ELSEVIER

Plant Science 155 (2000) 75-83

PLANT SCIENCE

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Four developmental stages identified by genetic dissection of pea (*Pisum sativum* L.) root nodule morphogenesis

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Received 30 September 1999; received in revised form 31 December 1999; accepted 19 January 2000

Abstract

Root nodule structural organisation of nine pea Fix⁻ mutants representing seven symbiotic loci has been studied. This set of mutants has revealed lesions at four developmental stages in the pea-*Rhizobium* symbiosis. (i) Mutant RisFixA is affected in infection thread differentiation in nodule tissue, infection droplet formation, bacteroid differentiation and nodule persistence. (ii) Allelic mutants RisFixL and RisFixO (*sym*32) are blocked in bacteroid differentiation. (iii) Mutants RisFixM (*sym*26), RisFixN, RisFixQ (*sym*27), RisFixT (*sym*26) show premature degradation of symbiotic structures. (iv) Mutant RisFixV shows similar defects in nodule persistence but, in addition, this mutant is characterised by an abnormal and severe increase in the thickness of infection thread walls during the process of infection thread 'maturation' and senescence. Combining our new data with previously published studies of pea mutant phenotypes has allowed us to create an integrated scheme for the sequential functioning of the late pea symbiotic genes identified and characterised to date.

Keywords: Plant-microbe interactions; Legume--*Rhizobium* symbiosis; *Pisum sativum* L.; Pea mutants; Symbiotic genes; Nodule ultrastructure; Sequential functioning of genes

1. Introduction

Legume -*Rhizobium* symbiosis is a complex process requiring the expression of numerous symbiotic genes of both partners in an integrated pattern of development. In order to analyse this process it is necessary to use the process of genetic dissection to identify discrete developmental stages, which are under the control of one or a small group of genes. For this purpose, numerous collections of symbiotically defective mutants have been created in different legume species [1], but the collection of pea (*Pisum sativum* L.) symbiotic mutants is currently the most extensive available. It consists of more than 200 independently obtained mutants that were isolated using seven different genotypes [2-8], (Due, Sagan, personal communication, Tsyganov et al., unpublished results). Many of these mutants have been used for complementation analysis and, as a result, more than 40 symbiotic loci have been identified in pea [9,10] (Due, Sagan, personal communication).

Comparative morphological analysis of a series of symbiotic mutants has revealed specific stages of symbiotic development that are blocked in different pea mutants [6,10-19]. As a result, eight discrete sequential stages of nodule development have been revealed. To classify those stages, a system of phenotypic codes has been developed [10,20,21]: Hac, root hair curling; Iti, infection thread growth initiation; Ith, infection thread differentiation inside root hair cells; Itr, infection thread differentiation inside root cortical cells; Itn, infection thread differentiation inside nodule tissues; Idd, infection droplet differentiation; Bad, bacteroid differentiation; Nop, nodule persistence.

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0168-9452/00/$ - see front matter
PII: S0168-9452(00)00207-7

Four stages (from Hac to Itr) are associated with early steps of symbiosis development. Mutants blocked at these stages have Nod$^-$ or Nod$^{+/-}$ phenotype. Mutants blocked at the following four stages (from Itn to Nop) have Fix$^-$ phenotype.

This study was aimed at a morphological analysis of a series of uncharacterised Fix$^-$ mutants obtained in cv. Finale [3]. The observed phenotypes were then integrated into a more general survey of how the sequence of nodule developmental stages is blocked by specific mutations.

2. Material and methods

2.1. Plant material

Fix$^-$ mutants RisFixA, RisFixK, RisFixL, RisFixO, RisFixM, RisFixN, RisFixQ, RisFixT and RisFixV [3] and initial cv. Finale used in this study were kindly provided by K.J. Engvild, Agricultural Research Department, Riso National Laboratory, Roskild, Denmark. These mutants represent seven different symbiotic genes: RisFixL, RisFixO-*sym32*; RisFixM, RisFixT-*sym26*; RisFixQ-*sym27* (Due, Sagan, personal communication); whereas mutants RisFixA, RisFixK, RisFixN and RisFixV, which were nonallelic to each other nor to any of the mutants above, represent four additional symbiotic genes, but these have no assigned gene symbols yet (Tsyganov et al., unpublished results).

2.2. Plant growth conditions and collection of the material for analysis

Plants were grown in growth cabinets Heraeus V6tch HPS2000 (day/night 16/8 h, temperature 21/19°C, relative humidity of 75%, photon irradiance of 490 μE/m^2 per s). Nutrient solutions and methods of seed inoculation were described previously [22]. The commercial effective strain CIAM 1026 of *Rhizobium leguminosarum* bv. *viciae* [23] was used as the inoculant. For microscopy the nodules were collected from the roots when plants were at the stage of early flowering (approximately 28 days after inoculation coinciding with planting). At this stage wild type nodules were mature enough and had no signs of degradation. The nodules were collected from certain zone of the root below the cotyledons to have nodules of approximately the same age. About five nodules from each of five plants of each genotype were collected in three independent experiments.

2.3. Microscopy

The techniques used for microscopy have been described previously [10].

3. Results

3.1. Cv. Finale

The nodules of cv. Finale demonstrated the histological zonation and ultrastructural organisation (Fig. IA-C) characteristic for typical wild-type pea symbiotic root nodules [10,14,19,22,25]. In zone III [26], each mature symbiosome consisted of one differentiated pleomorphic Y- or X-shaped bacteroid surrounded by symbiosome membrane (Fig. 113 and C).

3.2. *Mutant RisFixA*

Nodules of mutant RisFixA were characterised by alterations of histological differentiation (Fig. 2A). Zones I, II, were abnormally enlarged. There was no distinct interzone II-III. Nodules contained hypertrophied infection threads that frequently occupied the most part of a profile of the host cells in zone II (Fig. 2B). Endocytosis of bacteria was `delayed' (as manifested by an extended zone II) but was otherwise apparently normal, i.e. one by one (Fig. 2C). After endocytosis, the bacterial matrix was less electron-opaque than that of bacteria in infection threads, but these 'juvenile' bacteroids did not enlarge in size (Fig. 2C). Symbiosomes containing several bacteroids were frequently observed in infected cells in the central part of the nodule (Fig. 2D). Nodules of this mutant were characterised by 'early senescence', as manifested by the premature appearance of zone IV with degrading symbiotic structures compared to the nodules of the initial line Finale (Fig. 2E).

3.3. Mutants RisFixL and RisFixO

Nodule histological zonation of both mutants was found to be abnormal and similar to each other (Fig. 3A). There was no distinct interzone

II-III and a true zone III was lacking. Infected cells were significantly vacuolised (Fig. 3A), which is not characteristic for effective pea nodules [6]. Infection threads appeared normal and were indistinguishable from those in wild-type nodules (Fig. 3B and C). Bacteria were endocytosed one by one and 'juvenile' symbiosomes generally contained a single bacteroid (Fig. 3B). Symbiosomes containing several bacteroids were mainly observed in infected cells in the central part of the nodules (Fig. 3C). Degradation of the infected cells (zone IV) began with disintegration of symbiosome membranes (Fig. 3D).

3.4. Mutants RisFixK, RisFixM (sym26) RisFixN, RisFixQ (syn127) and RisFixT (sym126)

Nodule histological differentiation of these mutants was similar to that of wild-type nodules [10,14,19,22,24,25], except for zone IV that was abnormally enlarged (Fig. 4A). Bacteroids demonstrated morphologically pronounced differentiation (Fig. 4B and C), however symbiosomes could only be found in narrow zone corresponding to zone III. In zone IV symbiosomes were transformed into the lysosome-like compartments containing the 'ghosts' of bacteroids (Fig. 4D).

3.5. Mutant RisFix V

All morphological traits characteristic for the previous group of mutants were found in the nodules of this mutant (Fig. 5A and B). A distinguishing feature of this mutant, however, was the process of infection thread 'maturation' and senescence: there were changes in the structure and

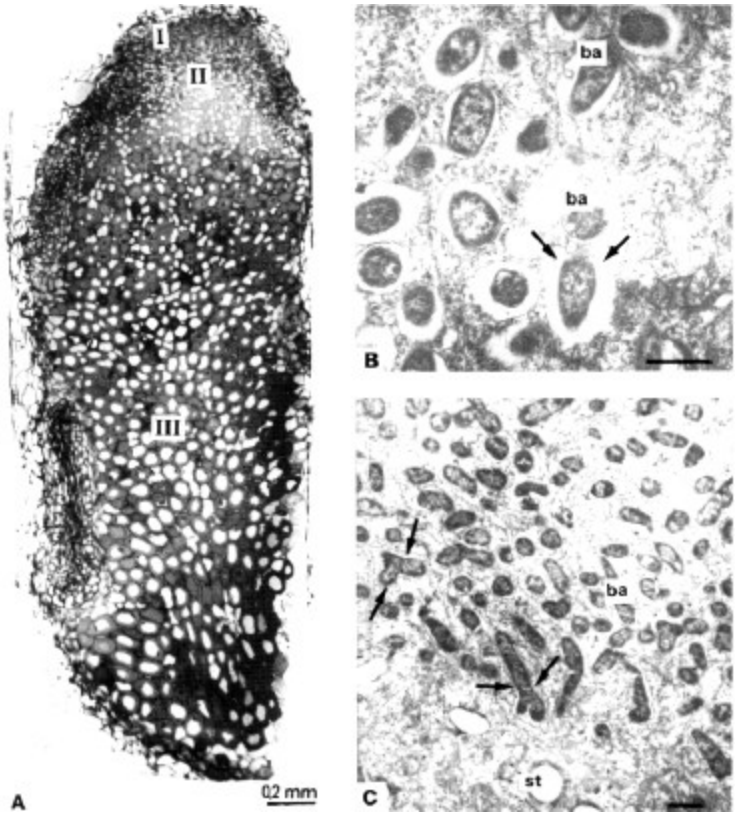

Fig. 1. Nodule structural organisation of cv. Finale: (A) longitudinal nodule section; (B) plant cells in zone 11 containing juvenile bacteroids; (C) plant cells in zone III containing differentiated bacteroids. I, II, III nodule histological zones; ba, bacteroids; st, starch granules; arrows, peribacteroid membranes. Bars in electron micrographs, 1 *m*m.

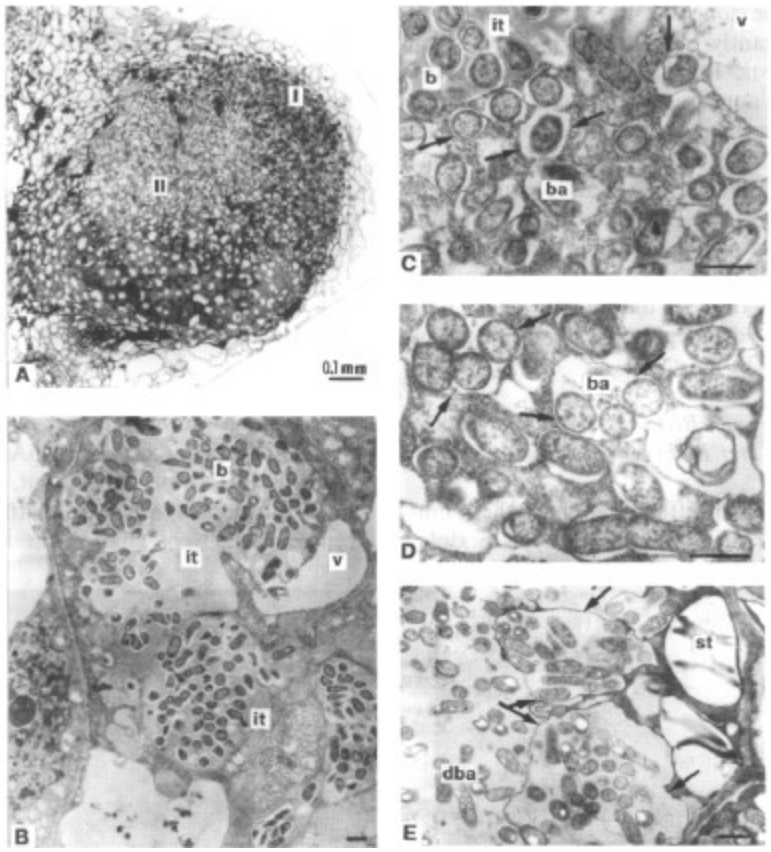

Fig. 2. Nodule structural organisation of mutant RisFixA: (A) longitudinal nodule section; (B) plant cells containing infection threads in histological zone 11; (C) bacteria endocytosed from infection thread: (D) infected cells of the central part of the nodule containing symbiosomes with several bacteroids; (E) lysosome-like compartments containing degrading bacteroids in nodule histological zone IV. 1, 11 nodule histological zones; it, infection thread; b, bacteria; ba, bacteroids; v, vacuole; st, starch granules; dba, degrading bacteroids; arrows, peribacteroid membranes and membranes of lysosome-like compartments. Bars in electron micrographs. 1 *m*m.

thicknesses of plant cell walls surrounding infection threads. Initially thickening of the infection thread wall could be observed in zone III (Fig. 5C). In zone IV, this process apparently continued and, in addition, the bacteria inside infection thread showed signs of degradation (Fig. 5D).

4. Discussion

Morphological analysis of nodules from nine Fix$^-$ mutants has shown that they are blocked at four different stages of nodule morphogenesis, as discussed below.

4.1. Mutant RisFixA

The mutant gene identified in line RisFixA apparently controls several stages of nodule development: Itn, Idd, Bad and Nop (for description, see introduction). A similar mutant phenotype was described for mutant SGEFix$^-$-1 (*sym40*) [10]. The only ultrastructural difference is that in nodules of RisFixA the bacteroids were of the same size and shape as bacteria inside infection threads whereas, in nodules of mutant SGEFix$^-$-1 (*sym40*), enlarged pleomorphic bacteroids could be observed and sometimes symbiosomes containing several bacteroids were found [10]. There were

also differences in the number of root nodules formed by these two mutants: mutant SGEFix$^-$-1 (*sym40*) showed an increased number of nodules (relative to wild type), which is a common host plant reaction to the formation of Fix$^-$ nodules [10]. However, in mutant RisFixA, a decreased number of nodules was described [27]. In addition to differences in mutant phenotypes, it was also shown that these mutants are not allelic to each other (Tsyganov et al., unpublished results). All the data represented above suggest that gene *sym40* and the symbiotic gene identified in line RisFixA control very close (if not the same) nodule developmental stages.

4.2. *Mutants RisFixL and RisFixO*

In nodule infected cells of the allelic mutants RisFixL and RisFixO, the only sign of bacteroid differentiation is a decreased electron density of the matrix. Bacteroids remain rod-shaped with no increase in size. As a rule, several bacteroids are surrounded by peribacteroid membrane. The phenotype of these allelic mutants can be described as Bad$^-$. Such an ultrastructural organisation of bacteroids is very similar to that of mutant line Sprint-2Fix$^-$ (*syn13l*) [6,22]. These data confirm the results, which were obtained by Novak et al. [19] except that, in the present study, hypertro-

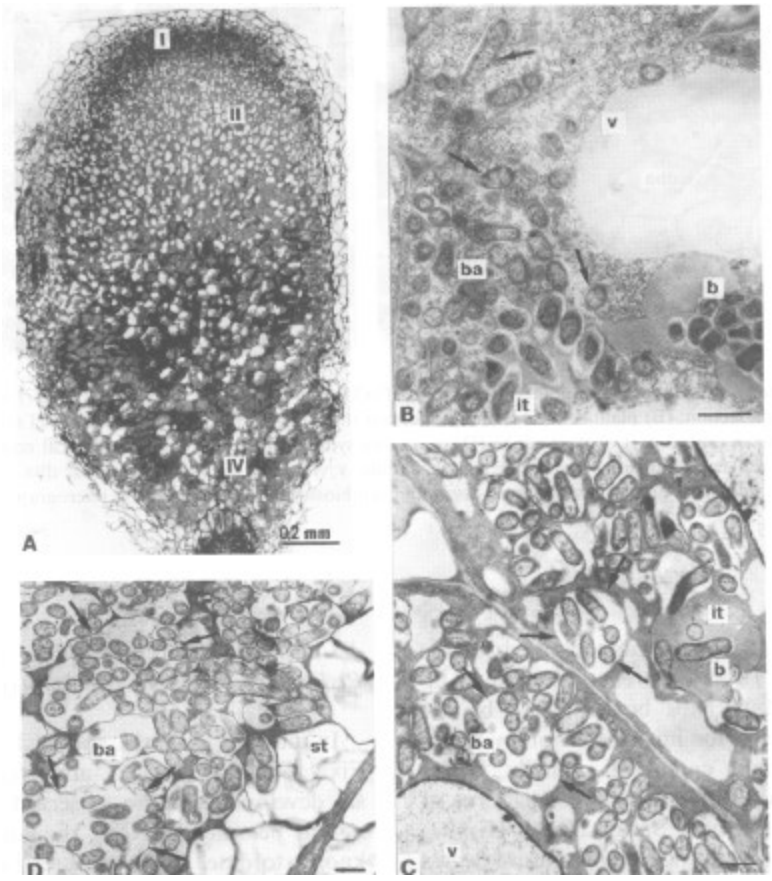

Fig. 3. Nodule structural organisation of mutants RisFixL and RisFixO: (A) longitudinal nodule section; (B) plant cells containing infection threads and 'juvenile' bacteroids; (C) Symbiosomes containing several bacteroids in central part of the nodule; (D) degrading symbiosomes in nodule histological zone IV. I, 11, IV nodule histological zones; it, infection thread; b, bacteria; ba, bacteroids: v, vacuole; st, starch granules; dba, degrading bacteroids; arrows, peribacteroid membranes and membranes of degrading symbiosomes. Bars in electron micrographs, 1 *m*m.

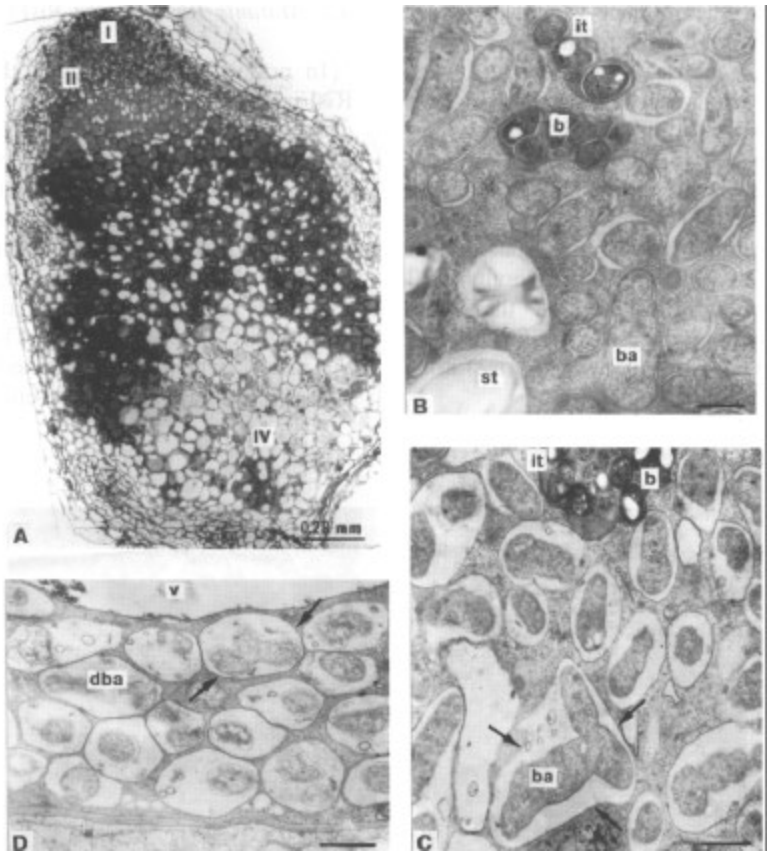

Fig. 4. Nodule structural organisation of mutants RisFixK, RisFixM (*sym26*) RisFixN, RisFixQ (*sym27*) and RisFixT (*sym26*): (A) longitudinal nodule section; (B) plant cells containing infection threads and 'juvenile' bacteroids; (C) symbiosome containing differentiated bacteroid in central part of the nodule; (D) degrading symbiosomes in nodule histological zone IV. I, II, IV nodule histological zones; it, infection thread; b, bacteria; ba, bacteroids; v, vacuole; st, starch granules; dba, degrading bacteroids; arrows, peribacteroid membranes and membranes of degrading symbiosomes. Bars in electron micrographs, 1 *m*m.

phied infection threads were not observed for the mutant RisFixO. These differences in nodule ultrastructure could perhaps be explained by the different strains used for inoculation or by variations in plant growth conditions. Finally, taking into account certain differences in the patterns of senescence between line Sprint-2Fix$^-$ (*sym31*) (which shows no signs of nodule premature degradation) and allelic mutants RisFixL (*syin32*) and RisFixO (*sym32*) (which have an increased histological zone IV) one can conclude that pea symbiotic genes *sym31* and *sym32* control very close (if not the same) stages of nodule development.

4. 3. Mutants RisFixK, RisFixM (syin26) RisFixN, RisFivQ (syn127) and RisRYT (sym26)

In mutants RisFixM and RisFixT (*sym26*), RisFixN, RisFixQ (*sym27*), abnormalities of symbiosis development were observed at the stage of nodule persistence (Nop), the latest stage of those known to date. Infection thread development, endocytosis of bacteria and morphologically pronounced bacteroid differentiation in infected cells were identical to those of wild-type nodules. However, nodule development was interrupted by premature degradation of symbiotic structures before

nodules could begin to function properly. This mutant phenotype has been described previously in pea and other legumes [13,14,19,28,29]. Because the Nop⁻ phenotype can be found quite frequently among legume symbiotic mutants, it suggests that many legume genes are involved in stabilisation of the nitrogen-fixing symbiosis and the avoidance of premature senescence.

4.4. Mutant RisFix V

Although the mutant phenotype of RisFixV was very similar to the group of 'early senescent' mutants discussed above, the difference in infection thread development made it possible to place this mutant in a different class (phenotype Itn⁻, Nop⁻). Abnormal development of infection threads in this mutant was described earlier by Novak and colleagues [19]. In this study it was discovered that cell walls around infection threads became much thicker during the process of infection thread 'maturation' and senescence. Further comparative study of mutant RisFixV will help to reveal the sequential functioning of the genes controlling the stages associated with Nop.

5. Conclusions

Combination of the results of the present study for mutants obtained in ev. Finale [3] with the data on morphological analysis of pea Fix⁻ mutants published previously [6,10,13,14,19,22] has made it possible to arrange all the pea Fix⁻ mutants in order, according to the hypothetical sequence in which these genes function in the late

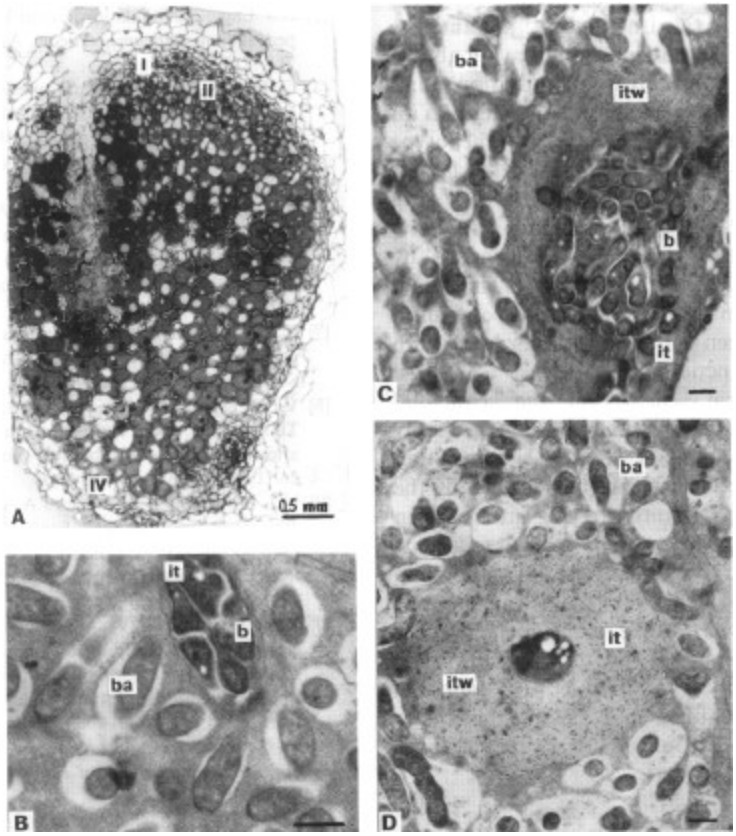

Fig. 5. Nodule structural organisation of mutant RisFixV: (A) longitudinal nodule section: (B) plant cells containing infection threads and 'juvenile' bacteroids; structure of infection thread with increased walls of surrounding plant cells in zone **II** (C) and IV (D). I, II, IV nodule histological zones: it. infection thread; b, bacteria: ba, bacteroids: v, vacuole: st, starch granules; dba, degrading bacteroids; itw, walls of the cells surrounding infection thread. Bars in electron micrographs, 1 *m*m.

stages of symbioitic development. Where a mutation was found to induce developmental abnormalities at several stages, we have applied the principle of the 'first morphological abnormality observed' in order to define the position of each mutant in this scheme. According to this principle, the earliest nodule developmental stage affected by the mutation represents the first point at which the expression of the gene function is required.

The predicted order of sequential gene functioning for late nodule development is as follows. Phenotype (i) Itn$^-$, Bar$^-$: the first position in the sequence is occupied by the mutant SGEFix$^-$-2 (*sym33*) [10]. Phenotype (ii) Itn$^-$, Idd$^-$, Bad$^-$, Nop$^-$: mutants SGEFix$^-$-1 (*sym40*) [10] and RisFixA are located in the second position. Phenotype (iii) Bad$^-$: mutants Sprint-2Fix$^-$ (*sym31*) [22], RisFixL (*sym32*) and RisFixO (*sym32*), with no differentiation of bacteroids occupy the third position. Phenotype (iv) Itn-, Nop-: mutant RisFixV is located in the next position, having abnormalities in infection thread 'maturation' and senescence along with premature degradation of symbiotic structures in infected cells. Phenotype (v) Nop$^-$: this position is occupied by the largest group of mutants (FN1 [13], E135f (*sym13*) [14], RisFixK, RisFixM (*sym26*) RisFixN, RisFixQ (*sym27*) and RisFixT (*sym26*), characterised by premature degradation of the nodules. Thus, as a result of the present study, seven more pea symbiotic genes have been classified in accordance with their sequential functioning.

This hypothetical scheme for the sequential functioning of pea symbiotic genes is based only on morphological analysis. Further confirmation of the order of the genes could be achieved by the creation of double mutants carrying mutations in genes with adjacent positions on the scheme derived from phenotypic analysis. This approach has already proved to be very fruitful in the case of Sym*13* and Sym*31* where the sequential functioning of these genes was recently confirmed [22]. Similarly, the analysis of double Fix$^-$ mutants created for all identified and classified pea late symbiotic genes will confirm the sequential functioning identified to date.

Acknowledgement

For VET, AYB and IAT this work was supported by the Russian Foundation for Basic Research (98-04-49883), Volkswagen-Stiftung, Germany (I/72 935), NATO Scientific and Environmental Affairs Division (HTECH.LG 971210). The authors are very grateful to N.J. Brewin for fruitful discussion and critical reading the manuscript and to L.E. Dvoryaninova and V.M. Semyonov for their excellent technical assistance.

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