Interaction of Two Genes Controlling Symbiotic Nodule Number in Pea (*Pisum sativum* L.)

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Abstract

Two lines of pea (*Pisum sativum* L.) forming excessive number of nodules have been genetically characterized and exploited for the construction of double mutant lines. Both the supernodulating parental line RisfixC and the hypernodulating parental line RisfixV (Engvild, 1987) form 5–6 times more nodules than the wild type when grown on low nitrate level. In RisfixC, the nodules are effective and their number is not influenced by nitrate, while the initiation of ineffective nodules of RisfixV is subject to nitrate suppression. In view of different response to nitrate, each line is hypothesized to carry a different fault in the feedback regulation of nodule initiation. Both mutations are inherited as single recessives and are non-allelic. No linkage between them was detected. The pleiotropic mutant features, namely, nitrate-tolerant nodulation, compact shoot, reduced nodulated root and lower specific nitrogenase activity in RisfixC and the ineffectiveness of RisfixV nodules, could not be dissociated from the primary character of nodule number. The detailed comparison of plants homozygous for both mutations with parental lines RisfixC and RisfixV suggests that the effect of the studied mutations on symbiotic nodule number is additive. Consequently, each mutation acts on a different regulatory pathway. Double recessive plants may provide an estimate of the potential number of nodules that can be induced in the pea root.

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Abbreviations

DM = dry mass, SNA = specific nitrogenase activity, TNA = total nitrogenase activity

1. Introduction

Symbiotic nodule number is one of the most important characteristics of the rhizobial symbiosis in legume plants. Symbiotic nodules are induced on the root of a particular plant host by nodule bacteria from the genera (*Azo-*, *Brady-*, and *Rhizobium*), which subsequently colonize the central tissue of these specialized plant organs. In a typical case, bacteria differentiate into the so-called bacteroids, which provide fixed atmospheric nitrogen to the host plant. According to our present knowledge, root nodules develop from meristematic regions that appear in the root cortex upon the action of rhizobial Nod factor(s) (for a review, see Franssen et al., 1992). The structure of Nod factor has been elucidated as a substituted chitooligosaccharide with structural peculiarities in different rhizobial species and biovars (Spaink, 1992). In spite of the assumed uniform action of Nod factor on the plant root tissue, induced meristematic regions are distinctly separated from each other and their number is obviously limited. In addition to Nod factor action, the number of nodules formed is homeostatically controlled by reverse-acting plant regulatory factors (Rolfe and Gresshoff, 1988).

The feedback action of these internal regulatory factors was shown to be abolished by specific plant mutations. These mutants are characterized by markedly increased number of nodules and by their uniform distribution throughout the root system (Jacobsen and Feenstra, 1984; Carroll et al., 1985; Duc and Messager, 1989). The abundantly nodulating mutants have been denoted as supernodulating (Carroll et al., 1985; Park and Buttery, 1988), hypernodulating (Gremaud and Harper, 1989; Olsson et al., 1989) or copious nodulating (Park and Buttery, 1988) according to the extent of phenotypic manifestation of a mutation. The known examples of supernodulation are associated with the nts ("nitrate-tolerant symbiosis") trait, which is manifested as the loss of the sensitivity of nodule initiation and development to the well-known inhibitory action of nitrate (Streeter, 1988).

In the present work, the inheritance of two recently characterized pea (*Pisum sativum* L.) mutants forming increased nodule number was investigated. Because the mutants are thought to be blocked at different regulatory circuits controlling nodule initiation (Novak et al., 1993b), the combined effect of both mutations in double mutant lines was exploited for testing the interaction of the two regulatory pathways.

2. Materials and Methods

Plant material

Pea (*Pisum sativum* L.) lines RisfixC and RisfixV originate from a set of nodulation mutants obtained with EMS mutagenesis in cv. 'Finale' (Engvild, 1987). Both lines were originally classified as Fix⁻. The subsequent detailed characterization led to the classification of RisfixC as a nitrate-resistant, supernodulating mutant, while RisfixV is a hypernodulating mutant which still responds to nitrate by reduced nodulation (Novak et al., 1993ab). The difference in response of the two mutants to nitrate is illustrated in Fig. 1a. The structure of nodules of RisfixC is not apparently disturbed (unpublished data) and the nodules are functional (Novak et al., 1993ab), while RisfixV nodules undergo a collapse resembling hypersensitive necrosis after differentiation of the late symbiotic zone (Novak et al., 1995). Consequently, the greater part of RisfixV nodules is ineffective.

Cultivation of plants

Seeds harvested from field-grown (if not indicated otherwise) plants were selected for the mean weight with 10% tolerance. After surface sterilization with 2% chloramine B for 30 min, the seeds were germinated in Petri dishes at 30°C for 3 d and planted into plastic cylinders (4.5 cm-diameter and 11.5 cm-tall) filled with perlite and irrigated with nutrient solution. At the moment of planting, the seedlings were inoculated with 3 ml suspension (2.3×10^7 cells/ml) of *Rhizobium leguminosarum* bv. *viciae* strain 248 (Josey et al., 1979) prepared by resuspending a 4-d yeast-mannitol plate culture in H_2O. Only data presented in Fig. 1a were obtained using strain 128C30 possessing similar symbiotic properties. Plants were grown in a Conviron S10H growth chamber at 16/8 h light regime, 21/14°C temperature, 77/87% relative humidity, and illumination 500 $\mu m/m^2/s$ of photosynthetically active radiation. Nutrient solution was changed twice a week. Other growing conditions were as previously (Novak et al., 1993b).

Two nutrient solutions were used for plant evaluation (Skrdleta et al., 1980): a low nitrate (0.625 mM) nutrient solution and a nutrient solution containing 10 mM NO_3^-. The concentration of 0.625 mM was preferred to a nitrate-free medium in view of the stimulatory effect of this nitrate level on nodulation (Fig. 1a). Nitrate-free solution (Skrdleta et al., 1980) was used for qualitative tests of symbiotic ineffectiveness, while a commercial complete nutrient solution (Hydroponix) was used for the recovery of plants raised on nitrate-free solution.
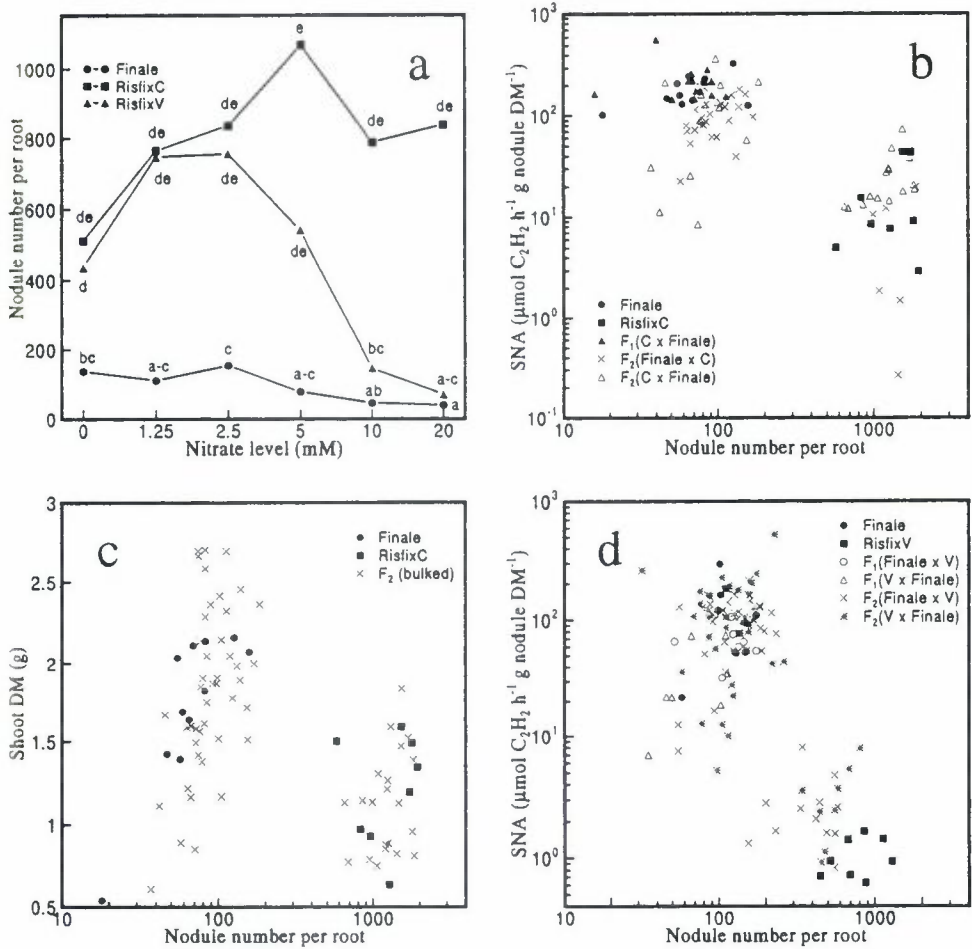
Figure 1.   Characterization of parental lines RisfixC and RisfixV: (a) behavior of the trait
of symbiotic nodule number in the studied pea lines as affected by supplied
nitrate level, (b), (c) distribution of F_1 hybrids and F_2 segregants from the crosses
'Finale' × RisfixC by nodule number, specific nitrogenase activity (SNA) and
shoot dry mass (DM), (d) distribution of F_1 and F_2 segregants from the crosses
'Finale' × RisfixV by nodule number and SNA. Plants were grown either on 10
mM (b, c) or 0.625 mM (d) nitrate. Data presented in (a) were partially
published earlier (Novak et al., 1993b). The F_1 from 'Finale' × RisfixC (b) was
not available. Tukey confidence intervals at 0.05 level are indicated in graph
(a).

For in planta nitrogenase assay, the plants were cultivated in plastic boxes
6.5×6.5×10 (height) cm with perforated bottom to provide the access for

nutrient solution. In this cultivation system, the shoot grew through a hole in the box cover.

Crosses were performed both in the field- and vegetation chamber-grown plants since 1992 season.

Plant evaluation

The plants were harvested 33–35 d after germination (29–32 days after inoculation). Total nitrogenase activity (TNA) was determined as acetylene reduction on detached nodulated roots that had been incubated in a closed-chamber system at 23°C (Skrdleta et al., 1987). Subsequently, nodule number after picking nodules from the root, nodule dry mass (DM), and DM of the root and shoot were determined. Chlorophyll content has been determined as a quantitative marker of symbiotic nitrogen supply in legumes (Mirza et al., 1990) in two discs (d = 8 mm) cut from the topmost fully expanded leaves of each plant. After quantitative extraction with 80% acetone, the chlorophyll a+b concentration was determined spectrophotometrically at 652 nm using isosbestic extinction coefficient according to Bruinsma (1961).

For the non-destructive assay of TNA *in planta*, the holes in the bottom of the cultivation boxes were closed with rubber stoppers, while the hole in the box cover was sealed around the stem with modeling clay. In this configuration, nodule number was counted on intact roots after harvesting seeds.

The significance of differences in growth and symbiotic traits was evaluated by Tukey confidence intervals using the sixth root of the original values.

3. Results

Since the genetic analysis of quantitative traits, like nodule number or nitrogenase activity, is based on the distribution of values, the data on individual plants are presented below in addition to average values.

Inheritance of the supernodulating mutant RisfixC

The analysis of 'Finale' × RisfixC and RisfixC × 'Finale' crosses was performed on high nitrate (10 mM) nutrient solution to maximize differences in nodule number between RisfixC and the wild type, according to Fig. 1a, and to facilitate the detection of a potential incomplete dominance. As shown in Table 1 and in Fig. 1b, F_1 plants were indistinguishable from the wild-type plants by selected growth and symbiotic traits, indicating full dominance of the wild-type phenotype.

Table 1.   Symbiotic and growth traits of F_1 hybrids and F_2 segregants in the crosses of
'Finale' and RisfixC. Plants were grown on 10 mM nitrate. Indices indicate
Tukey confidence intervals at 0.05 level.

Plants	Trait/(units)					
	Stem length[+] (cm)	Shoot DM (g)	Root DM (mg)	Total nodule DM (mg)	Nodule number	SNA [μmol C_2H_2/h /(g nodule DM)]
'Finale'	69.6 c	1.73 bc	269 b	12 a	75 a	179 cd
RisfixC	48.1 ab	1.21 ab	119 a	188 c	1328 b	17 ab
F_1(CxFi)[++]	65.3 bc	1.74 bc	267 b	16 ab	70 a	228 d
F_2(FixC)wt	65.0 c	1.92 c	304 b	21 b	97 a	107 c
F_2(CxFi)wt	60.7 bc	1.66 a–c	288 b	17 ab	88 a	130 c
F_2(FixC)sn	37.0 a	1.02 a	126 a	163 c	1345 b	7 a
F_2(CxFi)sn	43.9 a	1.17 a	149 a	166 c	1262 b	26 b

[+]Sum of the length of main and lateral stems (when present). [++]Reciprocal F_1 plants were
not available.

The distribution of F_2 plants by nodule number and specific nitrogenase
activity, SNA (Fig. 1b), yielded two classes resembling the parental
phenotypes. Judging from the bimodal distribution of nodule number in the F_2
generation, it seems that the nts trait and supernodulation cannot be
dissociated from one another. In the opposite case, we should have observed
four peaks originating from the combination of supernodulation +/– and nts +/–
traits. The characters of supernodulation and low SNA are inherited together
as well, as indicated by the absence of clusters of recombinant phenotypes (Fig.
1b). The trait of growth depression (compact plant growth), which is
characteristic of supernodulating lines (see Discussion), was expressed even on
high nitrate medium and could not be dissociated from supernodulation as well
(Fig. 1c). Analogical plots for the stem length and internode length confirmed
the complete linkage of the shortened stem and shortened internode characters
with high symbiotic nodule number, i.e. supernodulation (not shown). No
significant differences were found in average values of growth and symbiotic
characteristics between the distinguished classes of F_2 segregants and the
parental lines, with the one exception of nodule DM in the wild-type F_2 plants
from 'Finale' × RisfixC (Table 1). The F_2 phenotypes obtained in reciprocal
crosses were well comparable. The chi-square test in both directions (wild type

: mutant 42 : 13 and 44 : 12) and on the bulked data did not reject the 3 : 1 hypothesis (P = 0.815, 0.537, and 0.547, respectively), confirming monogenic recessive determination of the supernodulation character.

Inheritance of the hypernodulating mutant RisfixV

Plants from 'Finale' × RisfixV and RisfixV × 'Finale' crosses were analyzed on low nitrate (0.625 mM) nutrient solution to maximize the differences in nodule number and nitrogenase activity between genotypes according to Fig. 1a. F_1 plants resembled the wild-type plants in terms of nodulation traits (Table 2, Fig. 1d), indicating full dominance of the wild-type allele. However, the F_1 (RisfixV × 'Finale') plants were significantly weaker than the control as judged from growth traits and SNA. This discrepancy indicates a maternal plant effect.

Table 2. Symbiotic and growth traits of F_1 hybrids and F_2 segregants in the crosses of 'Finale' and RisfixV. Plants were grown on 0.625 mM nitrate. Indices indicate Tukey confidence intervals at 0.05 level.

Plants	Trait/(units)					
	Stem length+ (cm)	Shoot DM (g)	Root DM (mg)	Total nodule DM (mg)	Nodule number	SNA [μmol C_2H_2/h /(g nodule DM)]
'Finale'	34.6 bc	1.15 b–d	253 bc	32.6 ab	121 ab	113.6 c
RisfixV	24.1 a	0.46 a	164 ab	63.0 c	811 d	1.1 a
F_1(FixV)	38.8 bc	1.20 cd	265 c	40.4 bc	119 ab	71.4 bc
F_1(VxFi)	33.9 a–c	0.51 a	145 a	20.9 a	75 a	36.2 b
F_2(FixV)wt	38.3 bc	0.99 b–d	222 bc	35.4 b	131 b	96.1 c
F_2(VxFi)wt	41.7 c	1.18 d	270 c	37.9 b	125 ab	120.2 bc
F_2(FixV)hn	30.4 ab	0.73 ab	261 bc	36.8 b	429 c	2.6 a
F_2(VxFi)hn	34.1 bc	0.80 a–c	239 bc	38.8 bc	544 cd	3.4 a

+Sum of the length of main and lateral stems (when present).

In the F_2 generation, two clusters of plants could be distinguished according to the characters of nodule number and SNA, both classes tending to the

parental phenotypes (Fig. 1d). The cosegregation of high nodule number and low effectiveness characters supports their determination by a single mutation. In case of their determination by two different mutations (not tightly linked) we should observe two additional recombinant phenotypic classes, i.e. abundantly nodulating effective and low nodulating ineffective plants. No substantial differences in average values of the compared traits were detected between the distinguished classes of F_2 segregants and the parental lines (Table 2). However, the hypernodulated segregants grew better and formed significantly fewer nodules than the RisfixV control. This difference can be explained by the same maternal plant effect as that causing difference in the F_1 generation. The observed segregation ratio in the F_2 of both directions (29 : 13 and 43 : 8), as well as the bulked data, were not significantly different from 3 : 1 (P = 0.373, 0.125, and 0.590, respectively), indicating monogenic determination of the RisfixV phenotype.

Generation of double mutants

Lines RisfixC and RisfixV were crossed to prepare double mutant plants. Allelism of both mutations was excluded by the wild-type phenotype of F_1 plants (not shown). F_2 plants were analyzed on high nitrate (10 mM) levels to distinguish those expressing nitrate-resistant nodulation conditioned by the RisfixC mutation. At this concentration of nitrate, nodulation is partially suppressed in RisfixV, but still remains distinctly higher than in the wild-type plants (Fig. 1a). Hence, the used level of nitrate allowed us to separate the hypernodulated plants both from the supernodulated and from the wild-type plants. On the other hand, the high-nitrate nutrient solution prevented the use of nitrogen deficiency symptoms for unveiling the ineffectiveness conferred by the mutation of RisfixV. Therefore, nitrogenase activity was used as an auxiliary phenotypic marker. Nitrogenase assays were carried out non-destructively 38–40 days after germination so that seeds could be harvested from the plants of interest.

The segregation in the F_2 according to the distribution of nodule number and SNA yielded four classes of plants corresponding to the expected phenotypes (Fig. 2). However, these classes were not clearly separated from each other. The genotype of the putative double recessives can be denoted *ccvv* if we accept notation of *c* and *v* for the mutant alleles of RisfixC and RisfixV, respectively. These plants were expected to show combined features of the parental mutant lines, partly, nitrate resistance of nodulation as manifestation of the allele *c*, and further increased number of ineffective or low effective nodules due to the presence of the allele *v*. The correspondence of the observed segregation into approximate classes (33 wild type-like : 14 nitrate-resistant, effective : 11

hypernodulated, ineffective : 8 putative double recessives) to 9 : 3 : 3 : 1 (P = 0.215 in a chi-square test), as well as the correspondence of the wild-type recombinants/other classes ratio to 9 : 7 (P = 0.949), showed that the mutations are not linked each other.
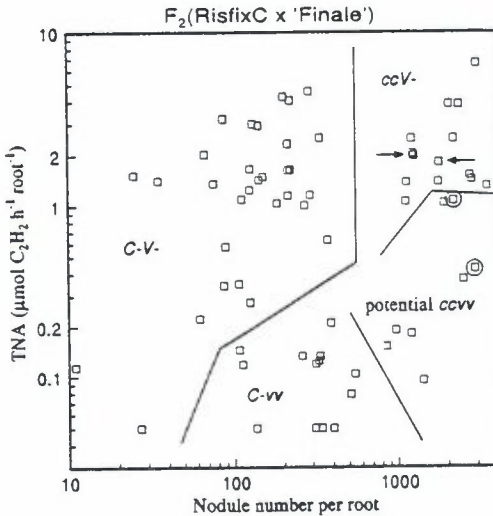

Figure 2.   Distribution of F_2 segregants from the cross RisfixC × RisfixV by symbiotic nodule number and total nitrogenase activity (TNA). Plants were grown on 10 mM nitrate. Putative classes of segregants are indicated. Positions of plants which progeny was used for preparation of stable double mutant lines are encircled or marked with arrows.

In view of the uncertain phenotype of *ccvv* recombinants, two independent strategies were used for the isolation of *ccvv* lines. In the first approach, the F3 progeny of two putative *ccvv* plants from the F_2 (encircled in Fig. 2) was tested for homogeneity and ineffectiveness using N-deficiency symptoms on nitrate-free nutrient solution. F_4 generation obtained after the F_3 plants rescue on complete medium was further tested for homogeneity and nitrate resistance of nodulation on high nitrate. In the second approach, the segregating F_3 progeny of two nitrate-resistant, but definitely effective F_2 plants (with assumed genotypes *ccVV* and *ccVv* in 1 : 2 ratio, marked with arrows in Fig. 2), was scored for ineffectiveness on nitrogen-free medium. The segregants showing nitrogen deficiency symptoms 25 days after germination were rescued on complete nutrient solution. The *ccvv* genotype of lines isolated in both ways and denoted as "CV" was confirmed by backcrosses to RisfixC and RisfixV.

Phenotype of double mutants

Obtained CV lines were used for the comparison of the double mutant phenotype with both parental lines and the wild-type plants. Recombinant and control seeds used had been harvested from growth chamber-grown plants except RisfixV seeds, which were of field origin. Plants from all CV lines formed a phenotypically homogeneous group which was clearly distinguishable from 'Finale', RisfixC, and RisfixV when grown on low nitrate (0.625 mM) and after being clustered by nodule number and SNA (Figs. 3a and 4). The average nodule number formed by CV plants on low nitrate (n = 1789) was higher than in RisfixC and RisfixV, and close to the sum of the number of nodules conditioned by both mutations separately (Table 3). On high nitrate (10 mM), the component corresponding to the v allele contribution disappeared, according to the behavior of the RisfixV mutant itself.

Other basic growth and symbiotic characteristics of CV plants are presented in Table 3. Like nodule number, total nodule mass was increased in CV when compared to the parental lines on low nitrate, although not significantly in respect of RisfixC. On high nitrate, CV plants paralleled RisfixC not only in nodule number, but also in other nodulation traits – total nodule DM and single nodule DM. The root DM of CV was reduced like in RisfixC on both nitrate levels, probably due to supernodulation. On the other hand, the increase in nodule number caused by the v allele was not associated with a decrease in root size.

The single nodule size of CV on low nitrate was slightly higher than in RisfixV, suggesting a better functional state of CV nodules. This assumption also corresponds to higher SNA and higher chlorophyll content in CV leaves in comparison with RisfixV.

Selected derived symbiotic traits that distinguish most clearly CV plants from both the parental lines are shown in Figs. 3b and 3c. The nodulation indices computed as total nodule DM/shoot DM ratio (Fig. 3b) and nodule number formed per g shoot DM (Fig. 3c) in CV plants were greater than the sum of the values of parental lines. RisfixC values were exceeded by factors of 3.41 and 4.93, respectively. However, this non-additivity originates from two different sources: increase in nodule number and nodule mass per root on one side and further deprivation of plant growth in CV on the other. Deeper growth depression in CV than in both parental lines is illustrated by stem length, leaf number, and shoot DM value decrease (Table 3) and by further shortening of internodes (Fig. 3d). However, the growth difference between CV and RisfixC, i.e. the component due to the presence of the allele v, was fully abolished by adding an excess of nitrate (Table 3, Fig. 3d), similarly to the behaviour of nodulation traits. Although the chlorophyll content in RisfixV and CV was
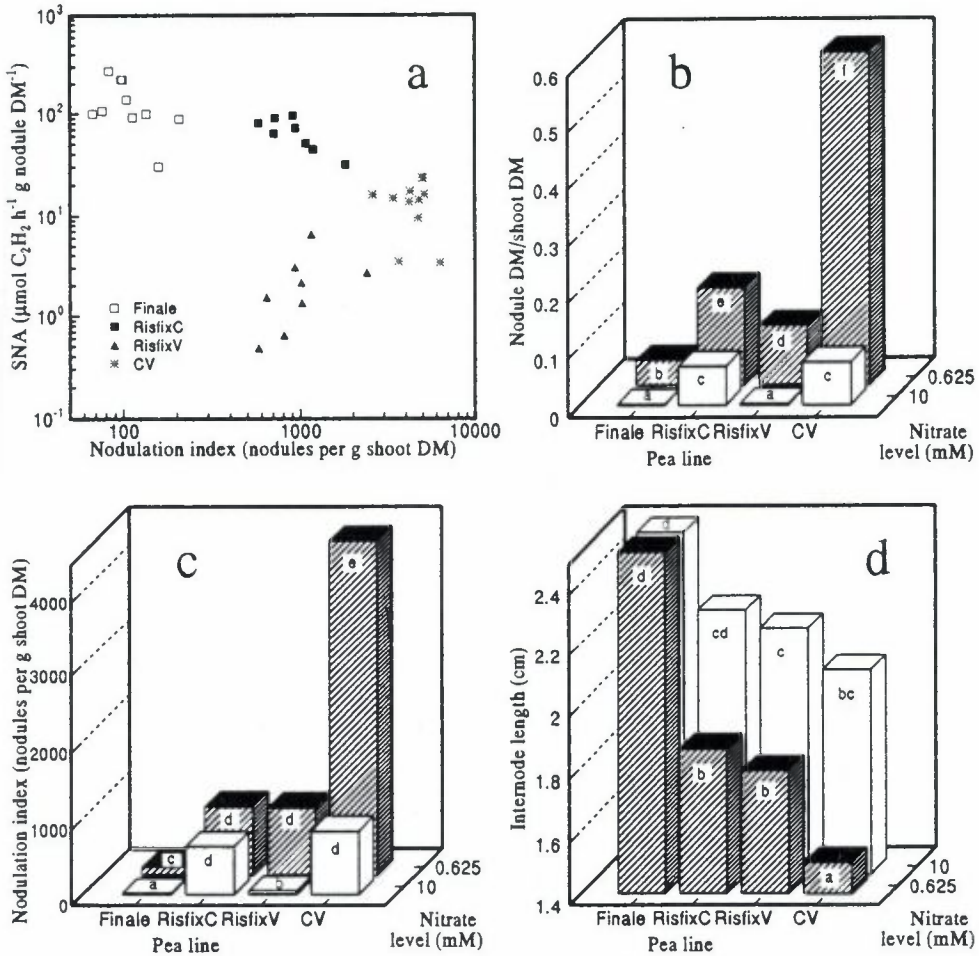
Figure 3.   Characterization of double mutant lines CV: (a) resolution of CV plants from paternal lines and the wild type by nodulation index and specific nitrogenase activity (SNA) on 0.625 mM nitrate; (b), (c) nodulation indices of CV plants as affected by supplied nitrate level; (d) internode length as affected by plant genotype and nitrate level in nutrient solution. DM = dry mass. Letters on bars indicate Tukey confidence intervals at 0.05 level.

also restored by nitrate availability, the data in Table 3 do not illustrate this response due to an unexplained terminal chlorosis appearing in RisfixV during fast growth on rich nutrient solution.

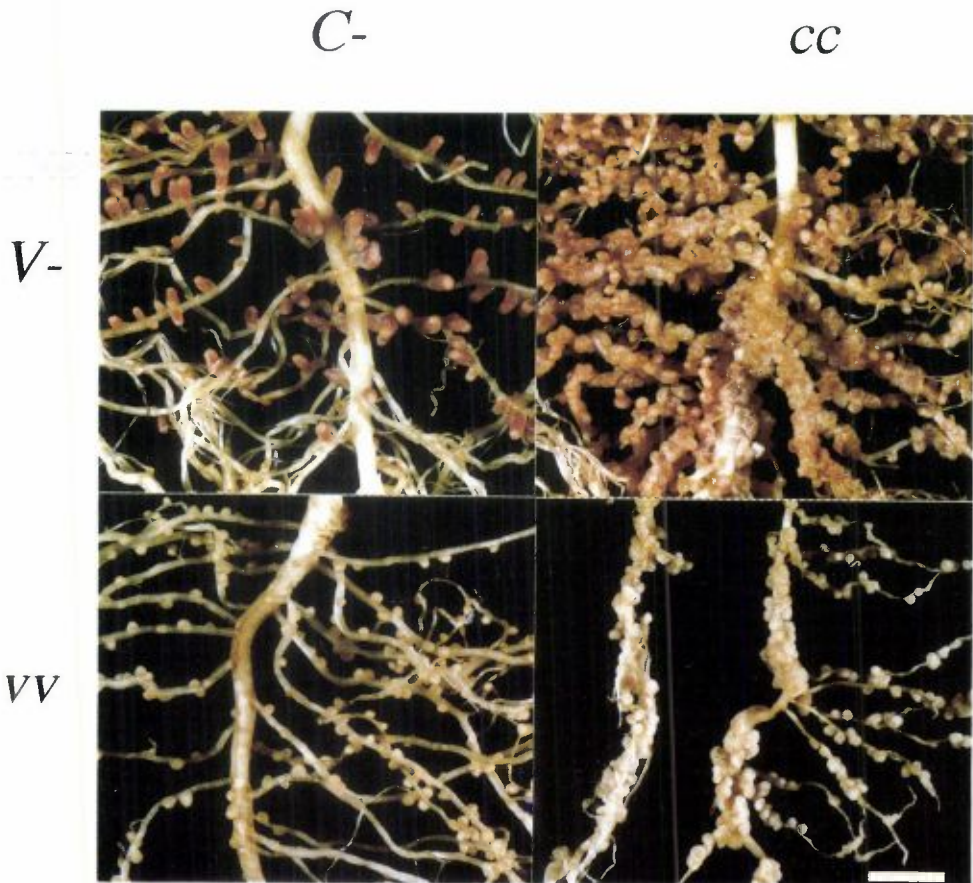$C-$ cc

$V-$

VV

Figure 4.   Appearance of nodulated root systems of pea of different genotypes 32 days
after inoculation. Plants were grown on low (0.625 mM) nitrate. Alleles in loci c
and v are indicated. Bar = 1 cm.

4. Discussion

We have shown that two mutations of pea that can increase the symbiotic
nodule number formed on the root of this plant are inherited as monogenic
recessives and are not allelic. The corresponding mutated alleles were
provisionally denoted c and v. Pleiotropic features of both mutations, i.e.
nitrate-resistant nodulation, compact plant growth, and lower SNA in RisfixC,
as well as the nodule ineffectiveness in RisfixV, were genetically inseparable

Table 3. Comparison of double mutant plants with parental lines RisfixC, RisfixV, and the wild type. Plants were grown on 10 mM nitrate. Indices indicate Tukey confidence intervals at 0.05 level.

Nitrate level (mM)	Plant line	Trait/(units)								
		Stem length+ (cm)	Leaf number++	Shoot DM (g)	Root DM (mg)	Total nodule DM (mg)	Nodule number	Single nodule DM (mg)	SNA [μmol C_2H_2/h /(g nodule DM)]	Chlorophyll content (μg/cm² leaf)
0.625	'Finale'	67.1 c	26.5 c	1.77 c	355 c	78 b	174 c	456 d	155.9 e	40.4 b
0.625	RisfixC	42.0 bc	22.6 c	1.15 bc	134 a	196 c	999 de	197 c	74.0 de	40.1 ab
0.625	RisfixV	27.4 b	15.3 ab	0.83 b	359 c	84 b	701 d	120 bc	2.3 ab	26.8 a
0.625	CV	16.9 a	11.3 a	0.42 a	96 a	244 c	1789 f	139 bc	13.5 c	36.0 ab
10	'Finale'	62.9 c	25.1 c	1.87 c	364 c	4 a	32 a	116 b	56.4 d	42.8 b
10	RisfixC	50.6 c	22.6 c	1.48 c	234 b	96 b	890 de	110 b	16.9 c	35.2 ab
10	RisfixV	60.6 c	27.9 c	1.37 c	353 c	4 a	72 b	56 a	3.5 b	27.9 a
10	CV	49.0 c	24.0 bc	1.44 c	245 b	102 b	1145 e	91 b	0.5 a	31.3 ab

+Sum of the length of main and lateral stems (when present). ++Total number of leaves on main and lateral stems.

from the primary character of high nodule number. Knowledge of the inheritance and of the physiological manifestation of both mutations allowed construction of double mutant lines, whose properties allow predictions about the mechanism of symbiotic nodule number determination in legumes.

Mutant RisfixC and other legume genes controlling nodule number

Most of the known major mutations conferring abundant symbiotic nodulation to legumes belong to the supernodulating/nts type, i.e. they condition markedly increased nodule number with uniform distribution throughout the root system and resistance to nitrate action (Delves et al., 1986). Supernodulating mutants have been obtained in soybean (Carroll et al., 1985; Kokubun and Akao, 1994), in pea cv. 'Rondo' (Jacobsen and Feenstra, 1984), cv. 'Frisson' (Duc and Messager, 1989; Sagan and Duc, 1996) and cv. 'Ramonsky 77' (Sidorova et al., 1995), common bean (Park and Buttery, 1988, faba bean (Duc, 1995), and *Medicago truncatula* (Sagan et al., 1995). The presence of the trait nts in all known supernodulators might be due to the wide use of nitrate-tolerant nodulation as a phenotypic selection marker for screening of mutagenized plant populations (Jacobsen and Feenstra, 1984; Carroll et al., 1985; Duc and Messager, 1989; Sagan and Duc, 1996). RisfixC mutant is similar to other described supernodulators in being associated with nts trait, although RisfixC was originally characterized by increased nodule number (Novak et al., 1993ab). Similarly, nts was confirmed subsequent to selection for nodule number in line f32, a supernodulator of faba bean (Duc, 1995). Although different supernodulating mutants increase the wild-type nodule number to different extent according to different species, allele, and growth conditions (Day et al., 1989; Sagan and Duc, 1996), the nodule number ratio ranges for pea supernodulators grown on low-nitrate medium between 4 and 6, independently of the way in which they have been generated (Jacobsen and Feenstra, 1984; Novak et al., 1993b; Sagan and Duc, 1996; present work). This value probably corresponds to the maximum nodule number that can be released by a single mutation of this type.

The pleiotropic features observed in RisfixC in addition to nts in the present work and earlier (Novak et al., 1993ab) have been already reported for other supernodulators: compact shoot (Gresshoff et al., 1988; Postma et al., 1988; Duc and Messager, 1989; Hansen et al., 1992; Sidorova et al., 1995; Sagan and Duc, 1996), reduced root of supernodulated plants (Gresshoff et al., 1988; Postma et al., 1988), and lowered SNA (Jacobsen and Feenstra, 1984; Day et al., 1987; Duc and Messager, 1989; Rosendahl et al., 1989; Wu and Harper, 1990).

As with other genetically characterized supernodulators (Delves et al., 1988; Postma et al., 1988; Kokubun and Akao, 1994; Sidorova et al., 1995; Sagan and Duc, 1996), RisfixC is conditioned by a single Mendelian mutation. In pea,

the supernodulating mutant nod3 has been attributed to the locus *nod3* (Postma et al., 1988), and mutant K301 to the locus *nod4* (Sidorova et al., 1995). On the other hand, two complementation groups of supernodulating lines obtained in cv. 'Frisson' have been ascribed to loci *sym28* and *sym29*, following the notation of nodule development mutants (Sagan and Duc, 1996). The relation of RisfixC to other pea supernodulating lines still has to be established.

In most of the reported cases, the pleiotropic features of supernodulating lines were inseparable from the nodule number character (supernodulation) when studied genetically: nitrate-tolerant nodulation in soybean mutant nts382 (Delves et al., 1988), the character of compact shoot in two complementation groups of pea supernodulating mutants originating from cv. 'Frisson' (Duc and Messager, 1989; Sagan and Duc, 1996), and stem fasciation in the pea mutants in locus *sym28* (Duc and Messager, 1989; Sagan and Duc, 1996) and in pea line K301 (Sidorova et al., 1995). These findings are consistent with our results in RisfixC. However, some cases of segregating traits were revealed among soybean supernodulating mutants: dwarfism (line nts 1007), male sterility (line nts 183), and leaf shape mutations in several other lines (Gresshoff et al., 1988).

The overnodulation in legumes is supposed to prevent full development of the root, to delay shoot development by excessive demand for photosynthates, and to lead to nodule immaturity and lower SNA (Day et al., 1987; Gresshoff et al., 1988). In spite of the failure in our crosses, an uncoupling of the valuable nts trait from supernodulation and associated undesirable pleiotropic effects might be interesting from the practical point of view (Gresshoff et al., 1988).

Link between ineffectiveness and nodule number in mutant RisfixV

The term hypernodulating has been used for mutants of soybean with only moderate increase of nodulation and weak nts (Gremaud and Harper, 1989; Olsson et al., 1989). This precedent led to an analogous notation of the RisfixV mutant, which exhibits increased nodule number without nitrate resistance (Novak et al., 1993ab). However, it seems that RisfixV has no obviously homologous mutation among other described Fix⁻ mutants of pea (Duc and Messager, 1989; Kneen et al., 1990; Borisov et al., 1992; Brewin et al., 1993; Novak et al., 1993ab) since it combines ineffectiveness with deregulation of nodule initiation. On the other hand, the nitrate-sensitive hypernodulation coded by loci *nod1* and *nod2* in pea was not associated with ineffectiveness (Gelin and Blixt, 1964; Postma et al., 1988). Certain homology can be assumed with the common bean mutant R32BS15 in view of the shared heterogeneity of nodule population and nitrate sensitivity of nodulation (Hansen et al., 1992). RisfixV should be considered as a nodule development rather than a nodule number mutant and as such might be ascribed to the next unoccupied symbiotic

locus in pea, presumably *sym30* since *sym28* and *sym29* were recently ascribed to the supernodulating lines from cv. 'Frisson' (Sagan and Duc, 1996).

Determination of both the ineffectiveness and the hypernodulation of RisfixV by one gene provides further evidence in favor of the causal relationship between these traits. The symbiotic tissue necrosis in RisfixV, as characterized formerly by optical and electron microscopy (Novak et al., 1995), was assumed to prevent production or transport of a nodule-derived factor, which suppresses nodule initiation in the proximity of an established nodule (Nutman, 1952; Caetano-Anollés et al., 1991). The possibility of a considerable increase in nodule number as a consequence of weakened dominance of certain types of ineffective nodules has been repeatedly considered by Nutman (1952) and Rolfe and Gresshoff (1988).

The observed maternal plant effect, i.e. poor growth and symbiosis development when the maternal plant was RisfixV, probably indicates a deficiency in a component essential for growth and nodulation in RisfixV plants. This assumption is in agreement with terminal chlorosis, often observed in this line. The maternal plant effect is not novel for plant nodulation genes. It has been observed already by Lie and Timmermans (1983) in crosses of pea lines carrying the allele *sym6* conferring strain-specific ineffectiveness.

The effect of ccvv genotype on nodulation

Two factors have been postulated to act on the nodule primordia once they are induced by rhizobial Nod factor: the shoot-derived systemic factor, which mediates the inhibitory action of initial nodules and of nitrate (Delves et al., 1986), and the already mentioned nodule-derived, short-range factor, produced by established nodules and root tips (Nutman, 1952; Caetano-Anollés et al., 1991). While mutant RisfixC is supposed to be blocked in production of the shoot systemic factor according to its behaviour on increasing nitrate level, RisfixV is probably blocked in the production of the nodule-derived factor. We can assume that both feedback pathways are interrupted in the double mutant lines CV. In other words, no inhibitory factor acts on the nodule initiation process, and all Nod factor-initiated nodules can develop to macroscopic size.

The observed additivity of the action of both mutations on nodule number in CV plants firstly confirms the notion about two different factors and secondly suggests that both factors act on different subsets of initial nodules. If both the mutations had interrupted the same regulatory pathway, we should not have observed a higher nodule number in CV than in RisfixC. On the other hand, it seems that the potential number of established nodules is not unlimited. The increased nodule number in RisfixC might correspond to the number of actual infections in the root. They have been defined in soybean as sites of cortical cell

divisions associated with rhizobial infection (Rolfe and Gresshoff, 1988). Although less than one tenth of these develop into distinct nodules in the wild-type plants of soybean (Rolfe and Gresshoff, 1988), the development of most of them was released in a supernodulating mutant nts382 as a consequence of a single mutation (Mathews et al., 1989). A similar mechanism of nodule number increase can be assumed in the supernodulator RisfixC. However, it is not obvious where the additional nodule fraction contributed by gene v in CV plants originates from. It might represent either the residual actual infections which are still arrested in RisfixC or activated pseudoinfections, i.e. meristematic foci that are not normally accompanied with a *Rhizobium* infection (Mathews et al., 1989). In addition, more latent preinfection foci from the root cortex might be realized in CV plants (Collins, 1983; Rolfe and Gresshoff, 1988).

The genotype of CV lines resembles constellation of the pea double mutant FN_1, which has been obtained by recurrent mutagenesis on the genetic background of the pea supernodulating mutant nod3 and which also combines supernodulation/nts with ineffectiveness. Similarly, further increase in nodulation of this line was interpreted as two mechanisms of nodule number determination (Postma et al., 1990). Our finding is also in agreement with evidence of the activity of nodule-derived factor on a supernodulating/nts background in soybean line nts382 (Caetano-Anollés et al., 1991).

Increased size and activity observed in CV nodules in comparison with RisfixV can be explained by a delay of nodule collapse, which is conditioned by the allele v, in view of slower nodule development, which is in turn conditioned by supernodulation (Day et al., 1987).

The obtained CV lines, like both parental mutant lines, are expected to be useful in further analysis of processes associated with symbiotic nodule initiation.

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