NGB1238) or 30-50 nodules (A5.6.9 x Rondo) were formed. Plants were scored as Nod⁻ if 0-5 nodules were present. For plants showing an intermediate nodulation (5-20 nodules) F3 plants were scored after inoculation with strain 248 or PRE.

Linkage analysis was performed using the program JOINMAP, version 1.4 [Stam, 1993].

RESULTS

nodX confers the ability to nodulate *sym1* and *sym2* containing pea lines

Since *Rlv* strain TOM can nodulate *sym1* containing Iran pea [Lie, 1984b], we studied whether the introduction of *nodX* into non-nodulating *Rlv* strains is sufficient to confer the ability to nodulate Iran pea and *sym1* containing introgression lines. We introduced *nodX* into *Rlv* strain 248 (248-X) and PRE (PRE-X) and found that strain 248-X produces two additional Nod factors due to the activity of NodX (Figure 1). This is consistent with the results of Firmin who reported two Nod factors (NodRlv-V(C18:4, Ac, Ac) and NodRlv-V(C18:1, Ac, Ac) being produced in addition to the Nod factors produced by *Rlv* strains lacking *nodX* [Firmin et al., 1993]. The amounts of Nod factors produced by strain PRE and PRE-X was too low to be detected (Figure 1).

Both *Rlv* strains 248-X and PRE-X nodulate Rondo pea as well as the original strains 248 and PRE. Inoculation of Rondo lines A5.4.3 and A5.6.9, carrying *sym2*, with strain 248-X or PRE-X resulted in nodulation comparable to that obtained with Rondo (Table 3). These results also indicate that the *nodX* construct is active in both *Rlv* strains. Furthermore, we tested whether these strains have the ability to nodulate Rondo peas carrying *sym1*. Inoculation of Rondo lines I1.3.10 and I6.2.2, carrying *sym1*, with strain PRE-X or 248-X resulted in nodulation comparable to Rondo (Table 3). Upon inoculation with strain 248 and PRE no or only a few nodules were formed on peas carrying *sym1* or *sym2*. These nodules were pink indicating that nitrogen fixation was taking place.

Table 3. *nodX* dependent nodulation of plants containing *sym1* or *sym2*

Pea lines	Rlv strains				
	TOM	248	248-X	PRE	PRE-X
Rondo	+	+	+	+	+
Afghanistan	+	-	+	-[1]	+[2]
Iran	+	-[3]	+	-	+
A5.4.3	+	-	+	-	+
A5.6.9	+	-	+	-	+
I6.2.2	+	-	+	-	+
I1.3.10	+	-	+	-	+

A plant was scored as + (Nod$^+$) or - (Nod$^-$) if 30-50 nodules or 0-5 nodules were formed, respectively. At least 25 plants were used for each inoculation.

[1] We never observed any nodules on Afghanistan pea after inoculation with strain PRE.

[2] About 5-15 nodules were formed on Afghanistan pea after inoculation with strain PRE-X.

[3] About 5-15 nodules were formed on Iran pea grown at 18 °C after inoculation with strain 248.

So, introduction of *nodX* into strain 248 and PRE allows these strains to nodulate *sym1* as well as *sym2* containing Rondo lines. Furthermore, *Rlv* strains 248-X and PRE-X are able to nodulate Iran and Afghanistan peas.
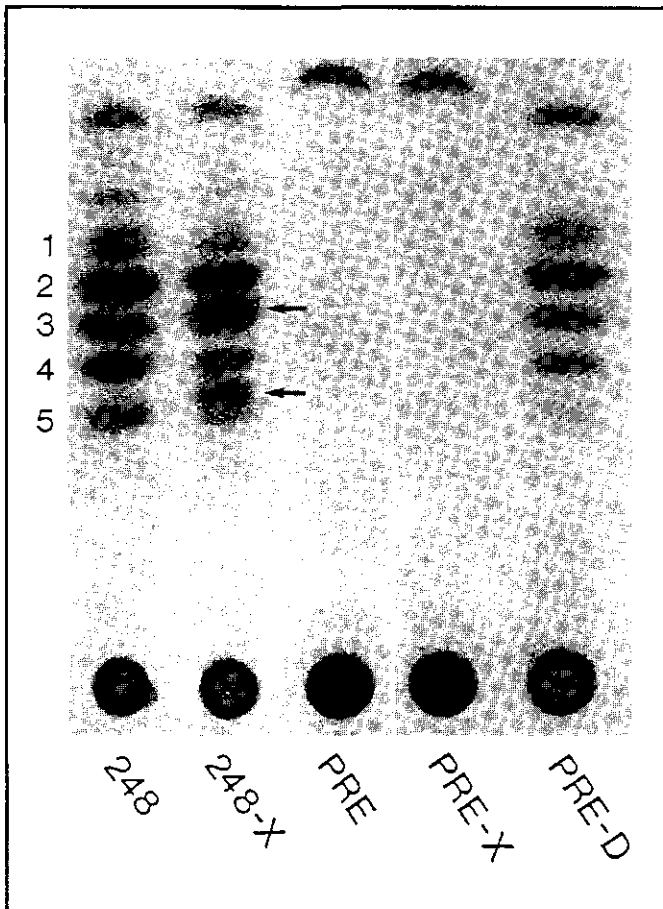
Figure 1: Thin layer chromatography (TLC) analysis of Nod factors production.
The Nod factors of different *Rlv* strains were labeled and analyzed on TLC according to Spaink [Spaink et al., 1992]. Strain 248 produces at least 5 major Nod factors (no. 1 to 5). After introduction of pMW1071 in strain 248 resulting in strain 248-X two additional Nod factors are produced (arrows). Strain PRE and PRE-X produce too low amounts of Nod factors to be detected. Introduction of pMP280 in strain PRE resulting in strain PRE-D raises the Nod factor production to the level obtained for strain 248.

Dominant nature of *sym1* and *sym2* is *Rlv* strain dependent

Lie described *sym1* and *sym2* behaving as dominant genes for the interaction with *Rlv* strain PRE [Lie, 1984b]. In addition, *sym2* behaved as a recessive or semi-dominant gene for *Rlv* strain PF2 in crosses between Afghanistan and Rondo pea [Lie, 1984b, Young, 1985].

To determine whether *sym1* and *sym2* behave similar for different *Rlv* strains

we performed nodulation experiments with the *Rlv* strains PRE, 248 and PF2 on plants heterozygous for *sym1* or *sym2*. F1 plants from crosses I1.3.10 x Rondo and A5.6.9 x Rondo were inoculated with these strains. Table 4 shows that the F1 plants are nodulated by *Rlv* strain 248 and PF2, but not by strain PRE, whereas none of these European strains has the ability to nodulate the lines I1.3.10 and A5.6.9.

Strain 248 as well as strain PF2 (data not shown) produce a much higher amount of Nod factors than strain PRE (Figure 1) indicating that nodulation of F1 plants requires *Rlv* strains producing a "high" amount of Nod factors. We introduced the *nodD* gene of strain 248 into strain PRE (PRE-D) and found that the production of Nod factors was raised to the level obtained for strain 248 (Figure 1). However, strain PRE-D was still unable to nodulate F1 plants (Table 4).

These results show that both *sym1* and *sym2* behave as recessive loci for strain 248 and PF2, and as dominant loci for strain PRE irrespective of the amount of Nod factors produced.

Table 4. Dominant or recessive nodulation phenotype of *sym1* and *sym2* plants is *Rlv* strain dependent

F1 plants	*Rlv* strains			
	248	PF2	PRE	PRE-D
I1.3.10xRondo	+	+	-	-
A5.6.9xRondo	+	+	-	-

A plant was scored as + (Nod$^+$) or - (Nod$^-$) if 30-50 nodules or 0-5 nodules were formed, respectively. At least 6 F1 plants were used for each inoculation.

Both *sym1* and *sym2* confer temperature sensitive nodulation to Rondo peas

Iran pea, carrying *sym1*, displays a temperature sensitive nodulation phenotype when inoculated with *Rlv* strain PRE [Lie, 1971a and 1971b]. To study whether *sym1* itself is responsible for the temperature sensitive nodulation of Iran pea, we inoculated Rondo lines I1.3.10 and I6.2.2, carrying *sym1*, with *Rlv* strain 248 and incubated the plants at 18°C (non-permissive temperature) or 26°C (permissive temperature). Only at the permissive temperature plants were nodulated (Table 5), showing that the introgressed region contains the genetic information for temperature sensitive nodulation.

Table 5. Temperature sensitive nodulation

Pea lines	temperature	
	18°C	26°C
Iran	-1	+
Afghanistan	-	-2
Rondo	+	+
I1.3.10	-	+
I6.2.2	-	+
A5.4.3	-	+
A5.6.9	-	+

A plant was scored as + (Nod$^+$) or - (Nod$^-$) if 30-50 nodules or 0-5 nodules were formed, respectively. Plants were inoculated with *Rlv* strain 248 and at least 25 plants were used for each inoculation.

[1] About 5-15 nodules were formed on Iran pea grown at 18°C.

[2] About 5-15 nodules were formed on Afghanistan pea grown at 26°C.

In all experiments performed so far *sym1* and *sym2* containing plants behave similarly. It was shown that Afghanistan pea can form a few nodules at 26°C [Lie, 1984b], hence it is possible that also *sym2* has a temperature sensitive nature, but that this has been masked in studies on Afghanistan pea due to the genetic background. Rondo lines A5.4.3 and A5.6.9, carrying *sym2*, were inoculated with *Rlv* strain 248 and incubated at 18°C or 26°C. As is shown in Table 5 these lines only form nodules at 26°C and not at 18°C, showing that the *sym2* like the *sym1* locus has a temperature dependent nature.

sym1 and *sym2* are allelic

The results described above showed that *sym1* and *sym2* have similar characteristics. This raised the possibility that these genes are allelic. To be alleles *sym1* and *sym2* should have the same map position.

Previously, it was shown that *sym2* is tightly linked to the leghemoglobin (Lb) cluster on linkage group I of pea, but only few markers were mapped in the vicinity of *sym2* [Weeden et al., 1990]. The map position of *sym1*, however, has not been determined before. We determined the position of *sym2* on the RFLP map of pea constructed by Ellis [Ellis et al., 1992] using two segregating F2 populations and markers 44, 164, 267 and Lb. The results are given in Figure 2a and the corresponding recombination frequencies are given in Table 6. These mapping studies showed that

sym2 is flanked by the markers 44 and 267 and maps close to the Lb cluster on linkage group I.
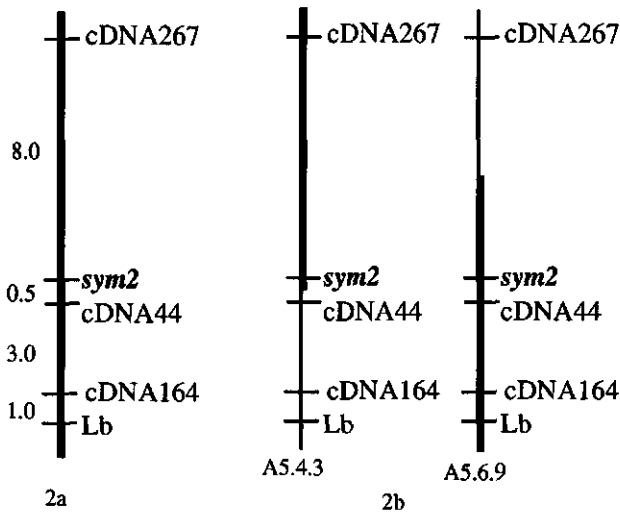
Figure 2: RFLP map of *sym2* locus and characterization of available backcross lines.
a. RFLP map of *sym2* locus. Distances between markers are designated in Morgan's units.
b. RFLP characterization of available backcross lines.
Bold line represents Afghanistan genome.

Table 6. Estimates of recombination percentages,
Pairwise data for crosses L-4 x NGB1238 and A5.6.9 x Rondo

Pair of markers	Recombination %	LOD
sym2 / Lb	4.6 ± 1.8	28.31
sym2 / cDNA164	3.3 ± 1.6	30.78
sym2 / cDNA44	0*	41.20
sym2 / cDNA267	6.3 ± 2.7	15.72
Lb / cDNA164	0.9 ± 0.7	45.93
Lb / cDNA44	3.6 ± 1.5	36.94
Lb / cDNA267	13.6 ± 3.9	10.11
cDNA164 / cDNA44	2.7 ± 1.3	39.62
cDNA164 / cDNA267	12.7 ± 3.8	10.64
cDNA44 / cDNA267	8.3 ± 3.0	14.72

* No recombination was found in our F2 populations.

In our F2 populations we did not detect a recombination between *sym2* and marker 44, since all plants homozygous for *sym2* were homozygous for marker 44. However, line A5.4.3 contains a crossover between *sym2* and marker 44 confirming the order of the loci as shown in Figure 2b. For line A5.4.3 the introgressed area from Afghanistan pea extends from 10-20 cM above to 0-1 cM below *sym2*. Line A5.6.9 has a recombination between *sym2* and marker 267 and the area below *sym2* is up to 20-30 cM (data not shown).

We used the markers 44, 164, and Lb for RFLP analyses of the *sym1* introgression lines I1.3.10 and I6.2.2. All three markers were of Iran origin (data not shown) indicating that *sym1* might have a similar map position as *sym2*. Since even accurate mapping can never provide the final proof that genes are allelic, we continued our studies by crossing *sym1* and *sym2* containing lines instead of doing a detailed mapping of *sym1*.

Rondo line I1.3.10, carrying *sym1*, was crossed reciprocally with Rondo line A5.6.9, carrying *sym2*, and the F1 progeny (8 plants for each cross) was inoculated with *Rlv* strain 248. As shown in Table 7 all F1 plants were Nod⁻. The F1 plants were selfed and the F2 progeny (60 plants) was again inoculated with strain 248. None of the plants formed nodules (Table 7). Hence, we conclude that *sym1* and *sym2* are allelic.

Table 7. Nodulation of F1 plants obtained after reciprocal crossings show that *sym1* and *sym2* are allelic

Rlv strain	pea lines and crosses				
	A5.6.9	I1.3.10	F1[*] A5.6.9xI1.3.10	F1[*] I1.3.10xA5.6.9	F2[+] (60 plants)
248	-	-	-	-	-

A plant was scored as + (Nod⁺) or - (Nod⁻) if 30-50 nodules or 0-5 nodules were formed, respectively.
[*] 8 plants were inoculated.
[+] 60 plants were inoculated.

DISCUSSION

We show here by crossing introgression lines that *sym1* and *sym2* are allelic. This observation is consistent with the fact that *sym1* and *sym2* introgression lines behave identical in all experiments performed. Furthermore, *sym1* and *sym2* map in

the same region on linkage group I as confirmed by recent studies of Weeden showing that they map within a distance of 0-4 cM (pers. comm.). The fact that *sym1* and *sym2* are allelic means that all genes conferring resistance to European *Rlv* strains identified in wild pea varieties in the area extending from Turkey to Tibet are alleles of *sym2*. Since *sym2* has been studied more extensively than *sym1* we propose that in future studies *sym1* shall be named *sym2*.

We here positioned *sym2* on linkage group I between markers 44 and 267 on the RLFP map of pea. This confirms the observation of Weeden [Weeden et al., 1990] that *sym2* is located in the vicinity of the Lb cluster on linkage group I. The relative distances between the markers around *sym2* were similar to the values of the RFLP map described by Ellis [Ellis et al., 1992], indicating that the presence of the introgressed region from Afghanistan does not influence the frequency of recombination.

We found that *sym2* displays a *Rhizobium* strain dependent recessive or dominant nodulation phenotype. *Rlv* strains 248 and PF2 can nodulate heterozygous *sym2* plants, whereas strain PRE is unable to do so. Strains 248 and PF2 produce a higher amount of Nod factors than strain PRE. But even strain PRE-D, producing similar amounts of Nod factor as strain PF2 and 248, is unable to nodulate heterozygous *sym2* plants. Therefore the recessive or dominant nature of *sym2* seems not to be correlated to the amount of Nod factors produced by the *Rlv* strain. Introduction of *nodX* in strain PRE confers the ability to nodulate heterozygous *sym2* plants, showing that the amount of Nod factor is not important when *nodX* is expressed (data not shown). The possibility remains that strain PF2 and 248 produce populations of Nod factors that are slightly different than those secreted by strain PRE allowing these strains to nodulate heterozygous *sym2* plants. Further studies are required to determine whether the strain dependent recessive or dominant nature of *sym2* is Nod factor related.

In previous genetic experiments by Lie [Lie, 1984b] and Young [Young, 1985] part of the progeny of crosses between Afghanistan pea and European cultivated peas (e.g. Rondo or Trapper) showed an intermediate nodulation phenotype (5-20 nodules) upon inoculation with e.g. strain PF2 (semi-dominance). Since we detected a clear 1:3 (Nod−:Nod+) segregation after crossing introgression lines carrying *sym2* with Rondo pea (data not shown), it seems that the genetic background of Afghanistan pea restricts nodulation independently from *sym2*.

Introduction of *nodX* into strain 248 (248-X) and PRE (PRE-X) allows these strains to nodulate *sym2* containing plants. The presence of *nodX* causes strain 248-X

to produce two additional Nod factors, presumably containing an acetate substitution at the C-6 position on the reducing sugar in accordance with the results of Firmin [Firmin et al., 1993], and therefore it is likely that *sym2* is involved in recognition of these Nod factors. Moreover, extensive search for nodulation resistance genes in wild pea cultivars has resulted in the identification of *sym2* alleles in at least ten different pea varieties [Young, 1985; Kneen and LaRue, 1984a]. Therefore it is obvious that this gene in some way plays an important role in the interaction between *Rhizobium* bacteria and its host, most likely in Nod factor recognition. A possibility is that *sym2* might encode (part of) a Nod factor receptor specifically recognizing the NodX modified Nod factors [Firmin et al., 1993; Heidstra et al., 1994; Ardourel et al., 1994]. A detailed analysis of the phenotype conferred by *sym2* and eventually the cloning of this gene will be the next challenges to reveal the function of *sym2*.

ACKNOWLEDGEMENT

CHAPTER 3

THE PEA EARLY NODULIN GENE *PsENOD7* MAPS IN THE REGION OF LINKAGE GROUP I CONTAINING *SYM2* AND LEGHEMOGLOBIN

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Abstract

The early nodulin gene, *PsENOD7*, is expressed in pea root nodules induced by *Rhizobium leguminosarum* bv. *viciae*, but not in other plant organs. *In situ* hybridization showed that this gene is transcribed during nodule maturation in the infected cells of the proximal part of the prefixation zone II. At the transition of zone II into interzone II-III, the level of *PsENOD7* mRNA drops markedly. *PsENOD7* has no significant homology to other genes. RFLP mapping studies have shown that *PsENOD7* is located in linkage group I between the leghemoglobin genes and *sym2*.

Key words: nodulins, *Pisum sativum*, RFLP map, *Rhizobium leguminosarum*.

Introduction

Rhizobium leguminosarum bv. *viciae* induces the formation of nitrogen fixing root nodules on the roots of *Pisum sativum* (pea). By mutagenesis and genetic studies several plant genes essential for normal nodule development have been identified and these genes have been named *sym* genes. In pea about 30 different *sym* genes have been described [Kneen et al., 1984; 1994] [Duc et al.,1989; Sagan et al., 1994]. The *sym* genes are distributed randomly on the seven linkage groups of pea [Kneen et al., 1994], but several *sym* genes, namely *sym2, sym5, sym19* and *nod3*, are clustered on linkage group I, near the major leghemoglobin (*Lb*) locus [Weeden et al., 1990. Temnykh et al., 1995].

The different stages of legume nodule development are accompanied by the expression of plant genes, the so-called nodulin genes. These genes, that are only expressed during nodule development, have been divided into early and late nodulin genes; the early nodulin genes are expressed before the bacteria start to fix nitrogen, whereas the late nodulin genes are induced around the start of nitrogen fixation [Nap and Bisseling, 1990].

Nodulin genes have been identified in several legumes like soybean, pea, *Medicago, Phaseolus, Sesbania, Vicia* and lupin [for reviews see Sanchez et al., 1991;

Franssen et al., 1992a]. In pea six early nodulin genes have been described, e.g. *PsENOD12* [Scheres et al., 1990a] and *PsENOD40* [Matvienko et al., 1994] and several late nodulin genes, e.g. glutamine synthetase (*GS*) [Tingey et al., 1987], leghemoglobin (*Lb*) [Nap, 1988] and *PsNOD6* [Kardailsky et al., 1993].

At present, it is unknown whether some of the pea *sym* genes encode nodulins. To answer the latter question it is essential to determine the positions of both *sym* and nodulin genes on genetic map and to check whether their position coincides.

In this paper, we describe the molecular characterisation of the early nodulin cDNA clone pPsENOD7, the *in situ* expression pattern of the corresponding gene, as well as the position of the gene on the genetic map.

Isolation of pPsENOD7

A λgt11 cDNA library, prepared from *Pisum sativum* cv. Sparkle root nodule RNA, was kindly provided by G. Coruzzi [Tingey et al., 1987] and seven early nodulin cDNA clones were isolated by differential screening [Scheres et al., 1990b]. Previously, we have described the characterisation of six clones, namely; pPsENOD2, pPsENOD3, pPsENOD5, pPsENOD12, pPsENOD14 and pPsENOD40 [Van de Wiel et al., 1990; Scheres et al., 1990a,b; Matvienko et al., 1994]. Here, we present the characterisation of pPsENOD7.

PsENOD7 is expressed only in nodules

Southern blot analyses revealed that the insert of pPsENOD7 hybridised to a single fragment in *Eco*RI (1 kb) or *Hin*dIII (8 kb) digested DNA from pea cv. Rondo (data not shown). These data indicate that PsENOD7 is encoded by a single gene.

We studied the expression of *PsENOD7* by Northern blot analysis of RNA from uninfected roots, from roots 4 and 8 days after sowing and inoculation with *R. leguminosarum* bv.*vicae* strain 248 and, from 15 days old nodules. *PsENOD7* mRNA had a length of 500b (Figure 1). *PsENOD7* mRNA was not detectable 4 days after inoculation, but it was present at a low level after 8 days and it accumulated to a markedly higher level in 15 days old nodules. The transcript was absent in shoots, hypocotyls, epicotyls, flowers, leaves, pods, cotyledons, and uninfected roots.
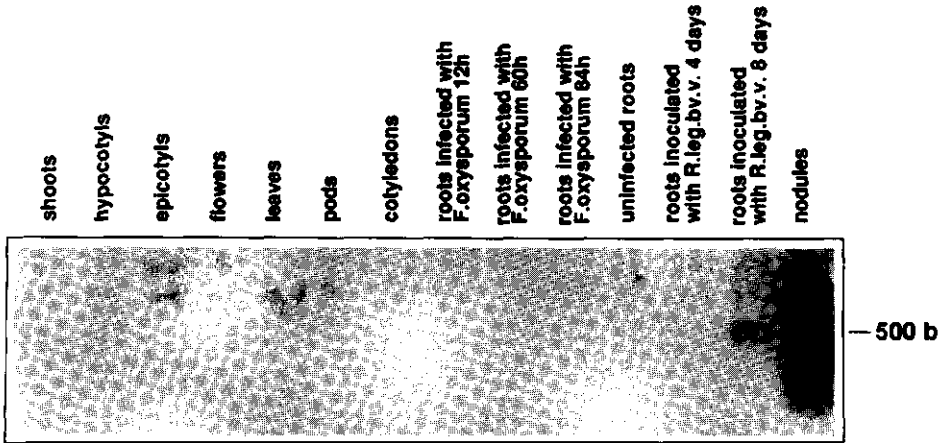
Figure 1.
Expression of *PsENOD7* in different plant organs.
Pea (*Pisum sativum* cv. Sparkle) plants were cultured and inoculated with *R. leguminosarum* bv.*viciae* strain 248 as described by Bisseling et al., [1978]. Plant organs were harvested from pea plants at different time points: shoots and cotyledons from 7 days old plants; hypocotyls, epicotyls and roots from 14 days old plants; flowers, leaves and young pods from 45 days old plants. Inoculated roots were harvested 4 and 8 days after inoculation; nodules were harvested 15 days after inoculation. Total RNA was extracted from plant tissues as described by Govers et al., [1985].
Fusarium oxysporum mycelium was inoculated in Czapek-dox medium and grown for 2 days at 30°C. Pea plants were inoculated with this suspension 3 days after sowing. *Fusarium* infected roots were harvested at 12, 60 and 84 hours after inoculation.

Furthermore, the gene was not induced in pea roots 12, 60 and 84 hours after inoculation with the fungal pathogen *Fusarium oxysporum* (Figure 1). Hence, *PsENOD7* appears to be a true nodulin gene [Van Kammen, 1984].

In situ localisation of *PsENOD7* mRNA

Pea forms nodules with an indeterminate growth pattern like most other temperate legumes. Thus a gradient of developmental stages is present from apex to root attachment point and consequently, the nodule central tissue can be divided in zones representing subsequent stages of development; zone I is the apical meristem, followed by prefixation zone II, interzone II-III and fixation zone III [Vasse et al., 1990; Franssen et al., 1992b]. At the transition of interzone II-III into fixation zone, amyloplast accumulation at the periphery of infected cells suddenly starts [Vasse et al., 1990; Franssen et al., 1992b].

Longitudinal sections of 14 days old pea nodules were hybridized with ^{35}S labelled antisense as well as sense *PsENOD7* RNAs. The sense probe gave no signal above background (result not shown), whereas the antisense probe hybridised with RNA present in infected cells [Fig 2a, b]. *PsENOD7* mRNA was first detectable in the proximal part of the prefixation zone II and reached its maximal level at the transition of the prefixation zone into interzone. At this transition the level of *PsENOD7* transcript suddenly dropped to a markedly lower level [Fig 2c, d].

It has been shown that at the transition of prefixation zone II into interzone II-III the expression level of several bacterial and plant genes rapidly changes. For example, the expression of *ropA* of *Rhizobium* is switched off, whereas the expression of the rhizobial *nif* genes is induced at this transition [Brito et al., 1995; De Maagd et al., 1994]. So the expression level of *PsENOD7* markedly drops when the bacteria acquire the ability to fix nitrogen. Together with the decrease of the expression of the *PsENOD7*, the expression of some other pea early nodulin genes e.g. *PsENOD5* and *PsENOD3* is down regulated, whereas the late nodulin gene *PsNOD6* [Kardailsky et al., 1993] and the alfalfa leghemoglobin genes are induced at this stage of development [De Billy et al., 1991]. Hence, the down regulation of *PsENOD7* at the prefixation zone/interzone transition provides additional evidence that at this transition a dramatic and rapid change in nodule development takes place.

Figure 2 (see next page).

In situ localisation of *PsENOD7* mRNA in a 14-day-old nodule of pea.
A - Bright field picture of a longitudinal section through a pea nodule. I = meristem (M), II = prefixation zone, II-III = interzone, III = fixation zone, R = root.

B - A combination of epipolarisation and bright field micrograph of the boxed area in C, showing the decrease in the level of *PsENOD7* mRNA at the transition of prefixation zone into interzone. Green dots are silver grains representing the signal. Amyloplasts in the infected cells are indicated by arrowheads; IC = infected cells, UC = uninfected cells.

C - A combination of epipolarisation and bright field micrograph of the part of the nodule indicated in A.

D - Epipolarization micrograph of C showing that *PsENOD7* mRNA accumulation starts in the proximal part of prefixation zone, and the level drops markedly at the transition of prefixation zone into interzone. (Bright-green dots are silver grains representing the signal).

The preparation of sections and hybridization conditions are according to a procedure described by Cox and Goldberg [1988] and Van de Wiel et al., [1990].
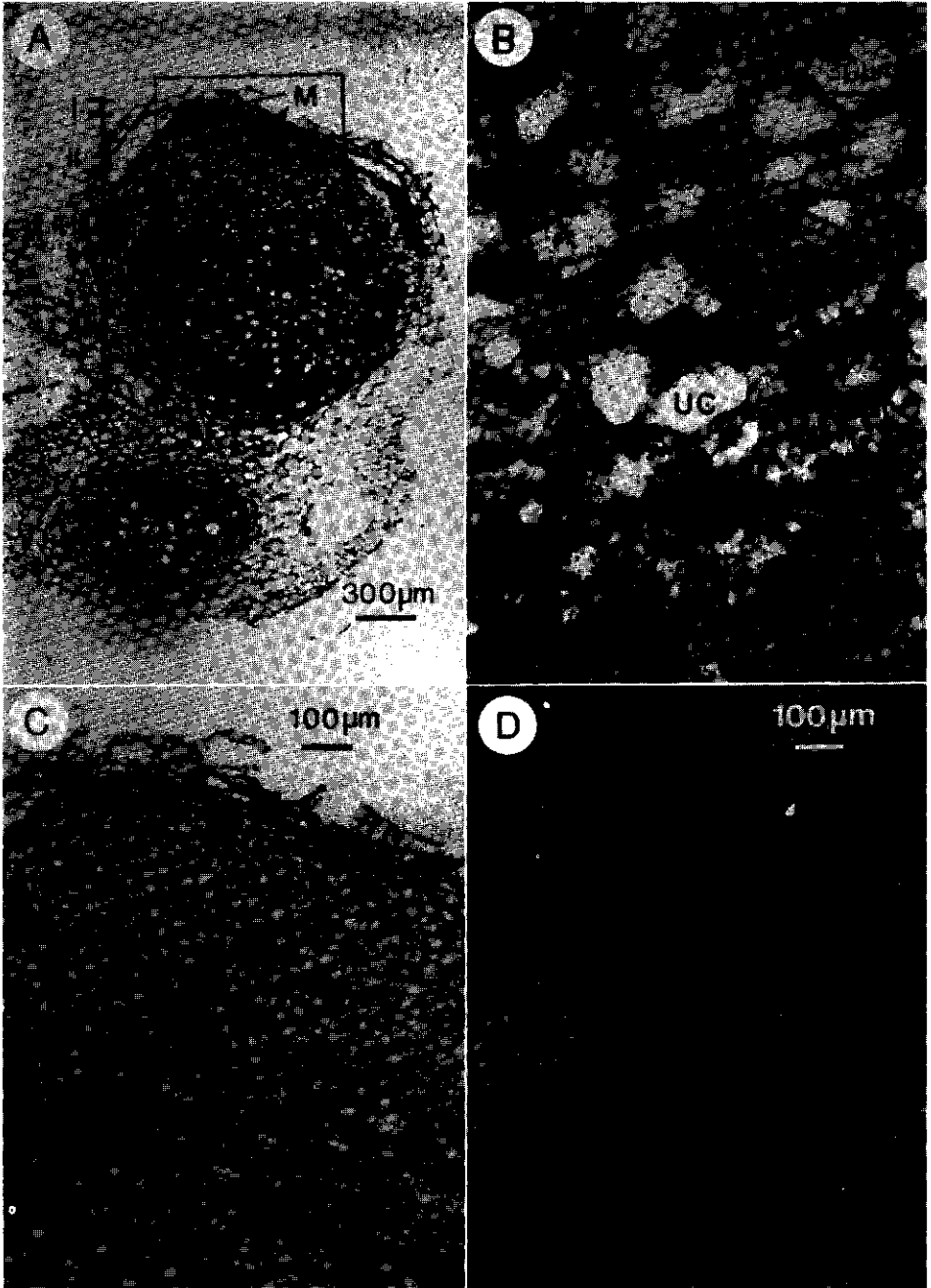
```
AGAAGAAACTCATCGTTGTAGCAATGATGAAAATCAAGCATGCTATCTTCTTATGCTTAT          60
                       M  M  K  I  K  H  A  I  F  L  C  L  C

GTGCAATGCTACTAATCTCTATTGTGGCAATTGAGCCTTATGAACACGAGAATCAATTTG         120
 A  M  L  L  I  S  I  V  A  I  E  P  Y  E  H  E  N  Q  F  G
                         Δ
                    -------------------        ----------------
GTGAAATAGAGAAACCAATGAGAAACATTGATGGAGTTGTAATACGTTTAACCAATGGTG         180
 E  I  E  K  P  M  R  N  I  D  G  V  V  I  R  L  T  N  G  E

 ----
AAGGCCGTGGCAGAAACGAGCCACTCTTTCCCGATTGCGAGAAAGACGGCGGCAGTGAAG         240
 G  R  G  R  N  E  P  L  F  P  D  C  E  K  D  G  G  S  E  G
    .
GTGGAAATTGTGGCGGACATGAGGTCGAGGAGGGCATCACTGAAAACGCCATTCCTATTC         300
 G  N  C  G  G  H  E  V  E  E  G  I  T  E  N  A  I  P  I  P

CTAACGGTGTAAGTCAAAGTCGTTGGTGGACACGCAAAGCACCAGTGGAGAAAATTCCTG         360
 N  G  V  S  Q  S  R  W  W  T  R  K  A  P  V  E  K  I  P  V

TGGAAAACTAGAAACGCATATACATGTATCATGTATTCATGGTGCAACAATATATAATGT         420
 E  N  *

CATAAGAAATGTAAAATAAAGATGGGACCATGTAGTTATTAAATTAAATAACAATTATAA         480
TAATATTTATGGAGTAAACTATC                                            503
```

Figure 3.

Nucleotide sequence of the insert of pPsENOD7 and deduced amino acid sequence. Position of the cleavage site of the putative signal peptide is indicated by Δ. The part of the sequence obtained after 5'-RACE is indicated in italics and the sequence present in both pPsENOD7 and the 5'-RACE clone is underlined. Two *PsENOD7* specific antisense oligonucleotides, and two universal primers (with multiple cloning sites): CTCGAGGATCCGCGGCCGC(T)$_{18}$ and GCTCGAGGATCCGCGGC were used to amplify the 5' region of *PsENOD7* mRNA. The antisense oligo's used for 5'-RACE are overlined.

Sequence of pPsENOD7

The insert of pPsENOD7 was sequenced using the dideoxy chain termination method with an automatic sequencer (Applied Biosystems model 373A). The cDNA insert of pPsENOD7 was 432 bp in length including a poly-A tail at the 3' end, while the *PsENOD7* mRNA had a size of about 500 b [see above]. Therefore, the missing 5' part of *PsENOD7* RNA was cloned. Using 5'RACE [Frohman et al., 1988] with the modifications by Kardailsky [1995], we obtained a clone of 184 bp containing 108 bp of the 5'-end of the insert of pPsENOD7 and 76 bp of the missing 5' end. The *PsENOD7* cDNA sequence contained a single large open reading frame with the first ATG codon at position 24. The putative ENOD7 polypeptide is 115 aa long (Fig. 3) with a Mw of 12kD. ENOD7 is a hydrophilic protein with a hydrophobic domain at

the N-terminal end, which may be part of a putative signal peptide [Von Heijne, 1983]. This suggests that ENOD7 is transported across a membrane and, hence, it might be a protein located in the space between rhizobia and the peribacteroid membrane or an extracellular protein. *PsENOD7* has no significant homology to other sequences present in the databases of the National Center of Biotechnology Information (NCBI), National Library of Medicine, NIH (Bethesda, MD). Database searches were performed using the BLAST algorithm [Altschul et al., 1990]. The DNA sequence of the *PsENOD7* can be found in the EMBL databank as accession number X93172.

Mapping of *PsENOD7*

The position of *PsENOD7* on the pea genetic map was determined in order to find out the relation of *PsENOD7* to previously identified *sym* genes. By using the segregating population of cross JI1794 x Slow [Weeden et al., 1994], we showed that *PsENOD7* is closely linked to the major *Lb* locus of linkage group I, in the region where *sym2* is also located [Weeden et al., 1990] (data not shown). *sym2* is the gene of Afghanistan peas which confers resistance to form nodules with *Rhizobium leguminosarum* bv *viciae* strains lacking the nodulation gene *nodX* [Lie, 1984; Firmin et al., 1993].
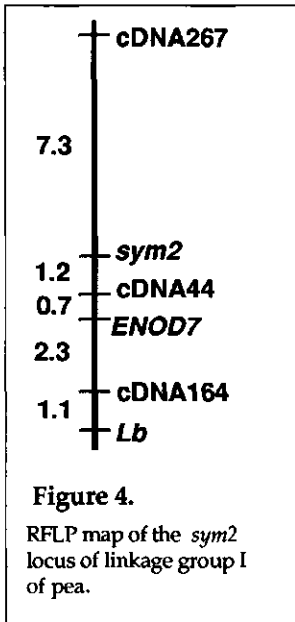
Recently, we have determined the position of *sym2* on the RFLP map of pea constructed by Ellis [1992] and shown that it is flanked by the RFLP markers 44 and 267 [Kozik et al., 1995]. We used segregating F2 and F3 (single seed descent from F2) populations of the cross L-4 x NGB1238 [Kozik et al., 1995] to position *PsENOD7* on the RFLP map. Linkage analysis was performed using the program JoinMap, version 1.4 [Stam, 1993]. The results presented in Table 1 and Figure 4, show that *PsENOD7* is located about 2 cM below *sym2* and 3.5 cM above the *Lb* locus. A confirmation of the order of markers in the *sym2* region was obtained by determining the sites of recombination in *Pisum sativum* Rondo lines containing an introgressed *sym2* area of pea

Figure 4.

RFLP map of the *sym2* locus of linkage group I of pea.

cv. Afghanistan [Kozik et al., 1995] (data not shown).

We have observed a single recombination between *PsENOD7* and *sym2* gene among 64 plants of the segregating F2 population of the cross L-4 x NGB1238, in a plant having no Afghanistan *sym2* allele. In the F3 offsprings derived from this plant, we found plants which were homozygous for the *PsENOD7* Afghanistan allele, as shown by RFLP analysis, but lacked the Afghanistan *sym2* allele (data not shown). This further demonstrates that *PsENOD7* does not coincide with *sym2*, while the low frequency of recombination shows that *PsENOD7* is tightly linked to this locus.

Table 1

Pairwise data for cross L-4 x 1238
(combined data for F2 and F3 populations)

Pair of markers	Recombination %	LOD
sym2 / Lb	5.1 ± 1.7	35.8
sym2 / cDNA164	4.0 ± 1.5	39.1
sym2 / cDNA44	1.0 ± 0.7	51.6
sym2 / cDNA267	6.6 ± 1.9	31.9
sym2 / ENOD7	1.7 ± 0.9	47.7
ENOD7 / cDNA267	9.1 ± 2.3	26.5
ENOD7 / cDNA44	0.7 ± 0.6	53.5
ENOD7 / cDNA164	2.2 ± 1.1	46.6
ENOD7 / Lb	3.2 ± 1.3	42.5
Lb / cDNA164	1.0 ± 0.7	51.9
Lb / cDNA44	4.0 ± 1.5	39.8
Lb / cDNA267	13.0 ± 2.8	19.6
cDNA164 / cDNA44	2.9 ± 1.3	43.5
cDNA164 / cDNA267	11.8 ± 2.6	21.5
cDNA44 / cDNA267	8.2 ± 2.2	28.6

To position *PsENOD7* on the RFLP map of pea [Ellis et al., 1992] we used the RFLP markers cDNA44, cDNA164, cDNA267 and Lb that are located around the *sym2* locus. Two segregating populations (F2 and F3 of cross L-4 (carrying *sym2*) x NGB1238) were used for mapping, each contains 64 plants. Genomic DNA was isolated from young pea leaves according to Rogers [1988] and digested with *Hind*III. Restriction enzyme digestion, gel electrophoresis, Southern blotting and filter hybridization (Hybond-N$^+$ membrane, Amersham) were performed by standard protocols [Sambrook et al., 1989]. The RFLP probes were labelled with α-^{32}P dATP using the random priming method [Feinberg and Vogelstein, 1983].

It is striking that two nodulin genes *PsENOD7* and *Lb,* and *sym2* map relatively close to each other. Furthermore, it has been shown that *sym* gene, *nod3,* is

also located in the vicinity of the *sym2* [Temnykh et al., 1995]. The *nod3* mutant has lost the ability to autoregulate nodule number and hence forms markedly more nodules than wild type peas [Jacobsen et al., 1984]. Although the exact position of *nod3* is still not known, *sym2* and *nod3* are not allelic [Kozik, Temnykh and Weeden, unpublished results]. Furthermore, it is unlikely that *PsENOD7* and *nod3* are allelic, since *PsENOD7* is expressed in the proximal part of the prefixation zone of the central tissue. It is not probable that a gene expressed at this stage of nodule development can control nodule number. Moreover, by Northern analysis we did not detect a difference in the level of *PsENOD7* expression in nodules of wild type pea cv. Rondo and *nod3* mutant (data not shown). Therefore, we conclude that *nod3* and *PsENOD7* are different genes and thus the region on linkage group I harbouring *PsENOD7* contains at least four genes involved in the *Rhizobium*-legume symbiosis; *sym2, nod3, PsENOD7* and *Lb*.

CHAPTER 4

DETAILED MAP OF THE *SYM2* REGION OF PEA LINKAGE GROUP I

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ABSTRACT

A detailed genetic map was constructed for the region of pea linkage group I containing *sym2, sym5, sym19, nod3,* and the early nodulin genes *PsENOD7, PsENOD40,* and leghemoglobin *(Lb)*. The *sym* genes and nodulin genes are located into two groups within 20 cM from each other. One group contains *sym2, nod3* and *ENOD7*, the second group contains *sym5, sym19* and *ENOD40*. Thus seven genes involved in symbiosis, four *sym* genes and three nodulin genes map within 20 cM, what is about 1% of the pea genome. In order to facilitate the cloning of *sym2* a search of new molecular markers for the *sym2* region was performed. Two flanking RAPD markers tightly linked to the *sym2* locus were found and converted into SCAR markers.

Key words: Pea *(Pisum sativum)*, *sym2*, RFLP map, RAPD and SCAR markers, *PsENOD40*.

INTRODUCTION

A detailed genetic map containing a high density of molecular markers tightly linked to a *sym* gene of interests is essential for the cloning of this *sym* gene via positional cloning.

sym2 is a gene conferring to the wild peas from Afghanistan nodulation resistance upon inoculation with European *Rhizobium leguminosarum* bv. *viceae (Rlv)* strains. *Rlv* strains from the Middle East, such as strain TOM can nodulate Afghanistan peas as well as European pea cultivars. The extended host range of strain TOM is due to an additional nodulation gene *nodX* [Davis et al., 1988]. NodX most likely is an O-acetyl transferase, since *Rlv* strains carrying *nodX*, produce an additional NodX modified Nod factor, which is acetylated at the C-6 position of the reducing N-acetyl glucosamine residue [Firmin et al., 1993]. Since pea lines, carrying *sym2*, display Nod factor dependent nodulation, it was postulated that *sym2* is involved in the recognition of Nod factor [Heidstra et al., 1993]. *sym2* was characterised and mapped previously [Lie, 1984; Young, 1985; Firmin et al., 1993; Kozik et al., 1995]. *sym2* locates about 7 cM below RFLP marker cDNA267 and 1 cM above cDNA44 (see Figure 1a) [Kozik et al., 1995].

In this paper we present a detailed genetic map of the part of pea linkage group I containing the *sym* genes, *sym2*, *sym5*, *sym19* and *nod3* and the nodulin genes *PsENOD7*, *PsENOD40* and leghemoglobin (*Lb*). Five new RAPD markers located in this region were mapped. Two of them, which are tightly linked to *sym2*, were cloned and converted to SCAR markers. The available *sym2* introgression lines were genetically characterised.

MATERIALS AND METHODS

Plant material and nodulation assay

The original pea lines and *sym2* introgression lines used in this study are described in Table 1.

sym2 introgression lines A5.4.3, A5.6.9 and L-4 were described previously [Kozik et al., 1995]. Line NGB1238-*sym2* was derived after two backcrosses of the *sym2* introgression line L-4 with NGB1238. A plant morphologically resembling NGB1238, but with a Nod⁻ phenotype upon inoculation with *Rlv* strain 248 was selected and named µ-3. Rondo-*sym2* lines A15.5 and A33.18 were derived by crossing A5.4.3 with Rondo and selection among F2 plants of this cross, using molecular markers (see below), for plants having the smallest *sym2* introgression region. The Sparkle-*sym2* introgression line was kindly provided by Dr TA. LaRue [Kneen et al., 1984].

The *nod3* mutant was obtained by EMS mutagenesis, described previously [Jacobsen and Feenstra, 1984]. Cross L-4 x Nod3 contained 128 F2 segregating individuals.

The F2 *sym2* segregating populations of the cross A5.6.9 x Rondo as well as F2 and F3 (single seed descent from F2) *sym2* segregating populations of the cross L-4 x NGB1238, described previously [Kozik et al., 1995; 1996] were used for mapping of molecular markers.

Nodulation tests were performed in modified Leonard jars according to Lie et al., [1988]. Plants were scored as Nod⁻ if 0-5 nodules were formed, Nod⁺ if 20-50 nodules were formed, and Nod⁺⁺⁺ (hypernodulating) if roots were completely covered by nodules (about 100-200 nodules per plant). The *Rlv* strains 248, 248-X, PRE and PRE-X used for inoculation were described previously [Josey et al., 1979; Lie, 1984; Kozik et al., 1995].

For each individual of the F3 segregating population of the cross L-4 × NGB1238 the *sym2* genotype was determined. A F3 plant was scored as *sym2* heterozygous if offspring from this F3 plant (at least 12 F4 plants were checked) upon inoculation with *Rlv* strain PRE showed segregation for Nod⁻/Nod⁺. Plants were scored as *sym2* homozygous if all offspring were Nod⁻ upon inoculation with *Rlv* strain PRE. Plants were scored as having no Afghanistan *sym2* allele if all offspring were Nod⁺ upon inoculation with *Rlv* strain PRE.

Table 1. Pea lines

Pea line	Origin	Number of back-crosses*	Phenotype upon inoculation with PRE/PRE-X	Reference
Rondo	European cultivar	-	Nod⁺ / Nod⁺	Lie, 1984
NGB1238 (= JI73)	multiple marker line, European origin	-	Nod⁺ / Nod⁺	Lamprecht, 1974
Afghanistan	wt Afghanistan, carrying *sym2*	-	Nod⁻ / Nod⁺	Lie, 1984
L-4	*sym2* introgression line; F8 of the cross Afghanistan x NGB1238	1	Nod⁻ / Nod⁺	Kozik et al., 1995
A 5.4.3	*sym2* introgression line; F8 of the cross Afghanistan x Rondo	3	Nod⁻ / Nod⁺	Kozik et al., 1995
A 5.6.9	*sym2* introgression line; F8 of the cross Afghanistan x Rondo	3	Nod⁻ / Nod⁺	Kozik et al., 1995
μ-3	*sym2* introgression line; F4 of the cross L-4 x NGB1238	3	Nod⁻ / Nod⁺	this study
A15.5, A33.18	*sym2* introgression lines; F4 of the cross A 5.4.3 x Rondo	4	Nod⁻ / Nod⁺	this study
Sparkle-*sym2*	*sym2* introgression line; F8 of the cross Afghanistan x Sparkle	7	Nod⁻ / Nod⁺	Kneen et al., 1984
Nod3	*nod3* (hypernodulating EMS mutant on the cv. Rondo)	-	Nod⁺⁺⁺ / Nod⁺⁺⁺	Jacobsen and Feenstra, 1984

*-number of backcrosses indicates how many times the original Afghanistan line was backcrossed with the Rondo or NGB1238 line.

Isolation of plant genomic DNA

Genomic DNA from pea leaves was isolated using the CTAB method [Rogers and Bendish, 1988] with some modifications.

Maxiprep: Fresh tissue (~10 g) was ground to a fine powder in liquid nitrogen in a porcelain mortar. The powder was transferred to a 50 ml plastic tube for centrifugation (Sarstedt) and 10 ml of 2xEB was added. (2x Extracton buffer (2xEB): 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% w/v CTAB, 20 mM 2-β-mercaptoethanol). The mixture was incubated at 60°C during 15-20 min with gently agitation. The mixture was extracted twice with chloroform-isoamylalcohol 24:1. The water phase was transfered to a new plastic tube for centrifugation and 1.5 - 2 vol. of precipitation buffer was added. The contents of a tube were mixed gently and a thread-like precipitate formed at room temperature during 5-10 min. (Precipitation buffer: 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% w/v CTAB). The precipitate was removed with a plastic rod, washed with water and then dissolved in 10 ml of 1.5 M NaCl During dissolvation 2.5 µl RNase A (10 µg/ml) was added. DNA was now precipitated by adding 2 vol. of 96% ethanol. The thread-like precipitate was again removed with a plastic rod, washed with 70% ethanol, dried in air and dissolved in 0.5-1.0 ml of water. This solution of DNA was used for enzymatic digestion with endonucleases, PCR and RAPD analysis.

Miniprep: An unexpanded top bud of a pea plant was ground in Eppendorf tube by pestle in 0.5 ml of 2xEB. The mixture was incubated at 60°C during 10-15 min with gently agitation and subsequently extracted twice with chloroform-isoamylalcohol 24:1. The supernatant after chloroform extraction was transfered to a new Ependorf tube and 2 vol. of precipitation buffer was added. The contents of the tube were mixed gently and a thread-like precipitate formed at room temperature during 10-15 min. The precipitate was collected by centrifugation (6 min, 10.000 rpm) and dissolved in 300 µl of 1.5 M NaCl to which 0.5 µl RNase A (10 µg/ml) was added. The solution was incubated at 50°C during 10-15 min. DNA was precipitated by adding of 2 vol 96% ethanol and the thread-like precipitate was collected by centrifugation, washed with 70% ethanol, dried in air and dissolved in 50 µl of water. Usually about 100-300 µg DNA is isolated from single top bud.

RFLP and linkage analyses

The pea early nodulin cDNA clones used were characterised previously; pPsENOD2 [Van de Wiel et al., 1990], pPsENOD3 [Scheres et al., 1990b], pPsENOD5 [Scheres et al., 1990b], pPsENOD7 [Kozik et al., 1996], pPsENOD12 [Scheres et al., 1990a], pPsENOD14 [Scheres et al., 1990b] and pPsENOD40 [Matvienko et al., 1994]. For the RFLP marker ENOD40, an EcoRI-HindIII fragment of the 5'-region of the insert of pPsENOD40 was used [Matvienko et al., 1994].

The use of RFLP markers cDNA44, cDNA164 and cDNA267 [Ellis et al., 1992] and Lb [Nap, 1988] for mapping of the *sym2* locus was described previously [Kozik et al., 1995].

Southern blotting and filter hybridization (Hybond-N$^+$ membrane, Amersham) were performed under standard conditions [Sambrook et al., 1989]. The RFLP probes were labelled with $[\alpha$-^{32}P]dATP using the random priming method [Feinberg and Fogelstein, 1983]. Linkage analysis was performed using the program JOINMAP, version 1.4 [Stam, 1993].

RAPD markers

The RAPD primers used in this study and the size of the amplified polymorphic bands are given in Table 2. The DNA sequences of the SCAR primers SCOPA1-A and SCOPA1-B containing the 10 bases of the original RAPD primer plus the next 11-14 internal bases from the end were designed according to Paran and MicHelmore, [1993]. The other SCAR primers were derived from internal sequences of the cloned RAPD fragments. SCAR primers are listed in Table 4.

Table 2. RAPD primers and size of amplified polymorphic fragments

RAPD marker	Primer sequence	Size (bp) polymorphic fragment in Afghanistan	Size (bp) polymorphic fragment in NGB1238
OPA1	5'-CAGGCCCTTC-3'	530	-
OPA4	5'-AATCGGGCTG-3'	-	450
P198	5'-CTGTTTGCGG-3'	850	1200
B374	5'-GGTCAACCCT-3'	-	1000
B474	5'-AGGCGGGAAC-3'	-	380

PCR and RAPD reactions

Amplification reactions were done in a DNA Thermal Cycler (Perkin Elmer Cetus). RAPD reactions were performed in 50 µl of 55 mM Tris-HCl pH 8.8, 16.5 mM $(NH_4)_2SO_4$, 4.95 mM $MgCl_2$, 25 mM KCl, 5.5 mM 2-β-mercaptoethanol, 1 mM of each dNTP (LKB-Pharmacia), 50 ng of each oligo's (OPERON), 0.5 U Taq-polymerase (Super Taq, HT Biotechnology LTD) and 100 ng of denaturated genomic DNA. Reaction mixtures were contained in 0.5 ml plastic tubes (Greiner) and overlaid with 60 µl of liquid paraffin.

The conditions for the amplification of RAPD fragments were; 94°C for 1 min, 35°C for 2 min, 72°C for 2 min (ramp between 35°C and 72°C was 3 sec/1°C) with 50 cycles. Amplification products were analyzed by gel electrophoresis in a 1.8% agarose gels and visualized by ethidium bromide staining.

For amplification with SCB374 primers the conditions were; 94°C for 3 min, 50°C for 2 min, 72°C for 2 min followed by 30 cycles 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. For amplification with SCOPA1 primers the conditions were; 94°C for 5 min followed by 35 cycles 94°C for 30 sec, 58°C for 1 min, 72°C for 30 sec. A 72°C incubation for 4 min was included as a final step. PCR reactions were performed in 50 µl of 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 0.1 mM of each dNTP (LKB-Pharmacia), 50 ng of each oligo, 0.5 U Taq-polymerase (Super Taq, HT Biotechnology LTD) and 100 ng of denaturated genomic DNA.

RESULTS

Mapping of early nodulin genes in linkage group I

Since *sym2* is active in early stages of nodule formation we wondered whether *sym2* might correspond with one of the cloned early nodulin genes. The genes *PsENOD2, 3, 5, 14* and *40* were checked for co-segregation with *sym2* using the *sym2* segregating populations L-4 x NGB1238 and A 5.6.9 x Rondo [Kozik et al., 1995]. We found that *PsENOD40* maps in linkage group I about 20 cM apart from *sym2*. Linkage analysis with RFLP markers surrounding *sym2* showed that the relative position of *PsENOD40* is at the same side as leghemoglobin (Lb) (figure 1a). The other tested early nodulin genes did not show linkage with the *sym2* locus. Previously, *PsENOD7* and *PsENOD12* were tested and it was then shown that

PsENOD7 maps about 2 cM apart from *sym2* while *PsENOD12* maps in linkage group III [Men et al., 1993; Kozik et al., 1994, 1996]. Therefore, among the seven early nodulin genes tested, two of them, *PsENOD7* and *PsENOD40*, map near the *sym2* locus in linkage group I, but none of the early nodulin genes coincide with *sym2*.

The hypernodulating locus *nod3* maps in linkage group I near *sym2*

The pea mutant Nod3 is affected in its nodule number regulating mechanism and has a hypernodulation phenotype, Nod[+++]. It is a monogenic and recessive EMS mutation in the cultivar Rondo [Jacobsen et al; 1984]. Recently it was shown that the hypernodulation gene *nod3* maps in linkage group I near *d*, which controls axillary pigmentation [Temnykh et al., 1995]. We performed crosses between pea lines L-4, carrying *sym2*, and Nod3 to obtain a double mutant homozygous for both *sym2* and *nod3* to check any epistatic relationship. Hundred-twenty-eight F2 individuals of the cross L-4 x Nod3 were analysed in search of a double mutant. After inoculation with *Rlv* strain 248-X, thirty F2 hypernodulating plants were selected. To find a double mutant the thirty plants were tested with the RFLP markers cDNA44 and cDNA267 previously demonstrated to flank *sym2* [Kozik et al., 1995]. Twenty-eight Nod[+++] plants were found to be homozygous for the markers cDNA44 and cDNA267, which proves that *nod3* is linked to *sym2* [Weeden and Temnykh, personal communication]. The other two hypernodulating plants showed a cross-over between markers cDNA267 and cDNA44, one plant being heterozygous for marker cDNA44, and the other heterozygous for cDNA267. These two heterozygous plants were backcrossed with the A5.6.9 introgression line. All F1 backcrossed plants were Nod[+] upon inoculation with *Rlv* strain 248. Since *sym2* in the interaction with *Rlv* strain 248 has a recessive nature, we conclude that the two heterozygous plants of the cross L-4 x Nod3 have no Afghanistan *sym2* allele. The results further show that *nod3* maps between RFLP markers cDNA44 and cDNA267.

Construction of integrated and detailed maps for the *sym2* region.

The RFLP map, previously constructed, based on the cross L-4 x NGB1238, contains only one marker, cDNA267, about 7 cM above *sym2,,* as depicted in Figure 1a [Kozik et al., 1995]. To find additional molecular markers we used the pairwise

data for linkage group I used for the construction of RFLP maps for all seven linkage groups of pea [Ellis et al., 1992; 1993]. In addition, unpublished raw data for linkage group I was used [Ellis, unpublished results]. The combination of the data for Recombinant Inbred Lines (RILs) of the cross IJ281 x IJ399 and the pairwise data for the F3 segregating population of the cross L-4 x NGB1238, by using the program JoinMap-1.4 [Stam, 1994], resulted in the integrated map shown in Figure 1b. This integrated map shows a RFLP marker cDNA40/3 that maps 2 cM above *sym2*. Unfortunately, this marker is not useful for further characterisation of the *sym2* region, because we were not able to detect a cDNA40/3 related polymorphism between Afghanistan and the European peas, Rondo and NGB1238 (data not shown).

The *sym2* region was screened for additional molecular markers, particularly between *sym2* and cDNA267, using over 450 RAPD decamer primers. This resulted in one RAPD marker, OPA1, tightly linked to *sym2*. An additional four RAPD markers, OPA4, P198, B374 and B474 (Table 2), shown to be linked to *sym2* [Weeden and Temnykh, unpublished results], were used for mapping. RAPDs OPA4, B374 and B474 only produced polymorphic fragments with NGB1238 and were dominant. RAPD marker P198 appeared to be a co-dominant marker, producing polymorphic bands with both Afghanistan and NGB1238 (Table 2). The five RAPD markers (Table 2) were mapped using F2 and F3 *sym2* segregating populations of the cross L-4 x NGB1238. The position of these markers on the map is shown in Figure 1c. Recombination frequencies between markers and pairwise data for the F3 segregating population of the cross L-4 x NGB1238 are given in Table 3. B374 is tightly linked to *sym2*, since we did find recombination in the F2 and F3 segregating populations of the cross L-4 x NGB1238. Further RFLP and RAPD analyses of the F4 generations (data not shown) confirmed that B374 maps in the interval *sym2*-cDNA44 as shown in Figure 1c. The RAPD marker OPA1 mapped between cDNA267 and *sym2*. We did not find recombination between *sym2* and OPA1 in the F2 and F3 segregating populations of the cross L-4 x NGB1238. The position of OPA-1 was confirmed by analysis of the Rondo *sym2* introgression lines A5.4.3 and A5.6.9 (see below, Figure 2) [Kozik et al., 1995]. In the line A5.6.9 a recombination has occurred between *sym2* and OPA1.
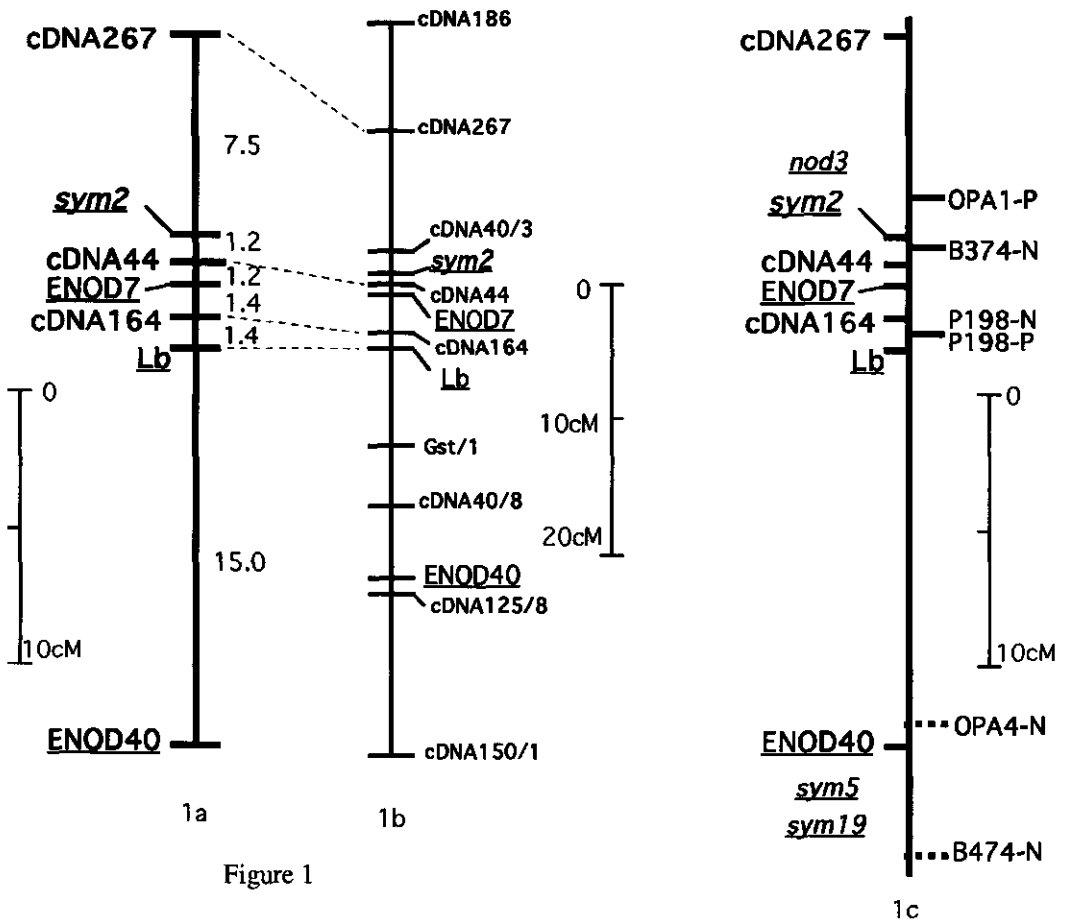
Figure 1

RFLP map (1a), integrated map (1b) and
detailed genetic map of the *sym2* region (1c)

1a - RFLP map of the *sym2* region. Map was constructed using pairwise data for F3 segregating
population of the cross L-4 x NGB1238. Precise position of *sym2* was determined by
co-segregation of *sym2* genotype with RFLP markers (details of determination of the *sym2*
genotype see in Materials and Methods). *sym2* and nodulin genes are underlined.

1b - Integrated map of the *sym2* region. Map was constructed by combining the pairwise data of
the crosses L-4 x NGB1238 (this study) and JI281 x JI399 [Elliset al., 1992]. Positions of RFLP
markers which were mapped directly on both populations (L-4 x 1238 and JI281 x JI399) are
connected by dashed lines. Note that maps on the Figures 1a and 1b have different scale.

1c - Detailed map of the *sym2* region. Newly added and mapped RAPD markers are shown at
the right side of the map. Positions of RAPD markers were determined by co-segregation with
RFLP markers in the cross L-4 x NGB1238. The *sym2* flanking RAPD markers OPA1 and B374
were converted into SCAR markers. *nod3* maps in between RFLP markers 267 and 44. For
RAPD markers OPA4 and B474 only relative positions are known and shown by dashed lines.
sym5 and *sym19* co-segregate with RAPD markers OPA4 and B474 (Weeden and Temnykh,
personal communication).

Table 3

	cDNA267 RFLP	OPA1-P RAPD	sym2 genotype	B374-N RAPD	cDNA44 RFLP	ENOD7 RFLP	cDNA164 RFLP	P198-N RAPD	P198-P RAPD	Lb RFLP	B474-N RAPD	OPA4-N RAPD
OPA1-P RAPD	3.4 ±2.5 (12.0)	*	-	-	-	-	-	-	-	-	-	-
sym2 genotype	6.6 ±2.0 (15.7)	0.00 (17.3)	*	-	-	-	-	-	-	-	-	-
B374-N RAPD	9.8 ±4.2 (8.0)	0.00 (5.5)	0.00 (18.8)	*	-	-	-	-	-	-	-	-
cDNA44 RFLP	8.1 ±2.1 (14.0)	2.2 ±2.0 (13.7)	1.2 ±0.8 (26.3)	0.00 (18.8)	*	-	-	-	-	-	-	-
ENOD7 RFLP	9.0 ±2.3 (12.8)	3.4 ±2.5 (12.0)	1.9 ±1.0 (24.4)	1.0 ±1.3 (16.7)	0.6 ±0.5 (27.8)	*	-	-	-	-	-	-
cDNA164 RFLP	10.7 ±2.6 (10.9)	5.9 ±3.5 (9.5)	3.2 ±1.3 (21.2)	2.0 ±1.8 (15.2)	1.9 ±1.0 (23.9)	1.2 ±0.8 (25.9)	*	-	-	-	-	-
P198-N RAPD	12.5 ±4.8 (6.3)	12.4 ±6.9 (3.1)	2.0 ±1.8 (14.7)	2.1 ±1.9 (15.2)	2.0 ±1.9 (14.7)	1.0 ±1.2 (16.7)	1.0 ±1.9 (19.3)	*				
P198-P RAPD	9.6 ±4.4 (7.3)	5.8 ±3.4 (9.7)	5.6 ±3.3 (10.1)	0.00 (5.1)	3.3 ±2.5 (12.2)	2.2 ±2.0 (13.5)	0.00 (17.1)	0.00 (5.9)	*			
Lb RFLP	12.3 ±2.8 (9.5)	7.1 ±3.8 (8.6)	4.5 ±1.6 (18.8)	3.0 ±2.2 (13.9)	3.2 ±1.3 (21.1)	2.5 ±1.2 (22.8)	1.2 ±0.8 (26.2)	1.0 ±1.3 (17.1)	1.1 ±1.4 (15.0)	*		
B474-N RAPD	25.9 ±7.4 (1.9)	19.2 ±8.4 (1.9)	12.5 ±5.0 (6.1)	12.6 ±4.9 (6.3)	12.2 ±4.8 (6.5)	10.7 ±4.4 (7.4)	9.4 ±4.1 (8.5)	10.0 ±4.3 (7.9)	0.00 (5.5)	10.6 ±4.3 (7.9)	*	-
OPA4-N RAPD	27.8 ±7.7 (1.5)	20.5 ±8.8 (1.7)	13.9 ±5.3 (5.3)	14.0 ±5.2 (5.5)	13.5 ±5.1 (5.7)	12.0 ±4.8 (6.6)	10.6 ±4.6 (7.7)	11.3 ±4.6 (7.0)	0.00 (5.1)	9.5 ±4.1 (8.4)	3.2 ±2.3 (13.7)	*
ENOD40 RFLP	29.8 ±4.3 (2.0)	25.8 ±7.2 (2.0)	21.8 ±3.7 (4.5)	13.8 ±4.8 (6.5)	20.6 ±3.6 (5.1)	19.4 ±3.4 (5.8)	17.5 ±3.2 (6.9)	11.3 ±4.3 (8.0)	17.3 ±5.7 (4.4)	15.7 ±3.1 (7.9)	3.2 ±2.3 (14.2)	0.00 (18.8)

Pairwise recombination percentage and LOD score (in brackets); data for F3 segregating population of the cross L-4 x NGB1238. RAPD markers (polymorphic DNA bands) which are amplified on the Afghanistan DNA (positive to sym2 RAPD markers) are designated with symbol -P (OPA1-P and P198-P). RAPD markers (polymorphic DNA bands) which are amplified on the NGB1238 DNA (negative to sym2 RAPD markers) are designated with symbol -N (B374-N, B474-N, OPA4-N and P198-N).

Construction of Sequence Characterized Amplified Regions (SCAR) primers

The RAPD markers OPA1 and B374 are at distance of approximately 1 cM or less from *sym2*. The OPA1 marker is in repulsion phase with *sym2*, which means that the polymorphic band is amplified on Afghanistan DNA and not on NGB1238 DNA (see Table 2). At the other hand the B374 marker is in coupling phase with *sym2*, which means that the polymorphic band is amplified on NGB1238 DNA but not on Afghanistan DNA. Consequently the use of marker B374 is limited to crosses involving pea line NGB1238, whereas OPA1 can be used to diagnose Afghanistan DNA. The DNA polymorphic bands obtained with OPA1 and B374 respectively were cloned and sequenced. The nucleotide sequences of both amplified marker bands did not show significant homology to other sequences present in the databases. Southern analysis using the cloned RAPD marker fragments showed that the OPA1 and B374 fragments are DNA repeats and they are therefore not useful for RFLP analysis.

To increase their specificity RAPD markers OPA1 and B374 were converted into SCAR markers. The SCOPA1 primer sequences consisted of the 10 bases of the original RAPD primer plus the next 11-14 internal bases of the cloned RAPD fragment. The designed SCB374 primer sequences consisted only of internal sequences of the cloned polymorphic RAPD fragment (seeTable 4). By using the SCB374 primers we were able to amplify a polymorphic band of 1 kb on NGB1238 DNA but not on DNA from other pea lines, like Afghanistan and Rondo. Using the SCOPA1 primers with genomic DNA from Afghanistan, Rondo and the introgression lines A5.4.3 and A5.6.9, a 530 bp DNA fragment was amplified. The 530 bp bands amplified from these DNA samples was cloned and sequenced. The fragment amplified from Rondo DNA showed a 9 bp deletion in comparison to the Afghanistan sequence. This deletion was used to design two new primers, one primer specific for the Rondo OPA1 sequence, SCOPA1-R primer, and one specific for the Afghanistan homologue, SCOPA1-A (Table 4). As a result, the primer pair SCOPA1-5'/SCOPA1-R specifically amplified only the Rondo OPA1 allele, and the primer pair SCOPA1-5'/SCOPA1-A only amplified the Afghanistan OPA1 allele (Table 4). These two pairs of SCAR primers are therefore useful to diagnose the presence of both Rondo and Afghanistan DNA from the *sym2* region in introgression lines.

Table 4. SCAR primers and size of amplified polymorphic bands

SCAR primer	primer sequence	amplified polymorphic DNA fragment
SCB374-5'	5'-TGTACCCAGGAAGGGTCCTGT-3'	SCB374-5'/SCB374-3'
SCB374-3'	5'-ACCCTCAGATAGGTACACAACAT-3'	amplifies a 1 kb fragment only on NGB1238 DNA
SCOPA1-5'	5'-CAGGCCCTTCCTACAAAAGAGATA-3'	SCOPA1-5'/SCOPA1-3'
SCOPA1-3'	5'-CAGGCCCTTCAGCTTGAAGA-3'	amplifies a 530 bp fragment on Afghanistan, Rondo, NGB1238 DNA
SCOPA1-R	5'-GAGGCCAGGTTCAGGTAT-3'	SCOPA1-5'/SCOPA1-R amplifies a 530 bp fragment on Rondo DNA.
SCOPA1-A	5'-GGTTCAGAGGGTTCAGGTAC-3'	SCOPA1-5'/SCOPA1-A amplifies a 530 bp on Afghanistan DNA

Characterisation of available *sym2* introgression lines

Molecular markers of the of the different parts of the *sym2* region (Figure 1c) were used to characterise the *sym2* introgression lines, A5.4.3 and A5.6.9 [Kozik et al., 1995], A15.5 and A33.18 [this study], Sparkle-*sym2* [Kneen et al., 1984] and μ-3 [this study] (Table 1). The *sym2* introgression lines originated from repeated backcrosses of Afghanistan pea with either Rondo, Sparkle or NGB1238 peas. The results of the RFLP and RAPD analyses of the different introgression lines are presented in Figure 2. The Sparkle-*sym2* was found to have a region, introgressed from Afghanistan, of about 3-5 cM. The Rondo-*sym2* lines A15.5 and A33.18 had even a smaller introgressed region of about 1-2 cM. The latter lines will are good starting material to be used for stratagies leading to cloning of *sym2*, e.g. positional cloning and differential display. The *sym2* introgression line μ-3 constructed in the NGB1238 genetic background has a relatively big *sym2* introgressed region. However, the multiple morphological markers of the line NGB1238 [Lamprecht, 1974] distributed randomly over the seven pea linkage groups allow easy detection during selection

which will facilitate the elimination of Afghanistan genome background in other regions than the *sym2* region.
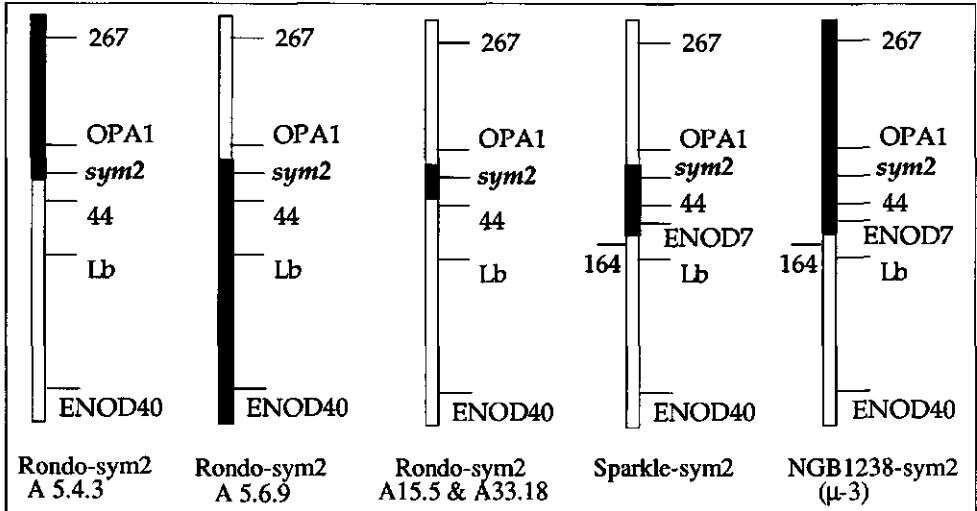
Figure 2. Genetic characterisation of available *sym2* introgression lines.
Black lines represent *sym2* introgressed region from Afghanistan pea. White lines represent the European pea genome (e.g. Rondo, Sparkle or NGB1238).

DISCUSSION

Two out of the seven pea early nodulin genes identified so far, i.e. *PsENOD7* and *PsENOD40*, map in linkage group I, where sym2 is located. Also in this region three other sym genes, *sym5*, *sym19* and *nod3* along with a cluster of Lb genes are located. The position of *PsENOD7* was previously established [Kozik et al., 1995] but we have now added *PsENOD40* to the map and found it to be located at a distance of about 15 cM from *sym2*. The early nodulin genes *PsENOD2*, *PsENOD3*, *PsENOD5*, *PsENOD12* and *PsENOD14* are divided over other linkage groups of the pea genome. It would have been a nice incidental circumstande if *sym2*, or any of the other *sym* mutant genes would have coincided with an (early) noduline gene as that greatly helps the identification of the nature of *sym* gene products, but so far this has not been found.

The part of linkage group I with the *sym* and nodulin genes comprises only about 30 cM of a total of 17000 cM of the pea genome. The location of seven genes in

involved in nodule formation and symbiosis is a conspicuous concentration of genes in this linkage group making this region an important part of the pea genome with regard to symbiosis. The region can be divided into two clusters, which are about 15 cM apart. One cluster includes the *sym2*, *nod3*, *PsENOD7* and *Lb* genes, while the other cluster comprises *PsENOD40*, *sym5* and *sym19*. The relative positions of *nod3*, *sym5* and *sym19* have yet to be determined precisely, but *nod3* was found closely linked to *sym2* whereas *sym5* and *sym19* are linked to *PsENOD40*.

We have searched for molecular markers closely linked to *sym2* as such markers are essential for positional cloning of *sym2* and the further characterisation of this gene. We integrated earlier published as well as unpublished data using the program Joinmap-1.4 (Stam 1994) and found one RFLP marker, *cDNA40/3*, that mapped 2 cM above *sym2*. Unfortunately, this marker did not give a polymorphism between Afghanistan and the European peas, Rondo and NGB1238, and therefore is not useful for further characterisation of the *sym2* region.

Furthermore, we have added five RAPD markers to the map of linkage group I (Fig. 1C). Two of the RAPDs were closely linked to *PsENOD40* and map in the cluster of genes around *PsENOD40*, whereas the other three map in the cluster of genes in the region of *sym2*. Two RAPDs, OPA1-P and B374-N are very tightly linked to *sym2* and both are found at a distance of 1 cM or less from *sym2*, each at a different side. These two RAPDs are very useful for characterising the area surrounding *sym2*. Both RAPDs were converted to SCAR markers to increase their specificity and these SCARs were used to define the introgressed region in different introgression lines. Especially usefull are the different SCOPA1 primers which can be used to diagnose Afghanistan or Rondo and Sparkle DNA. In this way we identified that the Rondo-*sym2* lines A15.5 and A33.18 have a very small *sym2* introgressed region of about 1-2 cM from Afghanistan pea. These lines are the preferred material to be used in cloning stratagies, such as positional cloning and differential display, resulting in the cloning of *sym2* and subsequent characterisation of its product.

CHAPTER 5

THE RHIZOBIAL *nodO* GENE DETERMINES
WHETHER THE PEA *sym2* GENE IS DOMINANT OR RECESSIVE

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INTRODUCTION

The symbiotic interaction of *Rhizobium* bacteria and legume plants starts with the exchange of signal molecules. Flavonoids extreted by the plant roots induce the expression of bacterial nodulation (*nod*) genes resulting in the synthesis of specific lipochito-oligosaccharides, named Nod factors [Lerouge et al., 1990]. The Nod factors of different rhizobia all have a similar structure. They consist of a tetra- or pentameric N-acetyl glucosamine backbone with a fatty acyl chain at the non-reducing terminal sugar moiety. Different substitutions at the terminal sugar residues and the structure of the acyl chain determine the differences in biological activity and host specificity [Spaink, 1992; Fisher and Long, 1992].

Nod factors elicit the *Rhizobium* induced early responses of the host, such as root hair deformation and mitotic reactivation of the root cortex [Vijn et al., 1993]. Since these responses are induced at concentrations as low as 10^{-9} - 10^{-13} molar, it has been proposed that Nod factors are recognised by receptors.

Recently, a protein binding Nod factor produced by *Rhizobium meliloti* has been identified in *Medicago truncatula* [Bono et al., 1995]. However, this protein has a relatively low affinity for Nod factors and, furthermore, the same affinity for Nod factors with or without a sulphate substitution at the reducing terminal sugar. Since the sulphate substitution is the major host specificity determinant in the *Medicago truncatula - Rhizobium meliloti* symbiosis, it is unlikely that this Nod factor binding protein is a Nod factor receptor. The identification of putative binding proteins is hampered by the hydrophobic nature of the Nod factor. Furthermore, Nod factor receptors might only occur in a specific tissue like the root epidermis. For these reasons, it might be almost impossible to isolate a Nod factor receptor by biochemical procedures and a genetic approach to identify the receptor could be an attractive alternative.

sym2 is a good candidate for a gene coding for a Nod factor receptor in pea [Heidstra et al., 1993; Kozik et al., 1995]. *sym2* was first identified in the wild pea variety Afghanistan where it confers nodulation resistance to European *Rhizobium leguminosarum* biovar (bv) *vicia* (*Rlv*) strains [Lie, 1984]. At the other hand, certain *Rlv* strains from the Middle East, like *Rlv* TOM, nodulate *sym2* harbouring pea lines as well as western pea cultivars lacking *sym2*. The extended host range of *Rlv* strain TOM is due to the additional *nod* gene *nodX* which encodes an acetyl transferase that specifically acetylates the reducing sugar moiety of pentameric Nod factors [Firmin et al., 1993]. It has been shown that *nodX* is the only additional gene required,

allowing European *Rlv* strains to nodulate *sym2* containing peas [Kozik et al., 1995]. Therefore it is probable that Sym2 is a protein involved in the recognition of NodX modified Nod factors.

Previously, we showed that *sym2* behaves either as a dominant or as a recessive gene depending on the rhizobial strain used for the inoculation. For example *sym2* is a dominant gene if *Rlv* strain PRE is used as inoculum , whereas it is recessive with *Rlv* strain 248 as inoculum. It was shown that the genetic nature of the Afghanistan *sym2* allele is not determined by the amount of Nod factor secreted by the bacteria [Kozik et al., 1995]. In this chapter we report the identification of the rhizobial gene controlling the dominant/recessive nature of *sym2* and show this gene to be *nodO*.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are described in Table 1. The differences between plasmids pIJ1089 and pMP225 are indicated in Figure 1. The plasmids pIJ1089 and pMP225 were mobilized from *E.coli* to Rlv strain 1391 using tripartite mating with pRK2013 as a helper plasmid [Ditta et al., 1980]. Rlv strain 248*nodO⁻* was made by crossing strains 1391 and A34*nodO⁻* (kindly provided by Dr J.A. Downie) as described by Beringer et al. [1978]. Selection of transconjugants was done on B⁻ medium [Spaink et al., 1991] with the appropriate antibiotics.
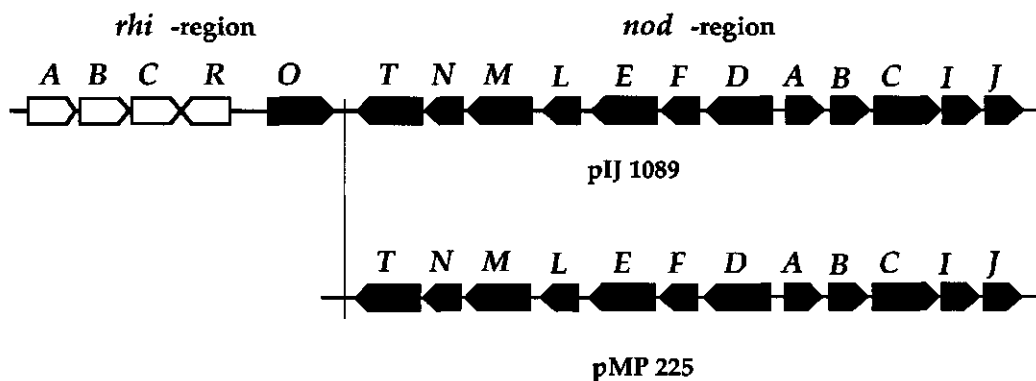
Figure 1. Plasmids pIJ1089 and pMP225 contain different parts of *nod* - region from pRL1JI.

Table 1. Strains and plasmids

Strains/plasmids	Relevant characteristics	Reference
248	wild type *Rlv* strain containing Sym plasmid pRL1JI	Josey et al., 1979
PRE	wild type *Rlv* strain	Lie, 1984
TOM	wild type *Rlv* strain containing Sym plasmid pRL5JI	Winarno and Lie, 1979 Brewin et al., 1980
1391	248 Rif^R cured from its Sym plasmid pRL1JI	Schlaman et al., 1992
A34*nodO*$^-$	8401/pRL1JI*nodO$_{94}$*::Tn5	Economou et al., 1994
248*nodO*$^-$	1391/pRL1JI*nodO$_{94}$*::Tn5	this study
1391.pIJ1089	1391 carrying pIJ1089	this study
1391.pMP225	1391 carrying pMP225	this study
pRK2013	helper plasmid	Ditta et al., 1980
pIJ1089	*nodABCIJDFELMNTO rhiABCR*	Downie et al., 1983
pMP225	*nodABCIJDFELMNT*	Spaink et al., 1987

Plant material

The original pea lines and the *sym2* introgression lines used in this study are presented in Table 2. The construction of the *sym2* introgression line A5.4.3 with Rondo genetic background was described previously [Kozik et al., 1995]. Heterozygous *sym2* plants used for nodulation experiments were derived from the cross A5.4.3xRondo.

sym2 introgression line R15-4 with NGB1238 genetic background was selected from the F2 segregating population of the cross L-4 x NGB1238 described previously [Kozik et al., 1995]. *sym2* homozygous F2 plant numbered R15 (see Appendix, pages: 108-109) showed a non-typical *sym2* phenotype. The R15 plant was Nod$^+$ upon inoculation with *Rlv* strain 248. The *sym2* homozygous genotype of this plant was confirmed by testing of its offspring, all of which were Nod$^-$ upon inoculation with *Rlv* strain PRE (twenty F3 plants were tested). However, after selfing of R15, twelve out of twenty four F3 plants tested still showed a Nod$^+$ phenotype (15-30 nodules per plant) upon inoculation with *Rlv* strain 248. The *sym2* homozygous line showing

a stable Nod$^+$ phenotype upon inoculation with *Rlv* strain 248 and a Nod$^-$ phenotype upon inoculation with *Rlv* strain PRE was selected and named R15-4.

Table 2. Pea lines

Pea lines	Relevant characteristics	Reference
Rondo	European cultivar	Lie, 1984
NGB1238	Multiple marker line	Lamprecht, 1974
A5.4.3	*sym2* introgression line, Rondo genetic background	Kozik et al., 1995
R15-4	*sym2* introgression line, NGB1238 genetic background	this study

RESULTS AND DISCUSSION

nodO determines the dominant/recessive nature of *sym2*

Depending on the *Rlv* strain used for inoculation, *sym2* behaves as a dominant or a recessive gene. For example, *Rlv* strain 248 formed nodules on *sym2* heterozygous plants, whereas *Rlv* strain PRE did not [Kozik et al., 1995]. Therefore, *Rlv* strain 248 contains additional genetic information facilitating the nodulation of heterozygous *sym2* plants. To examine whether this additional genetic information is located on the Sym plasmid *Rlv* strain 1391 was transformed with pMP225, containing the minimal set of *nod* genes essential for nodulation (Figure 1). In addition, *Rlv* strain 1391 was transformed with pIJ1089, which holds an additional 6kb of the Sym plasmid, containing *nodO* and the *rhi* genes (Figure 1). Inoculation of *sym2* heterozygous plants showed that both *Rlv* strain 1391.pIJ1089 and the wild type strain 248, resulted in the formation of 30-50 nodules, while strain 1391.pMP225 was unable to form nodules (Table 2). So the genetic information of *Rlv* strain 248 bringing about the nodulation of *sym2* containing peas, is located on this additional 6 kb, present in pIJ1089.

We checked whether *nodO* is responsible for the nodulation by *Rlv* strain 248 by inoculating *sym2* heterozygous peas with *Rlv* strain 248*nodO*$^-$. This mutant formed only 0-5 nodules, whereas, in the control experiment, the wild type strain 248 formed 30-50 nodules (Table 2). In contrast, European peas, lacking the Afghanistan *sym2*

Table 3. Nodulation of different pea *sym2* introgression lines and crosses

Rlv strain	Pea line / cross				
	Rondo $Sym2^C/Sym2^C$	NGB1238 $Sym2^C/Sym2^C$	A 5.4.3 $Sym2^A/Sym2^A$	F1 A543xRondo $Sym2^A/Sym2^C$	R15-4 $Sym2^A/Sym2^A$
TOM	30-50	20-40	30-50	30-50	20-40
248	30-50	20-40	0-5	30-50	20-30
PRE	30-50	20-40	0	0-5	0
1391.pIJ1089	30-50	20-40	0	30-50	20-30
1391.pMP225	30-50	20-40	0	0	0
248*nodO⁻*	30-50	20-40	0	0-5	0-5

At least ten plants were tested in each experiment. Numbers indicate amount of nodules per plant.
Nodulation tests were performed at 18°C, nodules were counted three weeks after inoculation.

allele, form 30-50 nodules with both *Rlv* strain 248 and strain 248*nodO⁻* (Table 2). So the recessive nature of *sym2* in the interaction with *Rlv* strain 248 is determined by *nodO*. *Rlv* strain PRE does not have a flavonoid inducible *nodO* [De Maagd et al., 1989], which explains why *sym2* is a dominant allele in the interaction with this *Rlv* strain. Since NodO is not involved in the biosynthesis of Nod factors, but more likely interferes with Nod factor activated signal transduction in the plant (see below), we conclude that *sym2* is a dominant gene which will further be designated as *Sym2*.

To distinguish the Afghanistan *Sym2* allele from other *Sym2* alleles we propose to name it $Sym2^A$. The *Sym2* allele of European cultivated peas will be named $Sym2^C$.

nodO as well as *nodX* help to overcome the nodulation block conferred by $Sym2^A$

NodO is a secreted protein that is not involved in Nod factor biosynthesis [Sutton, Lea & Downie, 1994]. When added to lipid bilayers, NodO forms channels that allow the passage of monovalent cations. Therefore, it has been suggested that NodO integrates into the plant plasmamembrane [Economou et al., 1994; Sutton et al., 1994]. *Rlv nodE⁻nodO⁻* mutants that have lost the ability to make Nod factors with a $C_{18:4}$ acyl group and only secrete Nod factors with an acyl fatty acid moiety $C_{18:1}$

[Spaink et al., 1991], are unable to form nodules on pea. The ability to nodulate is (partly) restored by the presence of *nodO* , since *Rlv nodE⁻nodO⁺* mutants induce the formation of nodules albeit at a 50% reduced level [Economou et al., 1994]. Therefore it has been postulated that NodO overcomes the absence of NodE modified Nod factors by amplifying Nod factor activated signal transduction, forming an ion channel in the plant plasmamembrane [Economou et al., 1994; Sutton et al., 1994].

To determine whether NodO compensates in a similar way for the lack of NodX modified factors, we inoculated *sym2^A* homozygous A5.4.3 with *Rlv* strains PRE, 248 and 248*nodO⁻*. Inoculation with *Rlv* strain 248 resulted in 0-5 nodules, whereas with strain 248*nodO⁻* and strain PRE no nodules were formed (Table 3). These results show that NodO can indeed partly restore the nodulation ability in *Rlv* strain 248, lacking *nodX* . Thus, *nodO* compensates for the Nod factor structure deficiency of a *Rlv nodE⁻* as well as a *nodX⁻* mutant, although the structural deficiency in both mutants is different.

We and others [Young, 1985; Kozik et al., 1995] have also previously observed that *Rlv* strains, e.g. strain 248, formed a few nodules on *sym2^A* homozygous pea lines. However, less than 5 nodules per plant was scored as Nod⁻ and consequently the difference in nodulation behaviour of *Rlv* strains 248 and PRE was overlooked [Lie, 1984; Kneen et al., 1984; Kozik et al., 1995]

NodO activity is influenced by the plant genotype

To study the nodulation phenotype conferred by *sym2^A* in different pea chromosomal backgrounds we introgressed *sym2^A* into the marker line NGB1238. The line R15-4 contains an introgressed region of at least 20 cM from the Afhanistan genome including *sym2^A* and all surrounding RFLP markers. The phenotype of this line was compared with that of the previously characterised introgression line A5.4.3 [Kozik et al., 1995; 1996]. Both introgression lines as well as the parental lines Rondo and NGB1238, respectively, were inoculated with the *Rlv* strains PRE, 248 and 248*nodO⁻*. Whereas no nodules were formed upon inoculation with *Rlv* strain PRE on either introgression line, *Rlv* strain 248*nodO⁻* was able to form 0-5 nodules on line R15-4. However, *Rlv* strain 248 formed 20-40 nodules on R15-4 plants, whereas, on A5.4.3 plants only 0-5 nodules were formed. All strains formed an equal number of nodules on the parental lines (Table 3).

Thus *sym2^A* had a similar phenotype in both genomic backgrounds when the

peas were inoculated with *Rlv* strains lacking a functional *nodO*. The fact that some nodules were formed with *Rlv* strain 248*nodO⁻*, may indicate that this line shows a less restricted nodulation phenotype. However, upon inoculation with *Rlv* strain 248, containing *nodO*, the nodulation behaviour was strikingly different, showing that NodO can efficiently compensate for the Nod factor structure deficiency in R15-4 plants, having a NGB1238 genetic background. Therefore we postulate that the pea genetic background can influence NodO activity.

CHAPTER 6

CONLUDING
REMARKS

Concluding remarks

The symbiotic association between *Rhizobium* and legumes is specific and requires close cooperation between the bacteria and the plant. The bacterial genes controlling nodulation and host specificity have been identified for the greater part and shown to be involved in the synthesis of Nod factors. One of the primary effects of Nod factors appears to be a modification of the pattern of plant cell growth causing root hair deformation, the development of infection threads and the induction of cell divisions in the root cortex. Hence unravelling the mechanisms by which plants perceive these rhizobial signal molecules and how these are transduced into the various plant resposes is a major challenge.

The very low concentrations that are involved in elicing the plant responses as well as the structural specificity of Nod factors required for the induction of responses, indicate that a receptor might be involved in the perception of Nod factors. Studying the various responses induced by *R meliloti* Nod factors in the epidermis of alfalfa roots showed that different structural demands are involved in the induction of these responses. For example, the induction of root hair deformation and *ENOD12* expression do not require a Nod factor with an acyl group with a specific structure (C16:2) nor the presence of the NodL dependent acetyl group at the non-reducing terminal sugar. These observations led Adourel et al. (1994) to propose that at least two different Nod factor receptors are present in the epidermis; a signalling receptor and an uptake receptor. The signalling receptor recognizes Nod factors, even if the non-reducing end is modified, and, upon binding, activates the induction of e.g. root hair deformation. The uptake receptor is activated only by the fully decorated Nod factor and upon binding, infection thread formation is initiated.

Among the *sym* genes of pea, *Sym2* has been shown to condition strain specific nodulation. The *Sym2* ^{A} allele of Afganistan pea had been shown to be responsible for the inability of European *R.leguminosarum* bv *viciea* strains to nodulate Afghanistan pea. Furthermore, it had been demonstrated that *R.l.* bv *viciea* strains carrying the additional *nodX* gene can nodulate Afghanistan pea. In this thesis, we extented these studies by showing that European pea lines containing the introgressed *Sym2* region of Afghanistan peas can be nodulated by rhizobia carrying *nodX* This observation strongly supports the hypothesis that Sym2 is involved in the recognition of Nod factors containing the NodX dependent acetyl substitution at the reducing sugar residue.

Whether *R.l.* bv *viciae* are carrying *nodX*, or not, in both cases the interaction

between *R.l.* bv *viciae* strains with or without *nodX*, and peas containing *Sym2^A* results in a similar induction of root hair deformation, cortical cell division and activation of *ENOD12*. However, the number of infections on *Sym2^A* peas was severely reduced compared to the infections on peas lacking *Sym2^A* as was shown by using *R.l.* bv *viciae* strain 248 containing a constitutively expressed *lacZ* gene. The few infections that took place were aborted in the epidermis (Geurts, Hadri & Heidstra, unpublished). In this respect, there is a striking similarity between the responses induced by the *R. meliloti nodFEL* mutants on alfalfa and those induced by *R.l.* bv *viciae nodX$^-$* strains on *Sym2 ^{A}* peas. So, it is possible that *Sym2* encodes a Nod factor receptor with the characteristics proposed for the uptake receptor.

The *Sym2^A* allele appears to be widely spread in the areas around the Mediteranian See. In all investigated pea lines that are resistant to European *R.l.* bv *viciea* , this resistance appears to be determined by the *Sym2^A* allele. This includes the *Sym1* gene of Iran peas that we showed to be *Sym2^A*. Hence it is probable that only two abundantly occurring natural variants of *Sym2* occur in wild and cultured pea varieties. We refer to these two *Sym2* variants as *Sym2^A* and *Sym2^C*; the latter does not require a *Rhizobium* carrying *nodX* for nodulation to occur.

We showed that nodulation resistance conferred by *Sym2^A* can (partly) be suppressed if rhizobia carry *nodO*, showing that *nodO* can complement for the absence of a functional *nodX* This explains why *Sym2^A* behaves as a dominant or recessive allele depending on the *Rizobium* strain used for the inoculation. When rhizobia containing a functional *nodO* gene were used *Sym2^A* acts as a recessive allele, whereas with rhizobia lacking *nodO* it is a dominant allele.

NodO is a secreted protein that is not involved in Nod factor biosynthesis (Sutton, Lea & Downie, 1994). When added to lipid bilayers, it forms channels that allow the movement of monovalent cations. Therefore, it has been suggested that NodO integrates into the plant plasmamembrane and this effects amplification of a Nod factor induced signal transduction pathway (Economou et al., 1994; Sutton et al., 1994). We postulate that NodO complements for a Nod factor structural deficiency not by affecting Nod factor structure but by amplifying the signal transduction cascade leading to infection.

In experiments using a *R.l.* bv *viciae nodE$^-$* mutant it was shown that the presence of *nodO* enables this strain to nodulate pea and vetch, although the number of nodules formed was cut down by half compared to the number formed after inoculation with the wildtype during the same time period (Economou et al., 1994). In the absence of *nodO* (*R.l.* bv *viciae nodE$^-$nodO$^-$* double mutant) this strain is unable

to nodulate pea and vetch, whereas its ability to induce root hair deformation is unaffected (Downie & Surin, 1990). The *nodE⁻* mutant produces Nod factors that only contain a C18:1 fatty acyl chain (Spaink et al., 1991), indicating that here *nodO* complements for the absence of NodE modified Nod factors.

Thus, *nodO* complements the Nod factor structure deficiency of a *R.l.* bv *viciae nodE⁻* as well as a *nodX⁻* mutant, enabling nodulation on $Sym2^C$ and $Sym2^A$ peas respectively, despite the fact that the structural deficiency in both mutants is different. Although, NodO more efficiently complements a *R.l.* bv *viciae nodE⁻* than a *nodX⁻* mutation (Chapter 5) as indicated by the amount of nodules formed by either mutant, this may be a gradual effect. This indicates that NodE and NodX modified Nod factors bind to a similar receptor. In other words, the $Sym2^C$ control receptor binds NodRlv-IV/V(Ac,C18:4) containing the NodE determined unsaturated fatty acid, whereas the $Sym2^A$ control receptor binds NodRlv-V(Ac,C18:4,Ac) containing the additional NodX determined modification. The complementation by NodO for Nod factor structure deficiency also supports the hypothesis that the *Sym2* alleles encode a control receptor.

The genetic studies on *Sym2* of Weeden and LaRue (Weeden, Kneen & LaRue, 1990) as well as the data presented in this thesis show that *Sym2* is located on linkage group 1 near the major leghemoglobin cluster. This is an interesting region of the pea genome, since it appears to contain a relatively high density of genes involved in the symbiotic interaction with *Rhizobium*, including the early nodulin genes *ENOD7* and *ENOD40*. This by itself justifies a further characterisation of this region. Furthermore, since Sym2 is a putative Nod factor receptor it is attractive to clone *Sym2*. Unfortunately, the size of the pea genome cautions the use of a positional cloning strategy involving YAC libraries and chromosome walking. PCR based techniques comparing either two genomes by AFLP-fingerprinting or comparing the mRNA pools of two plants by differential display reverse transcription PCR circumvent this problem. Presently differential display studies are being carried out. Near isogenic lines differing for a small introgressed region including the *Sym2* gene, which are generated from the genetic material described in this thesis, form the corner-stone of these studies.

REFERENCES

Altschul S.F., Gish W., Miller W., Myers E.W. and Lipman D.J. 1990. Basic local alignment search tool. Journal of Molecular Biology **215**, 403-410.

Ardourel M., Demont N., Debellé F., Maillet F., de Billy F., Promé J-C., Dénarié J. and Truchet G. 1994. *Rhizobium meliloti* liposaccharide nodulation factors: Different structural requirements for bacterial entry into target root hair cells and induction of plant symbiotic developmental responses. Plant Cell **6**, 1357-1374.

Arumuganathan K. and Earle E.D. 1991. Nuclear DNA content of some important plant species. Plant Molecular Biology Reporter **9** (3), 208-218.

Beringer J.E., Hoggan S.A. and Johnston A.W.B. 1978. Linkage mapping in *Rhizobium leguminosarum* by means of R plasmid-mediated recombination. Journal of General Microbiology **104**, 201-207.

Bisseling T., Van den Bos R.C. and Van Kammen A. 1978. The effect of ammonium nitrate on the synthesis of nitrogenase and the concentration of leghemoglobin in pea root nodules induced by *Rhizobium leguminosarum*. Biochimica et Biophysica Acta **539**, 1-11.

Blixt S. 1974. The pea. *In* Handbook of Genetics (edited by R.C. King). Vol. 2. Plenum Press, New York, pp. 181-221.

Blixt S. 1977. The gene symbols of *Pisum*. Pisum News Letters. Special volume, supplement.

Bono J.-J., Riond J., Nicolaou K.C., Bockovich N.J., Estevez V.A., Cullimore J.V. and Ranjeva R. 1995. Characterization of a binding site for chemically-synthesized lipo-oligosaccharidic NodRm factors in particulate fractions prepared from roots. Plant Journal **7**, 253-260.

Borisov A.Y., Morzhina E.V., Kulikova O.A., Tchetkova S.A., Lebsky V.K. and Tikhonovich I.A. 1993. New symbiotic mutants of pea (*Pisum sativum* L.) affecting either nodule initiation or symbiosome development. Symbiosys **14**, 297-313.

Brewin N.J., Beringer J.E. and Johnston A.W.B. 1980. Plasmid-mediated transfer of host range specificity between two strains of *Rhizobium leguminosarum*. Journal of General Microbiology **120**, 413-420.

Brito B., Palacios J.M., Imperial J., Ruiz-Argueso T., Yang W.C., Bisseling T., Schmitt H., Kerl V., Bauer T., Kokotec W. and Lotz W. 1995. Temporal and spatial co-expression of hydrogenase and nitrogenase genes from *Rhizobium leguminosarum* bv. *viciae* in pea (*Pisum sativum* L.) root nodules. Molecular Plant-Microbe Interaction **8**, 235-240.

Cannon W.A. 1903. Studies on plant hybrids: the spermatogenesis of hybrid peas. Bulleten of the Torrey Botanical Club **30**, 519-543.

Cooper J.B. and Long S.R. 1994. Morphogenetic rescue of *Rhizobium meliloti* nodulation mutants by trans-zeatin secretion, Plant Cell **6**, 215-225.

Cox K.H. and Goldberg R.B. 1988. Analysis of plant gene expression. *In* Plant Molecular Biology, A practical approach (edited by C.H. Shaw). IRL Press, Oxford, pp. 1-35.

Davis E.O., Evans I.J. and Johnston W.B. 1988. Identification of *nodX*, a gene that allows *Rhizobium leguminosarum* biovar *viciae* strain TOM to nodulate Afganistan peas. Molecular and General Genetics **212**, 531-535.

De Billy F., Barker D.G., Gallusci P. and Truchet G. 1991. Leghaemoglobin gene transcription is triggered in a single layer in the indeterminate nitrogen-fixing root nodule of alfalfa. Plant Journal **1**, 27-35.

De Haan H. 1930. Contributions to the genetics of *Pisum* (Flower colour factors, patched and dotted flowers). Genetica **12**, 321-439.

De Maagd R.A., Wijfjes H.M., Spaink H.P., Ruiz-Sainz J.E., Wijffelman C.A., Okker R.J.H. and Lugtenberg B.J.J. 1989. *nodO*, a new *nod* gene of *Rhizobium leguminosarum* biovar *viciae* Sym plasmid pRL1JI encodes a secreted protein. Journal of Bacteriology **171**, 6764-6770.

De Maagd R.A., Yang W.-C., Goosen-de Roo L., Mulders I.H.N., Roest H.P., Spaink H.P., Bisseling T. and Lugtenberg B.J.J. 1994. Down-regulation of expression of the *Rhizobium leguminosarum* outer membrane protein gene *ropA* occurs abruptly in interzone II-III of pea nodules and can be uncoupled from *nif* gene activation. Molecular Plant-Microbe Interaction **7**, 276-281.

De Vilmorin P. and Bateson W. 1912. A case of gametic coupling in *Pisum*. Proceedings of the Royal Society, London. **84** (Ser. B), pp. 9-11.

Denarie J. and Cullimore J. 1993. Lipo-oligosaccharide nodulation factors: a new class of signalling molecules mediating recognition and morphogenesis. Cell **74**, 951-954.

Ditta G., Stanfield S., Corbin D. and Helinski D.R. 1980. Broad host-range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proceedings of the National Academy of Sciences USA **77**, 7347-7351.

Downie J.A., Ma Q.S., Knight C.D., Hombrecher G. and Johnston A.W.B. 1983. Cloning of the symbiotic region of *Rhizobium leguminosarum*: The nodulation genes are between the nitrogenase genes and a *nif-A*-like gene. EMBO Journal **2**, 947-952.

Duc G. and Messager A. 1989. Mutagenesis of pea (*Pisum sativum* L.) and the isolation of mutants for nodulation and nitrogen fixation. Plant Science **60**, 207-213.

Economou A., Hawkins F.K.L., Downie J.A. and Johnston A.W.B. 1989. Transcription of *rhiA*, a gene on a *Rhizobium leguminosarum* biovar *viciae* Sym plasmid, requires *rhiR* and is repressed by flavonoids that induce *nod* genes. Molecular Microbiology **3**, 87-93.

Economou A., Davies A.E., Johnston A.W.B. and Downie J.A. 1994. The *Rhizobium leguminosarum* biovar *viciae nodO* gene can enable a *nodE* mutant of *Rhizobium leguminosarum* biovar *trifolii* to nodulate vetch. Microbiology **140**, 2341-2347.

Ellis T.H.N., Turner L., Hellens R.P., Lee D., Harker C.L., Enard C., Domoney C. and Davies D.R. 1992. Linkage maps in pea. Genetics **130**, 649-663.

Ellis T.H.N., Hellens R.P., Turner L., Lee D., Domoney C. and Welham T. 1993. On the pea linkage map. Pisum Genetics **25**, 5-12.

Engvild K.C. 1987. Nodulation and nitrogen fixation mutants of pea, *Pisum sativum*. Theoretical and Applied Genetics **74**, 711-713.

Fearn J.C. and LaRue T.A. 1991. A temperature sensetive nodulation mutant (*sym5*)

of *Pisum sativum* L. Plant, Cell and Environment **14**, 221-227.

Feinberg A. and Vogelstein B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry **132**, 6-13.

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. Molecular Microbiology **10**, 351-360.

Fisher R.F. and Long S.R. 1992. *Rhizobium* - plant signal exchange. Nature **357**, 655-660.

Franssen H.J., Nap J.P. and Bisseling T. 1992a. Nodulins in root nodule development. *In* Biological Nitrogen Fixation (edited by G. Stacey, R.H. Burris and H.J. Evans). Chapman and Hall, London, pp. 598-624.

Franssen H.J., Vijn I., Yang W.C. and Bisseling T. 1992b. Developmental aspects of the *Rhizobium*- legume symbiosis. Plant Molecular Biology **19**, 89-107.

Frohman M.A., Dush M.K. and Martin G.R. 1988. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proceedings of the National Academy of Sciences USA **85**, 8998-9002.

Gebhardt C., Oliver J.E., Forde B.G., Saarelainen R. and Miflin B.J. 1986. Primary structure and differential expression of glutamine synthetase genes in nodules, roots and leaves of *Phaseolus vulgaris*. EMBO Journal **5**, 1429-1435.

Gelin O. and Blixt S. 1964. Root nodulation in peas. Agri Hortique Genetica **22**, 149-152.

Götz R., Evans I.J., Downie J.A. and Johnston A.W.B. 1985. Identification of the host-range DNA which allows *Rhizobium leguminosarum* strain TOM to nodulate cv. Afghanistan peas. Molecular and General Genetics **201**, 296-300.

Govers F., Gloudemans T., Moerman M., Van Kammen A. and Bisseling T. 1985. Expression of plant genes during the development of pea root nodules. EMBO

Journal **4**, 861-867.

Govorov L.I. 1928. The peas of Afghanistan. Bull. Appl. Bot. Genet. Plant Breed. **19** (Nr. 2), 497-522.

Govorov L.I. 1937. Peas. *In* Flora of Cultivated Plants (edited by N.I. Vavilov and E.V.Wulff). Volume 4: Grain Leguminosae. Moscow and Leningrad, pp. 231-336.

Guinel F.C. and LaRue T.A. 1991. Light microscopy study of nodule initiation in *Pisum sativum* L. cv. Sparkle and its low-nodulating mutant E2 (*sym5*). Plant Physiology **97**, 1206-1211.

Heidstra R., Kozik A., Bisseling T. and Lie T.A. 1993. Pea Nod⁻ mutants and Nod factor perception. *In* New Horizons in Nitrogen Fixation (edited by R. Palacios, R. Mora and W.E. Newton). Dordrecht, Kluwer Academic Publishers, The Netherlands, pp. 267-268.

Heidstra R., Geurts R., Franssen H., Spaink H.P., Van Kammen A. and Bisseling T. 1994. Root hair deformation activity of nodulation factors and their fate on *Vicia sativa*. Plant Physiology **105**, 787-797.

Holl F.B. 1975. Host plant control of the inheritance of dinitrogen fixation in the *Pisum - Rhizobium* symbiosis. Euphytica **24**, 767-770.

Holl F.B. and LaRue T.A. 1976. Genetics of legume plant hosts. *In* Nitrogen fixation (edited by W.E. Newton and C.J. Nyman). Proceedings of the 1st International symposium. Washington State University Press. pp. 391-399.

Hombrecht G., Gotz R., Dibb N.J., Downie J.A., Johnston A.W.B. and Brewin N.J. 1984. Cloning and mutagenesis of nodulation genes from *Rhizobium leguminosarum* TOM, a strain with extended host range. Molecular and General Genetics **194**, 293-298.

Jacobsen E. 1984. Modification of symbiotic interaction of pea (*Pisum sativum* L.) and *Rhizobium leguminosarum* by induced mutations. Plant and Soil **82**, 427-438.

Jacobsen E. and Feenstra W.J. 1984. A new pea mutant with efficient nodulation in the presence of nitrate. Plant Science Letters **33**, 337-344.

Johnston A.W.B. and Beringer J.E. 1975. Identification of *Rhizobium* strains in pea root nodules using genetic markers. Journal of General Microbiology **87**, 343-350.

Josey D.P., Beylon J.L., Johnston A.W.B. and Beringer J.E. 1979. Strain identification in *Rhizobium* using intrinsic antibiotic resistance. Journal of Applied Bacteriology **46**, 343-350.

Kardailsky I., Yang W.C., Zalensky A., Van Kammen A. and Bisseling T. 1993. The pea late nodulin gene PsNOD6 is homologous to the early nodulin genes PsENOD3/14 and is expressed after the leghemoglobin genes. Plant Molecular Biology **23**, 1029-1037.

Kardailsky I. 1995. The *Rhizobium*-Legume interface in pea root nodules: studies of nodulins, glycoproteins and proteases. PhD thesis, University of East Anglia, Norwich, UK, pp. 20-21.

Kneen B.E. and LaRue T.A. 1984a. Peas (*Pisum sativum* L.) with strain specificity for *Rhizobium leguminosarum*. Heredity **52** (3), 383-389.

Kneen B.E. and LaRue T.A. 1984b. Nodulation resistant mutant of *Pisum sativum* (L.). Journal of Heredity **75**, 238-240.

Kneen B.E., LaRue T.A. and Weeden N. 1984. Genes reported to affect symbiotic nitrogen fixation by peas. Pisum News Letters **16**, 31-34.

Kneen B.E. and LaRue T.A. 1988. Induced symbiosis mutants of pea (*Pisum sativum*) and sweetclover (*Melilotus alba annua*). Plant Science **58**, 177-182.

Kneen B.E., LaRue T.A., Welch R.M. and Weeden N. 1990a. Pleiotropic effects of *brz*. Plant Physiology **93**, 717-722.

Kneen B.E., LaRue T.A., Hirsch A.M., Smith C.A. and Weeden N.F. 1990b. *sym13* - a gene conditioning ineffective nodulation in *Pisum sativum*. Plant Physiology **94**, 899-905.

Kneen B.E., Weeden N.F., LaRue T.A. 1994. Non-nodulating mutants of *Pisum sativum* (L.) cv. Sparkle. Journal of Heredity **85**, 129-133.

Kouchi H. and Hata S. 1993. Isolation and characterization of novel nodulin cDNAs

representing genes expressed at early stages of soybean nodule development. Molecular and General Genetics **238**, 106-119.

Kozik A., Men A.E., Kulikova O.A., Bisseling T. and Tikhonovich I.A. 1994. Pea (*Pisum sativum*) genes involved in symbiosis with nitrogen-fixing bacteria. IV. Study of gene inheritance with DNA probes. Molecular Biology (translated from Russian) **28**, 358-363.

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 Science **108**, 41-49.

Kozik A., Matvienko M., Scheres B., Paruvangada V.G., Bisseling T., Van Kammen A., Ellis T.H.N., LaRue T. and Weeden N. 1996. The pea early nodulin gene *PsENOD7* maps in the region of linkage group I containing *sym2* and leghemoglobin. Plant Molecular Biology, in press.

Krol A., Hontelez J., Van den Bos R.C. and Van Kammen A. 1980. Expression of large plasmids in the endosymbiotic form of *Rhizobium leguminosarum*. Nucleic Acids Research **8**, 4337-4347.

Lamprecht H. 1948. The variation of linkage and the course of crossing-over. Agri Hortique Genetica **6**, 10-48.

Lamprecht H. 1974. Monographie der Gattung Pisum. Steiermarkische Landesdruckerei Graz.

LaRue T.A. and Weeden N.F. 1992. The symbiosis genes of pea. Pisum genetics **24**, 5-12.

Le Gal M.F. and Hobbs S.L. 1989. Cytological studies of the infection process in nodulating and nonnodulating pea genotypes. Canadian Journal of Botany **67**, 2435-2443.

Lee K.H. and LaRue T.A. 1992. Pleotropic effects of *sym17*. Plant Physiology **100**, 1326-1333.

Lerouge P., Roche P., Faucher C., Maillet F., Truchet G., Promé J.-C. and Dénarié J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature **344**, 781-784.

Lie T.A. 1971a. Symbiotic nitrogen fixation under stress conditions. Plant and Soil, Special Volume, 117-127.

Lie T.A. 1971b. Temperature-dependent root-nodule formation in pea cv. Iran. Plant and Soil **34**, 751-752.

Lie T.A., Hille D., Lambers R. and Houwers A. 1976. Symbiotic specialisation in pea plants: some environmental effects on nodulation and nitrogen fixation. *In* Symbiotic nitrogen fixation in plants (edited by P.S. Nutman). International Biological Programme, vol. 7. Cambridge University Press, GB, pp. 319-333.

Lie T.A. 1978. Symbiotic specialization in pea plants: The requirement of specific *Rhizobium* strains for peas from Afghanistan. Annals of Applied Biology **88**, 462-465.

Lie T.A. 1981. Gene centres, a source for genetic variants in symbiotic nitrogen fixation: Host-induced ineffectivity in *Pisum sativum* ecotype *Fulvum*. Plant and Soil **62**, 125-134.

Lie T.A., Timmermans P.C.J.M. and Ladizinsky G. 1982. Host-controlled nitrogen fixation in the Legume-*Rhizobium* symbiosis: Incompatibility of *Pisum sativum* L. ecotypes *Elatius* Bieb. and *Abyssinicum* braun with European *Rhizobium leguminosarum* strains. Israel Journal of Botany **31**, 163-167.

Lie T.A. and Timmermans P.C.J.M. 1983. Host-genetic control of nitrogen fixation in the legume-*Rhizobium* symbiosis: complication in the genetic analysis due to maternal effects. Plant and Soil **75**, 449-453.

Lie T.A. 1984a. Gene centres, a source for genetic variants in symbiotic nitrogen fixation: The symbiotic response of the cultivated pea to *Rhizobium leguminosarum* strains from Europe and the Middle East. Plant and Soil **82**, 359-367.

Lie T.A. 1984b. Host genes in *Pisum sativum* L. conferring resistance to European *Rhizobium leguminosarum* strains. Plant and Soil **82**, 415-425.

Lie T.A., Goktan D., Engin M., Pijnenborg J. and Anlarsal E. 1987. Co-evolution of the legume-*Rhizobium* association. Plant and Soil **100**, 171-181.

Lie T.A., Pijnenborg J. and Timmermans P.C.J.M. 1988. Analysis of the host genes controlling the legume-*Rhizobium* symbiosis: Some technical problems and pitfalls. *In* Nitrogen Fixation by Legumes in Mediterranian Agriculture (edited by D.P. Beck and L.A. Materon). ICARDA, The Netherlands, pp. 93-100.

Markwei C.M. and LaRue T.A. 1992. Phenotypic characterization of *sym8* and *sym9*, two genes conditioning non-nodulation in *Pisum sativum* "Sparkle". Canadian Journal of Microbiology **38**, 548-554.

Matvienko M., Van de Sande K., Yang W.C., Van Kammen A., Bisseling T. and Franssen H. J. 1994. Comparision of soybean and pea ENOD40 cDNA clones representing genes expressed during both early and late stages of nodule development. Plant Molecular Biology **26**, 487-493.

Men A.E., Borisov A.Y., Rozov S.M., Tsygonov V.E. and Tikhonovich I.A. 1993. The early nodulin gene ENOD12 is in linkage group 3. Pisum Genetics **25**, 32-33.

Mendel G. 1866. Versuche uber Pflanzen-hybriden. Verhandlungen der Naturforschung Vereins in Brunn **4**, 3-47.

Mendel G. 1965. Experiments in plant hybridisation (edited by J.H. Bennet). Oliver and Boyd, London.

Nap J.-P. 1988. Isolation and analysis of a leghemoglobin gene from pea (*Pisum sativum*). *In* Nodulins in root nodule development. PhD thesis, Wageningen Agricultural University, The Netherlands, pp. 19-28.

Nap J.-P. and Bisseling T. 1990. Developmental biology of a plant-prokaryote symbiosis: The legume root nodule. Science **250**, 948-954.

Paran I. and Michelmore R.W. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theoretical and Applied Genetics **85**, 985-993.

Postma J.G., Jacobsen E. and Feenstra W.J. 1988a. Three pea mutants with an altered

nodulation studied by genetic analysis and grafting. Journal of Plant Physiology **132**, 424-430.

Postma J.G., Jacobsen E. and Feenstra W.J. 1988b. Experiments with mutants of pea (*Pisum sativum* L.) *In* Nitrogen fixation: Hundred years after (edited by H. Bothe, F.J. de Bruijn and W.E. Newton). Gustav Fisher Verlag, Stuttgart, pp. 629-633.

Postma J.G., Jager D., Jacobsen E. and Feenstra W.J. 1990. Studies on a non-fixing mutant of pea (*Pisum sativum* L.). I. Phenotypycal description and bacteroid activity. Plant Science **68**, 151-161.

Rogers S.O. and Bendish A.J. 1988. Extraction of DNA from plant tissues. *In* Plant Molecular Biology Manual (edited by S.B. Gelvin and R.A. Schilperoort). Dordrecht, Kluwer Academic publishers, A6, pp. 1-10.

Rozov S.M., Borisov A.Y., Tsyganov V.E. and Tikhonovich I.A. 1993. A new fix⁻ mutation in pea shows linkage with group III marker *M*. Pisum Genetics **25**, 45.

Sagan M., Messager A. and Duc G. 1993. Specificity of the *Rhizobium*-legume symbiosis obtained after mutagenesis. in pea (*Pisum sativum* L.). New Phytol. **125**, 757-761.

Sagan M., Huguet T. and Duc G. 1994. Phenotypic characterization and classification of nodulation mutants of pea (*Pisum sativum* L.). Plant Science **100**, 59-70.

Sambrook J., Fritsch E.F. and Maniatis T. 1989. Molecular Cloning, a laboratory manual, Second edition. Cold Spring Harbor Laboratorium Press.

Sanchez F., Padila J.E., Perez H. and Lara M. 1991. Control of nodulin genes in root-nodule development and metabolism. Annual Review of Plant Physiology and Plant Molecular Biology **42**, 507-528.

Scheres B., Van de Wiel C., Zalensky A., Horvath B., Spaink H., Van Eck H., Zwartkruis F., Wolters A.M., Gloudemans T., Van Kammen A. and Bisseling T. 1990a. The ENOD12 gene product is involved in the infection process during pea-*Rhizobium* interraction. Cell **60**, 281-294.

Scheres B., Van Engelen F., Van der Knaap E., Van de Wiel C., Van Kammen A. and Bisseling T. 1990b. Sequential induction of nodulin gene expression in the developing pea nodule. Plant Cell 8, 687-700.

Schlaman H.R.M., Lugtenberg B.J.J. and Okker R.J.H. 1992. The NodD protein does not bind to the promoters of inducible nodulation genes in extracts of bacteroids of *Rhizobium leguminosarum* biovar *viciae*. Journal of Bacteriology 174, 6109-6116.

Sidorova K.K. and Uzhintseva P. 1992. Use of mutants to detect genes controlling symbiotic characteristics in the pea. Genetics (Rus.) 28, 494-500.

Spaink H.P., Okker R.J.H., Wijffelman C.A., Pees E. and Lugtenberg B.J.J. 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Plant Molecular Biology 9, 29-37.

Spaink H.P., Sheely D.M., Van Brussel A.A.N., Glushka J., York W.S., Tak T., Geiger O., Kennedy E.P., Reinhold V.N. and Lugtenberg B.J.J. 1991. A novel highly unsaturated fatty acid moiety of lipooligosaccharide signals determines host specificity of *Rhizobium*. Nature 354, 125-130.

Spaink H.P. 1992. Rhizobial lipo-oligosaccharides: answers and questions. Plant Molecular Biology 20, 977-986.

Spaink H.P., Aarts A., Stacey G., Bloemberg G.V., Lugtenberg B.J.J. and Kennedy E.P. 1992. Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metabolites using thin-layer chromatography. Molecular Plant-Microbe Interaction 5, 72-80.

Stam P. 1993. Construction of integrated genetic linkage maps by means of a new computer package: JoinMap. Plant Journal 3 (5), 739-744.

Stokkermans T.J.W. and Peters N.K. 1994. *Bradyrhizobium elkanii* lipo-oligosaccharide signals induce complete nodule structures on *Glycine soja* Siebold et Zucc. Planta 193, 413-420.

Sutton M.J., Lea E.J.A. and Downie A.J. 1994. The nodulation-signalling protein nodO from *Rhizobium leguminosarum* biovar *viciae* forms ion channels in membranes. Proceedings of the National Academy of Sciences USA 91, 9990-9994.

Temnykh S.V., Kneen B.E., Weeden N.F. and LaRue TA. 1995. Localization of *nod-3*, a gene conditioning hypernodulation, and identification of a novel translocation in *Pisum sativum* L. cv."Rondo". Journal of Heredity (in press).

Tigney S.V., Walker E.L. and Coruzzi G.M. 1987. Glutamine syntetase genes of pea encode distinct polypeptides which are differentially expressed in leaves, roots and nodules. EMBO Journal **6**, 1-9.

Truchet G., Roche P., Lerouge P., Vasse J., Camut S., de Billy F., Promé J-C. and Dénarié J. 1991. Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature **351** 670-673.

Van de Wiel C., Scheres B., Franssen H., Van Lierop M.J., Van Lammeren A. and Van Kammen A. 1990. The early nodulin transcript ENOD2 is located in the nodule parenchyma (inner cortex) of pea and soybean root nodules. EMBO Journal **9**, 1-7.

Van Kammen A. 1984. Suggested nomenclature for plant genes involved in nodulation and simbiosis. Plant Molecular Biology Reporter **2**, 43-45.

Vasse J., De Billy F., Camut S. and Truchet G. 1990. Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. Journal of Bacteriology **172**, 4295-4306.

Vasse J., De Billy F. and Truchet G. 1993. Abortion of infection during the *Rhizobium meliloti*-alfalfa symbiotic interaction is accompanied by hypersensitive reaction. Plant Journal **4**, 555-566.

Vavilov N.I. 1951. The origin, variation, immunity and breeding of cultivated plants (English translation). Ronald Press Co., New York.

Vijn I., das Neves L., van Kammen A., Franssen H. and Bisseling T. 1993. Nod factors and nodulation in plants. Science **260**, 1764-1765.

Von Heijne G. 1983. Pattern of amino acids near signal-sequence cleavage sites. European Journal of Biochemistry **133**, 17-21.

Weeden N.F., Kneen B.E. and LaRue T.A. 1990. Genetic analysis of *sym* genes and other nodule-related genes in *Pisum sativum*. *In* Nitrogen Fixation: Achievements and

Objectives (edited by P.M. Gresshoff). Chapman and Hall, NY and London, pp. 323-330.

Weeden N.F. and Wolko B. 1990. Linkage map for the garden pea (*Pisum sativum*) based on molecular markers. *In* Genetic Maps. Locus maps of complex genomes (edited by S.J. O'Brein). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 6.106 - 6.112.

Weeden N.F., Muehlbauer F.G. and Ladizinsky G. 1992. Extensive conservation of linkage relationships between pea and lentil genetic maps. Journal of Heredity **83**, 123-129.

Weeden N.F., Ambrose M. and Swiecicki W. 1993. Pea (*Pisum sativum*). *In* Genetic maps. Locus maps of complex genomes (edited by S.J. O'Brein). Laboratory of Viral Carcinogenesis, National Cancer Institute. Cold Spring Harbor Laboratory Press. Sixth edition, pp. 6.24-6.34.

Weeden N.F., Timmerman G.M. and Lu J. 1994. Identifying and mapping genes of economic significance. Euphytica **73**, 191-198.

Winarno R. and Lie T.A. 1979. Competition between *Rhizobium* strains in nodule formation: interaction between nodulating and non-nodulating strains. Plant and Soil **51**, 135-142.

Yang W.C., Katinakis P., Hendriks P., Smolders A., de Fries F., Spee J., Van Kammen A., Bisseling T. and Franssen H. 1993. Characterization of GmENOD40, a gene showing novel patterns of cell-specific expression during soybean nodule development. Plant Journal **3**, 573-585.

Young J.P.W., Johnston A.W.B. and Brewin N.J. 1982. A search for peas (*Pisum sativum* L.) showing strain specificity for symbiotic *Rhizobium leguminosarum*. Heredity **48** (2), 197-201.

Young J.P.W. and Matthews P. 1982. A distinct class of peas (*Pisum sativum* L.) from Afghanistan that show strain specificity for symbiotic *Rhizobium*. Heredity **48** (2), 203-210.

Young J.P.W. 1985. Linkage of *sym2*, the symbiotic specificity locus of *Pisum sativum*.

Journal of Heredity **76**, 207-208.

SAMENVATTING

De symbiotische interactie tussen *Rhizobium* bacteriën en vlinderbloemige planten leidt tot de vorming van wortelknollen. In deze organen wordt de bacterie gehuisvest en daar zijn de bacteriën in staat atmosferische stikstof om te zetten in ammonia, die de plant kan gebruiken voor zijn groei. De vorming van wortelknollen omvat een aantal opeenvolgende stappen, die de expressie van zowel plante als bacteriegenen vereisen.

De *Rhizobium*-genen die absoluut essentieel zijn om tenminste de eerste stappen van de knolvorming te induceren zijn de zgn. nodulatiegenen (*nod*-genen). Deze genen zijn goed bestudeerd en er is aangetoond dat de Nod-eiwitten verantwoordelijk zijn voor de vorming van specifieke lipo-oligosacchariden (Nod-factoren), die in gezuiverde vorm de verschillende stappen van het knolvormingsproces kunnen induceren. Daarentegen zijn genen van de plant die essentieel zijn voor de knolvorming nog weinig bestudeerd. Genen die specifiek tot expressie komen bij de vorming van wortelknollen zijn de zgn. nodulinegenen. Van deze genen is kennis beschikbaar over het type eiwit waar ze voor coderen en in veel gevallen is ook bepaald in welk celtype deze genen tot expressie komen. Met behulp van genetische benaderingen is verder een groot aantal genen -de zgn. *sym*-genen- geïdentificeerd die essentieel zijn voor normale knolvorming, maar geen van deze genen is reeds gekloneerd.

In dit proefschrift is een van de *sym*-genen, *sym2*, bestudeerd. Dit gen is 25 jaar geleden door T.H. Lie geïdentificeerd in Afghaanse erwten. Wanneer dit gen ingebracht wordt in gecultiveerde erwten, dan verliezen deze het vermogen om knollen te vormen met *Rhizobium*-stammen die deze cultivars zeer efficiënt kunnen noduleren. A. Downie heeft aangetoond dat de rhizobia een extra *nod*-gen nodig hebben om knollen te maken op die *sym2* bevattende erwten. Dit is het *nodX*-gen en het eiwit waar het voor codeert zorgt er voor dat de Nod-factoren van een extra acetaatgroep worden voorzien. Deze resultaten maken het waarschijnlijk, dat het door *sym2* gecodeerde eiwit op een of andere manier betrokken is bij de herkenning van Nod-factoren.

Het doel van het werk dat wordt beschreven in dit proefschrift, is het karakteriseren van de erwtelijnen waarin het *sym2* gen geïntroduceerd is. Verder is er een gedetailleerde moleculaire kaart gemaakt van het gebied van koppelingsgroep 1, waar *sym2* gelocaliseerd is. Dankzij dit voorbereidende werk is het nu mogelijk om het *sym2*-gen te kloneren.

APPENDIX

Figure 1 (next page). Southern analysis of genomic DNA from different *sym2* introgression lines digested with *EcoRI* The blot were probed with labelled cDNA44, a molecular marker tightly linked to *sym2* (see Figure 2, page 37). The hybridisation reveal a cluster of cDNA44 homologous sequences tightly linked to *sym2*. All bands hybridising to the cDNA44 are inherited as a single Mendelian trait, recombination was not detected within the cluster of cDNA44 related bands. The *sym2* introgression lines L-4 and A5.6.9 have the Afghanistan cDNA44 pattern of bands, whereas the *sym2* introgresion line A5.4.3 shows the Rondo pattern. This means that line A5.4.3 has a recombination between *sym2* and the cDNA44 marker. The *sym2* introgression lines (previously referred to as *sym1* lines, before it was shown that *sym1* and *sym2* are allelic [Kozik et al., 1995]) I6.2.2 and I1.3.10 have the Iran cDNA44 pattern. The level of polymorphism between Rondo and Iran lines is lower than between Rondo and Afghanistan. Size of marker bands (λ-*Hind*III) are indicated in kbp.

Figure 2 (see pages 102-103). Examples of RFLP's linked to *sym2* among F3 plants (1W-16W) of a cross L-4 x NGB1238. Pea genomic DNA from the original parents Afghanistan, L-4 and NGB1238, an F1 plant from the cross L-4 x NGB1238, and segregating F3 individuals were digested by *Hind*III. The source of DNA is indicated at the top. The single blot was succesfully hybridised with the molecular markers cDNA267, cDNA44, ENOD7, cDNA164, Lb and ENOD40.

Abbreviations and designations: Afg - Afghanistan carrying *sym2* allele; L-4 - *sym2* introgression line; 1238 - NGB1238 line, European origin, lacking Afghanistan *sym2* allele; F1 - *sym2* heterozygous plant of the cross L-4 x NGB1238. A - plant is homozygous for Afghanistan type RFLP marker, B - plant is homozygous for NGB1238 type RFLP marker, H - heterozygous plants. Arrow-heads indicate Afghanistan polymorphic bands, asterisks indicate NGB1238 polymorphic bands.
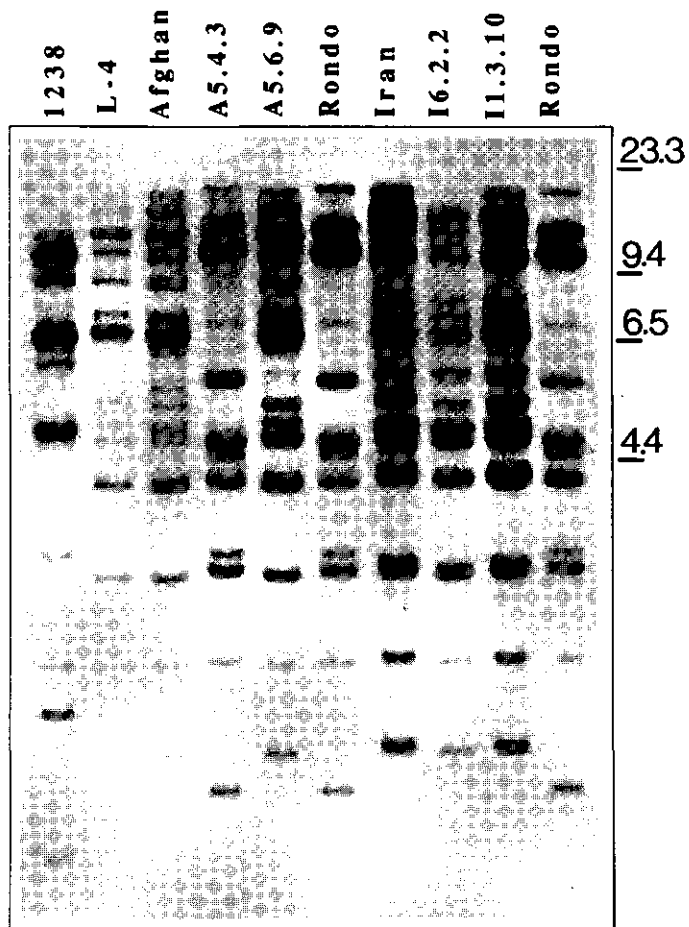
The hybridization patterns of cDNA44 probe to digests (EcoRI)
of genomic DNA from different *sym2* introgression lines
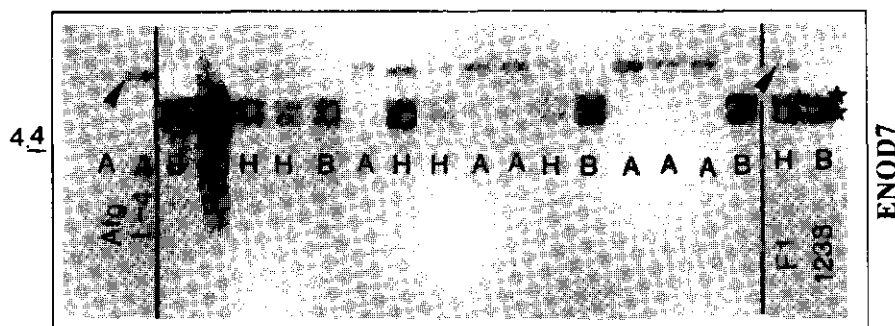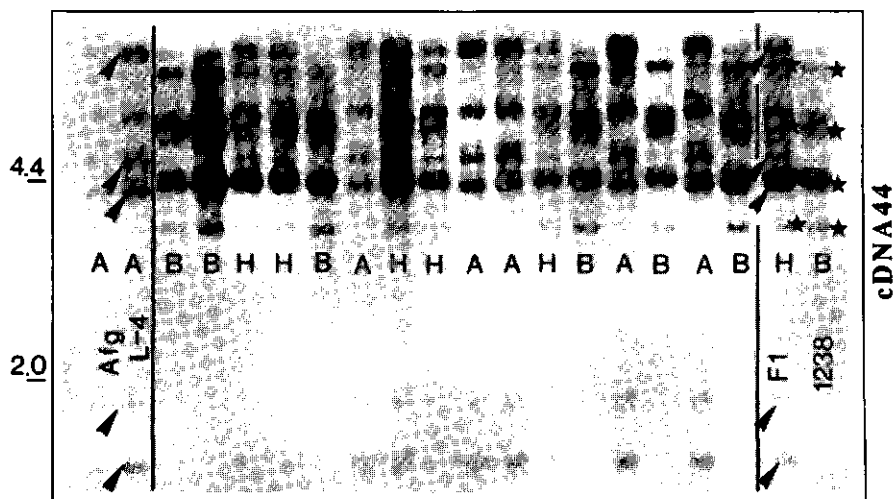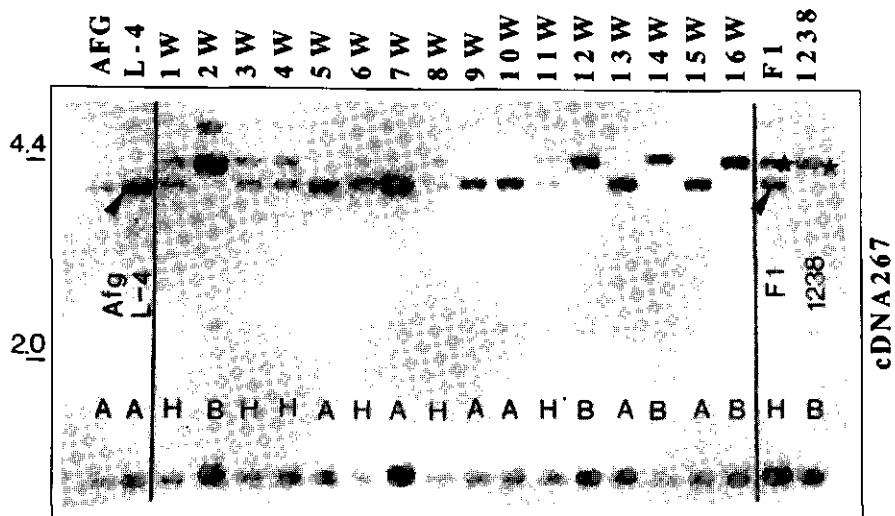
Examples of RFLP among F3 plants (1W - 16W) of a cross L-4 x NGB1238.
A - homozygous "Afghanistan", B - homozygous "1238", H - heterozygous plants.
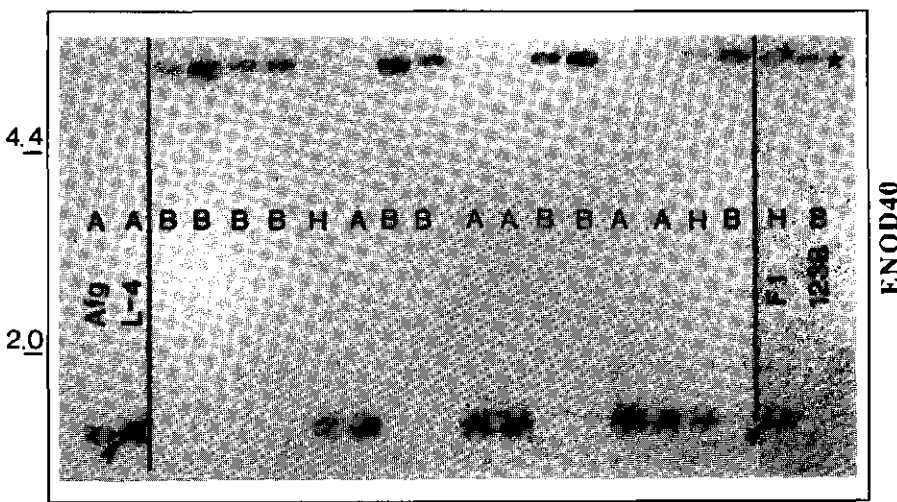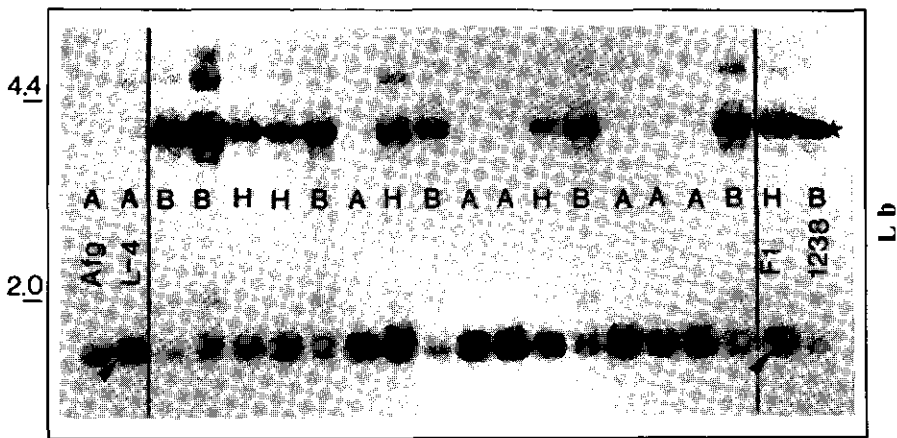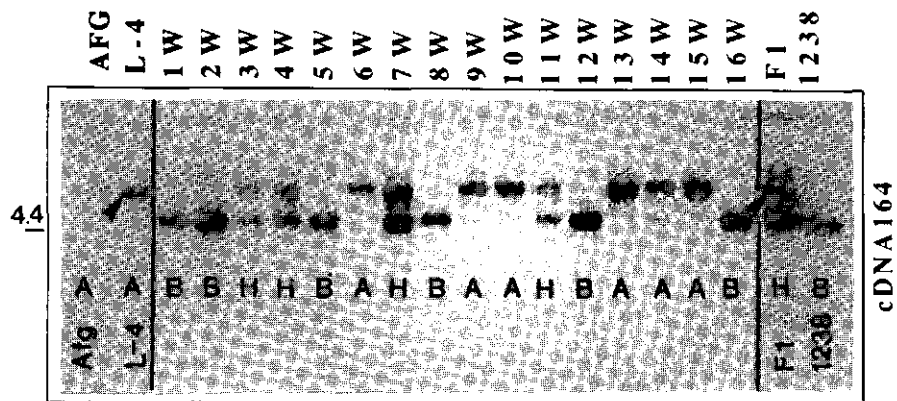
Examples of RFLP among F3 plants (1W - 16W) of a cross L-4 x NGB1238.
A - homozygous "Afghanistan", B - homozygous "1238", H - heterozygous plants.

Table 1 and 2 (pages 106-107). Complete raw data used for construction of the map of the *sym2* region described in Chapter 4.

Table 1. Data on the nodulation and RFLP/RAPD analyses of individual plants of the segregating F2 population of the cross L-4 x NGB1238.

Table 2. Data on the nodulation and RFLP/RAPD analyses of individual plants of the segregating F3 (single seed descent) population of the cross L-4 x NGB1238.

Designations and abbreviations:

First column: Plant number. The 48R plants were grown from round F2 seeds, the 16W plants were grown from wrinkled F2 seeds.

Second column: Number of nodules per plant produced after inoculation with *Rlv* starin 248. Plants with less than ten nodules were scored as − (=Nod⁻). Plants with more than ten nodules were scored as + (=Nod⁺). The number of nodules is underlined if the nodulation is contrary to the *sym2* genotype (see Chapter 5). Nodulation of homozygous *sym2* plants (Nod⁺) or Nod⁻ phenotype on heterozygous *sym2* plants upon inoculation with *Rlv* starin 248 is probably due to effect of genetic background of NGB1238 or Afghanistan pea lines (see Chapter 5).

mr - nodulation mostly on the main root. Plants nodulated on the main root in practically all cases in which the Afghanistan *sym2* is lacking.

lr - nodulation mostly on the lateral roots.

lmr - nodulation as well as on the main root as on the lateral roots. *sym2* heterozygous plants usually produced more nodules on the lateral roots than on the main root.

sml plnt - small plant, *sym2* phenotype was not determined

The following columns present the results of the RFLP/RAPD analyses using the RFLP molecular markers ENOD40, Lb, cDNA164, ENOD7, cDNA44 and cDNA267, and RAPD markers OPA1, B374, 198P, 198N, B474 and OPA4. The RAPD markers were checked for co-segregation with RFLP markers and mapped accordingly (Chapter 4).

Designations:

AFG - homozygous Afghanistan

1238 - homozygous NGB1238

htz - heterozygous locus

D - not NGB1238, i.e. locus is Afghanistan homozygous or heterozygous

C - not Afghanistan, i.e. locus is NGB1238 homozygous or heterozygous

Table I

Plant no.	Nodulation	ENOD 40	cDNA Lb	cDNA 164	ENOD 7	cDNA 44	sym2 nod-	RAPD A01	cDNA 267	RAPD B374	RAPD 198P	RAPD 198N	RAPD B474	RAPD OPA4
1R	+ 20-30	AFG	htz	htz	htz	htz	htz	D	htz	C	D	C	AFG	AFG
2R	+ 20-30	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
3R	+ 20-25	AFG	htz	htz	htz	htz	htz	D	htz	C	D	C	AFG	AFG
4R	+ 20-30	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
5R	- 3 ndls	htz	htz	htz	htz	htz	htz*	D	htz	C	D	C	C	C
6R	+ 20-30 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
7R	- 2 ndls	htz	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	C	C
8R	+ 20-25	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
9R	+ 20-30 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
10R	+ 20-25	htz	1238	1238	1238	1238	1238	1238	htz	C	1238	C	C	C
11R	+ 20-30	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
12R	+ 20-30	htz	htz	htz	htz	htz	htz	D	AFG	C	D	C	C	C
13R	+ 20-30 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
14R	+ 20-30	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
15R	+/- 15 ndls	AFG	AFG	AFG	AFG	AFG	AFG*	D	AFG	AFG	D	AFG	AFG	AFG
16R	- 1 ndl	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
17R	- 1 ndl	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
18R	+ 20-25	1238	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
19R	+ 30-40	AFG	AFG	AFG	htz	htz	htz	D	htz	C	D	C	AFG	AFG
20R	- 6 ndls	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
21R	+ 20-30	htz	htz	htz	htz	htz	htz	D	AFG	C	D	C	C	C
22R	+ 20-30 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
23R	+ 20-30	htz	htz	htz	htz	htz	htz	D	1238	C	D	C	C	C
24R	+ 20-25	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
25R	+ 20-30	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
26R	- 3 ndls	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
27R	+ 20-30 mr	AFG	htz	htz	htz	htz	htz	D	htz	C	D	C	AFG	AFG
28R	+ 20-25	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
29R	+ 20-30	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
30R	+ 20-30 mr	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
31R	- 1 ndl	htz	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	C	C
32R	+ 20-30 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
33R	+ 15-20	AFG	AFG	AFG	htz	htz	htz	D	htz	C	D	AFG	AFG	AFG
34R	+ 30-40	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
35R	+ 15-20	htz	htz	htz	htz	htz	htz	htz	-	C	D	C	C	C
36R	+ 20-30 mr	htz	htz	htz	1238	1238	1238	1238	1238	C	D	C	C	C
37R	+ 20-30	AFG	htz	htz	htz	htz	htz	D	1238	C	D	C	AFG	AFG
38R	- 4 ndls	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
39R	+ 15-20 mr	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
40R	+ 30-40	htz	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
41R	+ 20-30 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
42R	- 0	AFG	AFG	AFG	AFG	AFG	AFG	D	htz	AFG	D	AFG	AFG	AFG
43R	- 1 ndl	htz	htz	htz	AFG	AFG	AFG	D	AFG	AFG	D	C	C	C
44R	- 3 ndls	htz	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	C	C
45R	+ 20-25	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
46R	- 4 ndls	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
47R	+ 20-30	1238	htz	htz	htz	htz	htz	D	AFG	C	D	C	C	C
48R	+ 20-30 mr	htz	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C

Plant no.	Nodulation	ENOD 40	cDNA Lb	cDNA 164	ENOD 7	cDNA 44	sym2 nod-	RAPD A01	cDNA 267	RAPD B374	RAPD 198P	RAPD 198N	RAPD B474	RAPD OPA4
1W	+/- 10 ndls	1238	1238	1238	1238	1238	htz*	D	htz	C	1238	C	C	C
2W	+ 20-30 mr	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
3W	+ 15-20	1238	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
4W	+ 20-30	1238	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
5W	+ 20-30	htz	htz	htz	htz	htz	htz	D	AFG	C	D	C	C	C
6W	- 3 ndls	AFG	AFG	AFG	AFG	AFG	AFG	D	htz	AFG	D	AFG	AFG	AFG
7W	+ 20-30	1238	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
8W	+ 15-20	1238	1238	1238	htz	htz	htz	D	htz	C	1238	C	C	C
9W	+ 20-30	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
10W	- 5 ndls	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
11W	+/- 7 ndls	1238	htz	htz	htz	htz	htz*	D	htz	C	D	C	C	C
12W	+ 20-30 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
13W	+ 20-30	AFG	htz	htz	htz	htz	htz	D	htz	C	D	C	AFG	AFG
14W	+ 20-30 mr	htz	htz	htz	htz	1238	1238	1238	1238	C	D	C	C	C
15W	+ 20-30	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
16W	+ 30-40 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C

Table II

Plant no.	Nodulation	ENOD 40	cDNA Lb	cDNA 164	ENOD 7	cDNA 44	sym2 nod-	RAPD A01	cDNA 267	RAPD B374	RAPD 198P	RAPD 198N	RAPD B474	RAPD OPA4
1R	- 3 ndls mr	AFG	htz	htz	htz	htz	htz*	D	htz	C	D	C	AFG	AFG
2R	- 0	htz	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	C	C
3R	- 0	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
4R	- 3 ndls mr	AFG	htz	htz	htz	htz	htz*	D	htz	C	D	C	AFG	AFG
5R	- 8 ndls lr	1238	1238	htz	htz	htz	htz*	D	htz	C	D	C	C	C
6R	+ 20-25 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
7R	- 0	htz	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	C	C
8R	+ 20-25 lmr	htz	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
9R	+ 20-25 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
10R	+ sml plnt	htz	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
11R	- 0	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
12R	- 4 ndls lr	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
13R	+ 12-15 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
14R	+ 25-30 lr	htz	htz	htz	htz	htz	htz	D	AFG	C	D	C	C	C
15R	- 0	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
16R	- 0	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
17R	- 0	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
18R	+ 12-15 lmr	1238	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
19R	- 3 ndls lr	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
20R	- 4 ndls lr	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
21R	+ 12-15 lmr	htz	htz	htz	htz	htz	htz	D	APG	C	D	C	C	C
22R	+ 20-25 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
23R	- 4 ndls lr	AFG	AFG	AFG	AFG	AFG	AFG	D	1238	APG	D	AFG	AFG	AFG
24R	- 6 ndls lr	htz	htz	htz	htz	htz	htz*	D	htz	C	D	C	C	C
25R	+ 15-20 lr	1238	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
26R	- 4 ndls lr	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
27R	+ 20-25 mr	AFG	htz	htz	htz	htz	1238	1238	1238	C#	D	C	AFG	AFG
28R	- sml plnt	1238	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
29R	+ 20-25 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
30R	+ 20-25 lr	1238	htz	htz	htz	htz	htz	D	htz	C	D	C	C	C
31R	- 0	1238	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	C	C
32R	+ 20-25 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
33R	- 6 ndls	AFG	AFG	AFG	AFG	AFG	AFG	D	htz	AFG	D	AFG	AFG	AFG
34R	+ 20-25 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
35R	- 0	htz	htz	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG#	C
36R	+ 20-25 mr	AFG	AFG	AFG	1238	1238	1238	1238	1238	C	D	AFG	AFG	AFG
37R	- sml plnt	AFG	htz	htz	htz	htz	htz	D	1238	C	D	C	AFG	AFG
38R	- 4 ndls	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
39R	+ 15-20 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
40R	+ 25-30 mr	htz	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
41R	+ 20-25 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
42R	- 0	AFG	AFG	AFG	AFG	AFG	AFG	D	htz	AFG	D	AFG	AFG	AFG
43R	- 0	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
44R	- 0	htz	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG#	C
45R	+ 15-20 lr	AFG	AFG	AFG	AFG	AFG	AFG*	D	AFG	AFG	D	AFG	AFG	AFG
46R	- 0	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
47R	+ 25-30 lr	1238	htz	htz	htz	htz	htz	D	AFG	C	D	C	C	C
48R	+ 12-15 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C

Plant no.	Nodulation	ENOD 40	cDNA Lb	cDNA 164	ENOD 7	cDNA 44	sym2 nod-	RAPD A01	cDNA 267	RAPD B374	RAPD 198P	RAPD 198N	RAPD B474	RAPD OPA4
1W	+ 20-25 lr	1238	1238	1238	1238	1238	htz	D	htz	C	1238	C	C	C
2W	+ 20-25 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
3W	- 3 ndls	1238	htz	htz	htz	htz	htz*	D	htz	C	D	C	C	C
4W	- 4 ndls mr	1238	htz	htz	htz	htz	htz*	D	htz	C	D	C	C	C
5W	+ 20-25 mr	htz	1238	1238	1238	1238	1238	1238	AFG	C	1238	C	C	C
6W	- 0	AFG	AFG	AFG	AFG	AFG	AFG	D	htz	AFG	D	AFG	AFG	AFG
7W	- 5 ndls	1238	htz	htz	htz	htz	htz*	D	AFG	C	D	C	C	C
8W	- 3 ndls	1238	1238	1238	htz	htz	htz*	D	htz	C	1238	C	C	C
9W	- 0	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	C#	AFG
10W	- 8 ndls	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
11W	- 0	1238	htz	htz	htz	htz	htz*	D	htz	C	D	C	C	C
12W	+ 20-25 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C
13W	- 0	AFG	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	AFG	AFG
14W	+ 20-25 mr	AFG	AFG	AFG	AFG	1238	1238	1238	1238	C	D	AFG	AFG	AFG
15W	- 0	htz	AFG	AFG	AFG	AFG	AFG	D	AFG	AFG	D	AFG	C	C
16W	+ 20-25 mr	1238	1238	1238	1238	1238	1238	1238	1238	C	1238	C	C	C

CURRICULUM VITAE

Alexander Kozik was born in Pskow, North-West of Russia, on the 8th of March 1965. From 1972 to 1980 he enjoyed his primary and secondary school life. From 1980 to 1982 he received intensive basic high school education in the 45th Physical-Mathematical High School under supervision of the St.-Petersburg (Leningrad) State University. From 1982 to 1987 he studied Organic Chemistry and especially chemical synthesis of polypeptides during education in St.-Petersburg State University in Department of Organic Chemistry. M.Sc. project (diploma) was done on a chemical synthesis of polypeptide, a fragment of surface antigene of Hepatitis B virus. He started his study and work in Molecular-Biology field in 1987 in Institute of Agricultural Microbiology, Pushkin, St.-Petersburg, on a problem of Symbiotic Nitrogen Fixation. From 1989 to 1991 he was Ph.D. student of Institute of Agricultural Microbiology. He continued the Ph.D. education in Wageningen Agricultural University in Ton Bisseling's lab at the department of Molecular Biology, where he came on the December 1991. His scientific research last four years (1991-1995) were concentrated on the investigation of genetics of pea symbiotic genes. He finished his Ph.D. research on the end of 1995 and has been awarded by a Postdoctoral Fellowship at Waksman Institute at Rutgers, the State University of New Jersey to investigate signal transduction of pathogen-related genes in Arabidopsis.

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