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Twelve non-nodulating pea (Pisum sativum L.) mutants were studied to identify the blocks in nodule tissue development. In nine, the reason for the lack of infection thread (IT) development was studied; this had been characterized previously in the other three mutants. With respect to IT development, mutants in gene sym7 are interrupted at the stage of colonization of the pocket in the curled root hair (Crh+ phenotype), mutants in genes sym37 and sym38 are blocked at the stage of IT growth in the root hair cell (Ih+ phenotype) and mutants in gene sym34 at the stage of IT growth inside root cortex cells (Ic+ phenotype). With respect to nodule tissue development, mutants in genes sym7, sym14 and sym35 were shown to be blocked at the stage of cortical cell divisions (Cc+ phenotype), mutants in gene sym34 are halted at the stage of nodule primordium (NP) development (Np+ phenotype) and mutants in genes sym37 and sym38 are arrested at the stage of nodule meristem development (Nmd+ phenotype). Thus, the sequential functioning of the genes sym37, sym38 and the gene Sym34 apparently differs in the infection process and during nodule tissue development. Based on these data, a scheme is suggested for the sequential functioning of early pea symbiotic genes in the two developmental processes: infection and nodule tissue formation.

Key words: Pea–Rhizobium symbiosis, Pisum sativum L., root hair curling, infection thread, nodule primordium, nodule development, nodulation mutants, symbiotic genes.

INTRODUCTION

The interaction between legume plants and nodule bacteria of the genera Rhizobium, Sinorhizobium, Bradyrhizobium, Mesorhizobium and Azorhizobium, collectively termed rhizobia, leads to formation of symbiotic nodules on plant roots. The first clearly distinguishable stage of such interaction is root hair curling. Bacteria caught inside a pocket formed by a curled root hair penetrate the cell using a special tunnel structure called an infection thread (IT) (Brewin, 1991). In parallel to IT development, cortical cell divisions occur and a nodule primordium (NP) is formed (Brewin, 1991). Bacteria in the IT reach the NP and are engulfed (endocytosed) into the nodule cell cytoplasm. Bacteria then differentiate to specific nitrogen-fixing forms called bacteroids (Mylona et al., 1995). The bacteroid surrounded by the peribacteroid membrane is termed a symbiosome (Roth and Stacey, 1989; Brewin et al., 1993). In parallel to bacteroid differentiation, the NP develops into a nodule with typical histological zoning (Vasse et al., 1990; Brewin, 1991).

These processes are controlled by both microsymbiont and macrosymbiont genes. To help identify these symbiotic genes, large numbers of mutants of both partners blocked at different stages of symbiosis development have been collected (reviewed in Caetano-Anollés and Gresshoff, 1991; Fischer, 1994; Pueppke, 1996; Borisov et al., 2000). To date, the most numerous collections of symbiotic mutants for a plant partner have been obtained for pea (Pisum sativum L.) using chemical and physical mutagens (reviewed in Borisov et al., 2000). Intensive genetic and phenotypic analyses of a large number of these mutants have allowed identification of more than 40 pea genes involved in development of the symbiosis (reviewed in Borisov et al., 2000).

Phenotypic characterization of pea mutants incapable of forming nodules (Nod+ phenotype) has revealed that at least five genes, Sym8, Sym9 (Markwein and LaRue, 1992), Sym10, Sym19 and Sym30 (Sagan et al., 1994; G. Duc and M. Sagan, pers. comm.), control the earliest stage of infection (mutations in these genes result in peas whose root hairs cannot curl; root hair curling, Hac+ phenotype). At least nine genes, Sym2 (Degenhardt et al., 1976), Sym5 (Guinel and LaRue, 1991), Sym14, Sym35 (Tsyganov et al., 1999), Sym16 (Guinel and Sloetjes, 2000), Sym33, Sym40 (Tsyganov et al., 1998), Sym36 (Sagan et al., 1994; G. Duc and M. Sagan, pers. comm.) and Sym41 (Morzhina et al., 2000; Tsyganov et al., 2001), are involved in the subsequent development of the IT. Besides studying the influence of mutations on the infection process, some mutants have been studied with respect to the block in nodule tissue development.
development. All Hac\(^{-}\) mutants analysed have been characterized by a block in cortical cell division (Ccd\(^{-}\) phenotype) (Markwei and LaRue, 1992; Sagan et al., 1994). It has also been shown that mutants in the genes sym5 (Guinel and LaRue, 1991), sym16 (Guinel and Sloetjes, 2000) and sym36 (Sagan et al., 1994; G. Duc and M. Sagan, pers. comm.) are arrested at different stages of NP development. The latest blocks of nodule development have been described for mutants in the genes sym21 (Markwei and LaRue, 1997) and sym39 (Sagan et al., 1994; G. Duc and M. Sagan, pers. comm.) at the stage of young nodule emergence.

In parallel to Nod\(^{-}\) mutants, a large set of mutants unable to fix nitrogen (Fix\(^{-}\)) has been characterized and classified according to the stage at which fixation is blocked (Kneen et al., 1990; Postma et al., 1990; Borisov et al., 1992, 1997; Novák et al., 1995; Tsyganov et al., 1998; Morzhina et al., 2000). As a result, the sequential functioning of late symbiotic genes has been suggested and partially confirmed using double mutant analysis (Borisov et al., 1997; Tsyganov et al., 1998; Morzhina et al., 2000; Voroshilova et al., 2001).

In this study, 12 new Nod\(^{-}\) mutants were analysed using light microscopy to determine the block in both the infection process and nodule tissue development caused by mutation. Genetic dissection of the initiation of symbiotic root nodule development in pea (Pisum sativum L.) was performed using data obtained in this study and those already published in the literature.

### MATERIALS AND METHODS

#### Plant material

Parental Pisum sativum L. ‘Finale’ and six non-nodulating mutants: RisNod1 (sym34), RisNod4 (sym37), RisNod8 (sym35), RisNod14 (sym7), RisNod23 (sym34), RisFixF (sym38) (Engvild, 1987) (kindly provided by Dr K. J. Engvild, Agricultural Research Department, Riso National Laboratory, Roskild, Denmark), as well as laboratory line SGE (Kosterin and Rozov, 1993); and six non-nodulating mutants: SGENod\(^{-}\)1 (sym35), SGENod\(^{-}\)2 (sym14), SGENod\(^{-}\)3 (sym35), SGENod\(^{-}\)4 (sym38) (Tsyganov et al., 1994, 1999), SGENod\(^{-}\)6 (sym7) (Tsyganov et al., 2000) and SGENod\(^{-}\)8 (sym38) (Tsyganov et al., 1994) were used in this study. Gene symbols for mutants induced on gene gusA, the roots were stained as described previously (Voroshilova et al., 2001) 28 DAI (at 9 DAI in the case of nodule developmental stages in wild-type lines). To identify nodule developmental stages, roots were sliced into 70 \(\mu\)m sections using a vibratome VT 1000 S (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) before staining for 1 h.

#### Microscopic analysis

All samples were examined under Opton Axiovert-35 (Opton Feintechnik GmbH, Oberkochen, Germany) and Olympus BX50 [Olympus Optical Co. (Europa) GMBH, Hamburg, Germany] light microscopes.

#### Infection process

The infection process in the initial line SGE and in the non-nodulating mutants SGENod\(^{-}\)1 (sym35), SGENod\(^{-}\)2 (sym14) and SGENod\(^{-}\)3 (sym35) have been studied previously (Tsyganov et al., 1999); in the present study these mutant lines were characterized with respect to nodule tissue development only.

In roots of wild-type plants of cultivar Finale, all early stages of infection were identified. After curling of root hairs, ITs developed, followed by infected NPa (Fig. 2C).

The mutants proved to be blocked at different stages of the infection process. In allelic mutants RisNod14 (sym7) and SGENod\(^{-}\)6 (sym7) the infection process was blocked at the earliest stage: after root hair curling but before colonization of the pocket of the curled root hair (Crh\(^{-}\)) (Fig. 1A). The percentage of deformed and curled root hairs in mutants RisNod14 (sym7) and SGENod\(^{-}\)6 (sym7) was...
two-fold higher than in the corresponding wild-type lines (Table 1).

In mutant RisNod8 (sym35) the infection process was interrupted at the next stage, namely after colonization of the pocket of the curled root hair but before initiation of the IT (Iti+ phenotype) (Fig. 1B). In addition, in mutant RisNod8 (sym35), the percentage of deformed and curled root hairs was much higher than in wild-type Finale plants, reaching 60% by 23 DAI, i.e. 20-times more than in the wild type (Table 1).
The percentage of deformed and curled root hairs in mutant RisNod4 (sym37) was two-fold higher than that in cultivar Finale (Table 1), and in allelic mutants RisFixF (sym38), SGENod−4 (sym38) and SGENod−8 (sym38) the percentage of deformed and curled root hairs was five-fold higher than in the corresponding wild-type genotypes (Table 1). In all these mutants the infection process is arrested at the stage of IT growth inside the root hair (It− phenotype). Generally, growth of the IT inside the root hair was a very rare phenomenon in mutant RisNod4 (sym37), and ITs were blocked (Fig 1C) immediately after they began to grow (occasionally, however, a few nodules appeared on the roots of some plants). In mutants RisFixF (sym38), SGENod−4 (sym38) and SGENod−8 (sym38), the percent-
age of ITs was ten- to 30-fold higher than in the wild type (Table 2), and they could be blocked in different parts of the root hair (Fig. 1D).

The allelic mutants RisNod1 (sym34) and RisNod23 (sym34) were blocked at the latest stage of the infection process identified in this study. The percentage of deformed and curled root hairs in both mutants was two- to three-fold higher than in cultivar Finale (Table 1). IT formation was delayed in mutants and IT frequency was lower than in wild-type plants (Table 2). In addition, all the observed ITs of mutant lines RisNod1 and RisNod23 were aborted in the first layers of the root cortex cells (Itr± phenotype) (Fig. 1E).

Nodule tissue development

In this study of nodule tissue development, two wild-type genotypes and 12 symbiotic mutants were investigated. In both wild-type genotypes, all early stages of nodule tissue development were observed. After initiation of cortical cell divisions (data not shown), the NPa were formed and infected (Fig. 2C). Analysis of mutants SGENod±-1 (sym35), SGENod±-2 (sym35), SGENod±-3 (sym35), SGENod±-6 (sym7), RisNod8 (sym35) and RisNod14 (sym7) did not reveal any signs of nodule tissue development including cortical cell divisions (Ccd± phenotype) (data not shown).

In the allelic mutants RisNod1 (sym34) and RisNod23 (sym34), cortical cell divisions occurred but stopped after several divisions. As a result, the morphologically differentiated NPa did not develop (Npd± phenotype) (Fig. 2A). Cell division events were also delayed and the number of divisions was much lower than in Finale (Table 3).

In the allelic mutants RisFixF (sym38), SGENod±-4 (sym38) and SGENod±-8 (sym38), and in mutant RisNod4 (sym37), NPa developed but they were non-infected (Fig. 2B). The subsequent differentiation of those NPa was not observed and nodule meristem was not formed, i.e. these mutants were blocked at the stage of nodule meristem development (Nmd± phenotype). The percentage of NPa formed by all the mutants was less than in wild-type plants (Table 3).

DISCUSSION

In this study, a new set of non-nodulating pea mutants was analysed to reveal the developmental blocks caused by the mutations, both with respect to cellular infection and nodule tissue development.

Mutants characterized with respect to the infection process

The earliest block of the infection process was observed in mutants RisNod14 (sym7) and SGENod±-6 (sym7) arrested at the stage of colonization of pockets in curled root hairs (Crh± phenotype). It has recently been shown that the other mutant in gene sym7, E69, is blocked at an even earlier stage: no curled root hairs have been observed and its phenotype is therefore Hac± (Walker et al., 2000). However, in this study, clear deformations such as shepherd’s crook, but lacking bacteria, have been observed in two independent allelic mutants in gene sym7 (Fig. 1A). Moreover, the percentage of deformed and curled root hairs was two-fold higher than in wild types of both mutants (Table 1). The observed differences in phenotypes between the mutant E69 (sym7) on the one hand, and the mutants RisNod14 (sym7) and SGENod±-6 (sym7) on the other hand, may be explained by differences in genetic background, growth conditions and rhizobial strain used for inoculation. No pea mutants arrested at the stage Crh have been described to date. Thus, in this study a new stage of the infection process in pea has been identified by mutational analysis. A similar block of infection has been described in Medicago truncatula in three allelic mutants in the gene hel (Catoira et al., 2001).

Mutant RisNod8 (sym35) was blocked at a subsequent stage of the infection process, namely during IT growth initiation (Iti± phenotype) (Fig. 1B). Several pea mutants, SGENod±-1 (sym35), SGENod±-3 (sym35) and SGENod±-2 (sym14), have previously been shown to be blocked at this stage (Tsyganov et al., 1999). Thus, in pea, at least two genes are involved in initiation of IT growth. Mutant RisNod8 (sym35) was characterized by highly inflated numbers of deformed and curled root hairs (Table 1). The other mutants in this locus induced on line SGE, SGENod±-1 (sym35) and SGENod±-3 (sym35), are also characterized by a much higher percentage of deformed and curled root hairs (Tsyganov et al., 1999). The increased number of deformed and curled root hairs appears to be a positive feedback reaction by the plant following unsuccessful infections. In this respect the phenotype of pea mutants in gene sym35 is very similar to that of mutants in the gene nin of Lotus japonicus (Regel.). K. Larsen, which are characterized by excessive curling of root hairs (Schauser et al., 1999). The last fact may be very interesting for identification of possible pea genes homologous to the nin gene of L. japonicus, which is the first legume symbiotic gene cloned to date (Schauser et al., 1999). The mutant RisNod4 in gene sym37 and three allelic mutants RisFixF, SGENod±-4 and SGENod±-8 in gene sym38 were arrested at the stage of IT growth in the root hair. However, in mutant RisNod4 (sym37), ITs were aborted close to the site of infection (Fig. 1C); whereas in mutants RisFixF (sym38), SGENod±-4 (sym38) and SGENod±-8 (sym38), ITs often reached the middle (Fig. 1D) or even the base of the root hair before their growth was interrupted. A similar phenotype was described for Afghanistan peas carrying the sym2A allele. This is characterized by the formation of rare ITs arrested in the epidermis following inoculation by incompatible European Rhizobium strains (Degenhardt et al., 1976; Geurts et al., 1997). In mutant DK24 (sym36) (G. Duc and M. Sagan pers. comm.), ITs also aborted inside the root hair (Sagan et al., 1994). [DK24 (sym36) was induced on cultivar Finale and initially designated as RisNod24 (Engvild, 1987) like other RisNod mutants described in this paper.] The increased percentage of ITs in mutants in gene sym38 (Table 3) appears to be an attempt by the plant to compensate for unsuccessful infection and nodule formation. It is therefore likely that gene Sym38, as well as the above-mentioned gene Sym35, is involved in the positive regulatory feedback mechanisms of the number of infections.
### Table 1. The dynamics of root hair deformations and curling in the original and mutant lines

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DAI</th>
<th>Finale</th>
<th>SGE</th>
<th>RisNod14 (sym7)</th>
<th>SGENod−6 (sym7)</th>
<th>RisNod1 (sym34)</th>
<th>RisNod23 (sym34)</th>
<th>RisNod8 (sym35)</th>
<th>RisNod4 (sym37)</th>
<th>RisFixF (sym38)</th>
<th>SGENod−4 (sym38)</th>
<th>SGENod−8 (sym38)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>4.5 ± 0.5</td>
<td>0</td>
<td>7.9 ± 1.2</td>
<td>4.0 ± 0.6</td>
<td>8.0 ± 0.6</td>
<td>7.8 ± 0.7</td>
<td>9.1 ± 0.5</td>
<td>9.9 ± 0.7</td>
<td>8.2 ± 0.5</td>
<td>6.4 ± 0.6</td>
<td>15.9 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>4.6 ± 0.4</td>
<td>3.9 ± 0.4</td>
<td>9.6 ± 0.6</td>
<td>3.0 ± 0.3</td>
<td>9.6 ± 0.4</td>
<td>9.8 ± 0.4</td>
<td>39.5 ± 3.4</td>
<td>10.5 ± 0.4</td>
<td>17.4 ± 0.7</td>
<td>17.0 ± 0.9</td>
<td>12.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5.1 ± 0.5</td>
<td>3.0 ± 0.3</td>
<td>8.3 ± 0.4</td>
<td>6.5 ± 0.5</td>
<td>13.9 ± 0.6</td>
<td>17.3 ± 0.9</td>
<td>72.3 ± 2.6</td>
<td>8.1 ± 0.5</td>
<td>23.8 ± 1.8</td>
<td>18.7 ± 1.5</td>
<td>16.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>3.0 ± 0.2</td>
<td>2.9 ± 0.3</td>
<td>11.1 ± 0.6</td>
<td>4.2 ± 0.5</td>
<td>10.6 ± 0.5</td>
<td>14.5 ± 1.0</td>
<td>60.5 ± 2.1</td>
<td>5.3 ± 0.4</td>
<td>21.9 ± 1.2</td>
<td>21.6 ± 0.9</td>
<td>17.6 ± 0.6</td>
</tr>
</tbody>
</table>

Data are the average percentages (± s.e.) of deformed and curled root hairs in the visual field of the microscope. DAI, days after inoculation. All the mutants differ significantly (P > 0.95) from wild-type lines except mutant SGENod±-6 (sym7) at 9 DAI. Allelic mutants RisNod1 (sym34) and RisNod23 (sym34) do not differ significantly from each other at 5 and 9 DAI but differ (P > 0.95) at 15 and 23 DAI. Allelic mutants SGENod±-4 (sym38) and SGENod−8 (sym38) differ significantly at 5 and 9 DAI (P > 0.95) but do not differ from each other at other days of analysis.

### Table 2. The dynamics of IT growth in wild-type and mutant lines

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DAI</th>
<th>Finale</th>
<th>SGE</th>
<th>RisNod1 (sym34)</th>
<th>RisNod23 (sym34)</th>
<th>RisFixF (sym38)</th>
<th>SGENod−4 (sym38)</th>
<th>SGENod−8 (sym38)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>9.5 ± 6.6</td>
<td>6.3 ± 1.5</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td></td>
<td>9</td>
<td>10.5 ± 5.1</td>
<td>5.4 ± 1.3</td>
<td>0</td>
<td>0</td>
<td>3.2 ± 1.1</td>
<td>33.5 ± 5.8</td>
<td>84.6 ± 8.7</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>8.5 ± 2.1</td>
<td>3.1 ± 0.9</td>
<td>5.8 ± 1.8</td>
<td>3.0 ± 1.1</td>
<td>85.0 ± 11.6</td>
<td>19.8 ± 2.8</td>
<td>107.0 ± 6.8</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>16.1 ± 2.5</td>
<td>4.4 ± 1.2</td>
<td>7.0 ± 1.7</td>
<td>4.6 ± 1.3</td>
<td>94.5 ± 7.2</td>
<td>86.6 ± 7.5</td>
<td>40.6 ± 4.1</td>
</tr>
</tbody>
</table>

Data represent the number (± s.e.) of ITs in 100 visual fields of the microscope. DAI, Days after inoculation. All mutants differ significantly (P > 0.95) from the corresponding wild-type lines except mutant RisNod1 (sym34) at 15 DAI. There are no statistically significant differences between allelic mutants RisNod1 (sym34) and RisNod23 (sym34). The allelic mutants SGENod−4 (sym38) and SGENod−8 (sym38) differ significantly at 9, 15 and 23 DAI (P > 0.95).

### Table 3. The dynamics of cortical cell divisions (*) and NP development in wild-type and mutant lines

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DAI</th>
<th>Finale</th>
<th>SGE</th>
<th>RisNod1 (sym34)*</th>
<th>RisNod23 (sym34)*</th>
<th>RisNod4 (sym37)</th>
<th>SGENod−4 (sym38)</th>
<th>SGENod−8 (sym38)</th>
<th>RisFixF (sym38)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
<td>32.2 ± 3.8</td>
<td>47.6 ± 4.6</td>
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<td>0</td>
<td>2.2 ± 1.1</td>
<td>6.9 ± 1.9</td>
<td>7.7 ± 2.2</td>
<td>6.6 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>-</td>
<td>1.2 ± 1.2</td>
<td>1.8 ± 1.0</td>
<td>6.8 ± 2.0</td>
<td>3.4 ± 1.5</td>
<td>7.9 ± 2.0</td>
<td>7.1 ± 1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>-</td>
<td>1.3 ± 0.9</td>
<td>9.4 ± 2.7</td>
<td>7.4 ± 2.1</td>
<td>9.5 ± 2.3</td>
<td>2.9 ± 1.4</td>
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</tbody>
</table>

Numbers (± s.e.) of primordia in 100 visual fields of the microscope. DAI, days after inoculation.

*The nitrogen-fixing nodules had already formed and analysis of NPa was not carried out. All mutants differ significantly (P > 0.95) from corresponding wild-type lines. The allelic mutants RisNod1 (sym34) and RisNod23 (sym34), and SGENod−4 (sym38) and SGENod−8 (sym38) do not differ significantly from each other [except at 28 DAI when cortical cell divisions were not observed in mutant RisNod1 (sym34) but were observed in mutant RisNod23 (sym34)].
Thus, in pea, at least four genes control IT growth in the root hair, from the pocket of the curled hair to its base. It is interesting to note that similar blocks in IT growth in root epidermal cells have been observed for *Sinorhizobium*.
melliloti mutants, which are characterized by different abnormalities in sucrohexaose synthesis (Cheng and Walker, 1998). It is therefore possible that all the abovementioned genes, including Sym37 and Sym38, characterized in this study, are involved in the regulation of plant-bacterial surface interactions.

Two allelic mutants RisNod1 (sym34) and RisNod23 (sym34) were blocked at the latest stage of the infection process, during IT growth in root cortical cells (Iti<sup>−</sup> phenotype). A similar phenotype, i.e. arrest of the infection process in cortical cells, has been described previously in pea for mutant E2 in gene sym5 (Guinel and LaRue, 1991) and mutant R50 in gene sym16 (Guinel and Sloetjes, 2000). Thus, in pea, there are at least three genes controlling IT development inside the root cortex. A mutant defective at a similar stage has also been described for Lotus japonicus (Schauser et al., 1998).

Mutants characterized with respect to nodule development

In this study, mutants were also characterized with respect to the stage of nodule tissue development blocked by mutations. It was shown that two allelic mutants (SGENod<sup>−6</sup> and RisNod14) in the gene sym7, one mutant (SGENod<sup>−2</sup>) in the gene sym14, and three allelic mutants (SGENod<sup>−1</sup>, SGENod<sup>−3</sup> and RisNod8) in the gene sym35 lack any signs of nodule tissue development, including cortical cell divisions (Ccd<sup>−</sup> phenotype), but do show characteristic root hair curling (Hac<sup>−</sup> phenotype). The Ccd<sup>−</sup> phenotype had previously been described for pea mutants in genes sym8, sym9, sym10, sym19 and sym30, which were also impaired in the earlier stage of infection, i.e. Hac<sup>−</sup> phenotype (Markwe and LaRue, 1992; Sagan et al., 1994; G. Duc and M. Sagan, pers. comm.). Thus, in pea, the expression of at least eight genes is a prerequisite for the induction of cortical cell divisions, but cortical cell divisions are not a prerequisite for root hair curling and subsequent root hair colonization, as demonstrated in this study. Mutants unable to initiate cortical cell divisions have been described previously in different legumes: MnNC-1008 (NN) (nn<sub>1</sub>, nn<sub>2</sub>) in Medicago sativa L. (Dudley and Long, 1989); PM233B (nn<sub>1</sub>) in Cicer arietinum L. (Matthews and Davis, 1990); nin and Ljsym4 in Lotus japonicus (Regel.) K. Larsen (Schauser et al., 1999; Bonfante et al., 2000); and dmi1, dmi2, dmi3 and nsp in Medicago truncatula (Catoira et al., 2000). With the exception of the mutant in the nin gene (Schauser et al., 1999), all these mutants are also characterized by their inability to form curled root hairs.

In pea mutants in the gene sym34, initial cortical cell divisions occur, but they stop very quickly and an NP is not formed (Npd<sup>−</sup> phenotype) (Fig. 2A). Previously, it has been shown that mutants E2 in the gene sym5 (Guinel and LaRue, 1991) and R50 in the gene sym16 (Guinel and Sloetjes, 2000) are blocked at the stage of NP development. However, mutation in gene sym16 blocks NP development after formation of an initial ‘flat’ primordium (Guinel and Sloetjes, 2000). Because all these mutations lead to a block in NP formation, these three mutants have been combined into one group in the final scheme of sequential functioning of early pea symbiotic genes (Fig. 3). It is interesting to note that in Medicago truncatula mutants in gene hel blocked at a similar stage, i.e. after initiation of cortical cell divisions, the infection process is blocked before colonization of the curled root hair (Catoira et al., 2001).

In mutants in genes sym37 and sym38, an NP is formed but these mutants are arrested at the stage of nodule meristem development (Nmd<sup>−</sup> phenotype) (Fig. 2B). A similar phenotype was described for Afghanistan peas carrying the sym2<sup>−</sup> allele (Degenhardt et al., 1976; Geurts et al., 1997) and for mutant DK24 (sym36) (Sagan et al., 1994).

Thus, in pea, at least three genes (Sym5, Sym16 and Sym34) control NP development, whereas the expression of four other genes (Sym2, Sym36, Sym37 and Sym38) is required for subsequent nodule meristem development.

Sequential functioning of early symbiotic genes

The phenotypic characterization of a large set of non-nodulating pea mutants carried out in the present study allowed us to classify the symbiotic genes according to the stage of nodule development that they control. In addition, a scheme of sequential functioning of early pea symbiotic genes is suggested based on experimental data obtained in this study and data already published in the literature (Fig. 3).

It is interesting to note that mutations in genes sym2, sym36, sym37 and sym38 block IT development earlier than do mutations in genes sym5, sym16 and sym34. However, mutations in the former genes cause a block in nodule tissue development later than mutations in the latter genes (Fig. 3). These observations lead us to suggest that the genes are involved in two somewhat different, but parallel, programmes of nodule development: the infection process and nodule tissue development. The former group of genes (Sym2, Sym36, Sym37 and Sym38) controls IT development, whereas the second group (Sym5, Sym16 and Sym34) is involved in the control of nodule tissue development. To date, no pea mutants with the phenotype Iti<sup>−</sup>Ccd<sup>−</sup> or Iti<sup>−</sup>Ccd<sup>−</sup> have been identified. It seems that in pea, IT growth cannot be initiated before initial cortical cell divisions occur, and vice versa. However, it has been shown that in pea (Libbenha and Harke, 1973), as well as in Medicago (Dudley et al., 1987; Timmers et al., 1999; Catoira et al., 2001), cortical cell divisions precede the initiation of infection. Thus, it is thought that in mutants in genes sym7, sym14 and sym35, the interruption of infection is a result of the inability of those mutants to initiate cortical cell divisions.

In this study, we have identified three possible checkpoints for the programme of infection and one checkpoint for the programme of nodule tissue development. In the case of abortion of nodule tissue development at the first stage, i.e. cortical cell division (Ccd<sup>−</sup>), the infection process may be stopped at two stages: (1) after curling of root hairs but before colonization of curled root hairs (Crh<sup>−</sup>), as in the case of mutations in gene sym7; and (2) after colonization of curled root hairs but before initiation of IT growth (Iti<sup>−</sup>), as in the case of mutations in genes sym14 and sym35. Thus, during the period of initial cortical cell divisions, there are
two checkpoints for the infection programme. The existence of two checkpoints can explain differences which could be observed in blocks of cortical cell divisions caused by mutations in gene \( \text{sym7} \) on the one hand, and genes \( \text{sym14} \) and \( \text{sym35} \) on the other. For example, it is possible that in mutants in genes \( \text{sym14} \) and \( \text{sym35} \) some cell divisions do occur in the pericycle, similar to those described for \textit{Medicago} (Timmers et al., 1999); more refined morphological analysis of pea mutants might reveal these differences.

The third checkpoint for the infection programme is NP development. Up to now, two blocks of NP development have been identified in pea: after several cortical cell divisions in the case of mutations in genes \( \text{sym5} \) (Guinel and LaRue, 1991) and \( \text{sym34} \) (this study), and after formation of a ‘flat’ primordium in the case of a mutation in gene \( \text{sym16} \) (Guinel and Sloetjes, 2000). At this third checkpoint, the infection process is interrupted at sequential stages of IT growth inside the root cortex: mutation in gene \( \text{sym16} \) leads to a later block of IT growth inside the root cortex than do mutations in genes \( \text{sym5} \) and \( \text{sym34} \).

It seems that the only checkpoint for nodule tissue development identified to date involves IT growth in the epidermis. In cases where IT growth in the root hair is abolished, nodule tissue development is aborted after NP development and before nodule meristem formation. However, in the case of successful infections in plants homozygous in the \( \text{sym24} \) allele (Geurts et al., 1997) and mutant RisNod4 (\( \text{sym37} \)) (this study) that do occur occasionally, normal nitrogen-fixing nodules are formed. This situation somewhat contradicts results obtained in \textit{Medicago} where the meristem is formed even in the case of unsuccessful IT growth (Leigh et al., 1985; Yang et al., 1992; Timmers et al., 1999). These differences may be considered to be due to differences in the mechanisms of plant control over nodule development in different species. Such differences have been reported previously for \textit{Medicago} and \textit{Pisum} (Timmers et al., 1999). In these two species, different mechanisms of nodule development, especially in meristem development, may be supported by the fact that pseudonodules (nodule-like structures that form on roots without inoculation) have been reported in \textit{Medicago} (Truchet et al., 1989), but not in \textit{Pisum}. It is obvious that our suggested classification based on the logic of formal genetic analysis is relative. However, it may be very useful in future molecular-genetic studies, which are necessary to clarify the genetic mechanisms involved in nodule formation. For instance, the analysis of changes in intracellular calcium in root hairs as a response to Nod factor application has allowed refined genetic dissection of the earliest stage of the infection process, namely Hac in pea–Rhizobium symbiosis, and good candidates for the Nod factor receptor have been identified among the pea genes analysed (Walker et al., 2000).

Here we have summarized the results of studies aimed at the genetic dissection of the initiation of nodule formation in pea–Rhizobium symbiosis, a quarter of a century after the first report of pea plants showing abnormalities in IT growth in root hairs (Degenhardt et al., 1976). \textit{Pisum sativum} is the first legume plant to date for which refined and detailed genetic dissection of the early (Guinel and LaRue, 1991; Markwei and LaRue, 1992, 1997; Borisov et al., 1994; Sagan et al., 1994; Geurts et al., 1997; Tsyganov et al., 1999; Guinel and Sloetjes, 2000; this study) as well as late stages (Kneen et al., 1990; Postma et al., 1990; Borisov et al., 1992, 1997; Sagan et al., 1993; Tsyganov et al., 1998; Morzhina et al., 2000) has been performed. This genetic dissection has revealed the sequence of stages of symbiosis development controlled by pea symbiotic genes and has demonstrated a strong plant genetic control over nodule development during pea–Rhizobium symbiosis.

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