Symbiosis, 14 (1992) 297–313
Balaban, Philadelphia/Rehovot

New Symbiotic Mutants of Pea (*Pisum sativum* L.) Affecting Either Nodule Initiation or Symbiosome Development*

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Received February 12, 1992; Accepted June 17, 1992

Abstract

Following chemical (EMS) mutagenesis of pea (*Pisum sativum* L. variety Sprint-2), a number of mutants were isolated that were defective in symbiotic nitrogen fixation. Two mutants were resistant to inoculation (*Nod*$^-$) with a panel of 13 strains of *Rhizobium leguminosarum* bv *viciae*. In genetic crosses, the *Nod*$^-$ mutation in each of these two mutants was shown to segregate as a single Mendelian recessive character, and was found to be allelic with the previously described locus *sym8*. In addition, a single mutant which formed white (*Fix*$^-$) nodules without nitrogenase activity was isolated. The *Fix*$^-$ mutation of this mutant segregated as a single Mendelian recessive character which was non-allelic with the previously described locus *sym13*. Ultrastructural analysis of pea nodules revealed that the *Fix*$^-$ mutant was characterised by abnormal structure of the nitrogen-fixing units (symbiosomes): these contained several bacteroid-like cells per envelope rather than bacteroids being packed individually, which is more typical for pea. The *Fix*$^-$ phenotype of this mutant seems to be associated with a failure of bacteria to differentiate into nitrogen-fixing bacteroids, rather than with a premature bacteroid senescence as in other *Fix*$^-$ host-*Rhizobium* combinations. We propose that a new *sym*-locus has been described.

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0334-5114/92 /$03.50

Keywords: symbiosis, nitrogen fixation, mutants, *Pisum sativum* L., *Rhizobium*, symbiosome

Abbreviations: EMS: ethyl methane sulfonate, SM: symbiosome (peribacteroid) membrane, ITh: infection thread, PHB: poly-β-hydroxybutyrate, N: nucleus, No: nucleolus, IC: infected cell, UC: uninfected cell, B: bacteroid, b: bacteria, PBS: peribacteroid space, CW: cell wall, v: vacuole, st: starch

1. Introduction

Understanding of the basic principles of symbiosis depends to a large extent upon the knowledge of the intimate mechanisms of macro- and microsymbiont interactions. Formation of the functional nodules on the roots of legume plants is a complicated multi-stage process where the gene expression in one of the partners depends upon that in the other (Nutman, 1984; Govers et al., 1986). One important structural and functional unit of the symbiotic system is the symbiosome, a specific product of the host plant and bacteria interaction. Symbiosomes consist of differentiated functional bacteroids, i.e. a specially differentiated nitrogen-fixing form of *Rhizobium* bacteria, surrounded by a symbiosome (peribacteroid) membrane (Brewin, 1991). The role of each partner in the formation of this complex structure may vary in different stages of symbiosis. So differentiation of bacteroids and their contribution to the symbiosome is to a considerable extent under the host-plant control (Sutton, 1983), whereas the formation, stability and protein composition of the plant-derived peribacteroid membrane depends to a considerable extent on the nodule bacteria (Werner et al., 1991). Suitable genetic models are required in order to understand the well co-ordinated system of signal exchange between the macro- and microsymbionts that controls the formation and function of symbiosomes. Such models can be constructed using symbiotic mutants of both micro- and macrosymbionts. Bacterial mutant collections are available and data about the bacterial control of the formation and functioning of the symbiosome is fairly advanced (Simarov et al., 1990). In comparison knowledge about the role of the host-plant genome is relatively scarce. This is mostly due to the lack of spontaneous mutant alleles in host plant genes and also to a considerable difficulty in obtaining mutants in the late symbiotic genes. Thus, for example, out of 39 symbiotic mutants of pea in the collection of Dr. T.A. LaRue, 37 were *Nod*$^-$ and only two had alterations in the function of the nodules (T.A. LaRue, personal communication). Further difficulties include that nodulation defective mutants (*Nod*$^-$) can be screened more easily

than mutants having ineffective nodules (*Fix*$^-$), because in order to confirm their nature biochemical tests have to be employed.

The aim of this work was to isolate new symbiotic mutants of pea (*Pisum sativum* L.) and to use plant and bacterial mutants in order to understand the role of each partner in the formation and functioning of symbiosomes.

2. Materials and Methods

The induction of mutants was performed using the genetic line Sprint-2 (Berdnikov et al., 1989). This line has a shorter vegetative period. Medium sized nodules are pink and mostly on the main root. Ethyl methane sulfonate (EMS), a mutagenic agent known to be one of the most efficient for pea mutagenesis (Engvild, 1987) was used. Seeds were sterilized with concentrated sulphuric acid, then treated with 0.15% EMS for 10 hr and, after washing with tap water, were sown in soil for M_2 seed generation. Generation M_2 was inoculated with an effective *Rhizobium* strain and placed in aerated liquid medium with modified nitrogen-free mineral solution (Krasilnikov and Koreniako, 1940). After 4 weeks plants were visually screened for symptoms of N-deficiency (poor growth and chlorosis in the leaves). Prospective mutants of both types (*Nod*$^-$ and *Fix*$^-$) were transferred to medium supplied with nitrogen (1 g/l NH_4NO_3).

For the comparison and allelic tests of the newly isolated mutants, the following mutant lines of cultivar Sparkle from the Cornell University collection were used (courtesy of Dr. T.A. LaRue, Cornell University, USA):

- E2 – temperature sensitive nodulation, no or few nodules, locus *sym5*:
- R25 – non-nodulating mutant, locus *sym8*;
- E135f – ineffective mutant, locus *sym13*.

As controls the profusely nodulating parental line Sprint-2, the cultivar Sparkle as well as the genetic line L1714 (courtesy of Dr. S. Blixt, Weibullsholm Plant Breeding Institute, Sweden) were used.

As inoculants a commercial effective *Rhizobium leguminosarum* bv *viciae* CIAM 1026 strain, an ineffective CIAM 1064 strain and *A-1* capable of overcoming *sym2* determined resistance in Afganistan peas (Chetkova and Tikhonovich, 1986) were used (CIAM – Collection of Institute for Agricultural Microbiology). Seeds were inoculated at planting either with a bacterial suspension in water (10^7–10^8cells per plant) or with an adequate amount of the commercial peat inoculant "Rhizotorphin".

For the study of the strain specificity of the pea mutants a mixture of 13 efficient strains of various geographical origin was used (Strain collection of the All-Russian Research Institute for Agricultural Microbiology).

Plants were grown under hothouse conditions at a light intensity of 10000–12000 lux with a photoperiod of 14 hr. In summer, plants were grown in greenhouses with natural light and temperature.

The nitrogenase activity was determined on intact roots by a modified acetylene reduction technique (Alisova and Chunderova, 1976).

The total nitrogen content was determined in dry shoots by the Kjehldal method using Kjeltec-auto system (Tecator AB, Sweden).

Samples (pieces of nodules) for electron microscopy were fixed in 2.5% glutaraldehyde buffered with K, Na-phosphate (pH 7.2), washed with the same buffer and postfixed with 1.0% osmium tetroxide buffered by the same buffer and 1.0% gallic acid in distilled water. After washing with distilled water, the material was dehydrated through an ethanol series and absolute acetone and embedded in epon resin. Ultrathin sections were cut with a Reichert-Young ultratome, stained with uranyl acetate and lead citrate and examined with a Hitachi Electron Microscope H-300.

All measurements were carried out at the stage of early flowering when nitrogen fixation reaches its maximum level.

3. Results

Isolation of symbiotic mutants of pea

More than 5000 plants from 1050 M_2 families were screened in selective conditions. About 10% of families comprised chlorophyll-deficient mutants, while the amount of prospective families with nitrogen-deficient plants made up 0.1%, point to a rather high efficiency of EMS-mutagenesis. After screening, four lines with symbiotic defects were isolated. Three of them were non-nodulating — Sprint-2*Nod*$^-$-1, Sprint-2*Nod*$^-$-2, Spring-2*Nod*$^-$-3 although the latter *Nod*$^-$ phenotype was unstable. In Sprint-2*Nod*$^-$-1 chlorosis was evident even on full mineral nitrogen. Some plants of Sprint-2*Nod*$^-$-3 had a shoot with prominent sectoral structure. Visible sectors contained either necrotic tissue or had a lowered chlorophyll content. Both of these lines had a decreased seed productivity. The fourth mutant, line Sprint-2*Fix*$^-$-1 formed nodules but showed nitrogen deficiency symptoms even after inoculation and an increased reproductive ability when grown on mineral nitrogen. The nodules were white and smaller than those of the parent cultivar. They were situated mostly on the main root.

Physiological characteristics of symbiotic mutants

Two mutant lines and the parental cultivar were grown after inoculation with effective 1026 or ineffective 1064 *R. leguminosarum*. Three parameters were compared: nitrogenase activity of the nodules, total nitrogen content of the shoots and the dry shoot mass (Table 1). Nitrogenase activity in all *Fix*$^-$ variants was on the level of non-inoculated control while in the *Fix*$^-$ combination with strain 1026 — Sprint-2 + 1026 it reached about 26 mkmoles C_2H_4 per hr per plant.

The effective combination — Sprint-2 + 1026 was higher in both parameters than the other combinations which were ineffective either due to the strain or to plant mutations. This was especially evident in nitrogen content ($p=0.95$). The comparatively low prevalence of dry mass for Sprint-2 + 1026 over ineffective combinations may be explained by the fact that at the stage of early flowering high nitrogen fixation has not yet manifested itself in the growth of the shoot green mass. While non-fixing combinations perish soon after flowering the fixing ones continue to accumulate biomass.

Study of the strain specificity of Sprint-2*Nod*$^-$-2 and Sprint-2*Fix*$^-$ mutants revealed no supression of mutant phenotypes neither by any of the 13 strains of various geographic origin nor any of resident *Rhizobium* races from unsterile soil or even *A-1* strain.

Genetic analysis of symbiotic mutants

Results of the crossing of mutant Sprint-2*Fix*$^-$-1 with the parental line coincide with hypothesis of the recessive and monogenic determination of the mutant phenotype. The results are shown in Table 2.

Table 1. Dry shoot mass and total nitrogen content.

Line	Strain	Dry shoot mass g/plant	Nitrogen content mg/plant
Sprint-2	1026	0.327±0.019	9.64±1.07
Sprint-2	1064	0.216±0.018	3.80±0.91
Sprint-2*Fix*$^-$	1026	0.206±0.015	3.35±0.76
Sprint-2*Fix*$^-$	1064	0.261±0.015	4.59±0.71
Sprint-2*Nod*$^-$-1	1026	0.280±0.031	6.52±1.52
Sprint-2	Control	0.132±0.035	3.93±1.75
Sprint-2*Fix*$^-$	Control	0.199±0.035	3.45±1.45
Sprint-2*Nod*$^-$-1	Control	0.087±0.043	2.13±2.14

Table 2. Inheritance of symbiotic properties of mutant Sprint-2*Fix*$^-$.

Variant of crossing	Amount of plants						2 3:1
	F_1		F_2				
	Fix$^+$	*Fix*$^-$	*Fix*$^+$	*Fix*$^-$			
Sprint-2 × Sprint-2*Fix*$^-$	7	:	0	45	:	10	1.36
Sprint-2*Fix*$^-$ × Sprint-2	8	:	0	46	:	11	0.99
Total	15	:	0	91	:	21	2.33

Table 3. Functional tests for allelism. (F_1 phenotypes)

P1 / P2	Sprint-2 *Nod*$^-$-2	Sprint-2 *Fix*$^-$	Sparkle E2 (*sym5*)	Sparkle R25 (*sym8*)	Sparkle E135f (*sym13*)
Sprint-2 *Nod*$^-$-1	*Nod*$^-$	—	—	*Nod*$^-$	—
Sprint-2 *Nod*$^-$-2	—	—	*Nod*$^+$ *Fix*$^+$	*Nod*$^-$	—
Sprint-2 *Fix*$^-$	—	—	—	—	*Nod*$^+$ *Fix*$^+$
Sparkle E2 (*sym5*)	*Nod*$^+$ *Fix*$^+$	—	—	—	—
Sparkle R25 (*sym8*)	*Nod*$^-$	—	—	—	—
Sparkle E135f (*sym13*)	—	*Nod*$^+$ *Fix*$^+$	—	—	—

Tests for allelism with the mutants from Prof. T.A. LaRue (Table 3) showed that the *Nod*$^-$ mutations in Sprint-2*Nod*$^-$-1 and Sprint-2*Nod*$^-$-2 are allelic to the *Nod*$^-$ mutation in *sym8* locus (mutant R25), while mutation in Sprint-2*Fix*$^-$ turned out to be non-allelic to the *Fix*$^-$ mutation in the *sym13* locus (mutant E135f; Kneen et al., 1990).

Experimental distribution of the mutant phenotype does not differ significantly from the theoretical ratio (3:1).

Ultrastructural study of symbiotic mutants

Comparative ultrastructural analysis was carried out on pea nodules arising from the following combinations of macro- and microsymbionts (pea line + bacterial strain): L1714+1026, L1714+1064, Sprint-2+1026, Sprint-2+1064, Sprint-2*Fix*$^-$+1026, Sprint-2*Fix*$^-$+1064, Sparkle+1026, E135f+1026.

Combinations of effective symbiosis L1714+1026, Sprint-2+1026, Sparkle+1026 (Figs. 1a, 2a, 4a, 5a, 5c, 5e)

Nodules (3–5 up to 7–10 mm size) formed on the pea roots in these symbiotic pairs have a typical intensive pink colouring and similar ultrastructural organization. The nodule bacteroid zone is mostly filled with infective cells. In mature cells there is a large central vacuole that displaces the cytoplasm together with the nucleus towards the plasmalemma. The nucleus has a typical curved surface. The fragments of rough and smooth endoplasmic reticulum are distributed evenly in the cytoplasm, and mitochondria are nearer to the plasmalemma. Within the section of each infected cell there are several profiles of infection threads (ITh) containing electrondense bacteria. Numerous symbiosomes can be also visualized and the difference between bacteria in the ITh and mature differentiated bacteroids is clearly seen. Pleiomorphic Y-shaped bacteroids are 3–5 times larger than bacteria and have a lower electron density. In the bacteroids a non-condensed nucleoid and a well-developed ribosomal apparatus are seen. One nitrogen fixing unit (symbiosome) usually contains a single bacteroid surrounded with a symbiosome membrane (SM).

Ineffective combinations with strain 1064
L1714+1064 (Figs. 1b, 5b)

Early degradation of SM is a typical trait of this combination. The profiles of single bacteroids in the cytoplasm are surrounded with fragmentary SM or the latter is not seen at all. In this variant, numerous small (1.5–2.0 mm) white ineffective nodules are formed. Cells of the infected zone contain the nucleus shifted towards the cell wall, some small vacuoles and a smaller number of bacteroids in comparison with effective nodules. Pleiomorphic elongated or rounded bacteroids compared to bacteria in ITh have a medium electron density and a larger size (1.5–2.0 times). Bacteroids contain a non-condensed nucleoid and numerous ribosomes.

Sprint-2 + 1064 (Figs. 2b, 5d)

The main feature of this combination is the total degradation of SM. Bacteroid containing tissues of small (1–2 mm) white nodules bear typical signs of senescence. One can see degradation of all cytoplasmic structures of infected plant cells, on the profiles of which starch granules and ITh that have remained intact can be seen. Only a few bacteroids are lying freely among myeloid membrane structures, fragments of the degrading host-cell cytoplasm. They are about 1.5 times larger than bacteria in the ITh, contain granules of poly-β-hydroxybutyrate (PHB) and a condensed nucleoid.

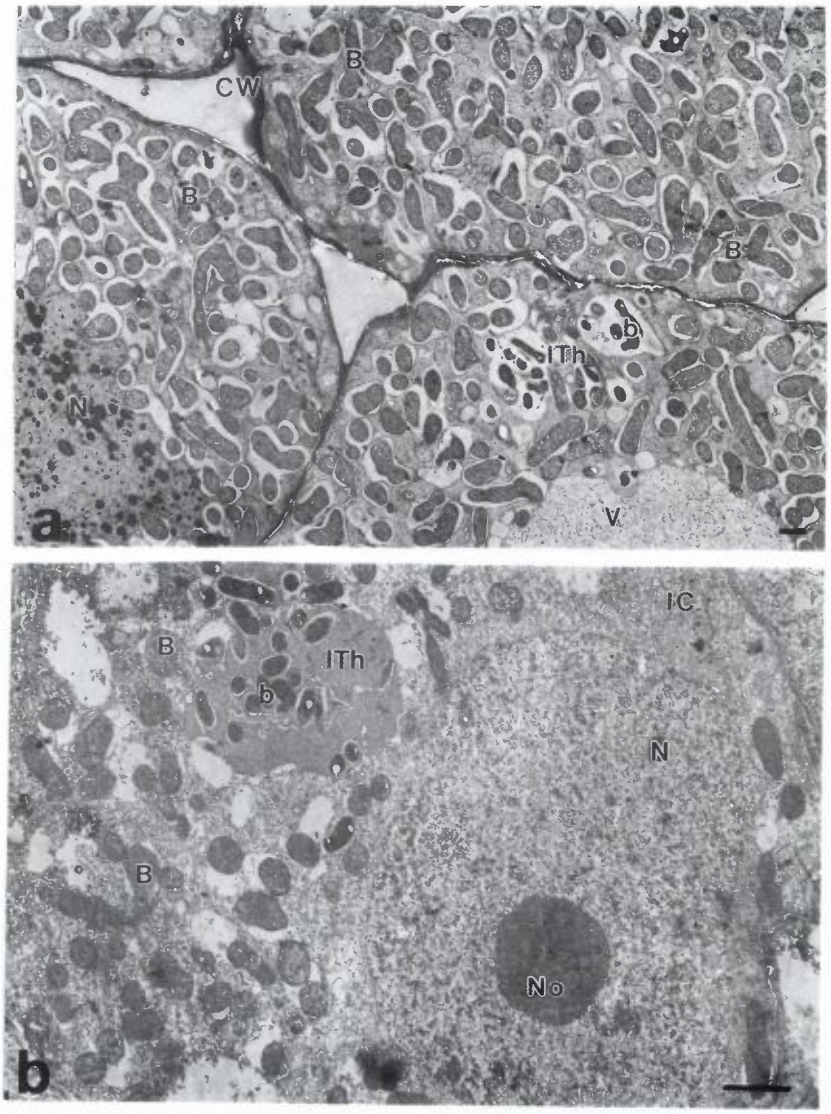

Figure 1. Ultrastructure of the nodule infected cells of the pea line L1714 (inoculation with strain 1026 (a) and 1064 (b). Bars represent 1 μm. N – nucleus, No – nucleolus, IC – infected cell, UC – uninfected cell, B – bacteroid, b – bacteria, SM – symbiosome membrane, PBS – peribacteroid space, CW – cell wall, v – vacuole, ITh – infection thread, st – starch.

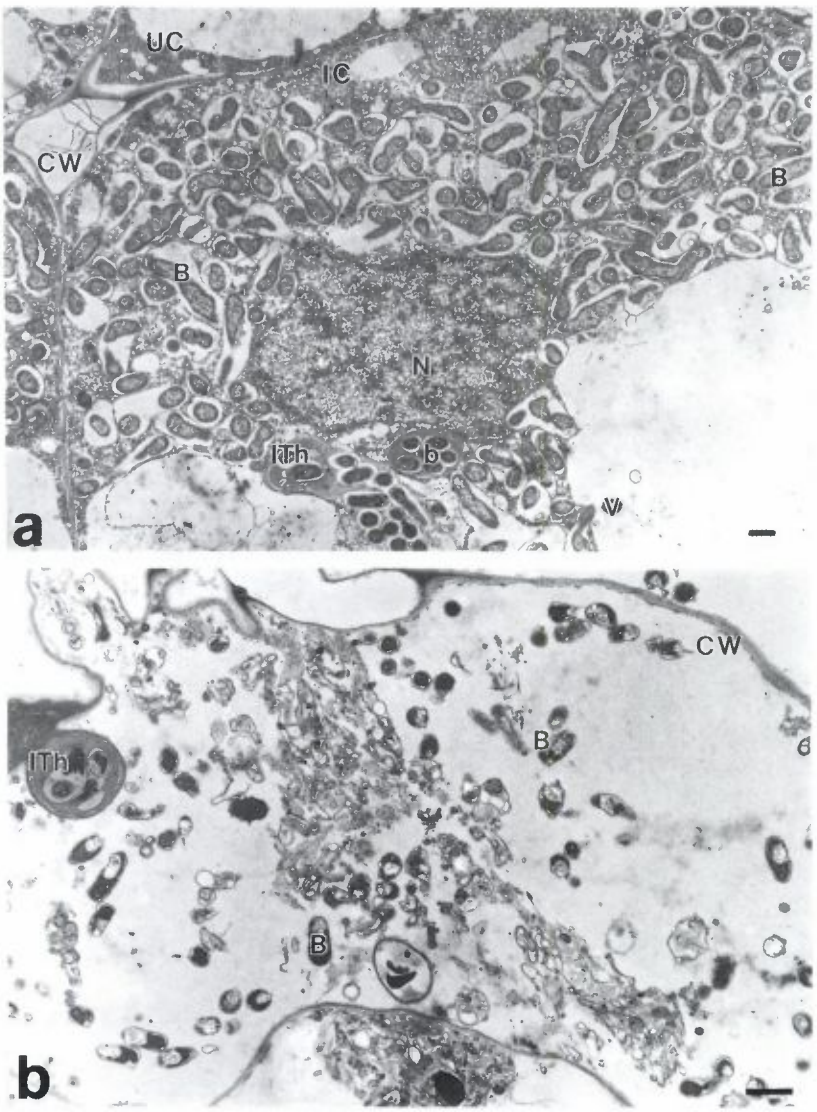

Figure 2. Ultrastructure of the nodule infected cells of the pea line Sprint-2 (inoculation with strain 1026 (a) and 1064 (b). Bars represent 1 μm.

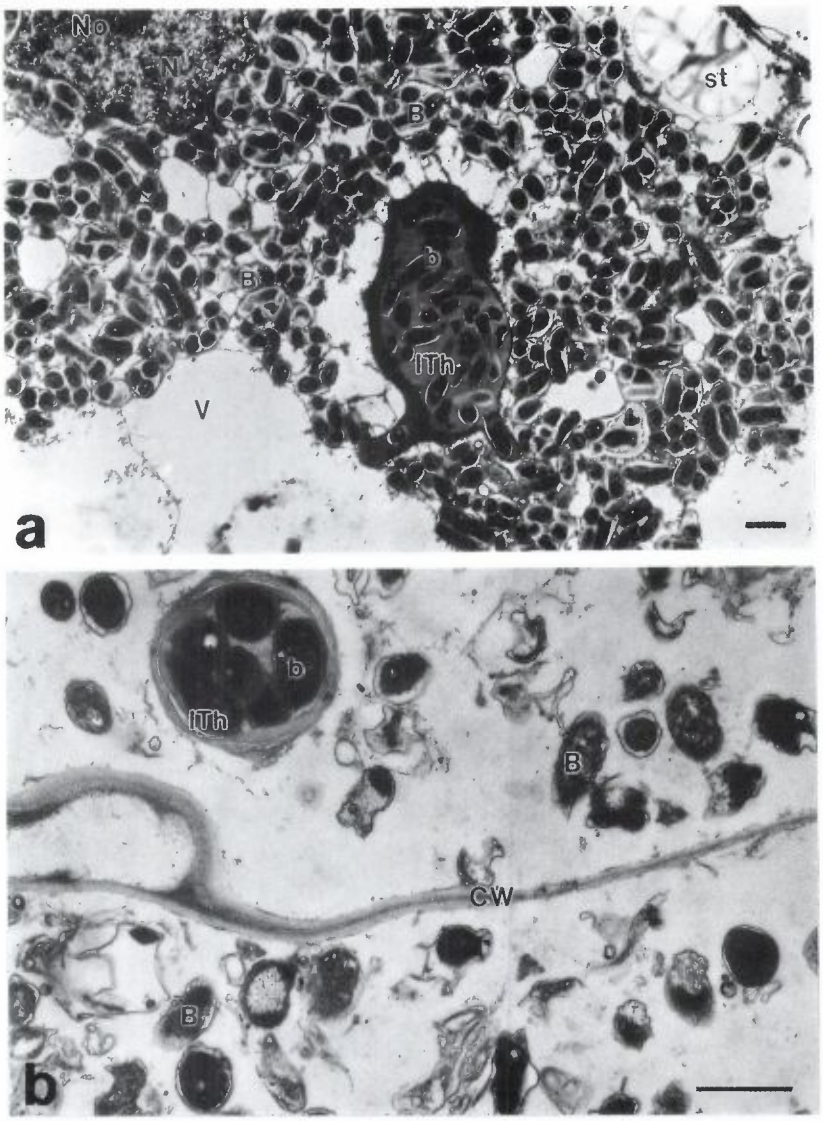

Figure 3. Ultrastructure of the nodule infected cells of the mutant pea line Sprint*Fix*$^-$ (inoculation with strain 1026 (a) and 1064 (b). Bars represent 1 μm.

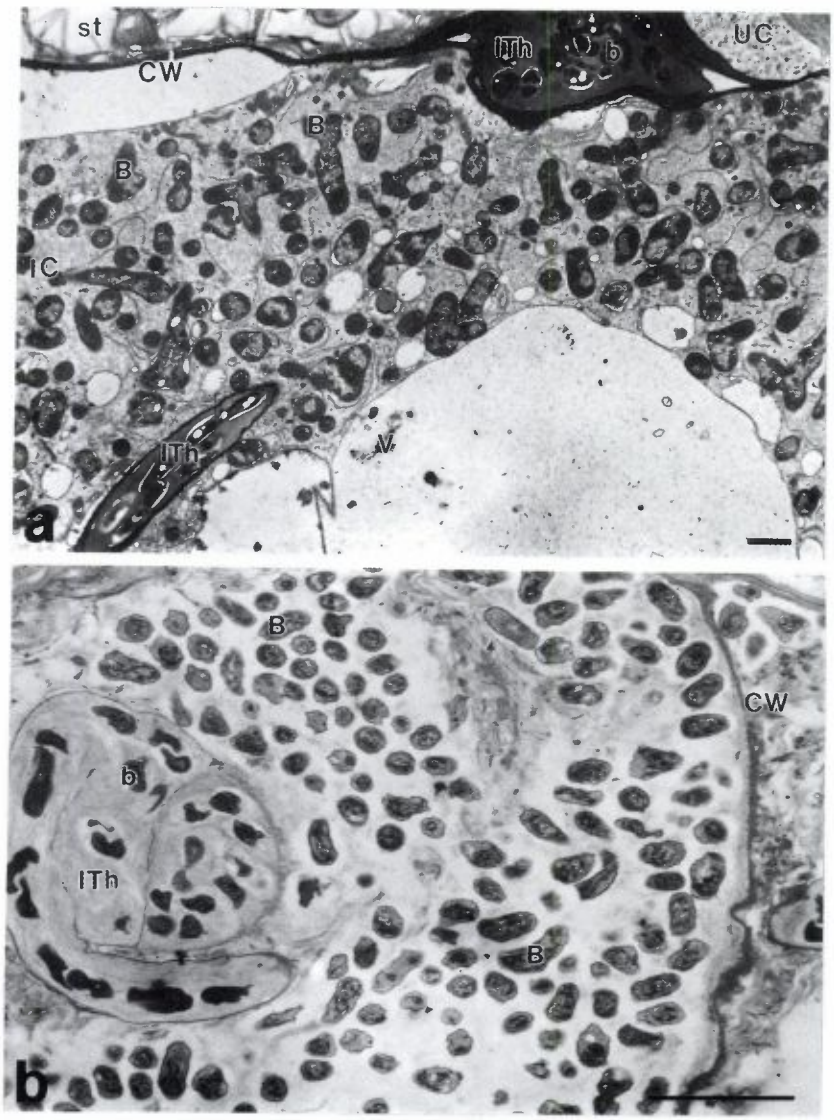

Figure 4. Ultrastructure of the nodule infected cells of the pea cultivar Sparkle (a) and mutant pea line E135f (b) (inoculation with strain 1026). Bars represent 1 μm.

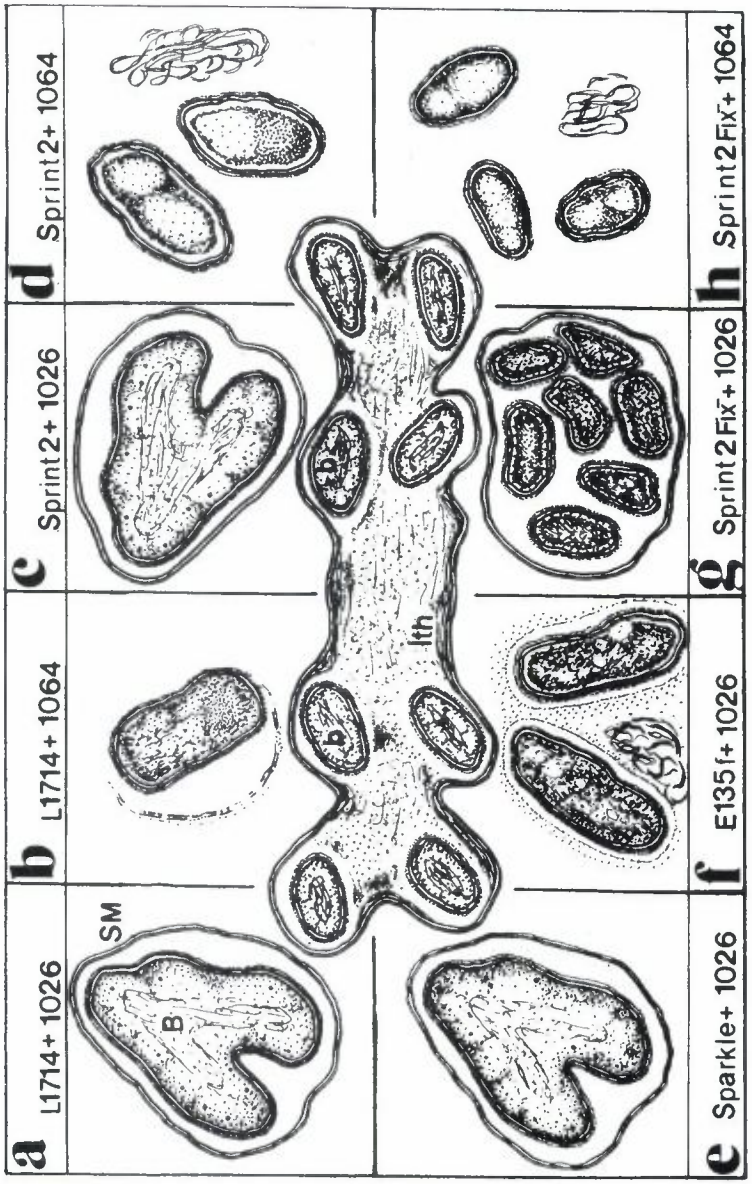

Figure 5. Symbiosome structure in the different combinations of micro- and macrosymbionts (Scheme). Bars represent 1 μm.

Ineffective combination with plant mutants

*Combination Sprint-2*Fix$^-$ *+ 1026 (Figs. 3a, 5g)*

The morphological peculiarity of this variant is the fact that bacteria in the ITh and bacteroids are indistinguishable from each other in form, in shape, and in electron density. In addition bacteroids are located in one SM not individually but in groups of 3–6 which is an abnormal structure for pea symbiosomes. Infected nodule cells contain an asymmetric vacuole, conglomerations of starch granules near the plasma membrane and non-structural electron transparent regions. Numerous abnormal symbiosomes and mitochondria are located in the cell cytoplasm. Bacteriods contain a condensed nucleoid and numerous ribosomes. The nodules formed in this variant are of medium size (3–5 mm) and white in colour.

Combination E135f + 1026 (Figs. 4b, 3f)

Morphologically the nodules (3–5 mm and white) and infected cells display traits of degradation and resemble those of ineffective variants Sprint-2 + 1064, L1714 + 1064. The few bacteroids are characterized by an elongated form, are larger (1.5 times) than the bacteria in the ITh and have a matrix of medium electron density. Bacteroids contain condensed nucleoids and ribosomes. Each bacteroid is surrounded with an electron transparent zone, corresponding presumably to remnants of the peribacteroid space. SM is not detected.

Ineffective variant with a plant mutant and strain 1064

*Combination Sprint-2*Fix$^-$ *+ 1064 (Figs. 3b, 5h)*

The structure of nodules and plant cells of the infected zone in this symbiosis combines morphological defects typical for both the plant line Sprint-2*Fix*$^-$ and strain 1064. Bacteria in the ITh and bacteroids are morphologically similar in form, size and electron density as shown for combination Sprint-2*Fix*$^-$ + 1026. In the sections one can also see clusters of bacteroids, comprising 10 cells and more, though SM is not detected the same as in variants of normal plants with ineffective strain (Sprint-2 + 1064, L1714 + 1064). Some bacterial cells contain PHB granules and the nucleoid in bacteroids is condensed. These data suggest that structural and functional organization of symbiosomes reflects the state of the symbiotic system as a whole.

4. Discussion

Based on the ultrastructural examinations we propose the following scheme reflecting the characteristic features of symbiosome structure in all symbiotic pairs studied (Fig. 5).

Both induced and natural symbiotically deficient mutants of pea FN-1 (Postma et al., 1990) and alfalfa (MnAg(In), MnSa(In), MnNc-3226(In) and MnNc-3811(In) (Vance and Johnson, 1983) studied until now and the pea mutant E135f (Keen et al., 1990) that we have studied in this work have common traits typical for ineffective symbiosis:

1. Early senescence of bacteroids,
2. Incomplete differentiation of bacteroids,
3. Abnormal or degrading symbiosome membrane (SM).

It is evident from this scheme that our plant mutant Sprint-2*Fix*$^-$ (Figs. 3a, 5g) has a set of unique traits. First of all the senescence time of bacteroids, symbiosomes and nodules is delayed or does not differ from that of the wild type. The second important trait is that there is almost no visible bacteroid differentiation. The third is that one symbiosome contains not one but several bacterial cells and that is not typical for pea symbiosomes (Robertson et al., 1978; Sutton, 1983). Formation of such abnormal symbiosomes is unlikely to be the result of the bacteroids' aggregating following the coalescence of normal symbiosome units (Fischer et al., 1986; Vance and Johnson, 1983). Perhaps the initial bacterial cell enclosed in SM continues to divide and as a result of a disturbance in synchrony, the synthesis of SM is not followed by partition into separate units. Alternatively several bacterial cells could be released together from the infection thread into cytoplasm and thus be enclosed within a single SM envelope as a result of the mutation. Thus in the cytoplasm of infected cells abnormal symbiosomes with a large amount of bacteroids would be formed.

Therefore our mutant can be defined phenotypically as being incapable of transforming bacteria into bacteroids. This fact allows us to postulate the existence of several specific signals from the plant that regulate the genetic system of bacteria during their differentiation into bacteroids. This period is critical for the development of the functional symbiotic system because the bacterial genes which are usually silent in free living state are induced resulting in the biosynthesis of an active nitrogenase complex. It is interesting to mention that structural nitrogenase proteins NifH, NifD, NifK were not visualized in the polyacrylamide gel of Sprint-2*Fix*$^-$ bacteroids (data not shown). Similar data were obtained in the study of another *Fix*$^-$ pea mutant (Postma et al.,

1987). It would be interesting in this connection to test the rate of expression of the main symbiotic promoters in the bacteroids of Sprint-2*Fix*$^-$.

We assume that the disturbance in the symbiotic relationship between Spring-2*Fix*$^-$ and the effective strain 1026 occurs at the stage when the bacteria come out from the infection thread. The different roles of the macro- and microsymbiont in forming the key structures of the symbiotic system are clearly seen from the study of mutants of both bacterial and plant origin. Comparison of the ultrastructural organization of symbiotic combinations L1714 + 1064 (Figs. 1b, 5b), Sprint-2 + 1064 (Figs. 2b, 5d) and literature data (Fischer et al., 1986; Spaink, 1989) suggests that early degradation of SM is a common phenotype for *Fix*$^-$ bacterial strains. Study of the nodule infected zone structure in the combination E135f + 1026 (Figs. 4b, 5f) revealed a similar degradation of SM, demonstrating the influence of the host-plant on the transformation of bacteria into symbiotically functional bacteroids. However, the combination Sprint-2*Fix*$^-$ + 1064 (Figs. 3b, 5b) shows that the role of the microsymbiont is also important, manifesting itself through the building up of symbiosome compartments. This double-mutant combination shows early degradation of bacteroids and the symbiosome membrane, thus pointing to the cross-control of this process.

Surveying the data of genetic analysis (Table 3) put together with the comparative ultrastructural description of *Fix*$^-$ mutant FN1 (Postma et al., 1990; Locus *sym20*; LaRue, personal communication) and that of Sprint-2*Fix*$^-$ we draw the conclusion that mutation in the line Sprint-2*Fix*$^-$ is Mendelian and recessive and not allelic to mutations in line E135f (Locus *sym13*; Kneen et al., 1990) and line FN1 (*sym20*). Therefore a new *sym*-locus of pea (*Pisum sativum* L.) has been described. Further work in obtaining and studying of symbiotic mutants of both micro- and macrosymbiont will allow further characterization of the process of symbiosome formation and functioning.

Acknowledgements

This work was financed by the Interbioazot Research Program (International Biological Nitrogen Research Program), and the Program of First Priority Directions of Genetics. We are grateful to Drs. S.M. Rosov, V.A. Berdnikov, and F.L. Gorel of the Institute of Cytology and Genetics, Siberia, for their help in dealing with plant material.

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