Effect of Mutations in *Pisum sativum* L. Genes Blocking Different Stages of Nodule Development on the Expression of Late Symbiotic Genes in *Rhizobium leguminosarum* bv. *viciae*

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In this report, the expression of late symbiotic genes (*furN*, *fixN*, and *nifA*) of *Rhizobium leguminosarum* bv. *viciae* was studied in nodules of mutant pea lines blocked at four successive stages of nodule development. Bacterial gene expression was analyzed in situ with transcriptional *gusA* reporter gene fusions. As a control, a constitutively expressed *gusA* gene was included. In the nodules of Nop⁻ (nodule persistence) mutants (mutant in gene *sym13*), which had not yet exhibited signs of premature senescence, the expression patterns observed were identical to those in wild-type nodules. Normal expression of fusions also occurred in nodules defective at the infection droplet differentiation stage (mutant in gene *sym40*) in which bacteria are endocytosed, but infection threads and infection droplets are hypertrophied. In contrast, in Itn⁻ (infection thread formation inside the nodule tissue) mutants (mutant gene *sym33*), in which there is no endocytosis of bacteria, expression of the constitutive fusion was only in infection threads and no activity was shown for the other fusions. From this it can be concluded that functionality of the plant gene *Sym33*, i.e., bacterial endocytosis, is a prerequisite for the expression of late symbiotic genes in the microsymbiont. No morphologically distinct interzone II–III could be detected in nodules blocked at the bacteroid differentiation stage (mutants in gene *sym31*). The constitutive fusion was expressed equally throughout the nodule tissue (except for the meristem), and the activity of fusions to late symbiotic genes increased gradually with a maximal expression level at the base of the nodule. This is consistent with an altered oxygen barrier previously reported for these nodules. By including double mutants, earlier results on sequential functioning of gene pairs *sym33*–*sym40* and *sym31*–*sym13* could be confirmed and it could be demonstrated that the developmental epistasis found at the morphological level also is reflected in the expression pattern of late symbiotic genes in the microsymbiont.

Legume plants are able to enter nitrogen-fixing symbioses with bacteria of the genera *Rhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium*, and *Bradyrhizobium* species. This symbiotic association is a very useful model system for studying general mechanisms of plant–microbe interactions because the development of the symbiotic organs, the root nodules, depends on molecular signal exchange between the two partners and on the coordinate expression of bacterial and plant genes (Schultze and Kondorosi 1998).

The role of rhizobial genes in these interactions has been studied intensively and, as a result, a number of bacterial genes involved in nodulation and symbiotic nitrogen fixation have been identified. The group of *nod* genes is required for the synthesis and secretion of factors (lipochitooligo-saccharides) that induce plants to form nodules (Schultze and Kondorosi 1998). The structure of these molecules determines the host specificity of nodulation (Schultze and Kondorosi 1996). The group of *nif* genes is necessary for the synthesis of the nitrogenase enzyme complex, whereas *fix* genes are essential for bacteria to function in the symbiotic state (Fisher 1994; Fisher and Long 1992; Van Rhijn and Vanderleyden 1995).

On the plant side, approximately 100 symbiotic genes have been described during the last 15 years in more than 10 legume species (Benaben et al. 1995; Borisov et al. 1999; Caetano-Anollés and Gresshoff 1991; Devine and Kuykendall 1996; Duc 1995; Esser-Monning et al. 1995; Kiss et al. 1995; Pedalino and Kipe-Nolt 1993; Pedalino et al. 1993; Pemberton et al. 1990; Penmetsa and Cook 1997; Sagan and Duc 1996; Sagan et al. 1995; Szczygłowski et al. 1998). In *Pisum sativum* L., more than 40 symbiotic genes have been identified to date (Borisov et al. 1999). Many pea mutants have been characterized at the morphological level (Borisov et al. 1992; Guinel and Sloetjes 2000; Kneen and LaRue 1984; Kneen et al. 1990; Markwei and LaRue 1992; Morzhina et al. 2000; Novák et al. 1995; Postma et al. 1988a; Postma et al. 1988b; Postma et al. 1990; Sagan et al. 1993; Sagan et al. 1994; Tsyganov et al. 1998; Tsyganov et al. 1999) and a block at a specific stage of nodule development has been shown for all of them. The mutants were classified with the phenotypic codes suggested by Vincent (1980) and modified by Borisov

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et al. (1999), Caetano-Anollés and Gresshoff (1991), and Tsyganov et al. (1998). On this basis, the process of nodule development was dissected into eight developmental stages: root hair curling (Hac), infection thread growth initiation (Iti), infection thread growth in root hair cells (Ith), infection thread growth inside root cortical tissue (Itr), infection thread differentiation inside nodule tissue (Itn), infection droplet differentiation (Idd), bacteroid differentiation (Bad), and nodule persistence (Nop) (Borisov et al. 1999).

The present study was aimed at the analysis of the expression of late symbiotic genes in *Rhizobium leguminosarum* bv. *viciae* in nodules of pea mutants blocked at four of these developmental stages: Itn, Idd, Bad, and Nop. To our knowledge, this is the first investigation of this kind. As a representative for late symbiotic genes, we chose the *fixNOQP* operon, which encodes a bacteroid-specific cbb3-type oxidase system essential for nitrogen fixation (Schlüter et al. 1995; Schlüter et al. 1997). This operon is duplicated in strain VF39 (*fixNc* and *fixNd*), and both copies are induced under free-living microaerobic conditions and in the nitrogen-fixation zone (histological zone III) of the root nodule (Schlüter et al. 1997). In addition, *nifA-gusA* and *fmrN-gusA* fusions were included in the study. NifA is the central nitrogen-fixation activator protein (Morett and Buck 1989; Morett et al. 1988) and FmrN is a regulator, which is necessary for induction of the *fixNOQP* operons (Colonna-Romano et al. 1990; Patschkowski et al. 1996; Schlüter et al. 1992).

RESULTS

Expression of bacterial fusions in wild-type nodules.

All three fusions behaved identically. Only the results obtained with the *fixN-gusA* fusions are presented below.

Inoculated plants of all wild-type lines (SGE, Sparkle, and Sprint-2) formed pink nodules. Expression of the constitutive fusion (Fig. 1E) occurred in all histological zones defined by Vasse et al. (1990), except for zone I (meristem), which does not contain bacteria. Zone IV, the area in which the symbiotic compartments are degraded, was not observed at this stage of development. The expression pattern of the *fixN-gusA* fusion (Fig. 1J), i.e., induction in interzone II–III and strong expression in the symbiotic zone (III) confirmed results described earlier for *Vicia hirsuta* nodules (Schlüter et al. 1997).

Expression of bacterial fusions in nodules blocked at the Nop stage.

Plants of line E135f (*sym13*) form small, greenish nodules, which develop all histological zones but are characterized by premature appearance of zone IV, i.e., the early degradation of symbiotic structures (Kneen et al. 1990). Figure 1D and I shows the expression of the bacterial fusions in mutant nodules that do not show signs of premature senescence and, therefore, exhibits the same expression pattern as wild-type nodules (compare with Fig. 1E and J, respectively). In nodules that already had developed a degradation zone, the staining was mosaic-like in the basal part as a result of the destruction of symbiotic compartments and bacteroids (data not shown).

Expression of bacterial fusions in nodules blocked at the Bad stage.

Plants of line Sprint-2Fix⁻ (*sym31*) form white, nonfixing nodules. The plant cells are infected but contain morphologi-

cally undifferentiated “juvenile” bacteroids (Borisov et al. 1992; Borisov et al. 1997a; Sherrier et al. 1997). In these mutant nodules, a morphologically distinct interzone II–III was not observed. The constitutive fusion was expressed equally throughout the nodule tissue, except for the meristem (Fig. 1C). Activity of the *fixN-gusA* fusion increased gradually with a maximal expression level at the base of the nodule (Fig. 1H), indicating that the symbiotic state of bacteria in which this fusion is induced was reached later, i.e., in deeper nodule cell layers (compare with the wild-type nodule, Fig. 1J). The double mutant RBT (*sym13*, *sym31*) (Borisov et al. 1997a) showed the same expression pattern (data not shown).

Expression of bacterial fusions in mutant nodules blocked at the Idd stage.

Plant mutant line SGEFix⁻1 (*sym40*) is characterized by the formation of abnormal, hypertrophied infection threads and infection droplets and as a result by abnormal endocytosis (Tsyganov et al. 1998). The expression of the constitutive and *fixN-gusA* fusions is shown in Figure 1B and G, respectively. The expression of the respective bacterial fusions is more or less identical to that observed in wild-type nodules, i.e., the constitutive fusion is expressed in zone II (the infection zone) and in zone III cells, which contain endocytosed bacteria (Fig. 1B), the *fixN-gusA* fusion is induced in interzone II–III and expressed throughout zone III (Fig. 1G).

Expression of bacterial fusions in mutant nodules blocked at the Itn stage.

As described earlier (Tsyganov et al. 1998), plants of the mutant line SGEFix⁻2 (*sym33*) form two types of nodules: white ones in which the infection threads do not form infection droplets and thus no endocytosed bacteria can be observed (except for some few plant cells) and pinkish ones in which the majority of nodule cells contain endocytosed bacteria, which are subject to premature degradation. Both types of nodules also could be observed after inoculation with the bacterial fusion strains. In contrast, plants of line RisFixU, carrying the same mutation (*sym33*) and the double mutant RBT3 (*sym33*, *sym40*) only formed white nodules in which endocytosed bacteria could not be detected. Figure 1A shows the expression of the constitutive fusion in nodules of mutant line RisFixU (*sym33*) where only the infection threads are stained. Expression of the *fixN-gusA* fusion could not be observed in this mutant (Fig. 1F). In nodules of mutant SGEFix⁻2 (*sym33*), however, expression of this fusion also occurred in those few cells where endocytosed bacteria could be found (Fig. 1K).

DISCUSSION

In the present study, a new approach was undertaken to investigate the expression of genes in the microsymbiont as a function of certain genes in the macrosymbiont. For this purpose, *gusA* transcriptional fusions of late rhizobial symbiotic genes (*fixN*, *fmrN*, *nifA*) were analyzed in pea mutants blocked at four different stages of nodule development. The absence of *gusA* activity in nodules of plant lines mutated in gene *sym33*, which is characterized by the lack of endocytosed bacteria, documents that the expression of late rhizobial *sym* genes such as *fixN*, *fmrN*, and *nifA* depends on the func-

tion of the plant gene *Sym33*, which controls bacterial endocytosis into the plant cell cytoplasm. This is confirmed by their expression in rare nodule cells containing bacteroids (SGEFix⁻-2) (Fig. 1K).

In addition, it was shown that the phenotypic manifestation of a mutation in gene *sym33* depends on genetic background. For instance, the phenotype of mutant SGEFix⁻-2 (*sym33*) obtained on the background of line SGE is leaky (some cells

Fig. 1. Expression patterns of a constitutively expressed *gusA* gene (Tn5-*gusA*) and a *gusA* transcriptional reporter gene fusions to late bacterial symbiotic genes (represented by *fixN-gusA*) in pea mutant nodules blocked at different developmental stages. **A–E**, Constitutive *gusA* fusion. **F–K**, *fixN-gusA* fusion. **A and F**, RisFixU (*sym33*). **K**, SGEFix⁻-2 (*sym33*). **B and G**, SGEFix⁻-1 (*sym40*). **C and H**, Sprint-2Fix⁻ (*sym31*). **D and I**, E135f (*sym13*). **E and J**, Wild-type line SGE; I–III: nodule histological zones (marked where identifiable). Arrows: infection threads; arrowheads: cells with endocytosed bacteria in nodules of line SGEFix⁻-2 (*sym33*); bar = 0.4 mm.

have endocytosed bacteria), whereas mutant RisFixU (*sym33*) derived from *P. sativum* L. cv. Finale has a stable phenotype (complete lack of endocytosis). Further phenotypic analysis is necessary to uncover the function of this gene and the nature of both mutations.

The differences observed in the expression pattern of late bacterial *sym* genes in nodules of pea lines carrying mutations in gene *sym31* as compared with wild-type plants (absence of a morphologically distinct interzone II–III and a gradually increasing expression toward the base of the nodule) can be explained by the fact that, in this mutant, the oxygen barrier appears to be altered. Instead of an abrupt drop, Romanov et al. (1995) showed that a slow and continual decrease in the oxygen concentration and a higher minimal level in Sprint–2Fix[−] (*sym31*) mutant nodules so that it can be assumed that the oxygen concentration necessary for the induction of late rhizobial *sym* genes is reached gradually only in the deeper cell layers of the nodule. At the same time, this observation confirms that oxygen is one of the major physiological signals for the expression these genes in *Rhizobium* spp. (Soupeine et al. 1995). In addition, the fact that expression of late rhizobial symbiotic genes does occur in Sprint–2Fix[−] (*sym31*) mutant nodules, in bacteroids that do not differ from bacteria in infection threads (Borisov et al. 1997a) demonstrates that complete differentiation of bacteroids is not necessary for induction of this group of bacterial genes.

By including double mutants, we confirmed previous results on the sequential functioning of the gene pairs *sym33*–*sym40* (Borisov et al. 1997b) and *sym31*–*sym13* (Borisov et al. 1997a) and demonstrate that the developmental epistasis found with respect to the structure of nodule symbiotic compartments also is reflected in the expression pattern of late symbiotic genes in the microsymbiont.

MATERIALS AND METHODS

Plant material.

Pea (*P. sativum* L.) mutant lines used in this study and their parental lines are listed in Table 1.

Bacterial strains.

The transcriptional fusion strains used in this study were derived from *Rhizobium leguminosarum* bv. *viciae* VF39SM (Priefer 1989). For constitutive *gusA* expression, a derivative that contained a Tn5–*gusA* insertion in the genome that was

verified to be expressed constitutively was chosen. The *fixN*–*gusA* fusion strain carried a *gusA* cassette fused to the 5′ part of *fixNc* and integrated into the *fixNOQP* operon on plasmid C (Schlüter et al. 1997). The *fmrN*–*gusA* fusion was constructed with an *EcoRV* restriction site in the center of *fmrN* (T. Patschkowski, unpublished). The *nifA*–*gusA* fusion was constructed as described by Patschkowski et al. (1996).

Bacteria were grown on TY agar medium (Beringer 1974) at 28°C. If necessary, antibiotics were added at concentrations as described by Patschkowski et al. (1996).

Plant inoculation.

P. sativum seeds were surface sterilized with concentrated sulfuric acid (30 min at room temperature). Bacteria were taken from plates, suspended in water (10⁸ to 10⁹ cells per ml), and 1 ml per seed was added at the moment of sowing the seeds in sterile vermiculite as a substrate. Nitrogen-free nutrient solution was used as previously described (Borisov et al. 1997a).

Plants were grown in growth chambers under the following conditions (day/night): 16 and 8 h, 21 and 19°C, relative humidity of 75%, and photon irradiance of 490 mE/m² per s.

Histochemical staining and microscopy.

Nodules from mutant and wild-type plants were harvested 4 weeks after inoculation. They were sliced into 70 µm sections by means of a VT 1000S vibratome (Leica, Wezler, Germany). Sections were stained in 50 mM sodium–phosphate buffer, pH 7.0, containing 0.1% Triton, 5 mM K₃Fe(CN₆), 5 mM K₄Fe(CN₆), and 0.02% 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid. In the case of the constitutive *gusA* fusion, the nodules were stained for 30 min, whereas nodules formed by the other fusion strains were incubated overnight. After staining, samples were fixed in 1.25% glutaraldehyde in 0.1 M phosphate buffer for 1 h and subsequently rinsed in water.

For light microscopy, sections were transferred onto glass slides. Photographs were taken with a Om-4 camera (Olympus, Hamburg, Germany) mounted onto an Olympus BX50 light microscope.

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Table 1. Plant material used in the study

Lines	Phenotype	References
SGE	Wild-type line	Kosterin and Rozov 1993
SGEFix [−] –1 (<i>sym40</i>)	Abnormal (hypertrophied) infection droplet formation (Idd [−])	Tsyganov et al. 1998
SGEFix [−] –2 (<i>sym33</i>)	No endocytosis of bacteria (Itn [−]), leaky phenotype	Tsyganov et al. 1998
RBT3 (<i>sym33</i> , <i>sym40</i>)	No endocytosis of bacteria (Itn [−])	Borisov et al. 1997b
Sparkle	Wild-type line	Kneen et al. 1990
E135f (<i>sym13</i>)	Early degradation of symbiotic compartments (Nop [−])	Kneen et al. 1990
Sprint–2	Wild-type line	Berdnikov et al. 1989
Sprint–2Fix [−] (<i>sym31</i>)	No bacteroid differentiation (Bad [−])	Borisov et al. 1997a
RBT (<i>sym13</i> , <i>sym31</i>)	No bacteroid differentiation (Bad [−])	Borisov et al. 1997a
Finale	Wild-type line	Engvild 1987
RisFixU (<i>sym33</i>) ^a	No endocytosis of bacteria (Itn [−])	Engvild 1987; this study

^a Gene symbol for the gene identified in this mutant line was assigned according to G. Duc and M. Sagan, *personal communication*. Mutant phenotype was described during present study.

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