Indian J. Genet., **63**(1): 1-4 (2003)

Site specific mutagenesis in nodulation genes of *Mesorhizobium ciceri* strain MC 18-7 using *in vitro* transposition in a heterologous cloned DNA

Vivek Chimote[1] and L. R. Kashyap

National Research Centre on Plant Biotechnology, Indian Agricultural Research institute, New Delhi 110 012

(Received: July 2002; Revised: December 2002; Accepted: December 2002)

Abstract

***Mesorhizobium ciceri* strain MC18-7, nodulating chickpea was used to generate *nod*-mutants to study primary nodulation responses. Transposon insertion was carried out in pRmSL-26 using *in vitro* transposition by EZ:: TN <KAN 2> insertion system. Transposon insertion sites in *nod* ABC and *nod* D genes in pRmSL-26 were used for marker exchanging in *M. ciceri* strain MC 18-7. In total three mutants, one *nod* ABC$^-$ mutant and two *nod* D$^-$ mutants were obtained. The common *nod* ABC$^-$ mutant showed complete lack of root hair infection/deformation, while *nod* D$^-$ mutants showed delayed/reduced primary modulation responses.**

Key words: Chickpea, *Mesorhizobium ciceri*, nodulation genes, *in vitro* transposition, marker exchanging

Introduction

Nodulation is a multi-step process requiring exchange and recognition of specific signals by symbiotic partners. Specific plant flavonoids/chalcones induce nodulation genes (i.e. *nod, nol* and *noe*) whose products are involved in synthesis of mono N-acylated chitooligosaccharides (LCOs) commonly known as Nod factors [1]. Nod factors are involved in induction of host specific nodulin genes resulting in nodule formation. The core Nod factor structure is synthesized by the activity of common *nod* ABC genes, which are structurally and functionally conserved in all rhizobia. Inactivation of the *nod* ABC genes abolishes the bacterial ability to elicit primary symbiotic responses on the host plant, regardless of the host, the mode of infection, type of nodule development, and the nodule's location [2]. The host-specific nodulation (*hsn*) genes determine the variation in host range of different rhizobia by modifying the core Nod factor in host specific manner. Regulation of nodulation genes requires the interaction between *cis* acting *nod* box region, *trans* acting Nod D protein and plant signals like flavonoid [3]. The Nod D protein is a bacterial gene activator of Lys R family, whose N-terminal is involved in *nod* box binding and C-terminal in host signal recognition [4, 5]. However, there are variations among rhizobia in the number of *nod* D copies present. Different copies of *nod* D interact differentially with plant signals and are required for efficient *nod* gene induction [6]. There is no correlation between the number of *nod* D copies and the broadness of host range.

Chickpea is an important pulse crop in India and rhizobia nodulating it (*M. ciceri, M. mediterraneum* and unclassified strains) have been studied intermittently at molecular levels leading to contradictory conclusions basically due to wide range of diversity in rhizobia modulating chickpea [7]. The structurally and functionally conserved *nod* ABC and *nod* D genes which are common to all rhizobia make them best candidates to initiate studies for understanding the whole process of nodulation in chickpea. The present investigation was therefore undertaken with a view to create nodulation defective mutants of *M. ciceri* strain MC18-7 as source of base material for further studies on nodulation process.

Material and methods

M. ciceri strain MC18-7 carries natural resistance to 5μg/ml of sodium azide. It is also resistant to nalidixic acid and chloremphenicol [8]. The source of heterologous nodulation genes was a 41.5 kb clone pRmSL-26 (a pRK290 based clone in PLAFR-1 cosmid) [9], which carries common *nod* ABC genes and regulatory *nod* D gene of *Sinorhizobium meliloti*. *In vitro* EZ::TN<KAN 2> insertion system was used to insert transposon in pRmSL-26 DNA. This system is based on *in vitro* transposition by combining the Tn5 transposase, the "Transposon" containing the 19 bp Tn5 outer ends, Mg^{2+} containing reaction buffer and the pRmSL-26 DNA as target molecules [10]. The 1.2 kb transposon carries kanamycin resistance marker for selection.

[1]Present address: Central Potato Research Institute, Shimla 171 001

Induction of mutation: The transposition system was used to generate *in vitro*, a library of random EZ::TN transposon insertion into a target DNA, pRmSL-26 in a simple one step reaction involving incubation at 37°C for 2 hrs. Reaction mixture was heated for 10 min. at 70°C and transformed into *Escherichia coli* strain DH5α. Selection was made on LB agar plates containing kanamycin (50μg/ml), tetracyclin (10μg/ml) and nalidixic acid (10μg/ml). Each kanamycin resistant colony was purified and maintained on LB agar plus kanamycin (50 μg/ml) and tetracyclin (10μg/ml). Culture of each transformant was grown overnight to isolate plasmid DNA from 5 ml LB broth with tetracyclin (10μg/ml). Each plasmid DNA sample was restricted with *Hin*d III and *Bgl* II, separately and analyzed on 0.8% agarose gel for presence of transposon insertion in desired fragments.

Marker exchanging: Log phase cultures of *E. coli* donor HB101 (pRmSL-26::EZ::TN), helper HB 101 (pRK2073) and *M. ciceri* recipient strain MC 18-7 were mixed together and mated on yeast extract mannitol plates at 30°C for 36 hrs. Resulting growth was scrapped, suspended in small amount of sterile water and plated onto *Rhizobium* minimal medium with nalidixic acid (30μg/ml), tetracyclin (10μg/ml) and neomycin (50μg/ml) to select *M. ciceri* transconjugants [MC18-7 (pRmSL26::EZ::TN)]. To get *nod* mutants of *M. ciceri*, a biparental mating was carried out on yeast extract mannitol plates at 30°C for 36 hrs using evacuation plasmid HB101 (pPH1JI) which is incompatible to pRK-290 derived plasmid vectors. The transconjugants were selected on *Rhizobium* minimal medium [nalidixic acid (30μg/ml), gentamycin (30μg/ml) and neomycin (50μg/ml)] and further purified. Purified transconjugants were checked for sensitivity to tetracyclin in order to ensure that incompatible plasmid pRmSL-26 was lost. Thus, these colonies carried transposon insertion in *nod* genes of strain MC 18-7.

Nodulation phenotyping: Nodulation phenotyping was done by microscopic examination of root hairs infected with mutants to ascertain primary symbiotic *hac*⁻ and *had*⁻ phenotypic responses. For this purpose, healthy seeds of chickpea cultivar Pusa 256 were sterilized by washing with 70% ethanol for 1 minute followed by 0.1% mercuric chloride treatment for 3 minutes. Traces of sterilants were removed by rinsing with sterile distilled water 5-6 times. Sterile seeds were placed in petriplates with 3-4 layers of wet sterile filter papers for 36 hours to allow seed germination. Germinated seedlings were inoculated with rhizobial culture and infection was allowed for 16-24 hours. The root tips were cut to 5mm length, stained with 0.01% methylene blue for 15 minutes on glass slide, washed and examined under a microscope at 400 × magnification.

Results and discussion

In this study, a reverse genetic approach was followed which provides a better insight in understanding the organization and expression of *nod* genes. Its requirement is the availability of heterologous clone carrying *nod* genes and then mutagenizing the gene to study the loss of function. A *S.meliloti nod* genes carrying clone pRmSL-26 fulfilled the requirement and was chosen for site-directed mutagenesis in the present study. Lambda:: Tn5 [11] and HB 101:: Tn5 [12] have been used as a source of transposon for mutagenesis in cloned DNA to generate mutants by reverse genetic approach. In present study Tn5 based *in vitro* EZ:: TN <Kan-2> insertion system [10] was used for transposon insertions in pRmSL-26. Efficiency of this system has been improved by inducing three mutations in Tn5 transposase and multiple changes in 19 hp outer end transposase recognition sequences. This method not only allows generating insertion mutations but also allows faster sequencing of large DNA molecules without subcloning by using primers based on transposon for amplification.

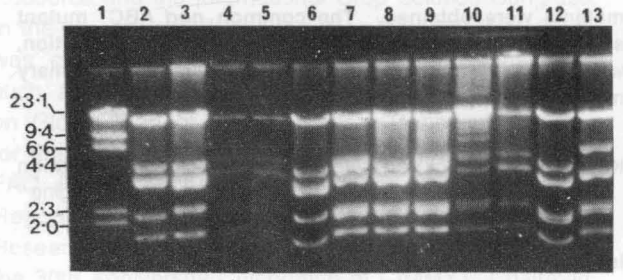

Fig. 1. Gel electrophoresis of transposon insertion clones digested with *Bgl* II. Lane 1: λ *Hin*d III digested marker; Lane 2-13: Transposon insertion clones digested with *Bgl* II

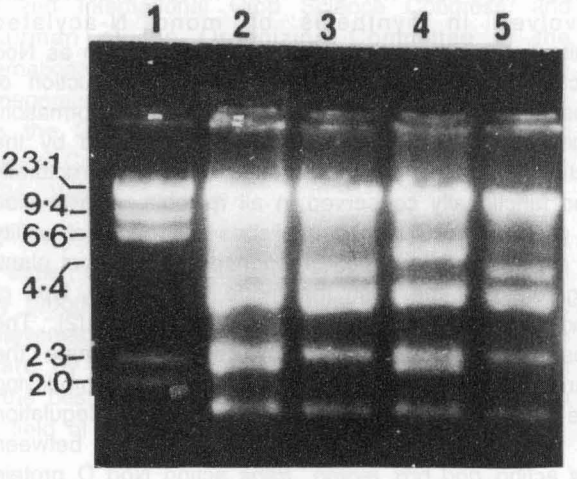

Fig. 2. Gel electrophoresis of transposon insertion clones (in *nod* region) digested with *Bgl* II. Lane 1: λ *Hin*d III digested marker; Lane 2: pRmSL-26 digested with *Bgl* II; Lane 3: pKTN-1 digested with *Bgl* II; Lane 4: pKTN-3 digested with *Bgl* II; Lane 5: pKTN-12 digested with *Bgl* II

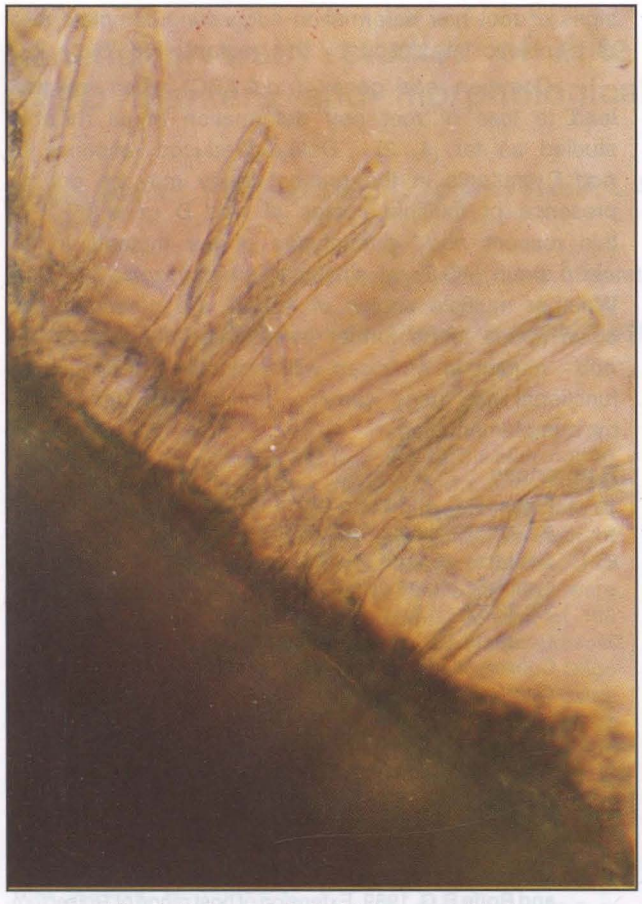

Fig. 3. Uninoculated chickpea root hairs

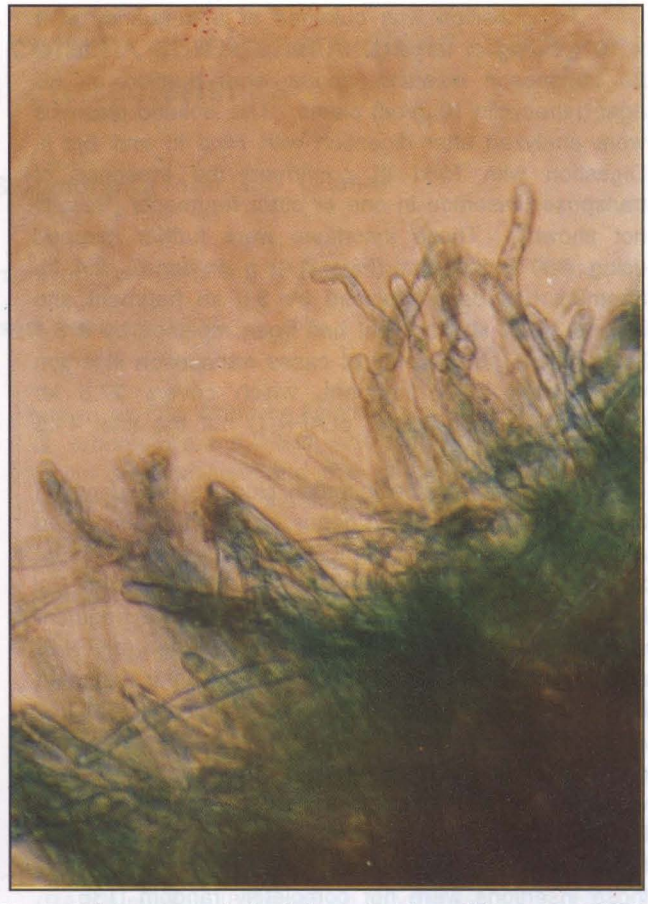

Fig. 5. Chickpea root hairs infected with *nod D*⁻ mutant KGN-

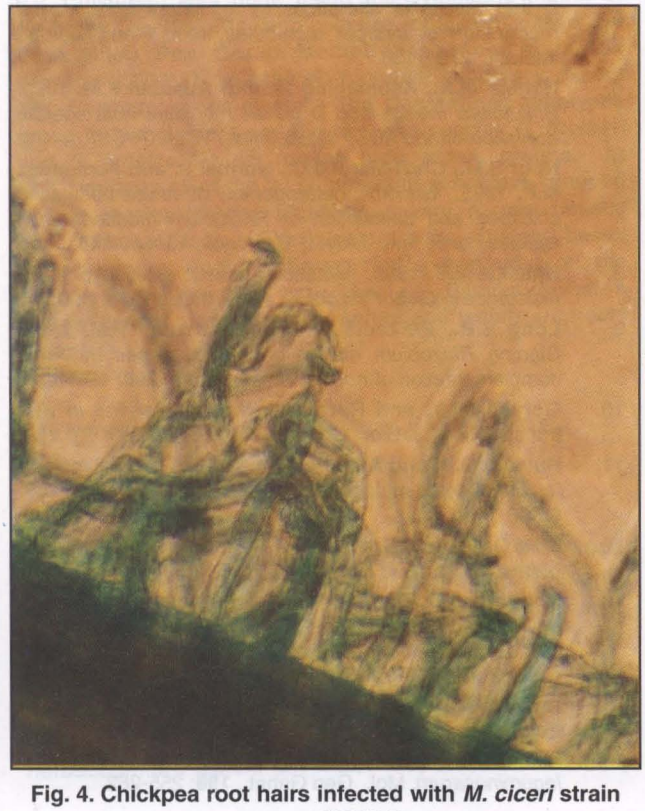

Fig. 4. Chickpea root hairs infected with *M. ciceri* strain MC18-7

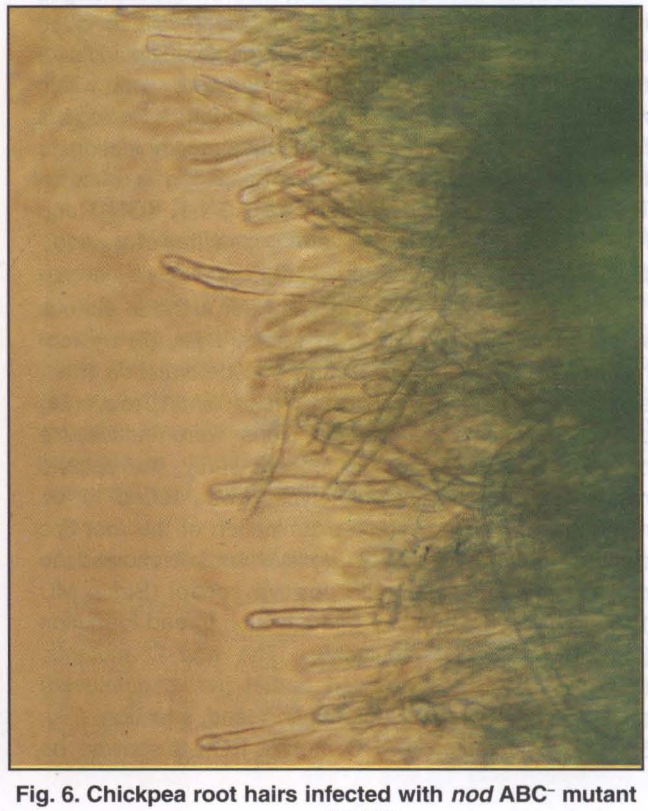

Fig. 6. Chickpea root hairs infected with *nod* ABC⁻ mutant KGN-3

Transposition was obtained at a frequency of 1 $\times 10^{-5}$ cfu/μg of target DNA i.e. pRmSL-26. A total of 24 transposon insertion clones were purified on LB agar (tetracyclin 10μg/ml) plates. The isolated plasmids were analyzed after digestion with *Hind* III and *Bgl* II. Digestion with *Hind* III confirmed the presence of transposon insertion in one or other fragments (Results not shown). These insertions were further mapped using *Bgl* II digestion (Fig. 1), e.g. in lane2, 2.4 kb fragment has been replaced by 3.6 kb fragment and in lane-3, 2.2 kb fragment has been replaced by a 3.4 kb fragment. In majority of cases transposon insertion was located in a fragment, which covers 27.5 kb (including 21.6 kb vector pLAFR-1) out the total 41.5 kb pRmSL-26 plasmid.

Three transposon insertion clones were identified having transposon in *nod* genes region and designated as pKTN-1, pKTN-3 and pKTN-12. As shown in Fig. 2, lane3 (pKTN-1) and lane5 (pKTN-12), a 2.2 kb *Bgl* II fragments has been replaced by a 3.4 kb fragment which carries *nod* D gene. Similarly in lane4 (pKTN-3) a 5.8 kb fragment has replaced a 4.6 kb fragment having *nod* ABC genes. Induction of mutations in *nod* ABC genes with low frequency may be due to some gaps in transposon insertion. More insertion mutants were needed for analysis to cover up these gaps. Earlier it has been reported that low frequency of transposon Tn5 insertions occur in *nod* region and those insertions were not completely random [13].

All three insertion clones were marker exchanged into *M. ciceri* strain MC 18-7 to get neomycin resistant and tetracycline sensitive transconjugants. This indicate that all three transposon insertions were well within *nod* genes of pRmSL-26, with enough homologous DNA sequences available for double recombination to take place on either side of transposon insertions. These mutants were designated as KGN-1, KGN-3 and KGN-12. These mutants were examined for *nod*$^-$ phenotype.

Nodulation phenotyping has been done in various ways (i) nodulation studies in Leonard jar, (ii) by root hair studies for curling, infection and deformation (*hac*$^-$ and *had*$^-$) and (iii) by thick and short root (*tsr*) phenotype assay. Since mutations were induced in common *nod* genes in the present study, the second method of modulation phenotyping was found to be most suitable. Microscopic examination of the root tips showed that uninoculated negative control showed no root hair deformation, while positive control (Strain MC 18-7) showed deformation and infection thread formation (Figs. 3 and 4, respectively). The *nod* D mutants, KGN-1 and KGN-12 showed partial (reduced/delayed) response to root hair deformation and infection (Fig. 5). As expected *nod* ABC mutant KGN-3 showed no signs of root hair deformation suggesting complete loss of symbiotic interaction by this mutant (Fig. 6).

Common *nod* genes (*nod* ABC) when mutated lead to loss of root hair deformation in all rhizobia studied so far [1, 2]. Delayed/reduced response in *nod* D mutants in the present study may be due to presence of multiple copies of *nod* D gene [6]. In this respect *nod*$^-$ phenotypes of the mutants in *M. ciceri* strain MI8-7 are similar to other rhizobia [13, 14]. Whether multiple copies of *nod* D are present in *M. ciceri* needs to be further investigated. Availability of *nod*$^-$ mutant in *M. ciceri* will facilitate identification of functional *nod* gene carrying clones from gene bank by complementation.

References

1. **Fisher R.F. and Long S.R.** 1992. Rhizobium-plant signal exchange. Nature, **357**: 655-660.
2. **Schultze M. and Kondorosi A.** 1998. Regulation of symbiotic root nodule development. Ann. Rev. Genet., **32**: 33-57
3. **Goethals K., van Montagu M. and Holsters M.** 1992. Conserved motifs in a divergent *nod* box of *Azorhizobium caulinodans* OPS571 reveal a common structure in promoter regulated by Lys R type proteins. Proc. Natl. Acad. Sci., USA, **89**: 1646-1650.
4. **Horwath B., Bachem C.W.B., Schell J. and Kondorosi A.** 1987. Host specific regulation of nodulation genes in *Rhizobium* is mediated by a plant signal, interacting with the *nod* D gene product. EMBO J., **6**: 841-848.
5. **McIver J., Djordievic M.A., Weinmann J.J., Bender G.L. and Rolfe B.G.** 1989. Extension of host range of *Rhizobium leguminosarum* bv *triffoli* caused by point mutation in *nod* D that results in alterations in regulatory function and recognition of inducer molecules, Mol. Plant Microbe Interact., **2**: 97-106.
6. **Honma M.A., Asomaning M. and Ausubel F.M.** 1990. *Rhizobium meliloti nod* D genes mediates host specific activation of *nod* ABC. J. Bacteriol., **172**: 901-911.
7. **Nour S.M., Cleyl-Marel J.C., Normal P. and Fernandez M.P.** 1995. Genomic heterogeneity of strains nodulating chickpea and description of *Rhizobium mediterraneum* species novel. Int. J. Syst. Bacteriol., **45**: 640-648.
8. **Bhaskar V.V.** 1999. Molecular genetic analysis of azide resistance in *Cicer-Rhizobium*. Ph.D. thesis, IARI, N. Delhi.
9. **Long S.R., Buikema W.J. and Ausubel F.M.** 1982. Cloning *Rhizobium meliloti* nodulation genes by direct complementation of *nod* mutants. Nature, **298**: 485-488.
10. **Goryshin I.Y. and Reznikoff W.S.** 1998. Tn5 *in vitro* transposition. J. Biol. Chem., **273**: 7367-7372.
11. **Ruvkun G.B. and Ausubel F.M.** 1981. A general method for site directed mutagenesis in prokaryotes. Nature, **289**: 85-88.
12. **Ditta G.** 1986. Tn5 mapping of *Rhizobium nitrogen fixation genes. Methods in Enzymol.*, **118**: 519-528.
13. **Kondorosi E., Banfalvi Z. and Kondorosi A.** 1984. A physical and genetic analysis of a symbiotic region of *Rhizobium meliloti*: Identification of nodulation genes. Mol. Gen. Genet., **193**: 445-452.
14. **Downie J. A., Rossen L., Knight C.D., Shearman C., Evans I.J. and Johnston A.W.B.** 1985. The structure and regulation of the nodulation genes of *Rhizobium leguminosarum*. Mol. Gen Genet., **198**: 255-262.