Initiation of a legume nodule with an indeterminate meristem involves proliferating host cells that harbour infection threads

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Summary

- A comparative analysis of nodule morphogenesis was carried out for three symbiotically defective pea (*Pisum sativum*) mutants that show abnormalities in nodule development.
- In the wild-type lines, resumption of cell proliferation in the pericycle and inner cortex results in the development of a nodule primordium, within which are found proliferating cells that harbour infection threads. However, this class of cell is not observed in the mutants RisFixA (*sym41*) and SGEFix$^-$-2 (*sym33*) where nodule development is arrested at the point of formation of the apical nodule meristem. It is proposed that the presence of proliferating cells harbouring infection threads is a prerequisite for normal formation of the nodule meristem.
- In mutant SGEFix$^-$-1 (*sym40*), nodule development does not differ from that of wild-type plants in the early stages but is blocked at the stage after nodule meristem persistence.
- A scheme is proposed for the sequential functioning of pea symbiotic genes *Sym33*, *Sym40* and *Sym41* in the programme of nodule development.

Abbreviations: IT, infection thread; DAI, days after inoculation; NM, nodule meristem; Anm, phenotypic code for the stage of apical nodule meristem formation; Nmp, phenotypic code for the stage of nodule meristem persistence.

Introduction

Symbiotic interactions between nodule bacteria (*Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Azorhizobium* etc.) and the roots of leguminous plants (*Pisum*, *Vicia*, *Medicago*, *Glycine*, *Lotus* etc.) lead to the formation of nodules as new plant organs that are physiologically specialized for biological nitrogen fixation. The process of nodule development is initiated by the exchange of molecular signals produced by both symbiotic partners (Schultze & Kondorosi, 1998). It continues with the co-ordinated expression of bacterial and plant genes (D'Haeze & Holsters, 2002; Oldroyd & Downie, 2008), leading to colonization of the host plant by rhizobia and differentiation of the symbiotic tissue of the nodule.

With over 16 000 species, the legume family (Fabaceae) shows great diversity in the morphology of legume/*Rhizobium* symbiotic structures and in the process of nodule development. Unfortunately, only a very limited number of *Rhizobium*–legume interactions have been studied in detail (Hadri *et al.*, 1998) but it is possible to identify at least two major types of root nodule, namely those with indeterminate and those with determinate nodule meristem (NMs) (for a review, see Sprent & James, 2007).

Symbiotic interactions between pea (*Pisum sativum*) and *Rhizobium leguminosarum* bv. *viciae* have been studied intensively (Bond, 1948; Libbenga & Harkes, 1973; Newcomb, 1976; Newcomb *et al.*, 1979; Brewin, 1991; Kijne, 1992; see Brewin, 2004 for a review) and *Pisum* can be considered typical for legumes forming indeterminate nodules (e.g. *Medicago*, *Trifolium* and *Vicia* spp.; Hadri *et al.*, 1998). The infection process induced by rhizobial Nodulation (Nod) factors includes the deformation and curling of root hairs, and the formation of transcellular infection threads (ITs) that grow in a 'cell-to-cell' manner (Brewin, 1998), delivering rhizobia into the plant

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root cortex. There is also a parallel process of cortical cell divisions resulting from resumption of the mitotic cycle in G0/G1-arrested plant root cells, leading to nodule primordium formation (Vasse *et al.*, 1990; Yang *et al.*, 1994; Hadri *et al.*, 1998; Foucher & Kondorosi, 2000). As a result, a plant organ – the nodule – develops with a complex histological structure. This includes a central nitrogen-fixing tissue comprising endoreduplicated plant cells differentiated into symbiotic forms (Truchet, 1978) and containing bacteria endocytosed into the host cell cytoplasm and also differentiated (both structurally and metabolically) into endoreduplicated symbiotic forms (Mergaert *et al.*, 2006), termed bacteroids (Brewin, 1991, 1998). The main characteristic of these cylindrical or branched nodules is that the NM is apically developed at the distal end of the nodule and is persistent in the sense that it functions for rather a long time. By contrast, legumes with spherical nodules and determinate NMs, for example *Phaseolus*, *Glycine* and *Lotus* spp., are characterized by a transient meristem in which mitotically active cells contain ITs and/or rhizobial cells that have been released into the cytoplasmic compartment as membrane-enclosed symbiosomes (Brewin, 1991).

The aim of the present study was to characterize pea (*Pisum sativum*) nodule development through an examination of three symbiotically defective mutants, SGEFix^{-}-2 (*sym33*), SGEFix^{-}-1 (*sym40*) and RisFixA (*sym41*), and the double mutant RBT3 (*sym33*, *sym40*). Previously, it was shown that the genes of interest control IT formation and nodule function (Tsyganov *et al.*, 1998; Morzhina *et al.*, 2000) at comparatively late stages of development. However, in the present study, particular attention was paid to early stages of nodule development (from the first cell divisions in the pericycle and root cortex to the stage of nodule emergence), and an analysis of the interrelationship between IT growth and the proliferation of host cells following infection by *Rhizobium* was performed.

Materials and Methods

Plant material and bacterial strains

Pea (*Pisum sativum* L.) mutant lines used in this study and their parental lines are listed in Table 1. For inoculation of plants, a derivative of strain VF39 of *Rhizobium leguminosarum* bv. *viciae* was used that contained a Tn*5-gusA* insertion constitutively expressing *gusA* (Voroshilova *et al.*, 2001). Bacteria were grown on TY agar medium (Beringer, 1974) at 28°C with the required antibiotics; streptomycin (Sm; 600 mg l^{-1}) and gentamycin (Gm; 40 mg l^{-1}).

Plant growth conditions and inoculation procedure

Plant seeds were surface-sterilized with concentrated sulfuric acid (30 min at room temperature). Germinated seeds were individually planted in plastic pots containing 200 ml of vermiculite and inoculated after 7 d. The composition of nitrogen-free nutrient solution and the method of inoculation were described previously (Borisov *et al.*, 1997a). Plants were grown in growth chambers (VB1514; Vötsch Industrietechnik GmbH, Balingen-Frommern, Germany) under the following conditions: day:night, 16 : 8 h; temperature, 21 : 19°C; relative humidity, 75%; photon irradiance, 490 μmol m^{-2} s^{-1}.

Collection of the material and statistical analysis

For analysis of nodule development, main root samples were collected at 5, 9 and 12 d after inoculation of seedlings (DAI). Nodules were collected at 12–28 DAI depending on the plant genotype. Five root samples and 10–20 nodule samples per variant were used. Wild-type nodules were collected at 42 DAI for analysis of the type of nodule branching.

χ^2 analysis (SigmaStat for Windows, version 2.3; SPSS Inc., Chicago, IL, USA) was used to compare numbers of nodule primordia between different lines.

Histochemical staining and microscopy

For visualization of ITs and cells colonized by bacteria in plant tissues, the main root pieces and nodules were sliced into 75-μm and 100-μm sections, respectively, using a vibratome VT 1000 S (Leica Microsystems GmbH, Wetzlar, Germany). Root and nodule sections were then stained, fixed as described previously (Voroshilova *et al.*, 2001) and mounted in distilled water on glass slides for light microscopy.

Table 1 Plant material used in the study

Line of *Pisum sativum*	Phenotype	References
SGE	Wild-type line	Kosterin & Rozov (1993)
SGEFix^{-}-1 (*sym40*)	Abnormal (hypertrophied) infection droplet development (Idd^{-}), leaky phenotype	Tsyganov *et al*. (1998)
SGEFix^{-}-2 (*sym33*)	No endocytosis of bacteria (Itn^{-}), leaky phenotype	Tsyganov *et al*. (1998)
RBT3 (*sym33, sym40*)	No endocytosis of bacteria (Itn^{-})	Borisov *et al*. (1997b); Voroshilova *et al*. (2001)
Finale	Wild-type line	Engvild (1987)
RisFixA (*sym41*)	Abnormal (hypertrophied) infection droplet development (Idd^{-})	Engvild (1987); Morzhina *et al*. (2000)

Itn, infection thread formation in nodule primordium.

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For conventional light microscopy, nodules were vacuum-infiltrated with Navashin's fixative (1% chromic acid:40% formalin:100% acetic acid, 10 : 4 : 1 (v/v/v) (Sass, 1958), with 0.1% Tween 20) or with 3% (w/v) paraformaldehyde with 0.1% Tween 20 in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4 and 5 mM KH_2PO_4, pH 7.3) for 30 min. Fixation was performed at 4°C for 12 h. After three washes in PBS for 20 min, each specimen was dehydrated in a graded ethanol series. Nodules were then counterstained overnight at 4°C in 1% (w/v) toluidine blue O dissolved in 95% ethanol. To embed the specimens in Steedman's wax (Vitha *et al.*, 1997), they were first placed in 100% ethanol for 1 h, and then in a graded wax/ethanol series at 37–40°C, followed by three changes of pure medium, each for 12 h. Serial 8-µm-thick sections were cut with a rotation microtome (RM2125RT; Leica Microsystems, Wetzlar, Germany) and then placed on silanized slides (coated with a 2% solution of 3-aminopropyltriethoxysilane (Fluka, St. Louis, MO, USA) in anhydrous acetone) coated with egg white and stretched by the addition of a small drop of double-distilled (dd) H_2O to one end of the ribbon. For Feulgen/alcian blue staining (Demchenko *et al.*, 2004), sections on slides were rehydrated in a graded ethanol series, equilibrated in dd H_2O, and incubated in 5 N HCl for 1 h, and then in Schiff's reagent for 2 h. Then, sections were washed in SO_3–water and in dd H_2O before being incubated for 2 h in 1% alcian blue 8GX (Sigma, St. Louis, MO, USA) in 3% acetic acid. Sections were washed in dd H_2O, dehydrated in a graded ethanol series, equilibrated in xylene and mounted with Eukitt™ (Kindler, Freiburg, Germany).

Pictures were taken using an Olympus BX51 microscope (Olympus Optical Co. Europa GmbH, Hamburg, Germany) equipped with a ColorView II digital camera and ANALYSIS® FIVE docu-image analytical software (Olympus Soft Imaging Solution GmbH, Hamburg, Germany).

Results

Wild-type pea lines SGE and Finale

In the two wild-type lines of pea used in the study (SGE and Finale), the sequence of events leading to nodulation was identical. Following root hair curling and the initiation of IT growth into the root hair cell, the IT starts to grow from cell to cell into the root cortex. Generally, the orientation of IT growth is radial (i.e. perpendicular to the root surface) and a certain amount of intracellular branching and bifurcation can be observed (Fig. 1a). Growth of the IT has the characteristic pattern of 'cell-to-cell' propagation (Brewin, 1998; Hadri *et al.*, 1998). Simultaneously, cells of the pericycle and inner cortex start proliferating, together generating the group of dividing cells of the nodule primordium (Fig. 1a,b). When the advancing IT enters the tissue of the nodule primordium, plant cells with endocytosed bacteria can be seen for the first time (Fig. 1c). Further growth of the infected nodule tissue leads to the formation of a young nodule that emerges from the root (Fig. 1c).

At the stage where developing nodules emerge from the root (Fig. 1c), they do not yet have the histological differentiation characteristic of indeterminate nodules as described by Vasse *et al.* (1990). Nevertheless, some preliminary analysis can be undertaken using visible histological patterns and taking into consideration the probable fate of certain nodule components. First, the 'main' IT is visible: this apparently forms the cellular pathway of 'entrance' to the root. Secondly, colonized nodule cells that already have endocytosed bacteria in the cytoplasmic symbiotic compartment are clearly seen. Thirdly, peripheral layers of nodule primordium cells not penetrated by ITs can be distinguished. Fourthly, on the distal side of the nodule primordium, a group of small dividing cells can be distinguished that appear to be the starting point for the apical NM. These cells are located directly above the plant cells already colonized by bacteria and surrounding the original 'entrance' pathway for the IT into the root (Fig. 1c). Actively proliferating plant cells can be observed at the distal end of this group (Fig. 1f), whereas, in the proximal cell layers, mitotic activity occurs concurrently with transcellular growth and branching of ITs (Fig. 1g,h). Further growth of the nodule is normally based on only one sector of the incipient NM because at a later stage of nodule development the 'entrance' to the nodule usually appears at an epicentric position (in approximately 70% of the nodules studied; Fig. 1d,e).

Mutant RisFixA (*sym41*)

The early stages of nodule primordium development of the mutant RisFixA (*sym41*) are similar to those observed in the roots of the wild-type line Finale, although the process is delayed (compare DAI in Figs 1 and 2 and Table 2). During this delayed nodule development, cells in the five to six cell layers of the outer cortex re-activate their mitotic cycle, as do cells of the inner cortex, but after several cycles of cell divisions they leave the mitotic cycle and ITs are formed within them later (at 19 DAI) (Fig. 2a,c). ITs continue growing into the root and cells of the inner cortex continue to proliferate, generating a nodule primordium (Fig. 2a). The highly branching trans-cellular ITs are never observed in cells of the nodule primordium, which can remain in the mitotic cycle for a longer period than the outer cortex cells (Fig. 2b,c). However, when cells of the central part of the nodule primordium have left the mitotic cycle, some ITs can be formed in them after they have stopped cycling (Fig. 2b).

At slightly later stages of nodule development (28 DAI), the nodule primordium grows around the plant cells containing ITs, forming a very abnormal lateral NM (Fig. 2d). The plant cells harbouring ITs are much larger than cells of the normal wild-type nodule primordium that are still in the cell cycle (Fig. 2b,c). The latter fact can be considered as an indication that, in this mutant, plant cells containing ITs have already

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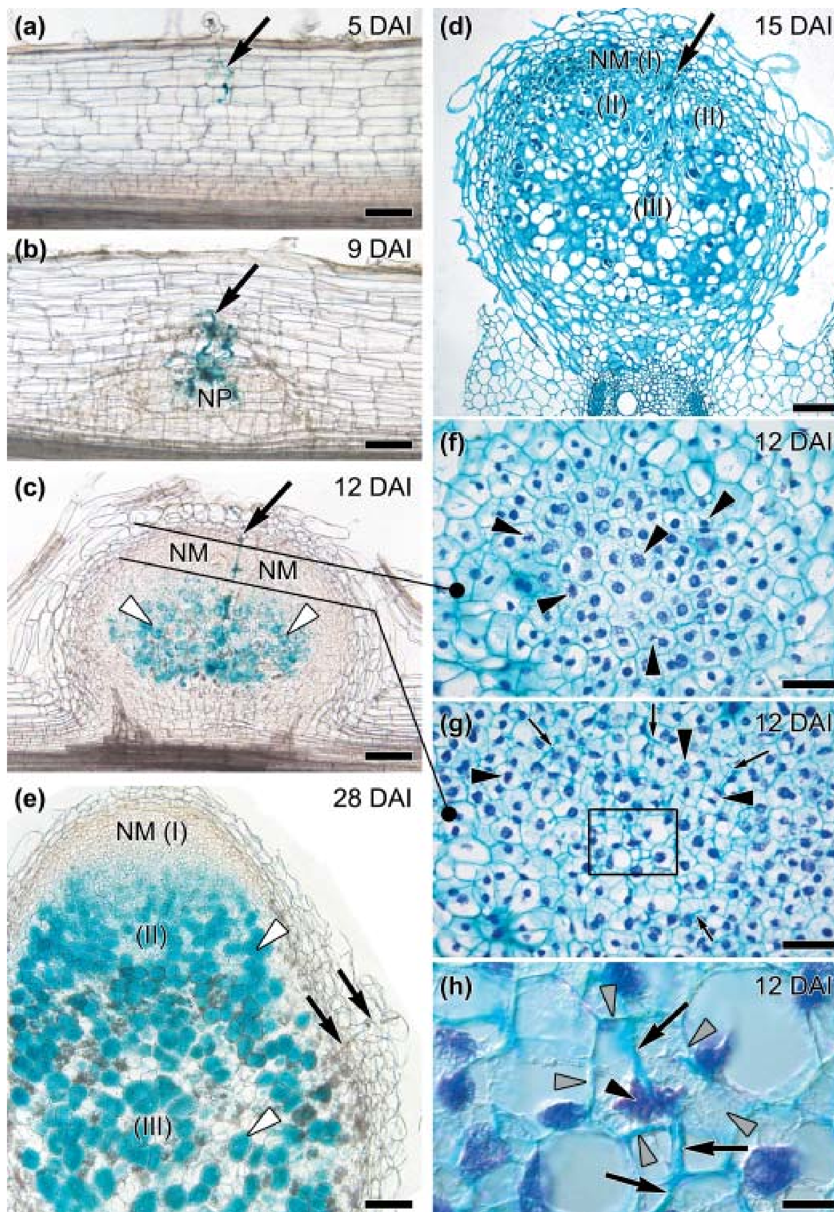

Fig. 1 Symbiotic root nodule development in wild-type line SGE of *Pisum sativum*. (a–c, e) Longitudinal vibratome sections of roots and nodules, with GUS staining of bacteria in infection threads (ITs) and colonized nodule cells. (d, f–h) Microtome longitudinal sections (d) and cross-sections (f–h) of nodules, with Feulgen/alcian blue staining. (a) Transcellular IT growth (black arrow) in the outer root cortex and resumption of cell proliferation in the inner cortex cells at 5 days after inoculation (DAI). (b) Nodule primordium at 9 DAI with proliferating cells harbouring ITs. (c) An emerging young nodule at 12 DAI. The black arrow indicates the nodule meristem penetrated in the middle by ITs, and the white arrowheads indicate plant cells in the infection zone with endocytosed bacteria. (d) A nodule at 15 DAI, showing normal histological organization. NM(I), nodule meristem/nodule histological zone I; (II), colonization zone of the nodule; (III), nitrogen fixation zone of the nodule. (f, g) Cross-sections in the NM region of a young nodule at 12 DAI; planes of the sections marked with lines in (c). Black arrowheads indicate dividing plant cells (mitoses), and arrows indicate transcellular ITs. (e) A nodule at 28 DAI. The 'IT entrance' to the nodule usually appeared at an epicentric position. (h) Magnified area demarcated by the rectangle in (g). Mitosis in the nodule meristem cell (cell wall indicated by grey arrowheads) that forms transcellular ITs. Neighbouring cells have the preinfection structures. NP, nodule primordium; NM, nodule meristem. Bars: (a, b, f, g) 50 μm; (c, d) 100 μm; (h) 10 μm.

left the mitotic cycle (before formation of ITs). No uptake of bacteria from ITs can be detected and the process of root nodule development in the mutant RisFixA (*sym41*) is normally blocked at this stage. Cells of the abnormal lateral NM cease cell proliferation and, as a result, the young nodule does not develop any further (Fig. 2c). Thus, it was revealed that the mutation in the gene *sym41* is associated with the absence of an ability to form ITs in proliferating cells and this apparently causes a block in apical NM development. Furthermore, the proliferative activity in the lateral NM is not maintained for a long period and nodule development is arrested.

Occasionally, the mutant RisFixA (*sym41*) forms a few white nodules as a result of sustained activity of the lateral NM (Fig. 2d) which grows around the cluster of cells harbouring the pathway for the 'main' trans-cellular branching IT (Fig. 2d).

Thus, this mutation in the pea symbiotic gene *sym41* prevents IT development in proliferating cells and, as a result, the development of the mature apical NM is blocked. In addition, the general ability to form nodule primordia is apparently affected (Np$^{+/-}$ phenotype) because the total number of nodule primordia in the mutant RisFixA (*sym41*) is reduced compared with wild-type plants (Table 2).

Mutant SGEFix$^-$-2 (*sym33*)

For mutant SGEFix$^-$-2 (*sym33*) at early stages of nodule development, cell divisions are induced in the inner root cortex, as in the wild-type pea line SGE. As a result, a nodule primordium develops underneath the cluster of cells through which the ITs have gained access to the root outer cortex

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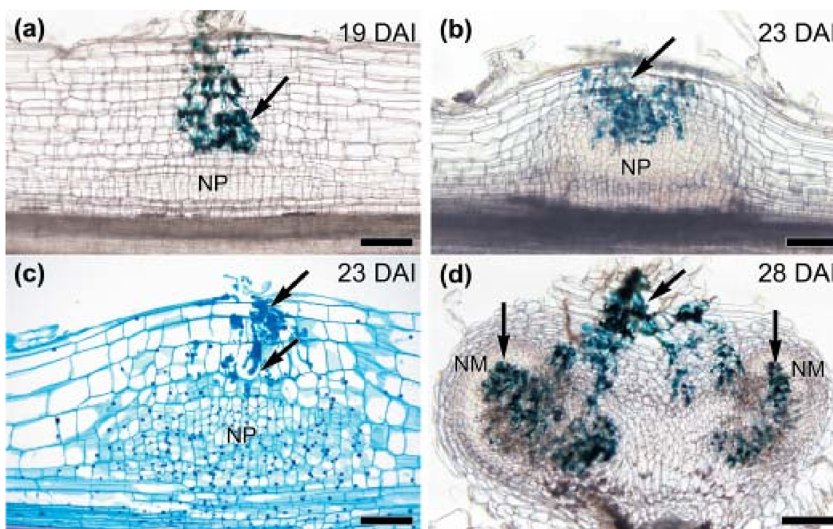

Fig. 2 Stages of root nodule development of the *Pisum sativum* mutant RisFixA (*sym41*). (a, b, d) Longitudinal vibratome sections of roots and nodules, with GUS staining of bacteria in infection threads (ITs). (c) Longitudinal microtome section of a root, with Feulgen/alcian blue staining. (a) Nodule primordium (NP) at 19 days after inoculation (DAI). ITs grow in the outer root cortex, and the NP forms from cells of the inner root cortex. (b, c) Emerging young nodule at 23 DAI; (d) nonfixing nodule at 28 DAI. ITs do not penetrate the proliferating cells of the NP. Arrows indicate transcellular ITs. NM, nodule meristem. Bars: (a, b, c) 100 µm; (d) 200 µm.

Table 2 The dynamics of nodule primordium development in wild-type and mutant lines of *Pisum sativum*

	Mean number of nodule primordia per plant Days after inoculation		
Genotype	5	9	12
Finale (wild-type)	3.80	18.40	20.70
SGE (wild-type)	1.20	22.20	30.00
RisFixA (*sym41*)	0.45[a]	3.10[a]	4.80[a]
SGEFix$^-$-1 (*sym40*)	3.65	16.10	38.35
SGEFix$^-$-2 (*sym33*)	0.45	8.55[a]	5.35[a]
RBT3 (*sym33*, *sym40*)	0[b]	11.00	21.15[b,c]

[a]The mean values in mutants differ significantly ($P > 0.95$) from those in the corresponding wild-type lines.
[b]The mean values in double mutant RBT3 (*sym33*, *sym40*) differ significantly ($P > 0.95$) from those in parental line SGEFix$^-$-1 (*sym40*).
[c]The mean value in double mutant RBT3 (*sym33*, *sym40*) differs significantly ($P > 0.95$) from that in parental line SGEFix$^-$-2 (*sym33*).

(Fig. 3a). The number of nodule primordia is lower at 9 and 12 DAI than in the wild-type plants (Table 2). The IT grows and branches in the outer cortex but does not penetrate the nodule primordium, similar to the situation in the mutant RisFixA (*sym41*) (Fig. 3b). Nodule primordium cells that are not penetrated by ITs continue proliferating and form a lateral NM growing around the 'main' ITs (Fig. 3c). Consequently, the emerging nodule contains host cells with ITs but lacks cells with intracellular bacteria (Fig. 3b,d). At later stages of nodule development, intracellularly branching ITs are found inside the mutant nodule tissue but they never penetrate the area of the lateral NM (Fig. 3c,f). Again, as with the mutant RisFixA (*sym41*), ITs could be observed both in longitudinal sections (Fig. 3d,f) and in cross-sections (Fig. 3e). These ITs are found only in large cells which, obviously, have left the mitotic cycle. The form of the white ineffective nodules of SGEFix$^-$-2 (*sym33*) is similar to that observed for the nodules of mutant RisFixA (*sym41*) (Fig. 3c), and their histological structure is abnormal, as described previously (Tsyganov *et al.*, 1998; Voroshilova *et al.*, 2001).

Thus, it was found that this mutation in the pea symbiotic gene *sym33*, similar to the mutation in RisFixA (*sym41*), is associated with a block of nodule morphogenesis at the stage of apical NM development, and this lesion apparently affects the general ability to form ITs in proliferating nodule primordium cells. Although only a single mutant allele was used for each locus, *sym33* and *sym41*, the fact that the same phenotype was observed in each case argues strongly that the phenotype is a consequence of the mutation rather than of a secondary effect.

Mutant SGEFix$^-$-1 (*sym40*) and double mutant RBT3 (*sym33*, *sym40*)

In the mutant SGEFix$^-$-1 (*sym40*), all early stages of NM development were apparently normal. As with the wild-type lines, co-ordinated IT growth in the root cortex and divisions of inner cortical cells at early stages of nodule development lead to the formation of a nodule primordium colonized by bacteria both inter- and intracellularly (Fig. 4a). The number of nodule primordia is quite similar to that found in wild-type lines (Table 2) and at the distal end of the infected nodule primordium, the differentiation of the NM surrounding the 'main' IT leads to the growth of the young nodule emerging from the root (Fig. 4b). As in wild-type nodules, further growth of the nodule is apparently supported by one sector of the NM only (Fig. 4b) while, in the proximal cell layers of NM, cells can divide and develop ITs simultaneously (Fig. 4d). Nevertheless, beginning from 12 DAI, the nodules were found to have abnormal histological structure: the NM of such nodules is either very narrow (flat) or cannot be found at all (Fig. 4c,e). Presumably, the cells of the NM in the nodules of SGEFix$^-$-1 (*sym40*) stop proliferating relatively early in nodule development

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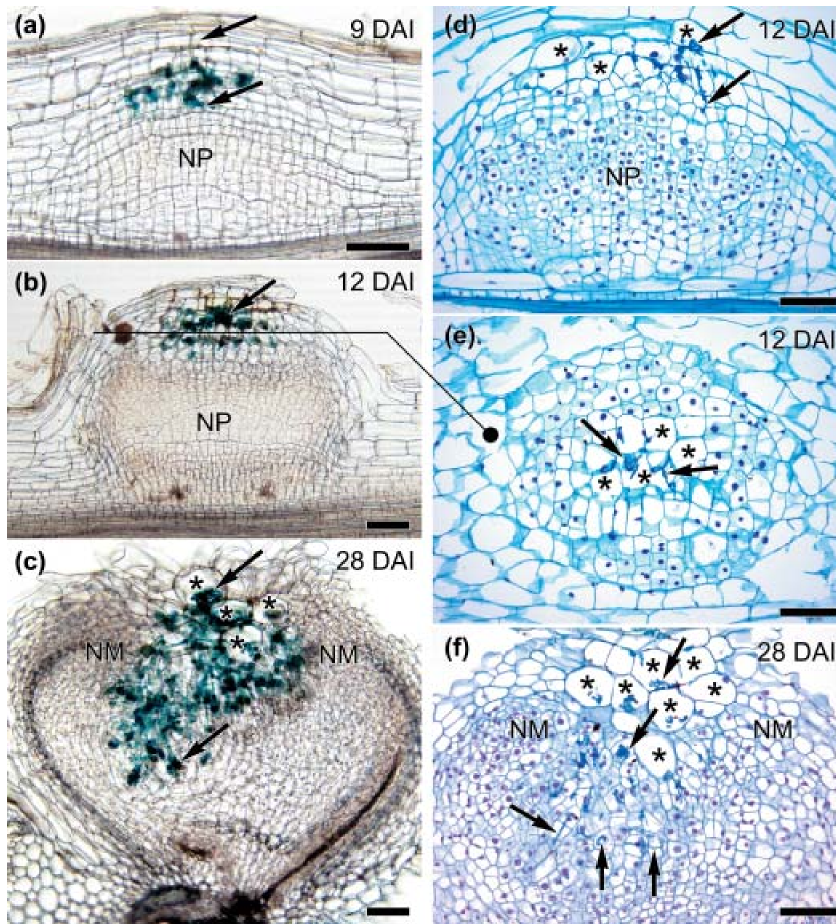

Fig. 3 Stages of root nodule development of the *Pisum sativum* mutants SGEFix^{-}-2 (*sym33*) (a–c) and RBT3 (*sym33*, *sym40*) (d–f). Panels (a) to (c) show longitudinal vibratome sections of roots and nodules, with GUS staining of bacteria in transcellular infection threads (ITs). Panels (d) and (f) show microtome longitudinal sections of nodules, and panel (e) a cross-section of a nodule, with Feulgen/alcian blue staining. (a) Nodule primordium (NP) and IT growth in the outer root cortex at 9 days after inoculation (DAI). ITs do not penetrate the proliferating cells of the NP. (b, d, e) Emerging young nodules at 12 DAI, with ITs forming only in the outer root cortex cells. The NP is not infected. Cells harbouring ITs (asterisk) can enlarge, but not divide. (c, f) A nonfixing nodule at 28 DAI. NP cells that continue proliferating are not penetrated by ITs and form a lateral nodule meristem (NM). Arrows indicate ITs. Asterisk, plant cells that have left the mitotic cycle. Bars, 100 μm.

(approximately 12 DAI) and consequently the majority of the cells in the apical zone of the nodule leave the mitotic cycle, undergo endoreduplication and become colonized by bacteria (Fig. 4c). As a result, the 'main' IT ('entrance' to the root) is situated centrally or slightly off-centre (Fig. 4c,e). It is important to note that, as in the wild-type, the 'main' IT crosses cell layers of the nearly fully developed NM, with the size of cells indicating that they are still in the mitotic cycle (Fig. 4f). In addition, the hypertrophic infection droplets described previously (Tsyganov *et al.*, 1998) can be easily observed in inner nodule cells of the mutant which have left the mitotic cycle (Fig. 4f).

Thus, it was found that, in the case of a mutation in the pea symbiotic gene *Sym40*, the early stages of co-ordinated cell proliferation of nodule primordium and IT development do not differ from those of wild-type plants. Similarly, the number of nodule primordia is equal to that found in the wild-type (Table 2). At later stages, ineffective nodules of the mutant SGEFix^{-}-1 (*sym40*) are characterized by a relatively short period of activity of the mature NM, that is, by a lack of NM persistence (the Nmp^{-} phenotype).

In addition, it was observed that root nodules of the double mutant RBT3 (*sym33*, *sym40*) showed the same phenotype as those of SGEFix^{-}-2 (*sym33*) plants (data not shown). However, in the double mutant, the first nodule primordia were observed only at 9 DAI (Table 2). Therefore the dynamics of nodule primordium formation is also affected in this double mutant line. At later stages (Table 2), the number of nodule primordia is higher than in the mutant SGEFix^{-}-2 (*sym33*) but lower than that observed for SGEFix^{-}-1 (*sym40*), so an intermediate/cumulative mutant phenotype was observed as a result of mutations in both pea genes *Sym33* and *Sym40*.

Discussion

Wild-type nodule morphogenesis

Various authors (e.g. Bond, 1948; Yang *et al.*, 1994; Timmers *et al.*, 1999) have demonstrated that, in indeterminate legume nodules, the development of the wild-type nodule starts with the resumption of cell proliferation in the pericycle and then in the inner root cortex. This results in the formation of the nodule primordium (NP). At the distal end of the NP, an apical NM develops that is 'uninfected', that is, free of ITs and cells containing intracellular bacteria. By contrast, in cells with determinate NMs, for example in *Phaseolus* and *Lotus*

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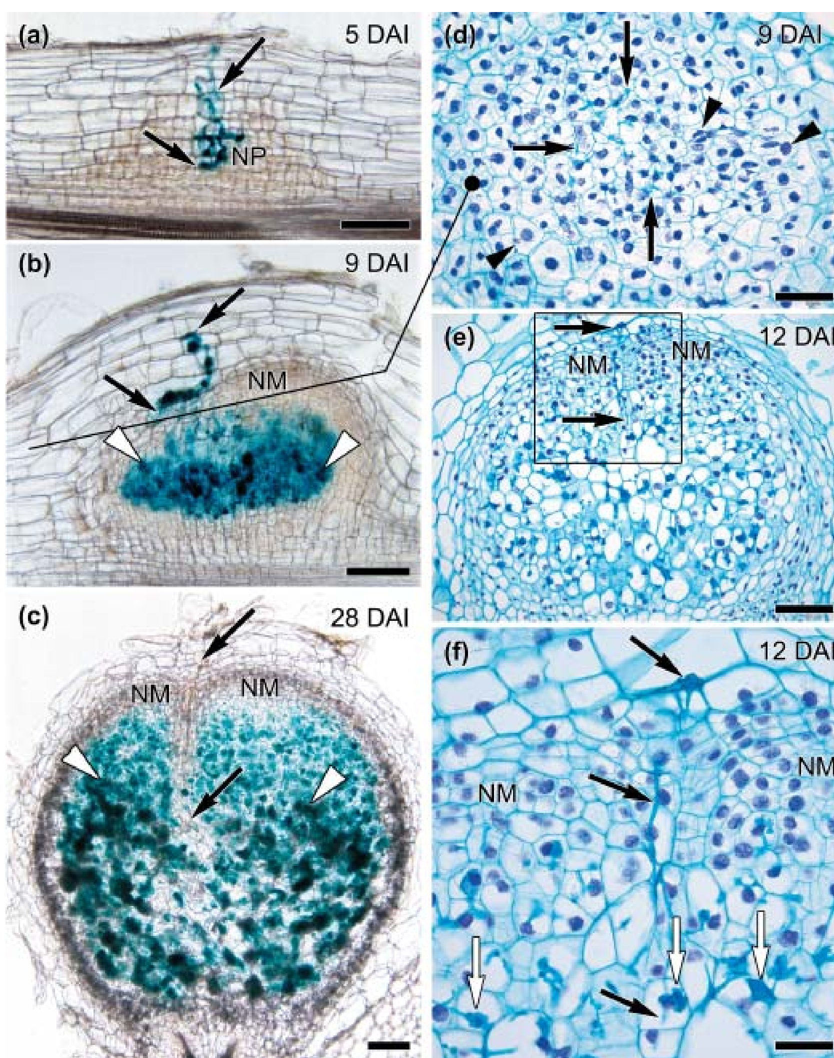

Fig. 4 Stages of root nodule development of the *Pisum sativum* mutant SGEFix$^-$-1 (*sym40*). (a–c) Longitudinal vibratome sections of roots and nodules, with GUS staining of bacteria in infection threads (ITs) and colonized nodule cells. (d–f) Microtome cross-section (d) and longitudinal sections (e, f) of nodules, with Feulgen/alcian blue staining. Panel (d) shows the plane of the cross-section marked with the line in (b). (a) Nodule primordium at 5 days after inoculation (DAI), (b, d) emerging young nodule at 9 DAI, (e, f) nonfixing nodules at 12 DAI and (c) nonfixing nodules at 28 DAI. Panel (f) shows a magnification of the area demarcated by a rectangle in (e). Black arrows indicate ITs; white arrows, hypertrophied infection droplets; black arrowheads, dividing plant cells; white arrowheads, plant cells with endocytosed bacteria. NP, nodule primordium; NM, nodule meristem. Bars: (a–c) 100 μm; (d, e) 50 μm; (f) 25 μm.

spp., the meristematic cells within the developing nodule are infected, in the sense that they harbour either ITs or released bacteria, or both (Brewin, 1991).

It has been demonstrated by genetic dissection that the developmental processes governing bacterial 'entry' and nodule organogenesis are closely co-ordinated but distinct (Tsyganov *et al.*, 2002). There are instances in which bacterial infection can occur in the absence of nodule organogenesis (Murray *et al.*, 2007), and, conversely, there are instances in which nodule organogenesis can occur in the absence of bacterial infection (Truchet *et al.*, 1989; Gleason *et al.*, 2006; Tirichine *et al.*, 2006). Furthermore, it has been postulated that the epidermal and cortical processes leading to nodule development must be closely coupled so that a nodule primordium occurs close to the site of bacterial infection in order to generate a nodule containing intracellular bacteria (Oldroyd & Downie, 2008).

In general, this model of nodule initiation has been confirmed in the present study, but with certain previously unobserved additions. By analysis of symbiotically defective pea mutants, it was discovered that IT development and plant cell proliferation are tightly coupled during the development of cylindrical nodules with apical uninfected meristems (indeterminate type). In order to develop a mature nodule with an active apical meristem, a group of dividing cells on the proximal face of the nodule primordium apparently have to be penetrated by an IT and undergo the consequent process of topological reorganisation. Therefore, a key condition for formation of the apical NM, discovered in this study and apparently absent from mutant lines SGEFix$^-$-2 (*sym33*) and RisFixA (*sym41*), is the ability of plant cells to form transcellular branching ITs without leaving the mitotic cycle (Figs 1g,h, 4d). These mutant lines also form abnormal lateral meristems instead of a normal apical NM.

At the transition from nodule primordium to a mature nodule, five histological zones were revealed in the present study (Fig. 1c):

- a zone of NM with uninfected distal cell layers providing nodule growth;

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• a proximal layer of NM with infected proliferating cells providing both nodule growth and propagation of ITs;
• the pathway of 'entrance' of the 'main' IT crossing plant cells which, based on their small size and presence of mitotic figures (Fig. 1f–h), are still in the mitotic cycle;
• the central zone of endoreduplicated plant cells containing intracellular bacteria taken up from ITs;
• peripheral tissues which were not analysed in detail in this study.

Based on the results of this study, it can be postulated that plant cells from the nodule primordium that are still in the mitotic cycle can start forming ITs when adjacent cortical cells have been invaded by the original IT. In the maturing nodule, cell-to-cell spreading of ITs through the nodule primordium culminates in the development of proximal layers of NM with similar properties (Fig. 1g,h), that is, they are proliferating cells harbouring ITs. It is clear from Fig. 1h that cells in the mitotic cycle can harbour ITs. This raises the topological issue of how a cell plate can be laid down at the same time as an IT, as the formation of both structures seems to involve components of the same cytoskeletal machinery. However, our data indicate that these two processes could probably occur simultaneously, or at least successively in a single host cell.

Furthermore, we postulate that proper functioning of the apical NM requires the continual presence of mitotic cells harbouring ITs. The existence of this interzone is thought to be a key condition for proper histological differentiation, development and functioning of the indeterminate nodule. It is interesting to note that, in spherical nodules with determinate meristems, the presence of infected proliferating cells has always been regarded as a key component of nodule development (Brewin, 1991). The molecular basis of the developmental processes governing cell proliferation and nodule development may be discovered when the genes corresponding to *Sym33* and *Sym41* have been cloned and sequenced. Furthermore, it is still unclear how the hormonal state of the tissues involved in nodule formation controls such a process, although it is obvious that hormone-mediated mechanisms of regulation could be involved (e.g. Ferguson *et al.*, 2005; Gonzalez-Rizzo *et al.*, 2006).

At the stage of the young nodule, the NM identity is being determined by means of at least two genetic factors (Voroshilova *et al.*, 2004; Combier *et al.*, 2006). Also, at this stage the ITs change the direction of growth: when the IT enters a nodule primordium the plant creates ITs in a different direction, with subsequent IT growth following behind the NM, colonizing proximal layers of it. Reversed growth of ITs was previously reported for indeterminate nodules of *P. sativum* (Libbenga & Harkes, 1973) and *Medicago truncatula* (Timmers *et al.*, 1999; Monahan-Giovanelli *et al.*, 2006). How these developmental processes, which are also observed in the mutants studied (data not shown), influence the formation of typical NM with proliferating cells harbouring ITs will be a subject of further studies.

Incidentally, in this paper we have tended not to use the term 'meristematic cells' because it is based on a topological definition rather than a functional definition. The term 'meristematic cells' simply implies a location within the region of the meristem. It could include cells in the mitotic cycle, cells undergoing endoreduplication, and even terminally differentiated cells. The term 'proliferating cells' is preferred because it provides a precise description for cells in the mitotic cycle. For example, nodule primordia contain 'proliferating cells' but these cannot be described as meristematic cells for the reasons described above.

Another new feature described in the present study is the position of the primary IT penetration site, relative to the apex of the mature nodule. It was shown in this study that, as a rule, the 'entrance' of an IT in a mature pea nodule is situated laterally (Fig. 1e). A similar position was found previously for pea nodules invaded by lipopolysaccharide-defective strains of *R. leguminosarum* (Perotto *et al.*, 1994). Accordingly, it is suggested that the growth of the nodule is usually supported by only one sector of the hemispheric NM of a young nodule.

Development of nodules in symbiotically defective mutants

In previous work, the term Nmd (for nodule meristem development) was used to describe mutants affected in NM formation (Tsyganov *et al.*, 2002), but the mutants analysed in the present study allow us to dissect this process still further. It is proposed that two new terms should be used to describe specific stages of NM development: (1) Anm, for development of the apical nodule meristem at the distal part of the nodule primordium, and (2) Nmp, for the subsequent stage of mature nodule meristem functioning (i.e. nodule meristem persistence).

It has been shown that, during interactions of the RisFixA (*sym41*) mutant with rhizobia, the nodule primordium consisting of the plant cells in the mitotic cycle is formed. However, proliferating nodule primordium cells are not penetrated by ITs. The only cells that are penetrated by ITs are cells in the five to six cell layers of the outer cortex of the root (Fig. 2c,d). As a result, the NM at the distal end of the nodule primordium does not arise (Anm^{-} phenotype). This can be explained by the observation that the mutation in the gene *Sym41* prevents IT formation in nodule primordium cells that are still in the mitotic cycle. Instead of forming an apical NM, an abnormal lateral NM grows around cells containing ITs (Fig. 2c), and nodule development is usually blocked at the stage of nodule emergence from the root as a result of the low 'mitotic potential' of this lateral NM.

A block at a similar stage of nodule development – 'emergence from the root' – was reported for Afghanistan peas carrying the *Sym2*^{A} allele (Degenhardt *et al.*, 1976; Geurts *et al.*, 1997) and for pea symbiotic mutants in the genes *Sym36* (Sagan *et al.*, 1994), *Sym37* and *Sym38* (Tsyganov *et al.*, 2002). It is

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Table 3 Sequential expression of phenotypes for regulatory *Pisum sativum* symbiotic genes during nodule formation

Subprogrammes of development	Phenotypic manifestation of mutations in the genes									
Differentiation of nodule tissues	Ccd$^-$			Anm$^-$	Npd$^-$	Anm$^-$ (?)	Anm$^-$	Nmp$^-$		
Colonization and symbiosome differentiation	Hac$^-$	Crh$^-$	Iti$^-$	Ith$^-$	Itr$^-$	?	Itn$^-$	Idd$^-$	Bad$^-$	Nop$^-$
Genes	*Sym8* *Sym9* *Sym10* *Sym19* *Sym30*	*Sym7*	*Sym14* *Sym35*	*Sym2* *Sym36* *Sym37* *Sym38*	*Sym5* *Sym16* *Sym34*	*Sym21* *Sym39*	***Sym33*** ***Sym41***	***Sym40***	*Sym31* *Sym32*	*Sym13* *Sym25* *Sym26* *Sym27* *Sym42*

Hac, root hair curling; Crh, colonization of curled root hair; Iti, infection thread growth initiation; Ith, infection thread differentiation inside root hair cells; Itr, infection thread differentiation in root tissue; Itn, infection thread formation in nodule primordium; Idd, infection droplet differentiation; Bad, bacteroid differentiation; Nop, nodule persistence; Ccd, cortical cell division; Npd, nodule primordium development; Anm, apical nodule meristem development; Nmp, nodule meristem persistence. Names of genes involved in this study are in bold.

important to note that, in the case of mutations in these genes, the growth of ITs was aborted in the root hair cells ('infection thread differentiation inside root hair cells' (Ith$^-$) phenotype) which led to a block in NM initiation. By contrast, ITs of the RisFixA (*sym41*) mutant develop fairly normally in root hairs and in the cells of the outer cortex but do not penetrate the nodule primordium. Moreover, occasionally, if the lateral NM persists, the mutant RisFixA (*sym41*) can form few nodules, but they are ineffective as a result of abnormal IT growth inside the young nodule and lack of bacteroid differentiation (Morzhina *et al.*, 2000).

In mutant SGEFix$^-$-2 (*sym33*), the development of nodule tissue is arrested at the same stage of meristem development as in the RisFixA (*sym41*) mutant (Anm$^-$ phenotype), but the development of mutant nodules is much more advanced because their lateral NMs have a higher potential for mitotic activity than those of RisFixA (*sym41*). Consequently, these nodules develop abnormally with the large plant cells in the central part filled with ITs and representing the area of penetration from an original IT (Fig. 3c). The same morphology was also observed for nodules that developed occasionally on the roots of the mutant RisFixA (*sym41*) (Fig. 3c). Therefore, in pea, apical NM formation is apparently (directly or indirectly) controlled by at least two genes: *Sym33* and *Sym41*. It is important to note that mutations in both these genes also cause impaired IT growth in comparatively mature nodules ('infection thread formation in nodule primordium' (Itn$^-$) phenotype) (Tsyganov *et al.*, 1998; Morzhina *et al.*, 2000; this study), demonstrating an inability to form ITs in cells in the mitotic cycle.

In the case of the pea mutant SGEFix$^-$-1 (*sym40*), the development of nodule tissue does not differ from that in the wild-type plants up to the stage of apical NM formation (Fig. 4a,b,d,e). However, the NM apparently stops functioning prematurely and the resultant nodules are much smaller than those of wild-type plants (Fig. 4c). Previously, it was shown that IT development of this mutant is blocked at the stage of infection droplet differentiation (Idd$^-$ phenotype) (Tsyganov *et al.*, 1998). Thus, the expression of the pea gene *Sym40* is not only important for IT functioning but also for supporting the persistence of the NM (Nmp$^-$ phenotype).

Sequential functioning of pea symbiotic genes with respect to nodule tissue development

In Table 3, the observations from the present study are set alongside other proposed schemes of development for nodules with indeterminate meristems, as suggested for early (Guinel & Sloetjes, 2000; Tsyganov *et al.*, 2002) and late developmental stages (Borisov *et al.*, 1997a,b; Tsyganov *et al.*, 1998; Morzhina *et al.*, 2000; Voroshilova *et al.*, 2001). Altogether, eight pea genes have been found to date to be co-operatively involved in NM formation. The genes *Sym2* (Degenhardt *et al.*, 1976; Geurts *et al.*, 1997), *Sym36* (Sagan *et al.*, 1994), *Sym37* and *Sym38* (Tsyganov *et al.*, 2002) control the colonization process, that is, the growth of ITs before they enter the nodule primordium (Tsyganov *et al.*, 2002), and mutations in them lead to the arrest of nodule primordium formation and NM formation. The genes *Sym21* (Markwei & LaRue, 1997) and *Sym39* (Sagan *et al.*, 1994) probably function in the programme of nodule tissue development.

In this research, it has been found that mutations in the pea genes *Sym33* and *Sym41* result in the inability of plant cells to form ITs while still in the mitotic cycle. In addition to abnormal growth of ITs in root cortical cells and young nodule tissue (Tsyganov *et al.*, 1998; Morzhina *et al.*, 2000; this study), the phenotype of such mutants is described as Anm$^-$ (Table 3). The gene *Sym40* functions at a later stage with respect to NM formation, at the stage of NM persistence (Nmp$^-$). The phenotype of the double mutant RBT3 (*sym33*, *sym40*) is basically similar to that of SGEFix$^-$-2 (*sym33*), indicating that *Sym33* is epistatic to *Sym40* with respect to its effects on nodule primordium development. However, these inferences are based on a single mutant allele at each locus and therefore the conclusions should be regarded as preliminary. Further

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characterization of these genes at the level of primary structure will reveal molecular mechanisms involved in the cellular function of these genes and how they control the interaction between cell proliferation and the formation of ITs.

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