Mol Gen Genet (1997) 254:592 – 598

ORIGINAL PAPER

A. Y. Borisov · S. M. Rozov · V. E. Tsyganov
E. V. Morzhina · V. K. Lebsky · I. A. Tikhonovich

Sequential functioning of *Sym-13* and *Sym-31*, two genes affecting symbiosome development in root nodules of pea (*Pisum sativum* L.)

Received: 28 October 1996 / Accepted: 22 January 1997

Abstract Two Fix$^-$ mutants of pea (*Pisum sativum* L.) which are unable to fix molecular nitrogen, E135f (*sym-13*) and Sprint-2Fix$^-$ (*sym-31*), were crossed to create the doubly homozygous recessive line, named RBT (*sym-13*, *sym-31*). The ultrastructural organization of nodules of the RBT line was compared with that of each of the two parental mutant lines and with the original wild-type genotypes of the cultivars Sparkle and Sprint-2. It was shown that the RBT line is similar to the mutant line Sprint-2Fix$^-$ in having abnormal symbiosome composition and bacteroids with relatively undifferentiated morphology. Because the phenotypic manifestation of the *sym-31* mutant allele suppresses the phenotypic manifestation of the *sym-13* mutant allele, it is concluded that the function of the gene *Sym-31* (which is mutated in the Sprint-2Fix$^-$ line) is necessary at an earlier stage of symbiosome development than the gene *Sym-13* (which is mutant in the E135f line).

Key words Plant-microbe interactions · Nitrogen fixation · Mutant nodules · Pea symbiotic genes · Nodule ultrastructure

Introduction

The legume-*Rhizobium* symbiosis has attracted considerable interest from biologists of different specializations because it provides a model for studying mechanisms of plant-microbial interactions. The legume root nodule represents an entirely new plant organ which is colonized by rhizobia. The endophytic bacteria differentiate during nodule development, becoming transformed into specialized endosymbiotic forms, termed bacteroids, which acquire the capacity to fix atmospheric nitrogen (Mylona et al. 1995). In pea (*Pisum sativum* L.) and many other temperate legumes, each bacteroid is individually enclosed by a plant-derived membrane termed the peribacteroid membrane (Brewin 1991); the entire functional unit is termed a symbiosome (Roth and Stacey 1989). The structure and development of symbiosomes is of fundamental importance to the *Rhizobium*-legume symbiosis, but very little is known about the plant-microbial interactions that control the differentiation of this organelle-like compartment (Verma 1992).

During the past two decades, genetic analysis of *Rhizobium* has begun to dissect the complex network of signals leading to nodule initiation and development (Dénarié and Cullimore 1993). For technical reasons, the genetic analysis of the host legume has proceeded more slowly than that of the bacterium, but this situation is now changing and, as a result of experimental mutagenesis programmes in various laboratories around the world, a large number of independent symbiotic mutants have been generated in different legume species. These studies have identified about 60 host-plant genes affecting symbiosis with nodule bacteria (Phillips and Teuber 1992). The comparative study of mutant phenotypes provides useful means of discovering new symbiotic abnormalities and classifying mutant alleles. However, more complex genetic models are sometimes needed to analyze the interactions of a group of genes that apparently affect fundamental elements of the development of morphological structure. Such combinatorial genetic models have not yet been described for symbiosis.

This paper describes the construction of a double-mutant pea line that carries two non-allelic, symbiotically defective Fix$^-$ mutations. When compared with the wild-type phenotypes of the corresponding parental lines (Sparkle and Sprint-2), each of the mutant alleles *sym-13*

Communicated by A. Kondorosi

A. Y. Borisov (✉) · V. E. Tsyganov · E. V. Morzhina
V. K. Lebsky · I. A. Tikhonovich
All-Russia Research Institute for Agricultural Microbiology, Podbelsky Chaussee 3, St. Petersburg-Pushkin 8, 189620 Russia

S. M. Rozov
Institute of Cytology and Genetics, Novosibirsk, 630090 Russia

(in line E135f) and *sym-31* (in Sprint-2Fix$^-$) was found to affect the development and function of bacteroids within the symbiosome compartment of the host cell. The results of these morphological and biochemical comparisons between mutant and parental lines have been published and discussed in previous papers (Kneen et al. 1990; Borisov et al. 1992; Suganuma and LaRue 1993; Romanov et al. 1995). It was shown that the mutation in *sym-13* determines the premature degradation of almost completely (in the morphological sense) differentiated bacteroids, while the mutation in gene *sym-31* is characterized by an abnormal symbiosome structure and reduced morphological differentiation of bacteroids.

Because the Sprint-2Fix$^-$ (*sym-31*) mutation results in less advanced differentiation of bacteroids than was observed with line E135F (*sym-13*), it seemed likely that the wild-type allele of *Sym-31* functions prior to *Sym-13* in the pathway of nodule development. In order to test this hypothesis, it was necessary to introduce both mutant alleles into the same pea line and to study the double-mutant phenotype to examine how the effects of the corresponding mutant alleles might interact in the same genome.

The goal of this research was therefore to determine when and how these two alleles interact, and also to extend the comparative morphological characterization of mutants E135f (*sym-13*) and Sprint-2Fix$^-$ (*sym-31*), which have been described previously (Kneen et al. 1990; Borisov et al. 1992).

Materials and methods

Plant material

The symbiotically defective mutant line E135f (*sym-13*) was kindly provided by Prof. T. A. LaRue (Kneen et al. 1990). Mutant line Sprint-2Fix$^-$ (Borisov et al. 1992) has recently been assigned the mutant allele designation *sym-31*, following allelism tests conducted by Drs. G. Duc and M. Sagan (personal communication). Sparkle was the parental genotype from which E135f (*sym-13*) was derived and the line Sprint-2 (Berdnikov et al. 1989) was the source of Sprint-2Fix$^-$ (*sym-31*).

Rhizobium strain

The commercial by available nodulating strain *Rhizobium leguminosarum* bv. *viciae* CIAM 1026 was used as an inoculant in all experiments (Safronova and Novikova 1996).

Genetic construction of the double mutant line

After crossing the mutant lines E135f (*sym-13*) and Sprint-2Fix$^-$ (*sym-31*), the F_2 generation was obtained by self-pollination of F_1 plants. The F_2 progeny were each used in a series of repeated backcrosses with both parental mutant lines, and in each case these progeny were allowed to self-pollinate. In order to screen for deficiencies in symbiotic nitrogen fixation, backcross (F_b) plants (after each cross of each F_2 plant with each parental line) were grown under nitrogen-free conditions and analyzed for signs of nitrogen starvation at the early flowering stage. These plants were then uprooted and visual inspection of the root systems was performed. Thus, by the analysis of test cross progeny phenotypes, it was possible to deduce the genotype of the parental F_2 plant. It was expected that the plant phenotypes of F_b families would be of the following types: only Fix$^+$ plants; Fix$^+$ and Fix$^-$ plants (approximately 1:1); and only Fix$^-$ plants. This would be consistent with the following states for the corresponding locus in the F_2 plants: dominant homozygous; heterozygous; and recessive homozygous. By performing this analysis after back-crosses between the F_2 individuals and each of the mutant lines E135f and Sprint-2Fix$^-$, it was possible to identify the F_2 individuals with a genotype carrying both recessive alleles in the homozygous condition.

Plant growth conditions and analysis of root nodules

For multiplication and crosses of the lines the various growth conditions described earlier were used (Borisov et al. 1992). For analysis the plants of all lines studied were grown in growth chambers as follows: 16 h day (21° C) and 8 h night (19° C), relative humidity, 75%; photon flux 490 $\mu E/m^2$ per s; the plant nutrient solution used was modified after Krasilnikov and Korenyako (1940) and contained (in mg per liter of water): K_2HPO_4, 1000; $Ca_3(PO_4)_2$, 200; $MgSO_4 \cdot 7H_2O$, 1000; $FeSO_4$, 30; H_3BO_3, 5; $(NH_4)_2MoO_4$, 5; KBr, 0.6; KI, 0.5; $CuSO_4 \cdot 5H_2O$, 0.4; NaCl, 0.3; $Al_2(SO_4)_3 \cdot 18H_2O$, 0.3; $ZnSO_4 \cdot 7H_2O$, 0.2; $CoCl_2 \cdot 6H_2O$, 0.2; $NiSO_4$, 0.2; $MnSO_4$, 0.2; Li_2SO_4, 0.2, pH about 7.0; sterile vermiculite and sand were used as substrates.

The method of seed inoculation and the techniques of electron microscopy were described previously (Borisov et al. 1992). The nodules of the double mutant genotype (*sym-13*, *sym-31*) were collected for microscopic analysis several times during selection of the final genetic type: from generation F_3 to generation F_9.

Results

Construction of double mutant line RBT (*sym-13*, *sym-31*)

Following a cross between E135f (*sym-13*) and Sprint-2Fix$^-$ (*sym-31*) lines, 30 F_2 plants were obtained by self-pollination of the plants of F_1 generation. Each of these 30 individuals was then back-crossed with each of the two parental mutant lines. Progeny obtained from each of these backcrosses was tested for the Fix$^-$ phenotype; it was found that only one of the F_2 individuals yielded a consistent Fix$^-$ phenotype in all progeny from backcrosses with both parental lines E135f and Sprint-2Fix$^-$. This plant (number 14) was presumed to be recessive homozygous for both loci (*sym-13* and *sym-31*). After self-pollination of this plant for 10 generations seeds were selected to create the "double Fix$^-$" line named RBT. The genotype of the RBT line (*sym-13*/*sym-13; sym-31*/*sym-31*) was confirmed by further control backcrosses. The plants of this line form 80 ($\pm$ 14) white or greenish, medium sized nodules under the growth conditions described in Materials and methods. In this paper, the nodule phenotype of the RBT line is compared with the parental mutant lines, E135f and Sprint-2Fix$^-$, and with the initial wild-type genotypes Sparkle and Sprint-2.

Infection thread development and endocytosis

Ultrastructural analysis of RBT (*sym-13*, *sym-31*) nodules revealed the following morphological characteristics, revealing common traits or differences between this line and the other lines tested. Bacteria in infection threads showed little sign of morphological differentiation in RBT (*sym-13*, *sym-31*) or in any of the other pea lines tested (Fig. 1A). At the point of endocytosis (Fig. 1B), bacterial cells showed a slight decrease in electron density of the matrix and a slight increase (1.5–2-fold) in average size. However, no differences could be detected between any of the pea lines at this developmental stage. Similarly, the process of endocytosis appeared normal in the RBT (*sym-13*, *sym-31*) line, with the bacteria being engulfed individually by plasma membranes so that juvenile symbiosomes contained single bacteroids (Fig. 1C). This was also true for all the other pea lines examined (data not shown).

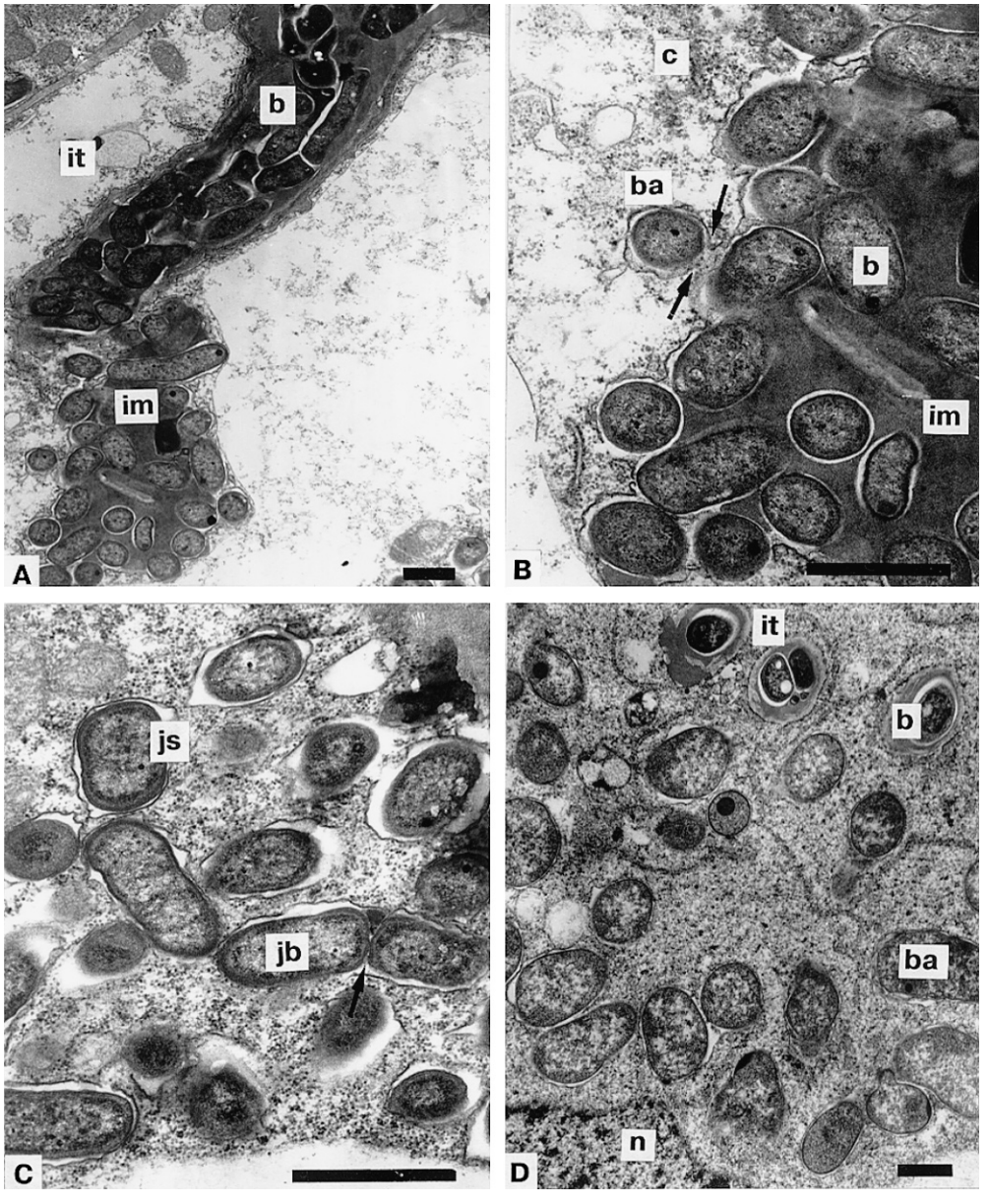

Fig. 1A–D Ultrastructural analysis of nodules from the double mutant pea line RBT carrying *sym-13* and *sym-31* as homozygous mutant alleles. **A** Structure of the infection thread (it) within the host nodule cell, showing relatively normal morphology. Within the lumen of the infection thread dividing bacteria (b) are embedded in infection thread matrix (im). Bacteria released into the plant cytoplasm are also visible. **B** Release of bacteria from an infection thread droplet into the host cell cytoplasm (c). Following endocytosis, each bacteroid (ba) is individually enclosed by a peribacteroid membrane (arrows). **C** Section of an infected host cell showing the division of a juvenile bacteroid (jb) inside the peribacteroid membrane (*arrow*). At this stage, each juvenile symbiosome (js) contains only one bacteroid. **D** Section of an infected host cell in the wild-type line Sparkle. Bacteroids (ba) in host plant cytoplasm are distinctly different from bacteria (b) in infection threads with respect to cell size, cell shape and the electron density of the cell matrix. *Bar* represents 1.0 μm

Development of symbiosome structures

Following endocytosis, the further development of bacteroids appeared to diverge in the various lines tested. This was examined by following the time course of symbiosome differentiation along the longitudinal axis in the invasion zone of the nodule. When the most juvenile symbiosomes were examined in all the pea lines, it was observed that the enclosed bacteroids were undergoing cell division (Fig. 1C). In the wild-type lines, Sparkle (Fig. 1D) and Sprint-2 (Fig. 2C), division of the bacteroids in the invasion zone was apparently followed by partitioning of the peribacteroid membrane, because the mature symbiosomes usually contained only a single bacteroid. This arrangement was also observed for E135f (*sym-13*) (data not shown), although in this case the bacteroids appeared to progress prematurely into the senescent phase (Kneen et al. 1990; Vasse et al. 1990). Very rarely, symbiosomes with two or three bacteroid profiles were observed in the infected cells of the symbiotic zone in the wild-type and E135f (*sym-13*) nodules: these views, however, were probably the result of taking a glancing section across the branched part of a pleiomorphic bacteroid.

As indicated previously (Borisov et al. 1992), a different pattern of symbiosome development was observed for Sprint-2Fix$^-$ (*sym-31*) nodules (Fig. 2A, D). As in wild-type nodules, it was possible to observe profiles of juvenile symbiosomes in which bacteroids were actively dividing (Fig. 2A). In addition, however, the profiles of other symbiosomes suggested that they themselves might be in the process of dividing or fusing (Fig. 2A, arrowheads). Thus, in the mutant Sprint-2 Fix$^-$ (*sym-31*), the balance between symbiosome fusion and symbiosome division seems to be shifted such that, in the central infected tissue of the nodule, bacteroids are enclosed in groups of 6–8 (Fig. 2D) rather than individually, as in E135f (*sym-13*; Kneen et al. 1990) and the wild-type lines (Fig. 2C).

Finally, it was observed that in the infected cells of RBT (*sym-13*, *sym-31*) nodules (Fig. 2B) the morphology of symbiosomes resembled that of Sprint-2Fix$^-$ (*sym-31*) nodules (Fig. 2D) and not that of E135f (*sym-13*) (Kneen et al. 1990). In these cells, there was no evidence that the division of juvenile bacteroids was accompanied by division of the symbiosome compartment (Fig. 1C): on the contrary, the observed profiles suggested a tendency towards symbiosome fusion. Thus, in the central symbiotic zone of such nodules, symbiosomes contained 6–8 bacteroids surrounded by one symbiosome membrane (Fig. 2B).

Morphological differentiation of bacteroids

In wild-type lines, the proliferation of juvenile bacteroids and concomitant division of symbiosomes (observed in the infection zone) is followed by the process of bacteroid differentiation which first becomes manifest in the early symbiotic region termed the interzone (Vasse et al. 1990; Mylona et al. 1995). For wild-type nodules, bacteroid differentiation is associated with a further decrease in electron density of the cell matrix, a progressive increase in cell size and a change of shape from rod-like to pleiomorphic (Kneen et al. 1990; Borisov et al. 1992). At maturity, in the nitrogen-fixing zone of the nodule, the bacteroids in symbiosomes differ from bacteria in the infection threads by a considerably lower matrix electron density and a 3- to 5-fold increase in cell size (Fig. 2C). In the mutant E135f (*sym-13*), bacteroids apparently develop into pleiomorphic forms in the normal way (Kneen et al. 1990). However, the further development of nitrogen-fixing capability is blocked by the premature degradation of symbiosomes and infected cell structures and ultimately by the senescence of the nodule as a whole (Kneen et al. 1990).

In contrast to the wild-type lines Sprint-2 and Sparkle, and the Fix$^-$ mutant E135f (*sym-13*), the bacteroids observed in the symbiotic zone of the mutant line Sprint-2Fix$^-$ (*sym-31*) (Borisov et al. 1992) did not differ significantly from bacteria in infection threads, either in the electron density of their matrix, or in cell size and shape (Fig. 2A, D). Similarly, in the central symbiotic zone of line RBT (*sym-13*, *sym-31*), the symbiosomes usually contained several bacteroids (Fig. 2B) and these showed very few signs of morphological differentiation after proliferation inside the symbiosome membrane. Thus, in terms of bacteroid morphology, the double mutant RBT (*sym-13*, *sym-31*) more closely resembles Sprint-2Fix$^-$ (*sym-31*) than E135f (*sym-13*).

Nodule degradation

The last stage of nodule development is the programmed senescence and degradation of symbiotic structures. The published data on comparative ultrastructural analysis for E135f (*sym-13*), Sprint-2Fix$^-$ (*sym-31*) and their parental lines (Kneen et al. 1990; Borisov et al. 1992) demonstrate quite distinct differences in this parameter between the mutant lines. Normal development of E135f (*sym-13*) symbiotic structures is interrupted by their early degradation, while this is not the case with Sprint-2Fix$^-$ (*sym-31*). Although the degradation of bacteroids was not as obvious in RBT (*sym-13*, *sym-31*) as in E135f (*sym-13*), it was difficult to draw any meaningful conclusions about the rate of degradation of symbiotic structures in the RBT (*sym-13*, *sym-31*) line because its genetic background was a mixed one (Sprint-2/Sparkle) in all generations of selection studied, and the rate of nodule development and histological differentiation is strongly dependent on the genotype of the host plant.

Discussion

In this study, the morphological effects of the *sym-13* and *sym-31* mutant alleles were examined, both individually and in combination in the RBT (*sym-13*, *sym-31*) line.

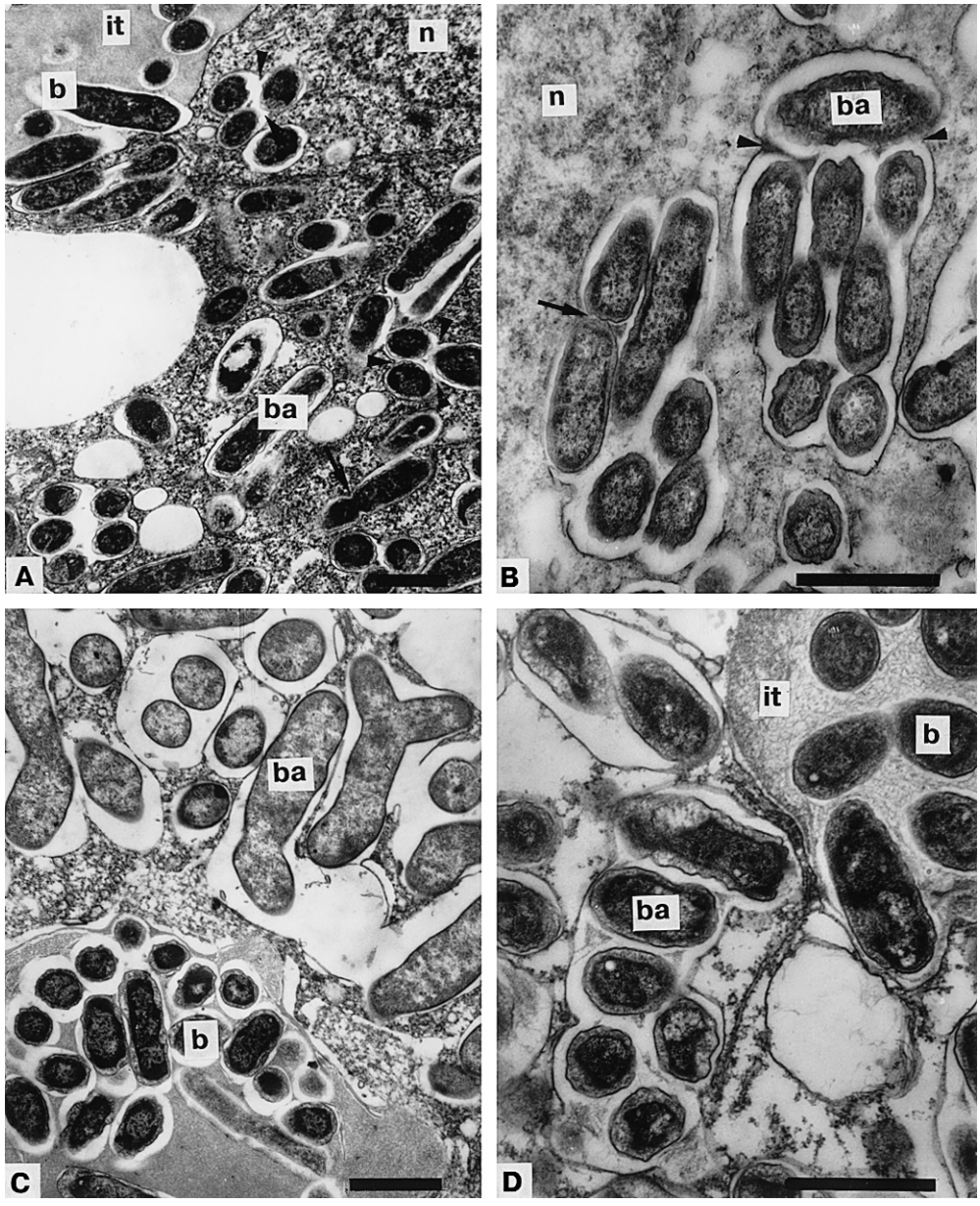

Fig. 2A–D Ultrastructural comparison of infected cells from nodules of RBT (*sym-13*, *sym-31*), Sprint-2Fix$^-$ (*sym-31*) and Sprint-2 (wild-type). **A** Sprint-2Fix$^-$. Bacteroids (ba) in host plant cytoplasm are morphologically similar to bacteria (b) in infection threads (it). The *arrow* shows the division of bacteroids within the symbiosome. *Arrowheads* show the fusion or division of several symbiosomes. Vacuole (v) and nucleus (n) are indicated. **B** Structure of symbiosomes in nodules of the double mutant line RBT. Each symbiosome consists of 6–8 bacteroids surrounded by a peribacteroid membrane. The *arrow* shows division of bacteroids; *arrowheads* show profiles of fusing or dividing symbiosomes. **C** Structure of symbiosomes in the wild-type line Sprint-2. Fully differentiated, pleiomorphic Y-shaped bacteroids are clearly different from bacteria in infection thread: bacteroids are 3–5 times larger and contain a matrix of low electron density. The peribacteroid membrane encloses only one or two bacteroids in each symbiosome unit. **D** Structure of symbiosomes in nodules of Sprint-2Fix$^-$ (*sym-31*). Most bacteroids are morphologically similar to bacteria in the infection thread. Each symbiosome contains 6–8 bacteroids. *Bar* represents 1.0 μm. Note that micrographs **A** and **C** are printed at higher magnification than micrographs **B** and **D**

Previous studies with E135f (*sym-13*) and Sprint-2Fix$^-$ (*sym-31*) and their parental lines Sparkle and Sprint-2 indicated that symbiosome development and morphology showed quite distinct differences in the two mutant lines (Kneen et al. 1990; Borisov et al. 1992). In E135f (*sym-13*) nodules, symbiosome development apparently proceeds normally up to the point where large pleiomorphic bacteroids are formed, but further development of the nitrogen-fixing state is interrupted by the early degradation of these apparently mature bacteroids. By contrast, in the case of nodules formed by mutant Sprint-2Fix$^-$ (*sym-31*), bacteroid development is largely arrested in the juvenile state, in which their size and shape is barely distinguishable from those of bacteria in

the lumen of infection threads. Furthermore, in the central symbiotic tissue of Sprint-2Fix$^-$ (*sym-31*), the symbiosomes enclose groups of 6–8 bacteroids rather than single bacteroids, as in the wild-type and E135f (*sym-13*) lines. Because of these ultrastructural differences, the two Fix$^-$ mutants have been assigned to different classes: E135f (*sym-13*) to a class of mutants showing early degradation of symbiotic structures, and Sprint-2Fix$^-$ (*sym-31*) to a mutant class showing early arrest of nodule development (Sagan et al. 1993). In the present study, all these earlier observations were confirmed in relation to the differences between E135f (*sym-13*) and Sprint-2Fix$^-$ (*sym-31*) lines.

The origin of the abnormal symbiosomes of Sprint-2Fix$^-$ (*sym-31*) was further investigated. In this mutant, the mechanism of uptake of bacteria from the infection thread lumen appeared to be normal (Fig. 2A). Endocytosis always seemed to involve the uptake of individual bacteria from the infection thread droplet. A form of endocytosis involving the simultaneous enclosure of several bacteria by one membrane was never observed with Sprint-2Fix$^-$ (*sym-31*), nor with any of the other plant lines studied. So, at present, we consider single-cell endocytosis to be the only possible mechanism of uptake for pea, and probably for other legumes with nodules having indeterminate apical meristems. For the first time, it has been shown that bacteria undergo slight morphological differentiation in the infection thread before endocytosis. This phenomenon, which was observed not only in wild-type nodules but also in all the mutants tested, was manifested by a slight increase in bacterial cell size at the point of endocytosis, coupled with a slight reduction in electron density of the bacterial cell matrix (Fig. 1A, B).

Released bacteria (juvenile bacteroids) continued to divide within the lumen of the symbiosome compartment. This was true both for Sprint-2Fix$^-$ (*sym-31*) (Fig. 2A) and for all other pea lines tested (Fig. 1B). However, at this point in symbiosome differentiation, the pathway of development for Sprint-2Fix$^-$ (*sym-31*) nodules seemed to diverge from that observed for E135f (*sym-13*) and the wild-type lines examined. In Sprint-2Fix$^-$ (*sym-31*), bacteroid division was not accompanied by concomitant division of the peribacteroid membrane (Fig. 2A). Instead, it seemed that the symbiosomes were fusing together to create larger conglomerate structures enclosing 6–8 bacteroids within a single peribacteroid membrane envelope. This process was not observed in E135f (*sym-13*) nor in any of the wild-type nodules examined. A tendency for symbiosomes to fuse together into aggregates has already been reported for aging infected cells of soybean and alfalfa nodules (Fischer et al. 1986; Vance and Johnson 1983). However, in the case of pea nodule cells, we interpret our observations as indicating that the division or fusion of symbiosomes may represent an equilibrium process. Displacement of the equilibrium to one side or the other could perhaps depend on the modification of pathways of membrane biogenesis and vesicle targeting. Maintaining the equilibrium between symbiosome division and symbiosome fusion could prove to be an important aspect of the mechanism by which proliferating bacteria are able to colonize host cells inside symbiosomes. It could also affect the extent to which the host cell develops a defense response to counteract this colonization process.

It is interesting to note that the symbiosome structure observed for the Sprint-2Fix$^-$ (*sym-31*) line is somewhat similar to that observed for wild-type nodules of many tropical legumes which have determinate (spherical) meristems. In nodules of *Lotus*, *Glycine* and *Phaseolus* spp., for example, it is normal for mature symbiosomes to enclose 6–12 bacteroids that are morphologically undifferentiated (Brewin 1991; Roth and Stacey 1989; Verma 1992). Thus, in an evolutionary sense, the *Sym-31* allele may function in pea nodules to suppress the development of this more primitive form of symbiosome morphology.

Comparative ultrastructural analysis of the RBT (*sym-13*, *sym-31*) line showed that it resembled the parental mutant line Sprint-2Fix$^-$ (*sym-31*) with respect to bacteroid differentiation and symbiosome structure. In nodules of the RBT (*sym-13*, *sym-31*) line, differentiation of juvenile bacteroids was arrested before they developed into mature pleiomorphic bacteroid forms (Fig. 2B) and symbiosomes always contained several bacteroids, as was observed for Sprint-2Fix$^-$ (*sym-31*). Moreover, the bacteroids of RBT (*sym-13*, *sym-31*) nodules did not appear to undergo premature senescence (Fig. 2B), as was observed in nodules of E135f (*sym-13*). Therefore the phenotypic manifestation of the *sym-31* mutant allele apparently suppresses manifestation of the *sym-13* mutant allele with respect to these parameters. What is not yet clear, however, is the extent to which RBT (*sym-13*, *sym-31*) may express (at least partially) the phenotype of premature symbiosome senescence that is observed with E135f (*sym-13*). RBT (*sym-13*, *sym-31*) nodules were analysed in plants from several generations (from F_3 to F_9) of our selection scheme and the same results were obtained with respect to nodule ultrastructure in spite of morphological changes observed in plants selected. But it is still difficult to separate the effects of the mutant alleles under study from possible background effects of host genotype. Unfortunately, it will be a labor-intensive and time-consuming process to construct sets of isogenic lines which differ only with respect to the alleles of these two genes. Hopefully, however, the RBT (*sym-13*, *sym-31*) line can still be used to investigate this problem further by making use of molecular markers and probes which can distinguish between the states of the symbiosome compartment established with each of the mutant alleles *sym-13* and *sym-31* (Brewin et al. 1995; Tikhonovich et al. 1995).

In summary, the sequence of steps leading to the formation of mature symbiosomes has been investigated by genetic analysis, in combination with the ultrastructural analysis of mutant nodules. The morphological effects of mutant alleles *sym-13* and *sym-31* were first examined in lines E135f (*sym-13*) and Sprint-2Fix$^-$

(*sym-31*). From this analysis, it was concluded that the *Sym-31* gene probably functioned at an earlier stage in symbiosome development than did the *Sym-13* gene. This hypothesis was further tested by constructing the line RBT (*sym-13*, *sym-31*) which carries both mutant alleles in the homozygous condition. In this double mutant, the expression of the mutant symbiosome morphology associated with *sym-31* appeared to be unimpaired by the presence of the *sym-13* mutant allele. On the other hand, the phenotype of premature bacteroid senescence, which was associated with *sym-13* in the line E135f, was not observed in the double mutant line RBT (at least, not to the same extent). Therefore, it is concluded that *Sym-31* functions in advance of *Sym-13* in the developmental pathway of symbiosome differentiation and that the proper functioning of this gene is required for the subsequent expression of the mutant phenotype associated with the *sym-13* mutant allele.

This is one of the first examples of the use of combinations of mutations to determine the functional sequence of action of two symbiotic genes whose mutations each result in Fix^- phenotypes. Further genetic construction of such models will make it possible to classify the symbiotic genes of pea according to the order in which they function during nodule development. It may even be possible to relate the time-course of appearance of symbiotic gene functions during nodule development to the time-course for the progressive evolution of the legume-*Rhizobium* interaction, which must have arisen by the step-by-step modification of plant systemic defense reactions leading to the development of increasingly intimate host-*Rhizobium* symbiotic interactions.

Acknowledgements This work was supported by the Russian State Research Program INTERBIOAZOT-2000, the Russian Foundation for Fundamental Research (95-04-12573 and 96-04-50361), INTAS (94-1058), NATO (HITEC.LG 941297 and HITEC.EV 960309) and the Russian State Program Frontiers in Genetics. The authors are very grateful to N. J. Brewin for fruitful discussion and critical reading of the manuscript, to S. A. Tchetkova for help during the preparation of the manuscript and to L. E. Dvoryaninova and V. M. Semyonov for their excellent technical assistance

References

Berdnikov VA, Rosov SM and Bogdanova VS (1989) Construction of a series of laboratory pea lines. In: Abstracts of Conference on Genetics May 23–25, Kiev, USSR. Vol 2 (Genetics of plants), pp 26–27

Borisov AY, Morzina EV, Kulikova OA, Tchetkova SA, Lebsky VK, Tikhonovich IA (1992) New symbiotic mutants of pea (*Pisum sativum* L.) affecting either nodule initiation or symbiosome development. Symbiosis 14:297–313

Brewin NJ (1991) Development of the legume root nodule. Annu Rev Cell Biol 7:191–226

Brewin NJ, Bolanos L, Dahiya AP, Gardner CD, Hernandez LE, Kardailsky IV, Rathbun EA, Sherrier DJ (1995) Differentiation of the symbiosome compartment in pea nodule cells. In: Tikhonovich IA, Provorov NI, Romanov VI, Newton WI (eds) Nitrogen Fixation: fundamentals and applications. Kluwer Academic Publishers, Dordrecht, Boston, London, pp 455–460

Dénarié J, Cullimore J (1993) Lipo-oligosaccharide nodulation factors: a new class of signaling molecules mediating recognition and morphogenesis. Cell 74:951–954

Fischer HM, Alvares-Morales A, Hennecke H (1986) The pleiotropic nature of symbiotic regulatory mutants: *Bradyrhizobium japonicum nifA* gene is involved in control of *nif* gene expression and formation of determinate symbiosis. EMBO J 5:1165–1173

Kneen BE, LaRue TA, Hirsch AM, Smith CA, Weeden NF (1990) *Sym-13* – a gene conditioning ineffective nodulation in *Pisum sativum*. Plant Physiol 93:717–722

Krasilnikov NA, Korenyako AI (1940) On methods for determining the root nodule bacteria count in soil. Mikrobiologiya 9:27–31

Mylona P, Pawlowski K, Bisseling T (1995) Symbiotic nitrogen fixation. Plant Cell 7:869–895

Phillips DA, Teuber LR (1992) Plant genetics of symbiotic nitrogen fixation. In: Stacey G, Burris R, Evans HJ (eds) Biological Nitrogen Fixation. Chapman and Hall, New York, pp 200–235

Roth LE, Stacey G (1989) Bacterium release into host cells of nitrogen-fixing soybean nodules: the symbiosome membrane comes from three sources. Eur J Cell Biol 49:13–23

Romanov VI, Gordon AJ, Minchin FR, Witty JF, Scøt L, James CL, Borisov AY, Tikhonovich IA (1995) Anatomy, physiology and biochemistry of root nodules of Sprint-$2Fix^-$, a symbiotically defective mutant of pea (*Pisum sativum* L.) J Exp Bot 46:1809–1816

Safronova VI, Novikova NI (1996) Comparison of two methods for root nodule bacteria preservation: lyophilization and liquid nitrogen freezing. J Microbiol Meth 24:231–237

Sagan M, Huguet T, Barker D and Duc G (1993) Characterization of two classes of non-fixing mutants of pea plants (*Pisum sativum* L.). Plant Sci 95:55–66

Suganuma N, LaRue TA (1993) Comparison of enzymes involved in carbon and nitrogen metabolism in normal nodules and ineffective nodules induced by a pea mutant E135 (*sym-13*) Plant Cell Physiol 34:761–765

Tikhonovich IA, Borisov AY, Chvabauskene JA, Kamardin NN, Kravchenko LV, Lebsky VK, Minchin F, Morzhina EV, Romanov VI, Skot L, Tchetkova SA, Tsyganov VE (1995) Plant genetical control at the crucial steps of symbiosis. In: Tikhonovich IA, Provorov NI, Romanov VI, Newton WI (eds) Nitrogen Fixation: fundamentals and applications. Kluwer Academic Publishers, Dordrecht, Boston, London, pp 461–466

Vance CP, Johnson LE (1983) Plant-determined ineffective nodules in alfalfa (*Medicago sativa*): structural and biochemical comparisons. Can J Bot 61:93–106

Verma DPS (1992) Signals in root nodule organogenesis and endocytosis. Plant Cell 4:373–382

Vasse J, de Billy F, Camut S, Truchet G (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J Bacteriol 172:4295–43