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Immunocytological evidence for abnormal symbiosome development in nodules of the pea mutant line Sprint2Fix$^-$ (*sym*31)

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Summary. Using a series of antibody probes as markers of symbiosome development, we have investigated the impaired development of symbiosomes in nodules formed by the plant mutant line Sprint2Fix$^-$ (*sym*31). In wild-type pea (*Pisum sativum* L.) nodules, bacteria differentiate into large pleiomorphic, nitrogen-fixing bacteroids and are singly enclosed within a peribacteroid membrane. In the *sym*31 mutant, several small undifferentiated bacteroids were often enclosed within one peribacteroid membrane, or were found within a vacuole-like compartment. In wild-type nodules, the monoclonal antibody JIM18, which recognizes a plasmalemma glycolipid antigen, bound to the juvenile peribacteroid membrane, and did not recognize the mature peribacteroid membrane. However, in the mutant, the antibody bound to all peribacteroid membranes within the nodule, suggesting that differentiation of the peribacteroid membrane was arrested. Another antibody, MAC266, recognized plant glycoproteins which normally accumulate in symbiosomes at a late stage of nodule development. Binding of this antibody was much reduced within mutant nodules, labelling only a few mature cells. Similarly, MAC301, which normally recognizes a lipopolysaccharide epitope expressed on differentiated bacteroids prior to the induction of nitrogenase, failed to react with rhizobial cell extracts isolated from nodules of the *sym*31 mutant. On the basis of these developmental markers, the symbiosomes of *sym*31 nodules appeared to be blocked at an early stage of development. The distribution of infection structures was also found to be abnormal in the mutant nodules. Models of symbiosome development are presented and discussed in relation to the morphological and developmental lesions observed in the *sym*31 mutant.

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Introduction

The soil microbe *Rhizobium* can interact with roots of legumes to form a new plant organ, the symbiotic root nodule (Mylona et al. 1995). Formation of the root nodule is accompanied by the expression of nodule-specific genes and the differentiation of specialized cell types. In indeterminate nodules, such as those formed on pea (*Pisum sativum* L.) roots, nodule tissue is derived from a persistent apical meristem. Immediately beneath the meristem, in the tissues of the invasion zone, infection threads grow and ramify through the cells, and bacteria are released into the host-plant cytoplasm from an unwalled infection droplet. Subsequent progression of nodule development is largely characterized by the generation and differentiation of the symbiosome compartment, a specialised component of the endomembrane system within which rhizobia develop the capacity for biological nitrogen fixation (Brewin 1991).

The symbiosome of indeterminate nodules is formed when bacteria are released individually into the host-plant cytoplasm by an endocytosis-like process (Basset et al. 1977). Bacteria (now termed bacteroids) remain enclosed in a plasmalemma-derived membrane termed the peribacteroid membrane (pbm): bacteroids and the plant-derived pbm are the primary components of the symbiosome. In pea nodules, the newly formed symbiosome continues to divide within

the plant cytoplasm. Subsequently, the bacteroids stop dividing and begin to develop the capacity for biological nitrogen fixation. Each differentiated bacteroid is singly enclosed by the highly specialized peribacteroid membrane (Brewin et al. 1996).

In determinate nodules, such as those formed on beans (*Phaseolus vulgaris*), the development and morphology of root nodules is different from that of indeterminate nodules. In determinate nodules, the meristem is only transiently active and the meristematic cells are invaded by infection threads which release rhizobia into the host-cell cytoplasm at an early stage (Rae et al. 1992). The infected cells continue to divide forming the central tissue mass of the nodule. At the same time, the intracellular symbiosomes also divide until they occupy most of the cytoplasmic volume. Ultimately, the host cells of determinate nodules are characterized by symbiosomes containing multiple bacteroids (Brewin 1991, Mylona et al. 1995).

Because nodule development is mediated by signals from both the plant and the microbe, the study of symbiotically defective mutants of either symbiont can help to elucidate important developmental pathways (Schultze et al. 1994). In pea, a wide range of symbiotically defective mutants has been described (Brewin et al. 1993). These have been classified as nodulation-deficient (Nod^-) (Kneen and LaRue 1986, Kneen et al. 1990, Jacobsen 1984, LaRue and Weeden 1992, Sidorova and Uzhintseva 1992), fixation-defective (Fix^-) (Weeden et al. 1990, Holl 1975, Kneen et al. 1990a, Duc and Messager 1989), supernodulating or hyponodulating (Kneen et al. 1990b, Gelin and Blixt 1964, LaRue and Weeden 1992, Sidorova and Uzhintseva 1992). As an example of a fixation-deficient mutant, the *sym*1 nodulation mutant of *Vicia faba* appears to be aberrant in the stage of bacterial release from infection threads or in bacteroid development (Häser et al. 1992). However, the cytological study of plant mutants with clear blocks in nodule development is still very limited (Novák et al. 1995, Häser et al. 1992, Sagan et al. 1993). It was shown by ultrastructural analysis that a mutation in gene *Sym*13 (line E135F) determines the premature degradation of almost completely differentiated bacteroids (Kneen et al. 1990a), while a mutation in *Sym*31 (line Sprint2Fix^-) determines abnormal symbiosome structure and reduced morphological differentiation of bacteroids (Borisov et al. 1992).

The *sym*31 mutant was originally obtained by EMS mutagenesis of the Sprint2 pea line and a stable mutant pea line (Sprint2Fix^-) was thus established (Borisov et al. 1992). Genetic characterization established that the phenotype could be ascribed to a single recessive mutation and the *Sym*31 allele has been assigned (G. Duc and M. Sagan pers. comm.). Furthermore, the construction of a double-mutant line indicated that *Sym*31 apparently functions earlier than *Sym*13 in symbiosome development (Borisov et al. 1997). In a recent physiological study of nodules from the *sym*31 mutant pea line (Romanov et al. 1995), it was shown that the bacteroids were still active in respiration and that the central tissues of the nodule still occupied a low oxygen environment. However, these nodules contained extremely low levels of hemoglobin and glutamine synthetase and reduced levels of sucrose synthetase. Alterations in carbon metabolism were suggested by the observation that nodules of the mutant line contained 15 times less ononitol than wild-type nodules, although the content of sucrose and starch was unchanged (Romanov et al. 1997).

In the present study, we examine the development of root nodules formed by the pea mutant *sym*31 (previously described as the Sprint2Fix^- line) which appears to be blocked at an early stage in the development of symbiosomes. To determine more precisely the extent of development within the mutant nodule, we used a series of monoclonal antibody probes, previously characterised as markers for symbiosome differentiation (Kardailsky et al. 1996, Perotto et al. 1991, VandenBosch et al. 1989). The results obtained confirm previous reports that the nodules of the mutant do not develop the capacity for nitrogen fixation (Borisov et al. 1992, Romanov et al. 1995), and suggest that the normal differentiation of the symbiosome compartment is interrupted before this stage.

Material and methods

Plant material and nodule production

Seeds of *Pisum sativum* L. (variety "Sprint2") and the *sym*31 mutant (Borisov et al. 1992) were surface sterilized and transferred into Ehrlenmeyer flasks containing Fahreus N-free medium solidified with 0.5% (w/v) agar (Brewin et al. 1983, 1985). After germination for 7 days in the dark, the peas were inoculated with *Rhizobium leguminosarium* bv. *viciae* strain B556 (Sindhu et al. 1990) or 3841 (Wang et al. 1982). Seedlings were grown for 2–3 weeks in a cabinet with a programmed cycle (20 °C, 16 h light / 16 °C, 8 h darkness), and nodules were then harvested for tissue fixation.

Tissue fixation

Nodules were harvested from roots and transferred directly into fixative. Whole nodules or slices of nodules were fixed overnight at

4 °C in 1% glutaraldehyde, 4% formaldehyde in 100 mM phosphate buffer, pH 6.9. Preserved specimens were dehydrated in an ethanol series and infiltrated and embedded in London Resin White, using benzoin methyl ether as a catalyst for UV polymerization at –20 °C. Alternatively, specimens were fixed with potassium permanganate for 15 min or 1% OsO_4 for 1 h, rinsed with buffer and propylene oxide, and were embedded in Agar100 followed by heat-mediated polymerization.

Immunocytochemistry at the light microscopic level

Sections (0.5 μm thick) were cut with a glass knife and collected on gelatin-coated slides. Specimens were incubated in blocking buffer (2% w/v bovine serum albumin, 1% normal goat serum, in TBS [Tris-buffered saline; 200 mM NaCl in 10 mM Tris (hydroxymethyl)aminomethane-HCl, pH 7.4] with 0.02% NaN_3) for 0.5–1 h at room temperature. Sections were incubated in diluted primary antibody for 1 h at room temperature or overnight at 4 °C. Specimens were rinsed with filtered TBS, and incubated with secondary antibody colloidal gold conjugates for 1 h at room temperature, and finally rinsed again with TBS. For visualization of labelling, specimens were rinsed with filtered water, and silver enhanced with IntenSE M silver enhancement kit (Amersham Int., Amersham, U.K.) according to the manufacturer's instructions. Some sections were stained with 1% basic fuschin to visualize all tissues. Samples were observed with bright-field optics or by phase contrast on a Zeiss Axiophot and photographed with Technical Pan film (Kodak).

Electron microscopy

Ultra-thin (90 nm) sections were cut with a diamond knife and collected on uncoated gold mesh grids. Samples were counterstained with 0.5% (w/v) uranyl acetate (aqueous) and alkaline lead citrate. To provide additional section strength, grids were coated with a thin film of carbon. Specimens were visualized and photographed on a JEOL 1200 EX transmission electron microscope at 80 kV.

Nodule fractionation and dot blot analysis

Membranes and bacteroids were isolated from nodules derived from homogenates of pea nodules (three weeks post-infection). The fractionation procedure involved centrifugation through sucrose density cushions as described by Perotto et al. (1991). An additional fraction containing endomembranes and plasma membranes (nodule membranes) was obtained from nodule homogenates, after removal of symbiosomes and cell wall debris, by ultracentrifugation at 100,000 **g** for 40 min in a Beckman TLA-100 rotor (Perotto et al. 1995).

For dot immunoassay, bacteroids, pbm and nodule membrane fractions were resuspended in TBS at a concentration of approximately 1 mg/ml. Aliquots (1 μl) were applied to sheets of nitrocellulose and, after blocking with 3% (w/v) BSA, the sheet was divided into strips and treated with individual monoclonal antibodies. Color development involved a second antibody conjugated to alkaline phosphatase (Perotto et al. 1995).

Results

Morphology of the sym31 mutant nodules

The extent of nodule growth on *sym*31 plants was found to be quite variable. Most nodules developed to the size of wild-type nodules (although they remained white). However, in many cases, another class of small white nodules developed, and these nodules had no obvious apical meristem after growth for 2–3 weeks in controlled environment conditions (data not shown). In the present study, the cytological examination of *sym*31 nodules was confined to those specimens that had a persistent apical meristem and exhibited a developmental gradient in the median longitudinal axis. Such nodules made it possible to examine the progressive differentiation of the symbiosome compartment in infected cells of increasing age.

Light microscopic analysis of the *sym*31 nodule showed gross morphological differences compared to the Sprint2 wild-type (Fig. 1). The most obvious change was the extent of bacterial invasion within the central tissue. As expected for the wild-type nodule, the central zone contained both infected and uninfected cells (Fig. 1 A). The highly cytoplasmic infected cells contained thousands of pleiomorphic bacteroids. In the mutant, many fewer host cells were filled by bacteroids, and the infected cells did not exhibit the normal increase in cell size (Fig. 1 B). Additionally, the bacteroids within these cells appeared small and relatively undifferentiated (data not shown).

To examine more closely the extent of bacteroid development, bacteroids were isolated from mature mutant and wild-type nodules (Fig. 1 C, D). Wild-type bacteroids exhibited characteristic branching, accumulation of protein in dense bodies, and increase in cell volume. In general, the mutant bacteroids appeared to be undifferentiated and they were approximately the same size as free-living bacteria. However, a few bacteroids showed some degree of branching and accumulation of protein bodies (data not shown).

Immunocytochemical analysis of plant antigens

To investigate the degree of tissue differentiation within the mutant nodule, we examined the distribution of a series of developmental markers recognized by monoclonal antibody (MAb) probes, as revealed by silver-enhanced immunogold staining of tissue sections without counter-staining (Fig. 2). JIM18 recognizes a glycolipid component present on the plant plasmalemma and on newly formed peribacteroid membranes (Perotto et al. 1995). Biochemical differentiation of the pbm is clearly indicated by the regulation of this antigen, which is visible even at the level of light microscopy but could be confirmed, where necessary, by electron microscopy (Perotto et al. 1995). In wild-type Sprint2 nodules, the anti-

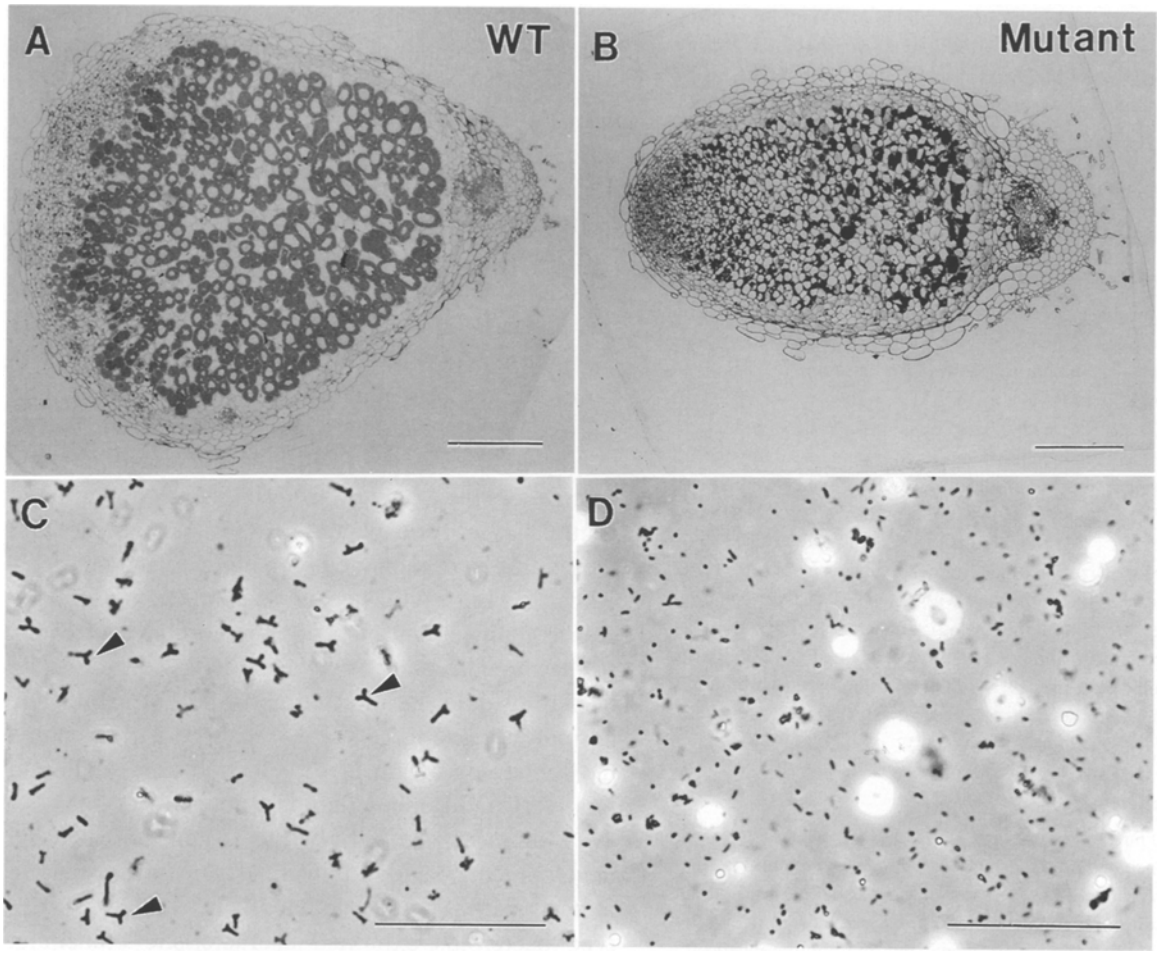
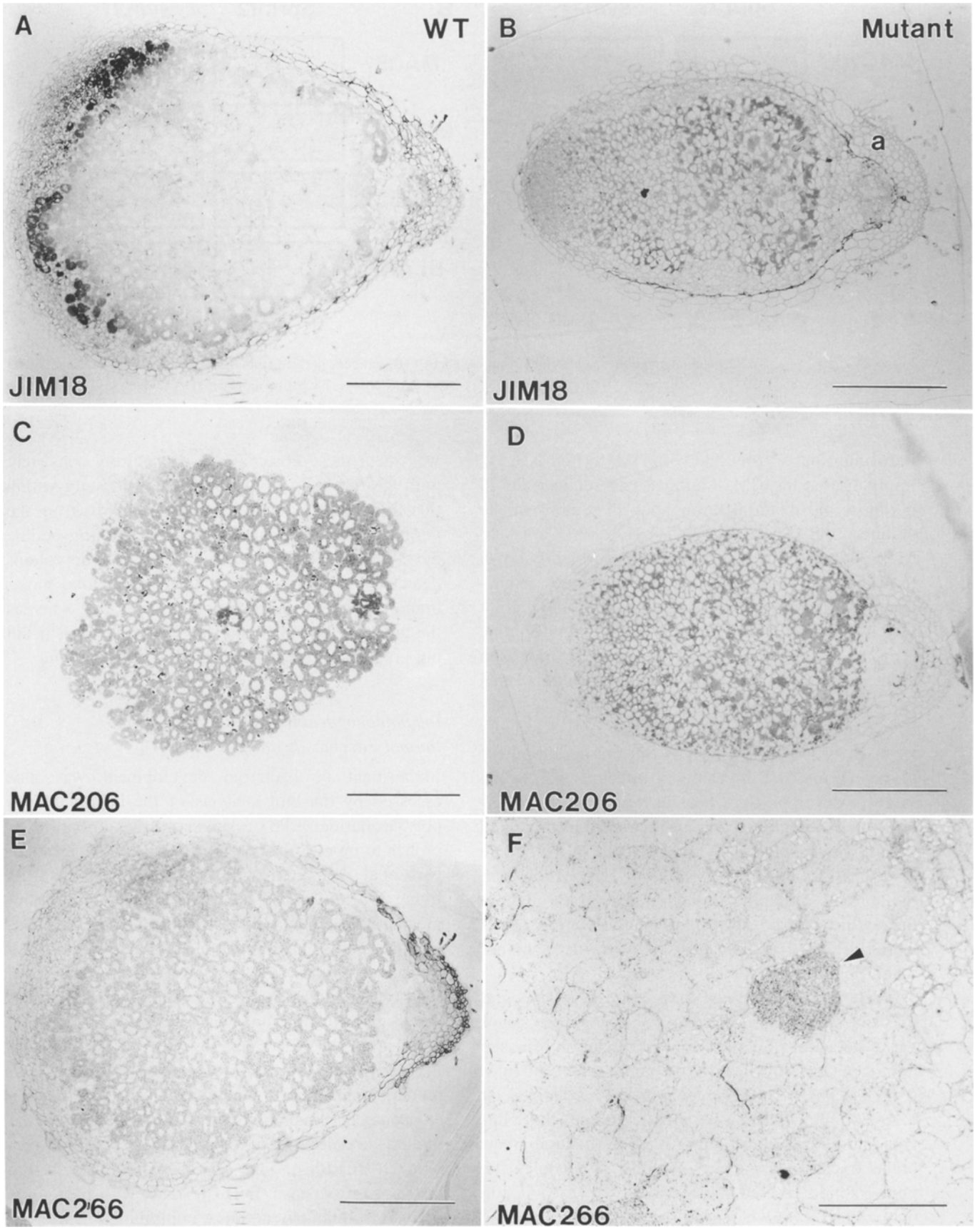


Fig. 1 A–D. Light micrographs of Sprint2 wild-type and *sym*31 mutant nodules and of isolated bacteroids. **A** and **B** Median longitudinal sections counterstained with basic fuschin to show all tissue types; **C** and **D** purified bacteroids visualized by phase contrast microscopy. **A** Wild-type nodule, **B** mutant nodule. **C** Bacteroids from wild-type nodules, showing characteristic branching (arrowheads); **D** bacteroids from mutant nodules. Bars: A and B, 0.5 μm; C and D, 50 μm

body recognized juvenile peribacteroid membrane in cells close to the nodule apex (Fig. 2 A), but was apparently absent from the mature pbm associated with cells in the central tissue, confirming the observations of Perotto et al. (1995) using a different pea line. Within nodules of the *sym*31 mutant, the cytoplasmic membranes (i.e., the peribacteroid membranes) of infected cells in the central tissue were apparently labelled uniformly, implying that there was no loss of JIM18 antigen from the peribacteroid membranes (Fig. 2 B). This observation was confirmed by examination of immunostained tissue at higher magnification (data not shown).

MAC206 also recognizes a glycolipid component of the pbm (Perotto et al. 1991). Within the wild-type Sprint2 nodule, peribacteroid membranes of all developmental stages were recognised by this probe (Fig. 2 C), in contrast to JIM18 which was differentially expressed (Fig. 2 A). Within nodules of the *sym*31 mutant, the pattern of labelling with MAC206 was

Fig. 2. Light micrographs (without counterstaining) distinguishing the distribution of developmental markers in wild-type (A, C, and E) and mutant (B, D, and F) nodules, as visualized by immunogold labeling with silver enhancement. (Because the sections were not counterstained, all the dark areas in the tissue represent regions of immunogold-silver staining.) **A** and **B** Distribution of JIM18 antigen, a marker for the juvenile form of peribacteroid membrane in wild-type nodules. **C** and **D** Distribution of MAC206 antigen, a marker for all symbiosome membranes. **E** and **F** Distribution of MAC266 antigen, indicating the degree of symbiosome maturation. Bars: A–E, 0.5 μm; F, 50 μm


A
WT
JIM18
B
Mutant
a
JIM18
C
MAC206
D
MAC206
E
MAC266
F
MAC266

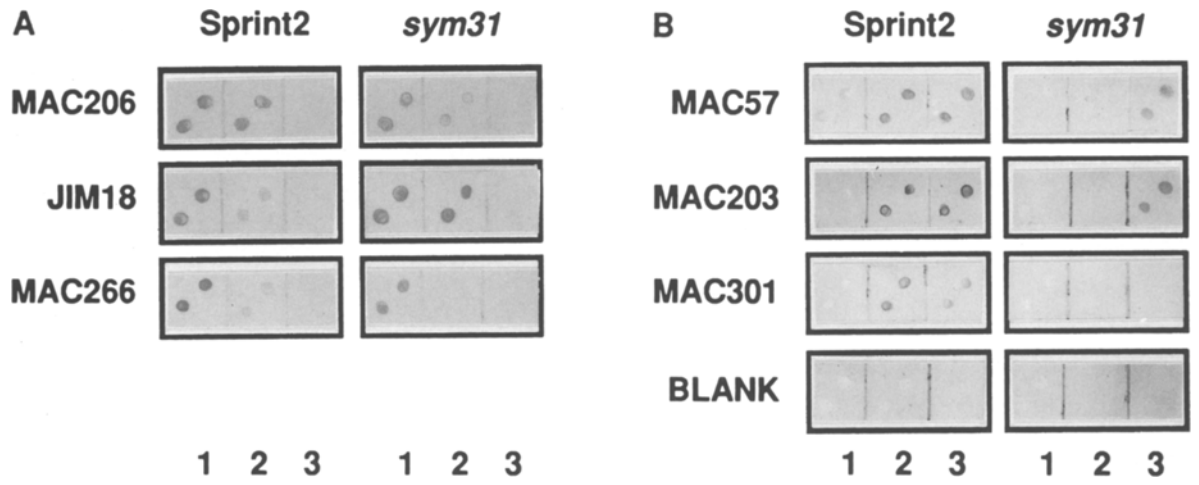


Fig. 3. Dot immunoassay for plant (**A**) and microbial symbiosome-associated (**B**) antigens derived from homogenates of Sprint-2 and *sym*31 mutant pea nodules. *1* Nodule membranes; *2* peribacteroid membranes; *3* bacteroids. Each dot represents an aliquot (1 μl) containing approximately 1 μg protein

indistinguishable from that with JIM18 (Fig. 2 B, D), implying that the JIM18 antigen was retained on all peribacteroid material throughout all regions of the nodule.

MAC266 recognizes a group of glycoproteins shown to accumulate in symbiosomes in late stages of bacteroid development (Kardailsky et al. 1996), all of which apparently carry the same carbohydrate epitope (Perotto et al. 1991). The corresponding MAC266 antigen can therefore be used as an indicator of this late stage of symbiosome differentiation, as was shown in earlier studies (Perotto et al. 1991, Kardailsky et al. 1996). In a wild-type Sprint2 nodule, these glycoproteins first appeared in infected cells at the stage in development where bacteroids were beginning to differentiate, and the antigen accumulated within infected cells in the mature zone of the nodule (Fig. 2 E). In nodule sections from *sym*31, however, the antibody binding was extremely low throughout the nodule (data not shown) although a very few cells were weakly recognized, as illustrated at higher magnification (Fig. 2 F).

The relative abundance of these plant developmental markers was also verified by dot blot analysis of various fractions obtained by centrifugation of tissue homogenates from wild-type and mutant nodules (Fig. 3). The glycolipid components recognized by MAC206 and JIM18 were present in both the wild-type and mutant pbm fractions and in the total membrane fraction. However, in wild-type nodules the pbm fraction reacted more strongly with MAC206 than with JIM18, whereas in the mutant the converse was seen: these observations are entirely consistent with the results of immunocytochemistry suggesting that in mutant nodules JIM18 is never lost from the peribacteroid membrane at any stage during nodule development. Similarly, the family of glycoproteins detected by MAC266 was identified in total membrane fractions from both nodule types but, whereas the antibody bound weakly to wild-type pbm, it did not give any signal with the mutant pbm fraction.

Immunochemical analysis of Rhizobium lipopolysaccharide antigens

Impairment of bacteroid development was also assessed by dot-blot analysis of the bacterial lipopolysaccharide (LPS), using specific antibodies as probes to monitor bacteroid differentiation (Kannenberg et al. 1994). MAC57 recognizes LPS on free-living *Rhizobium* and on bacteroids of all developmental stages: hence this antibody was used to confirm that equal quantities of material had been loaded for dot immunoassay of bacteroids from mutant and wild-type nodules. MAC203, reacts with LPS produced by rhizobia under low O_2 conditions and, because the MAC203 epitope was equally abundant in preparations of bacteroids from wild-type and mutant nodules, it is concluded that a low oxygen environment prevails in the central tissues of the mutant nodules. However, MAC301, which recognizes an LPS epitope expressed just prior to the onset of nitrogenase, failed to react with bacteroids derived from mutant nodules, implying that bacteroid

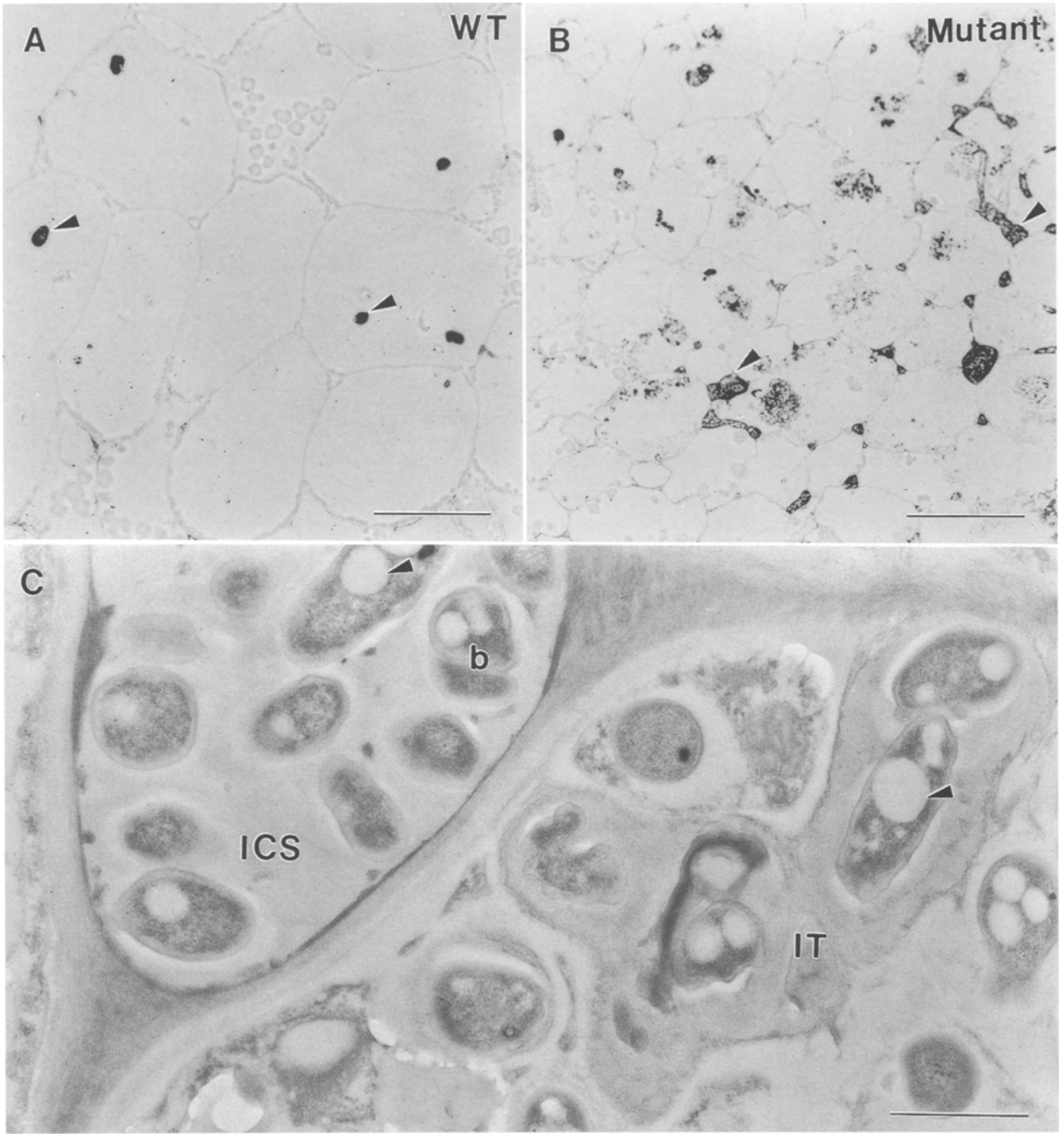


Fig. 4 A–C. Infection thread structures in the most mature zone of wild-type and mutant nodules. **A** Light micrographs showing distribution of remnant infection threads (arrowheads) in the most mature region of a wild-type nodule, visualized by labelling with MAC265 and immunogold/silver staining. **B** MAC265 labeling of threads in the most mature region of mutant nodule. Arrowheads indicate unusual thread structures. **C** Electron micrograph of an infection thread from a *sym*31 mutant. LR White sections were stained with uranyl acetate and lead citrate before observation. Large inclusion bodies, frequently seen within bacteria and bacteroids of the mutant nodules, are indicated by arrowheads. *ICS* Infection thread moving between two cells; *IT* infection thread; *b* bacteria. Bars: A and B, 50 μm; C, 1 μm

development had been arrested prior to the expression of nitrogen fixation capacity. It is also interesting to note that the LPS antigens derived from bacteroids of Sprint2 wild-type were strongly represented in the peribacteroid membrane fraction, whereas this was not the case for the pbm material derived from mutant nodules.

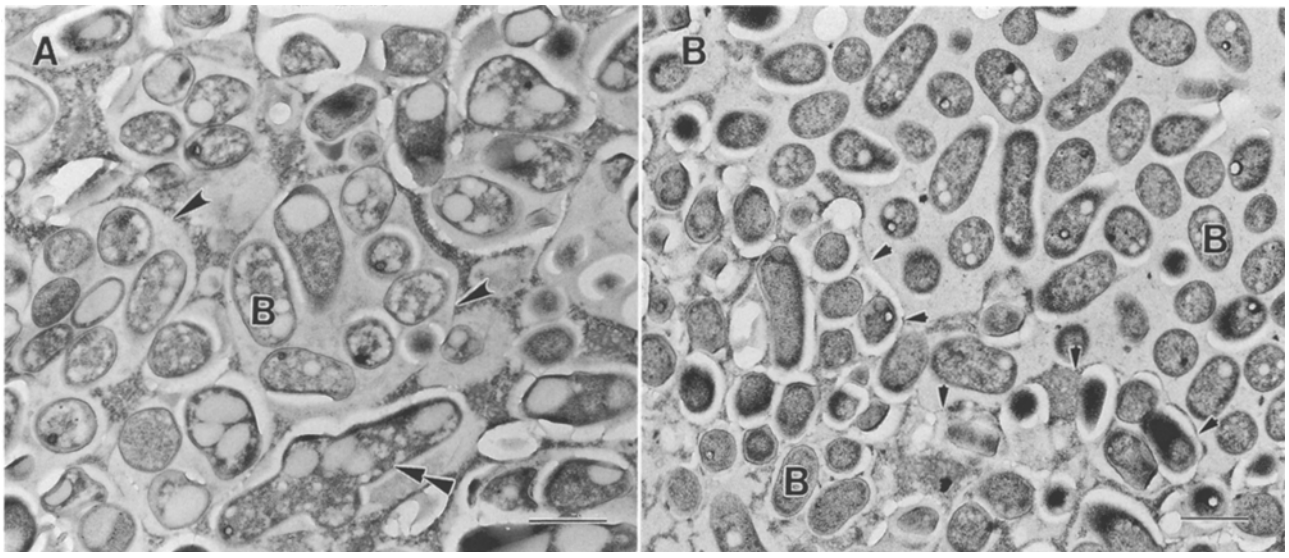


Fig. 5 A, B. Types of infected cells in *sym*31 mutant nodules. LR white sections were stained with aqueous uranyl acetate and alkaline lead citrate before observation. **A** Infected cell containing symbiosomes with multiple bacteroids (arrowheads). A rare branched bacteroid is indicated by a double arrowhead. **B** A second type of infected cell, showing bacteria within a vacuole-like compartment (delineated by arrowheads). *B* Bacteroid. Bars: 1 μm

Structure of the infection thread

Antibody MAC265 was used to examine the structure and distribution of infection threads in mutant nodule tissue. This antibody recognizes a glycoprotein within the infection thread matrix (Rae et al. 1992, VandenBosch et al. 1989). Within wild-type nodules, the antibody identified infection structures in the invasion zone (data not shown), as well as remnant threads within the mature zone (Fig. 4 A). Similarly in the mutant, the antibody recognized threads within the invasion and most mature zones. In addition, in the oldest part of the nodule the probe highlighted an unusual abundance of infection threads positioned between two primary cell walls. Characteristically, these penetrated only a short distance within individual infected cells (Fig. 4 B).

Analysis by transmission electron microscopy confirmed that within the *sym*31 nodule tissue there were indeed a large number of threads with normal morphology, passing through walls and cells. It also confirmed the presence of aberrant infection structures. Figure 4 C shows several short infection threads, passing only a short distance within the infected cell instead of penetrating more deeply into the host cytoplasm.

Electron microscopic analysis of the peribacteroid membrane

The impaired expression of plant developmental markers associated with maturation of the symbiosome and the unusual development of bacteroids suggested that the differentiation of the pbm of the mutant was abnormal. We therefore used conventional electron microscopy to examine the development of the pbm. Two types of infected cells could be distinguished within the mature zone of *sym*31 nodules (Fig. 5). In the first cell type, bacteroids were multiply enclosed within a single peribacteroid membrane, as previously described (Borisov et al. 1992). Very few bacteroids were branched (Fig. 5 A), unlike those within a wild-type nodule. In the second cell type, some bacteroids were singly enclosed, whereas others were multiply enclosed within a pbm. However, the majority of bacteroids within the second type of infected cell were not associated with symbiosomes but were enclosed within a large vacuole-like compartment (Fig. 5 B). The contents of this vacuole-like compartment were not recognized by MAC265 nor was each bacteroid surrounded by any form of membrane which could be detected by heavy metal staining (Fig. 5 B) or by JIM18 immunostaining (data not shown). Few bacterial cells were branched. The morphology of both of these host cell types was dif-

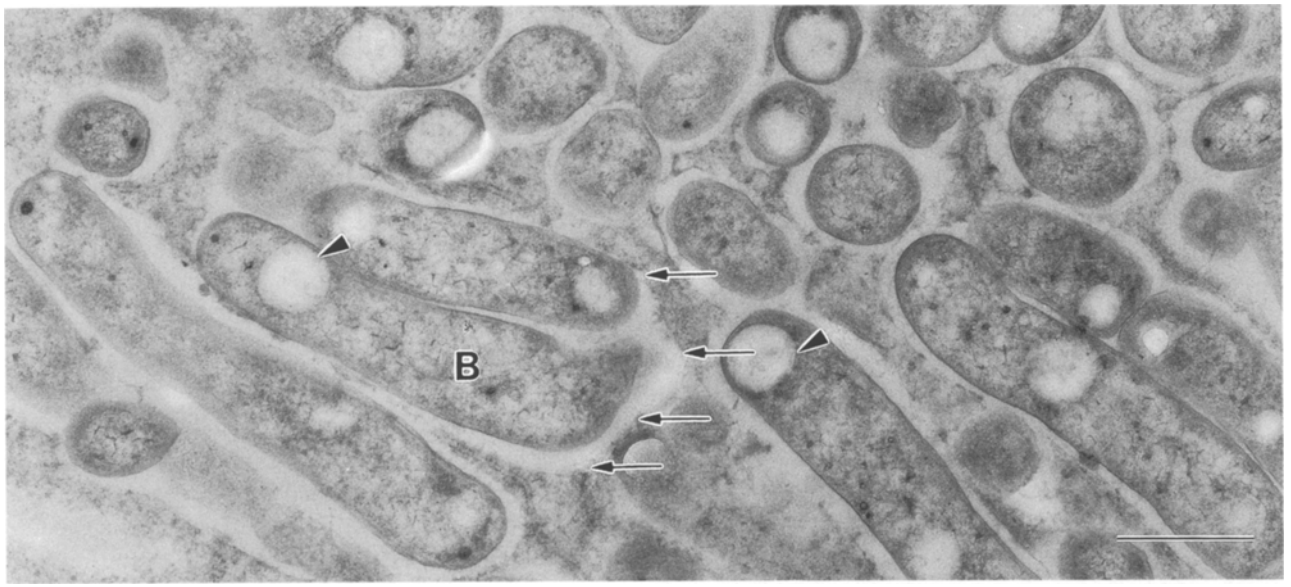


Fig. 6. Electron micrograph showing rod-shaped bacteroids (*B*) from a tissue section of a pink *sym*31 nodule. Tissue was embedded in Agar100 and sections were stained with uranyl acetate and lead citrate before observation. Arrows mark the position of peribacteroid membrane enclosing more than one bacteroid. Arrowheads indicate inclusion bodies, a common feature of bacteroids from *sym*31 nodules. Bar: 1 μm

ferent from mature infected cells characteristic of wild-type nodules. However, the morphology of cells with multiply enclosed bacteroids is more similar to the infected cells found closer to the invasion zone in wild-type nodules, where the bacterial cells are still dividing.

Variations in the phenotype of sym31 root nodules

Under the growth conditions, plants carrying one or more pink nodules were occasionally observed with *sym*31 (representing about 0.5% of the mutant plants examined). In these rare cases, one or a cluster of several pink nodules were found on a root system that otherwise carried small white nodules typical of the mutant phenotype. This suggests a somewhat leaky phenotype for the *sym*31 allele. Immunogold staining revealed that cells within these pink nodules reacted with leghemoglobin and nitrogenase antibodies (data not shown). However, despite the expression of leghemoglobin in these pink *sym*31 nodules, EM analysis revealed that bacteroids in the most mature infected cells were not fully differentiated. The bacteroids were elongated, but not branched like bacteroids from wild-type nodules and in many cases there still seemed to be several bacteroids within a single peribacteroid membrane envelope (Fig. 6).

Discussion

Symbiosomes are derived initially by endocytosis of bacteria through the plasmalemma of host cells (Verma and Hong 1996). The surrounding membrane subsequently develops into a unique membrane type, the peribacteroid membrane (pbm), which is no longer associated with the plant cell wall, although it may be in intimate contact with the bacterial cell wall. In the present study, we used microscopy and immunocytochemistry to investigate the lesions in symbiosome development for the pea nodulation mutant Sprint-2Fix$^-$ (*sym*31; Borisov et al. 1992). A number of previously characterized monoclonal antibodies were used as probes to monitor the progressive differentiation of the symbiosome compartment (Kardailsky et al. 1996; Perotto et al. 1995, 1991; VandenBosch et al. 1989; Kannenberg et al. 1994). It was shown that, in the nodules formed by the *sym*31 mutant, the symbiosomes retained several juvenile characteristics. Furthermore, the development of infected cells and infection threads was shown to be aberrant.

To study the developmental progression of symbiosomes, the antibody JIM18 was used because it recognizes a glycolipid component of the plant plasma membrane and newly formed symbiosome compartments (Perotto et al. 1995). As the symbiosome

membrane differentiates, the epitope can no longer be detected on the symbiosome membrane in wild-type nodules (Perotto et al. 1995). However, in nodules formed by the mutant plant, labeling with JIM18 was uniform throughout the infected cells of the nodule (Fig. 2), indicating the retention of immature membrane characteristics of the pbm.

Following the loss of JIM18 epitope from the pbm, further maturation of symbiosomes is normally accompanied by the accumulation of a nodule lectin (PsNLEC) and related glycoproteins identified by virtue of a carbohydrate epitope recognized by MAC266 (Perotto et al. 1991, Kardailsky et al. 1996). Whereas this epitope was expressed in infected cells of wild-type nodules (Fig. 2 E), it could only be detected in very low amounts in a few cells of the *sym*31 root nodules (Fig. 2 F). Probably because the level of MAC266 antigens was low in symbiosomes, these antigens could not be detected by dot-blot analysis of purified PBM from mutant nodules, although a strong signal was still obtained for MAC266 antigen in the nodule membrane fraction (Fig. 3). These data suggest either that MAC266 antigens are not delivered to the juvenile-type symbiosomes that prevail in mutant nodules or, alternatively, that this material is turned over very rapidly within the symbiosome compartment of the mutant nodules.

The observations that morphology and biochemical composition of the symbiosome are unusual suggest that targeting to the pbm may be atypical in the *sym*31 mutant. However, targeting of plasmalemma components in *sym*31 plants appears to be normal, and aberrant nodule formation is the only detected plant phenotype. The presence of the glycolipids recognized by JIM18 or MAC206 demonstrates normal targeting of components of the plasmalemma and juvenile form of pbm. However, the later stages of pbm development may be impaired in ways that were not observed for pea line E135f (Kneen et al. 1990a), a symbiotically defective pea line where symbiosomes apparently develop normally but senesce prematurely.

Clearly, symbiosome development and differentiation involve the transit of plant components through the plant endomembrane system. However, the mechanism for targeting of plant components to the symbiosome is unknown. Small GTP-binding proteins have been demonstrated to be important in vesicle-mediated protein transport (Verma et al. 1994), and these proteins are apparently required for the biogenesis of the pbm (Cheon et al. 1993). Antisense experiments with plant homologs of Rab1p resulted in smaller infected host cells with fewer bacteroids, with some bacteroids being released into the central vacuole of infected cells (Cheon et al. 1993). Interestingly, all of these characteristics were also seen in nodules formed by the *sym*31 mutant reported here. However, there is still no direct evidence that vesicle targeting is altered within these plants.

An alternative interpretation of the mutant phenotype suggests that the *sym*31 nodules may exhibit features more characteristic of determinate nodules. In determinate nodules, such as those formed by French beans (*Phaseolus vulgaris*). The development and morphology of the nodule is somewhat different from that of indeterminate nodules, as seen in legumes such as peas, clover, and vetch. Determinate nodule meristems are active only transiently, resulting in a spherical nodule of delimited cell number. Infection threads grow through and between host cells, and a large proportion of the threads only penetrate a short distance into the plant cell before bacteria are released (Rae et al. 1992). As the intracellular bacteroids mature and differentiate, multiple bacteroids remain enclosed within each pbm. It is interesting to note that nodules formed on the *sym*31 plants exhibit several features which are normally associated with determinate nodules: the nodule meristem aborts in many of the developing nodules; many of the infection threads are extracellular or penetrate only a short distance into the host cell (Fig. 4); bacteroids are morphologically undifferentiated (Fig. 1 D); and bacteroids are multiply enclosed within symbiosome units (Borisov et al. 1992), particularly in nodules where meristematic activity is arrested. These observations are intriguing, because they suggest that the fundamental difference between symbiosome morphology in determinate and indeterminate nodule types may represent a simple developmental switch.

The phenotype of nodules formed on *sym*31 plants was found to be somewhat variable between nodules and even variable between different host cell types within the same root nodule, suggesting that expression of the ultrastructural phenotype may be dependent on the physiology of the host cells. In some cases, multiple bacteroids were enclosed within one peribacteroid membrane; in other cases they were found within a vacuole-like compartment, perhaps representing a fusion product of several symbiosomes. This cell-specific variability was not previously reported (Borisov et al. 1992, Romanov et al. 1995), but may have been revealed in the present

study because the plants were grown under different environmental conditions.

In conclusion, the use of antibody probes has provided evidence that bacteroid and peribacteroid membrane differentiation are arrested at an early stage of symbiosome development in *sym*31. Ultrastructural analysis has identified two abnormal compartments harboring these undifferentiated bacteroids. Further analysis of the biogenesis of cytoplasmic, symbiosomal and vacuolar membranes from *sym*31 nodules may help to identify the consequences of the developmental lesion in these nodules, and hence explain the anatomical and biochemical features that have previously been described (Borisov et al. 1992, Romanov et al. 1996). In addition, further characterization of the *sym*31 allele is an important challenge for genetic analysis, in order to understand the biochemical nature of this developmental lesion.

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