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A. Schneider · S.A. Walker · M. Sagan · G. Duc
T.H.N. Ellis · J.A. Downie

Mapping of the nodulation loci *sym9* and *sym10* of pea (*Pisum sativum* L.)

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Abstract Several mutants defective in the nodulation process during rhizobial or endomycorrhizal endosymbiosis of pea have been identified previously. We have integrated the map positions of two such nodulation mutations, *sym9* and *sym10*, into the molecular map of pea by applying molecular-marker techniques combined with bulked segregant analysis (BSA). Lines P2 and P54 were found to carry alleles of *sym9*, line P56 carried an allele of *sym10*. F2 populations were derived from crosses of P2, P54 and P56, to JI281 and JI15, two of the parental lines that have been used previously to generate a molecular map of pea. *sym9* was located on linkage group IV by AFLP-BSA analysis and subsequently mapped by RFLP in both F2 populations, P2 × JI281 and P54 × JI281. RFLP-BSA analysis was applied to assign *sym10* to linkage group I. The RFLP marker locus, *chs2*, cosegregates with *sym10* in the F2 population of P56 × JI15.

Keywords *Pisum sativum* · Legume · Nodulation · Mapping · *Rhizobium*

Introduction

When infected with *Rhizobium leguminosarum* bv *viciae*, the pea root develops a new plant organ, the root nodule. Early events of the nodulation process comprise root hair-curling and deformation; later events are infection thread development and nodule initiation (Schultze and Kondorosi 1998; Downie and Walker 1999).

An important step for the molecular analysis of the nodulation process is the identification of the plant genes involved. A series of mutants defective in this process has been obtained in the course of several mutagenesis programs (Engvild et al. 1987; Sagan et al. 1994; Kozik et al. 1996 and references therein). Several nodulation-defective loci have been identified, but only a few of them show a complete lack of nodules (Nod$^-$): *sym7*, *8*, *9*, *10*, *11*, *14* and *19*. Mutations at four loci (*sym8*, *9*, *10* and *19*) affect also root-hair deformation and all subsequent events, and three of them (*sym8*, *10* and *19*) are in addition defective for Nod-factor-induced changes in intracellular calcium concentration (calcium spiking) in root hairs (Walker et al. 2000). The mutation at *sym19* prevents the pea root from establishing endomycorrhizal symbioses; its precise map position on the molecular map (Hall et al. 1997) was determined by a combined AFLP-RFLP analysis (Schneider et al. 1999).

The precise map positions of the remaining nodulation loci have to be determined. The use of different pea populations for mapping purposes as well as the dearth of "anchor" loci (Weeden et al. 1996 and references therein) has hampered efforts to integrate the map positions of nodulation mutants into one single reference map. Approximate map positions on a genetic map of pea were established for *sym7* (linkage group III), *sym8* (linkage group VI), *sym9* (linkage group IV A), *sym10* (linkage group I B), *sym11* (linkage group VII) and *sym14* (linkage group II and I A) (Weeden et al. 1996).

Here we report on the integration of the map positions of *sym9* and *sym10* into the molecular map established by Hall et al. (1997) by applying amplified fragment length polymorphism – bulked, segregant analysis (AFLP-BSA) and restriction fragment length polymorphism – bulked segregant analysis (RFLP-BSA) for general assignment of loci to linkage groups, followed by RFLP- and AFLP-analysis of individuals for fine mapping.

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A. Schneider (✉) · S.A. Walker · T.H.N. Ellis · J.A. Downie
John Innes Centre, Norwich Research Park, Colney,
Norwich NR4 7UH, UK
e-mail: Anja.Schneider@uni-koeln.de
Tel.: +49-221-470-6596, Fax: +49-221-470-5039

M. Sagan · G. Duc
INRA Dijon, BV 1540, F-21034 Dijon Cedex, France

Present address:
A. Schneider, Botanisches Institut, Universität zu Köln,
Gyrhofstrasse 15, D-50931 Köln, Germany

Materials and methods

Plant material

Pisum sativum cv Frisson line P2 (Duc and Messager 1989; Sagan et al. 1994) was crossed to *P. sativum* JI281 and JI15 (Ellis et al. 1992; Hall et al. 1997), and the F2 progenies were grown in a greenhouse under nitrogen limitation and monitored for nodulation. Line P54 of *P. sativum* cv Frisson was crossed to *P. sativum* JI281, and P56 to JI15. F2 progenies were propagated under the same conditions. The cross P2 × JI281 resulted in 126 F2 plants of which 31 were Nod–. These 31 Nod– plants were grouped into three bulks of 10, 10 and 11 plants, respectively. Two control bulks, containing ten WT plants each, were generated. Plant material was collected separately, frozen in liquid nitrogen and stored until further use. The cross P2 × JI15 resulted in 138 F2 plants. From these, the 37 Nod– plants were bulked in three groups of ten and one of seven plants. Also a WT bulk with ten plants was generated. The cross P54 × JI281 resulted in 123 F2 plants harboring 37 Nod– individuals. The cross P56 × JI15 yielded 150 F2 plants containing 39 Nod– plants, which were grouped into three bulks of 10, 10 and 9 plants. In addition, one bulk containing ten wild-type plants was generated.

AFLP marker analysis

To determine the map position of *sym9*, the AFLP marker system adopted for pea by Lu et al. (1996) and Hall et al. (1997) was used. Genomic DNA was extracted as described (Ellis et al. 1998), and digested and ligated to *Pst*I and *Mse*I adapters as described (Lu et al. 1996; Hall et al. 1997). After selection of biotinylated DNA fragments, PCR was performed using primers PstA (5′-GACTGCGTACATGCAGCC-3′) and Mse5 (5′-GATGAGTCCTGAGTAATAT-3′) according to Lu et al. (1996) and Hall et al. (1997). PCR products were fractionated on a 4.5% (w/v) polyacrylamide gel, exposed to a phospho-image plate and the image was recorded using a Basis 1000 computer.

RFLP analysis

Forty micrograms of genomic DNA were digested with *Eco*RI, fractionated on a 0.8% (w/v) agarose gel, transferred to nylon membranes, hybridized and washed as described (Schneider et al. 1999). The peasqua-, cDNA 243-, cDNA 194/2-, chs2- and chs1-probes (Hall et al. 1997) were prepared as described (Ellis et al. 1992). The filters were exposed to a phospho-image plate and the image recorded using a Basis 1000 computer.

Mapping

AFLP and RFLP data were analyzed using the MAPMAKER (Lander et al. 1987; UNIX version /EXP3.0b) program. The allelic state of AFLP and RFLP bands was controlled independently twice.

Results and discussion

Complementation analysis of *sym9* lines

The mutant pea lines P2, P54 and P53 are unable to form nodules and the mutations responsible were reported to fall into two genetic complementation groups, P2/P53 and P54 (Duc and Messager 1989). However, when complementation analyses were repeated with at least nine independent crosses for each of the six combinations (P2 × P53; P2 × P54; P54 × P2; P54 × P53; P53 × P2; P53 × P2), the data were consistent with P2, P54 and P53 all being in one complementation group.

To determine if the corresponding mutation is allelic to any of the known nodulation loci, P54 was crossed to the tester lines E2 (*sym5*), E69 (*sym7*), R25 (*sym8*), R72 (*sym9*), N15 (*sym10*), N24 (*sym11*), E135 N (*sym14*), NEU5 (*sym19*) and R30 (*sym20*). At least three individuals from at least two independent crosses were tested. All crosses resulted in F1 progenies that were Nod+, with the exception of P54 × R72, of which the F1 progenies were all Nod–. Subsequently, P2 and P53 were also crossed to R72 and again the progenies were all Nod–, demonstrating that P2, P53 and P54 carry alleles of *sym9*.

AFLP-bulked segregant analysis for *sym9*

To determine the map position of *sym9* 126 F2-plants of cross P2 × JI281 were analyzed. Thirty one plants were Nod– and the leaf tissue of these was combined into three bulks. The DNA of these bulks and of two WT-bulks was subjected to AFLP analysis. Since wild-type bulks contained also heterozygous plants, only those bands present in the WT parent, but absent in the mutant parent, were informative. Therefore the number of informative bands detected by this type of analysis is only 50%, compared to the number of polymorphic bands obtained by using individual plants.

The primer combination PstA/Mse5 yielded three informative polymorphic fragments in P2 × JI281. The fragments A5/16, A5/14 and A5/11 derived from JI281 co-segregated with the mutant phenotype (Fig. 1A). The corresponding loci, *A5/16*, *A5/14* and *A5/11*, were previously mapped onto the molecular map to linkage group IV (Hall et al. 1997). Therefore, *sym9* from line P2 is located on linkage group IV, confirming the approximate localization described by Weeden et al. (1996).

Molecular markers detecting loci nearby the positions of *A5/16*, *A5/14* and *A5/11* in the molecular map, such as the RFLP probe 'peasqua' (Hall et al. 1997), were useful to confirm the localization of *sym9* in the mapping population used. The peasqua-probe detects a polymorphism between P2 and JI281 (Fig. 1B). The wild-type bulks inherited both two parental *peasqua* alleles, whereas the mutant bulks inherited mainly the allele derived from the mutant parent P2. However, a faint signal is detectable in mutant bulk 1, indicating a few recombination events present between *peasqua* and *sym9*.

The map position of *sym9* was confirmed by crossing P2 to JI15, another line that has been used to generate the molecular pea map. The peasqua-probe detected again a polymorphism between the two parents (Fig. 1C). Only the P2-derived allele was identified in the mutant bulks indicating the absence of recombination events between *peasqua* and *sym9* in this cross.

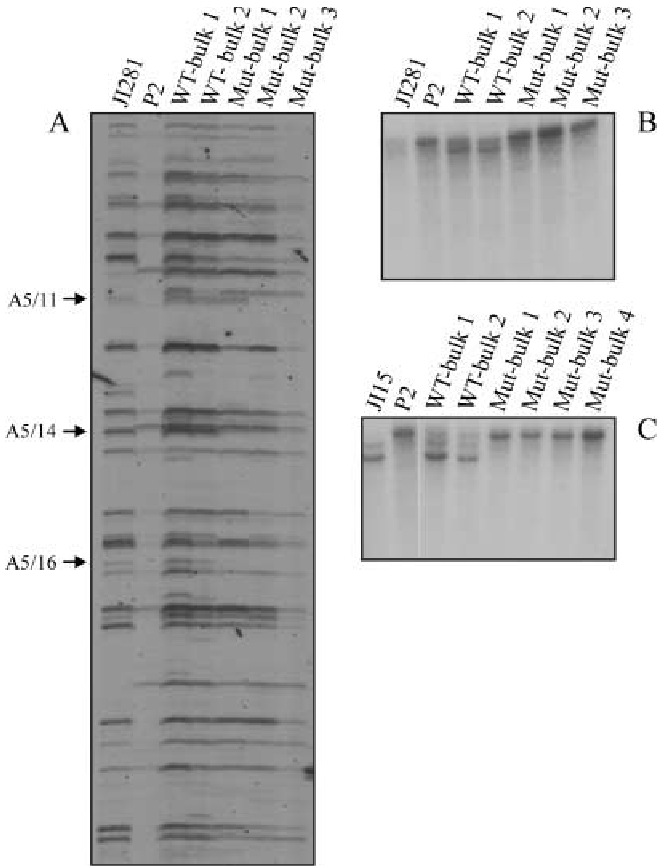

Fig. 1A–C BSA analysis for *sym9*. **A** Co-segregation of *sym9* with the AFLP loci *A5/11, A5/14* and *A5/16*. The polymorphic bands *A5/11*, *A5/14* and *A5/16* are indicated by *arrows*. They are present in the parental line JI281 (*WT*), absent from P2 (*sym9*) and from the mutant bulks except for *A5/11*, which is present in mutant bulk 1. This indicates at least one recombination event between *sym9* and *A5/11* within this bulk. **B** BSA-RFLP analysis for *sym9*. The fragment pattern for the 'peasqua' probe of the parental lines JI281, P2, two WT bulks and three mutant bulks is shown. Note that mutant bulk 1, besides the upper band derived from the mutant parent P2, also contains a faint lower band derived from JI281. **C** DNA polymorphisms were detected as described in (**C**) for parents JI15 and P2, two WT and four mutant bulks. Note that in this cross no recombination event between *peasqua* and *sym9* was detected in the bulks

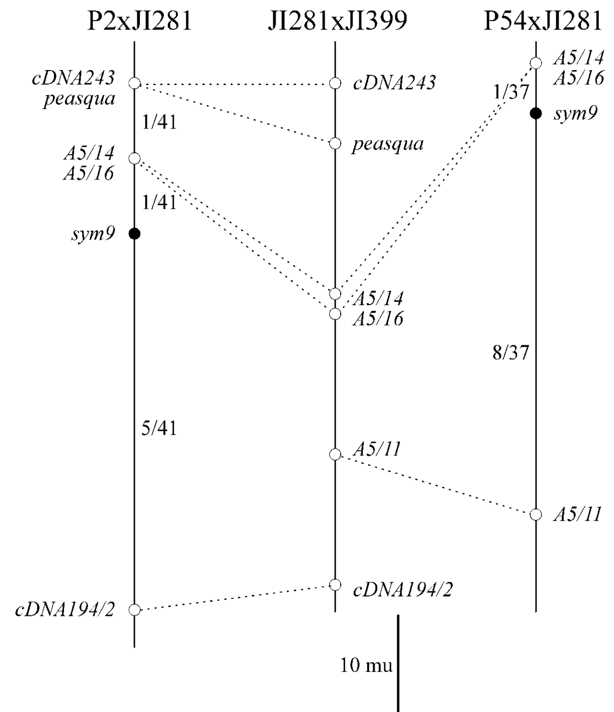

Fig. 2 Map position of *sym9* on linkage group IV. Distances between marker loci obtained by the cross JI281 × JI399 are given as map units (*mu*) according to Hall et al. (1997), while distances in the crosses P2 × JI281 and P54 × JI281 are given as the ratio of the number of recombinants and the total number of plants analyzed

Fine-mapping of *sym9*

We examined the individual plants of all three mutant bulks (31 plant lines) and of one WT bulk (ten plants) of the cross P2 × JI281 using the cDNA markers 243 and 194/2 (Hall et al. 1997) that flank the *peasqua* region on linkage group IV (Fig. 2). Seven individuals were identified with recombination events between *peasqua* and *cDNA194/2*, all of them were Nod^{-}.

Lines with recombination events in this region are listed in Table 1. Two individuals (nos. 129 and 133) inherited both parental alleles of *peasqua* and *cDNA243*, and five individuals inherited both parental alleles of *cDNA194/2* (nos. 51, 59, 9, 89 and 96). Thus, all seven individuals were heterozygous for either *peasqua/cDNA243* or *cDNA194/2*.

DNA of lines 129 and 133 containing also the JI281-derived *peasqua* allele was originally used for mutant bulk 1, thus giving rise to the faint band visible in the BSA-RFLP analysis (see Fig. 1B). This demonstrates that the sensitivity of this analysis was sufficient to detect as few as two recombination events within a bulk of ten plants.

AFLP analysis of the seven recombinant plant lines showed that line no. 133 carried a recombination event between *sym9* and *A5/14*, because it inherited the JI281-derived alleles of *A5/14* and *A5/16*. The data obtained with the RFLP probes and the AFLP markers *A5/14* and *A5/16*, in combination with the previous mapping in JI281 × JI399 (Hall et al. 1997), allowed us to conclude that *sym9* is located between *cDNA194/2* and *A5/14*, 1.2 cM from *A5/14* and *A5/16*, which were not resolved in this cross (Table 1 and Fig. 2). The segregation of *A5/11* was inconsistent with the other data, because the JI281-derived allele is present in lines nos. 9, 89, 96, 133 and 129. Therefore, it was not possible to place *A5/11* either between *sym9* and *A5/14* or between *sym9* and *cDNA194/2*. One possible explanation for this abnormal segregation could be that the *A5/11* marker is derived from two or more closely linked DNA sequences, for example as a member of a repetitive sequence family. Another explanation could be that methylation of the DNA sequence generating *A5/11* is different in the parental lines P2 and JI281, and changes occasionally (Knox and Ellis 2001).

Table 1 Allelic situation of individual plant lines out of the crosses P2 × JI281 and P54 × JI281. (a) P2 × JI281: plant lines nos 51, 59, 9, 89, 96, 129 and 133 show recombination events between *cDNA243* and *cDNA194/2*. The 34 plant lines not recombinant in this region are not shown. Note that *A5/11* shows an irregular pattern as described in the text. (b) P54 × JI281: plant lines nos 30, 59, 80, 88, 92, 95, 104, 121 and 85 show recombination events between *A5/16, A5/14* and *sym9* on the one side and *A5/11* and *sym9* on the other side. The 28 plant lines not recombinant in this region are not shown. –/–, homozygosity for P2 allele; +/–, heterozygosity; +/+, homozygosity for JI281 allele; +/. one JI281 allele and the second allele uncertain; nd, not determined

Plant no.	Markers						
	cDNA243	peasqua	A5/16	A5/14	sym9	cDNA194/2	A5/11
(a) P2 × JI281							
51, 59	–/–	–/–	–/–	–/–	–/–	+/–	–/–
9, 89, 96	–/–	–/–	–/–	–/–	–/–	+/–	+/.
129	+/–	+/–	–/–	–/–	–/–	–/–	+/.
133	+/–	+/–	+/.	+/.	–/–	–/–	+/.
(b) P54 × JI281							
30, 59, 80, 88, 92, 95, 104, 121	nd	nd	–/–	–/–	–/–	nd	+/.
85	nd	nd	+/.	+/.	–/–	nd	–/–

The location of *sym9* was confirmed using 125 F2 plants of the cross P54 × JI281. The 37 Nod⁻ progenies were analyzed individually and a total of nine were found to carry recombination events between *sym9* (from P54) and either *A5/11* or *A5/16*, *A5/14* (from JI281). One Nod⁻ plant (no 80) carried the *A5/16* and *A5/14* alleles of JI281 and eight plants carried the *A5/11* allele of JI281 (Table 1). On the basis of these data, we calculated that *sym9* is located 1.4 cM from *A5/16* and *A5/14*, confirming the conclusions drawn from the analysis of the cross P2 × JI281.

Bulked segregant analysis of *sym10*

The mutant lines P5, P56, P9 and P10 are also unable to form nodules, and the mutations responsible were reported to fall into one complementation group (Duc and Messager 1989). To determine whether they were allelic to any known nodulation locus, with line P56 the same type of analysis was performed as described for line P54 (see above). The cross P56 × N15 (*sym10*) resulted in F1 progenies that were Nod⁻, indicating that line P56, and therefore also P5, P9 and P10, carry alleles of *sym10.*

sym10 has previously been located near the morphological markers *i* and *af* on linkage group I (Weeden et al. 1996). To verify this map position on the molecular map (Hall et al. 1997), bulked segregant analysis was performed. A mapping population (P56 × JI15) was generated and the DNA of F2 individuals was pooled into one WT and four mutant bulks. A probe specific for *chs2* (Harker et al. 1990) located at the bottom of linkage group I was hybridized to Southern blots of *Eco*RI-restricted genomic DNA. One band was present in the wild-type bulk and absent in all mutant bulks (Fig. 3A). A control Southern analysis used a probe specific for *chs1*, which maps to another linkage group (Hall et al. 1997) (Fig. 3B). Although polymorphisms were present between the parental lines, P56 and JI15, no co-segregation of the corresponding band with the mutant bulks

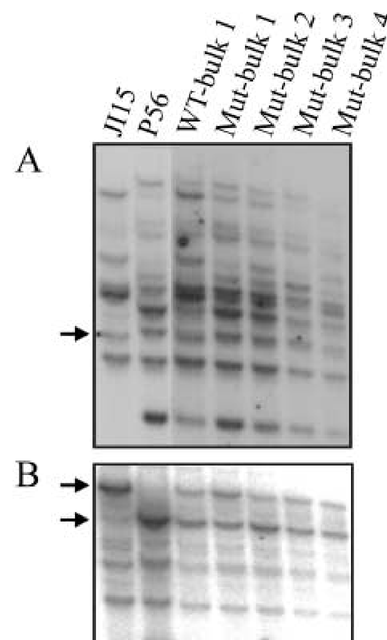

Fig. 3A, B BSA-RFLP analysis of *sym10*. **A** DNA polymorphisms were detected for parental lines JI15 (*WT*) and P56 (*sym10*), one WT and four mutant bulks using the 'chs2' probe. The polymorphic band segregating with *sym10* is indicated by an *arrow*. **B** The same filter as in **A** was hybridized with the 'chs1' probe. Polymorphisms between the parental lines JI15 and P56 (indicated by *arrows*) do not co-segregate with *sym10*

was observed. Because no recombination event between *chs2* and *sym10* was detected, no further fine-mapping was performed. On the basis of the resolution of the RFLP-BSA analyses observed for P2 × JI281 (two out of ten, see above), we calculate that *sym10* is within 6 cM of *chs2*.

The nodulation signalling pathway is also being dissected in *Medicago truncatula* and *Lotus japonicus*, both being small-genome legumes suitable for positional gene cloning (Stougaard 2001). In *L. japonicus*, the two loci

LjSym5 and *LjSym1* are involved in the early stages of signalling and root-hair deformation (Stougaard 2001). However, mycorrhizal symbiosis is not affected in *ljsym5* and *ljsym1* mutants. Either of these mutations might be equivalent to the pea *sym10* mutation mapped here, because *sym10* mutants of pea can also enter symbiosis with mycorrhiza (Duc et al. 1989), but are defective for the earliest events in nodulation signalling (Sagan et al. 1994; Walker et al. 2000). In *M. truncatula*, mutations affecting the *Dmi1* and *Dmi2* loci block root-hair de-formation, as well as Nod-factor-induced calcium spiking and mycorrhization (Wais et al. 2000; Catoira et al. 2001). Therefore these loci may be equivalent to the pea *Sym8* and *Sym19* loci, of which mutations also block Nod-factor-induced calcium spiking (Walker et al. 2000). *M. truncatula* mutants for the *Dmi3* locus are defective for the normal root-hair deformation response, although Nod-factors can still induce calcium spiking in root hairs. Therefore *dmi3* mutants show a phenotype equivalent to that of *sym9* mutants mapped here. The mapping and characterization of these genes will be an important step towards understanding of signalling in nodulation and its conservation in different legumes.

In summary, we have mapped the nodulation-defective loci *sym9* and *sym10* to the molecular map of pea. Closely linked, or even co-segregating, markers were identified that are useful for further high-resolution mapping in large populations and subsequent positional cloning of the mutated genes.

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