



Isolation and characterization of symbiotically defective pyrimidine and amino acid auxotroph of *Mesorhizobium ciceri* in chickpea

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Abstract

Transposon Tn5 induced, four *Mesorhizobium ciceri* auxotroph were isolated and characterized. Unlike its wild type parent (TL 620), all four mutants were found defective for amino acid and pyrimidine biosynthesis. The auxotroph mutants were characterized and found TL130 as cytosine and uracil, TL 196 for guanine, cytosine, uracil and riboflavin, TL 141 as serine and TL 38 as arginine defective. Symbiotic characterization of these mutants revealed phenotypic deformities and deficiencies in biological nitrogen fixation. All the four auxotrophic mutants were characterized as *nod⁻/fix⁻* nature with reduced nitrogenase activity of 42.2, 26.3 and 17.13% respectively as compared to the wild type which is further supported by sub cellular examination of the nodules section by TEM study.

Key words

Auxotroph, Symbiotic, Transposon mutagenesis, *Mesorhizobium ciceri*

Introduction

Chickpea (*Cicer arietinum* L) is a cool season grain legume of exceptionally high nutritive value and most versatile food used. It is mostly grown under rainfed conditions in arid and semi-arid areas around the world. In India, chickpea was grown on 8.75 million ha land producing 8.25 million tones grain with 943 kg ha⁻¹ productivity during 2010-2011. Most of the legume crops have ability to fix atmospheric nitrogen (N₂) by symbiotically association between groups of microbes called *Rhizobium* (Sprent, 2001). Nitrogen is one of the essential nutrients for plant growth but due to extensive use of chemical fertilizer in agriculture is under debate to environment concern and fear for consumer health (Yadav *et al.*, 2011). Growing concern about the environmental, over population, energy nutrition and agricultural sustainability has drawn the attention of scientist towards the need for environmentally sustainable practice and organic farming system (Lee and Song, 2007). The extending role of biofertilizer like

Rhizobium will certainly reduce the use of chemical fertilizer and decrease adverse environment effect.

Symbiotic nitrogen fixation is a major source of nitrogen for legume crops. Numbers of genes are known to be associated with host-*rhizobium* interaction with well defined *nif*, *nod*, *fix* and many other genes involved in biosynthesis of different metabolites. Symbiotic phenotype varied with the type of mutation for example, *Rhizobium leguminosarum* bv. *viciae* mutants unable to transport branched-chain amino acids via the two main amino acid ABC transport complexes AapJQMP and BraDEFGC produce a nitrogen starvation phenotype when inoculated on *Pisum sativum* plants (Prell *et al.*, 2010). Some *Sinorhizobium meliloti* mutant's genes involved in isoleucine, valine and leucine biosynthesis were previously described as being unable to induce nodule formation on host plants (Peltzer *et al.*, 2008). Borjigen *et al.* (2011) reported proline auxotrophs (ProC) of *Bradyrhizobium japonicum* were found underdeveloped nodules (*nod⁻*) in

soybean. Mutation in the *lysA* gene (Lysine) impairs the symbiotic properties of *Mesorhizobium ciceri* (Das et al., 2010). Different workers with several *Rhizobium* species have isolated mutants with various kinds of nutritional requirements (Yadav et al., 2007). Analysis of such mutants has helped in identifying different metabolites and various biosynthetic pathways involved directly or indirectly in the effective symbiotic relationship (Newman et al., 1994; Vineetha et al., 2001; Das et al., 2006).

In order to establish the possible path of nitrogen flow in the legume symbiosis, transposon Tn5 mediated auxotroph (which depend on nutrients supplement for its growth) were generated in *Mesorhizobium ciceri* strain TL620 to understand the biological nitrogen fixation (BNF) process.

Materials and Methods

Mesorhizobium ciceri, a wild type strain TAL620 with relevant phenotype Nx^+ , Sm^r , Neo^s , Cm^s , Tc^s , Rif^s was used as parent to generate mutants, and *E. coli* with relevant phenotypes Ap^r , Cm^r , Km^r , pBR325 containing suicide plasmid S-17 (pSU5011) was used for bi-parental cross as recipient and donor strain respectively. The chickpea variety C-235 seeds were procured from the National Seed Laboratory, Genetics Division, IARI, New Delhi and employed for studying symbiotic properties of the different *Rhizobial* mutants.

Bacterial conjugation, isolation and screening of Tn5 auxotrophs : To generate random mutants of *M. ciceri*, a total of 6 ml log phase culture of donor *E. coli* S-17 (pSU5011) and recipient *M. ciceri* TAL 620 was grown in LB medium containing antibiotic. Both the cultures were mixed in 1:1 ratio in a patch on TY medium free from antibiotic and incubated at 30°C for 12 hrs (Kondorosi et al., 1977). After 12 hrs, the bacterial growth was scrapped with the help of sterile spatula and suspended in 0.08% saline water and 0.1ml suspension of mating mixture was spread on YEMA selecting medium containing naladixic acid (25 µg ml⁻¹) and neomycin (100µg ml⁻¹). A 0.1 ml of donor strain suspension was also spread on selective medium as control. Incubation was done at 28° C for 6-7 days. A total of seven hundred fifty neomycin and nalidixic resistant derivative of Tn5 transposon insertion in wild type strain called transconjugants were selected and each transconjugant was purified separately by repeated single colony isolation in the same selective medium for further use.

Transconjugants derived from Tn5 induced mutagenesis were screened for putative auxotrophs by streaking them on Yeast Manitol and Agar (YEMA) medium and Rhizobium Minimal Medium (RMM) medium. After 4 days of inoculation at 28°C, the growth was recorded. The Tn5 derivatives which showed growth on YEMA but not

on RMM agar medium were assumed to be auxotrophs and each of these were purified and used in further studies. The purified auxotrophs were restreaked on RMM agar medium supplemented with modified Holiday pools (Holiday, 1995) and growth patterns were observed after incubation for 40 hrs at 28°C to know the individual defective mutants deficit of nutrients.

Plant inoculation and nodulation studies : The chickpea variety C-235 was used to inoculate with wild and auxotrophic mutants. The full grown bacterial cultures were scrapped from slant culture and suspended in petri plates containing sterile distilled water and final solution containing 10⁸ bacteria ml⁻¹. The appropriate numbers of healthy seeds were soaked in water for 12 hrs and transferred onto Petri plates containing water-soaked blotting paper for germination. The germinated seeds were inoculated and planted aseptically in autoclaved pots containing vermiculite quartz sand mixture (1:1). The whole setup of *Rhizobium-Plant* interaction experiments were conducted under controlled environmental condition in the national phytotrone facility with a 12 hr photoperiod and light intensity of 250 µEm⁻² S⁻¹. The day and night temperature was 23°C and 15°C, while the relative humidity was 60%. After 3 days of planting, seeds germinated into small seedlings and after 7 days of seed germination, cotyledon was removed from the seedling so that plant could not get the nitrogen source other then biological nitrogen fixation. The chickpea seedlings were regularly supplied with nitrogen-free nutrient solution as described by McKnight (1949). Further, the plants were uprooted and nodules were taken for observation.

Acetylene reduction assay : The nitrogenase enzyme responsible for N₂-fixation was accessed by acetylene reduction assay (ARA) (Dilworth, 1996).

Transmission electron microscopy : The section of nodules induced by the wild strain (TAL620) and its three defective strains (TL130, TL141 and TL38) were subjected to microscopic examination. The nodules for each strain were collected after six weeks of inoculation and processed as per protocol (J.C.TU, 1974) for examining under transmission electron microscopy (TEM) in AIIMS, Biotechnology Division, Electron Microscopy Facilities, New Delhi.

Results and Discussion

Type of defective auxotroph mutants : Frequency of Tn5 insertion mutagenesis for auxotrophy was observed as to be 1% with *Rhizobium meliloti* (Swaminathan et al., 1992) but with *Mesorhizobium ciceri* was observed to be 0.05% only (Das et al., 2006). After further screening of hundred twenty transconjugants, only five defective auxotrophic mutant namely TL38, TL130, TL141 and TL196 were identified.

Morphological and enzymatic assay : Phenotypic variation in plants inoculated with defective mutants showed stunting behavior of growth with yellowing of leaf as compared to plants inoculated with wild type which had green leaves. The nodules induced by these auxotrophs were conical/oval in shape, white in color and were mostly located on lateral roots. The nodules produced by wild type were pink in colour, round shape and present on both primary and lateral roots. The plants inoculated with these mutants (TL38, TL130 and TL141) were preliminary examined and found to be *nod⁺/fix⁺* phenotype whereas TL196 showed *nod⁻/fix⁻* phenotype. The nitrogenase enzymatic assay of this auxotrophic mutant also showed reduction in activity with 42.2, 26.34 and 17.13% respectively as compared to the wild type (Lodwing *et al.*, 2003) as shown in (Table 1).

Mutants with various nutritional requirements auxotroph were isolated. The TL38 arginine defective mutant showed irregular pattern of white nodules formation with reduced nitrogenase activity (19.5 nmoles plant⁻¹ hr⁻¹) as compared to the wild type *M. ciceri* TAL 620 (33.74 nmoles plant⁻¹ hr⁻¹) (Table 1). The sequence analysis of gene showed 322-residue polypeptide that were homologous to C-terminal moiety of the *Saccharomyces cerevisiae* ARG5, 6 gene product N-acetylglutamate (Floriano *et al.*, 2006). While TL130 was a unique auxotrophic mutant with multi requirement of cytosine and uracil symbiotically defective in nitrogenase activity (8.89 nmoles plant⁻¹ hr⁻¹). This kind of multi nutritional auxotroph may be due to single gene mutation that affects the metabolic pathway in such a way that a simultaneous requirement is created for cytosine and uracil. These results indicated that some of the intermediates /or enzymes of pyrimidine biosynthetic pathway of *M. ciceri* play a key role in bacteroidal transformation and nodule development (Jasdeep *et al.*, 2011). Further investigation in this direction could lead to un-revealing role of new genes in symbiotic pathway. Although mutant TL196 was defective (Cyt⁻, Ura⁻, Gua⁻, Ribo⁻) in host invasion and unable to form the nodules in root supports the finding of purine auxotroph in *M. loti* (Noel *et al.*, 1988). Similar kind of auxotrophy (TL9) was reported in *M. ciceri*. This mutant was able to grow even in presence of any one requirement which could be that they have a common intermediate leading to each of

these requirements. The inverse PCR studies of flanking region of the Tn5 insertion in TL9 auxotroph revealed the involvement of cystein proteinase like gene in symbiotic pathway (Jasdeep *et al.*, 2011).

The auxotrophic mutants observed defective in nodules formation showed drastically reduced ARA activity. The nodules induced were white indicating that mutation was in the development process, and drastically disrupted, either due to impairment of the nitrogenase synthesis which leads to the formation of nodules was affected.

TEM investigation of the symbiotic defect auxotrophs : The sub-cellular examination of wild type and two auxotrophic mutant (TL130 and TL141) was carried out by TEM which revealed higher concentration of bacteroids in cortical cells of wild type (Fig.2A) than auxotrophic mutant. Hence, there is increased level of N₂ fixation in wild type, whereas mutant shows reduce bacteroids (Fig.2C) with reduce nitrogenase activity. In the wild type strain, (Fig. 2B) the PBH bodies were very prominent while they were scarce in ultrathin section of TL130 and TL141. The result of TEM showed that the presence of bacteroids in the nitrogen fixation zone of nodule; induced nodules were mostly spherical or oval unlike the elongated bacteroids in the nitrogen fixation zone of the wild strain. This implies that nodule development was affected in the auxotrophic mutant leading to symbiotic deficiencies.

The TEM photographs do not indicate that at what stage these defects of development occurred, it could be that bacteroids are formed normally but they are physiologically not as active as the bacteroids of the wild strain TAL620. This would be true for TAL620, which shows PBH bodies while these are absent in section of nodules of TL130 and TL141 auxotrophic mutant (Cevellos *et al.*, 1996). In most of the auxotrophic mutants, variation in the ARA values clearly showed disruption of nitrogenase synthesis, which led to deformation of nodules formation. Serine defective auxotroph indicated that mutation was in the development process which was drastically disrupted either by nitrogenase synthesis or nodules developments (Das *et al.*, 2006). In fact, a reduced level of ARA may be due to the

Table 1 : Characterization of wild strain and defective mutants for symbiotic association

Auxotrophic mutants	Strains	Nodule number	Nodule color	Nodule shape	Dry wt. of nodule	Nitrogenase activity (n mol pt ⁻¹ h ⁻¹)	Nitrogenase activity (% reduction)	Phenotype Nod/Fix	
Wild type	TAL620	17	Pink	C/O	0.023	33.74	-	+	+
Arg ⁻	TL38	13	White	C/O	0.031	19.5	42.2	+	+
Cyt ⁻ /Ura ⁻	TL130	15	White	C/O	0.036	8.89	26.34	+	+
Ser ⁻	TL141	13	White	C/O	0.035	5.78	17.13	+	+
Cyt ⁻ , Ura ⁻ , Gua ⁻ , Ribo ⁻	TL196	NA	NA	NA	NA	NA	NA	-	-

C/O- conical and oval; ARA-acetylene reduction assay; Nod- Nodulation; Fix-Fixation

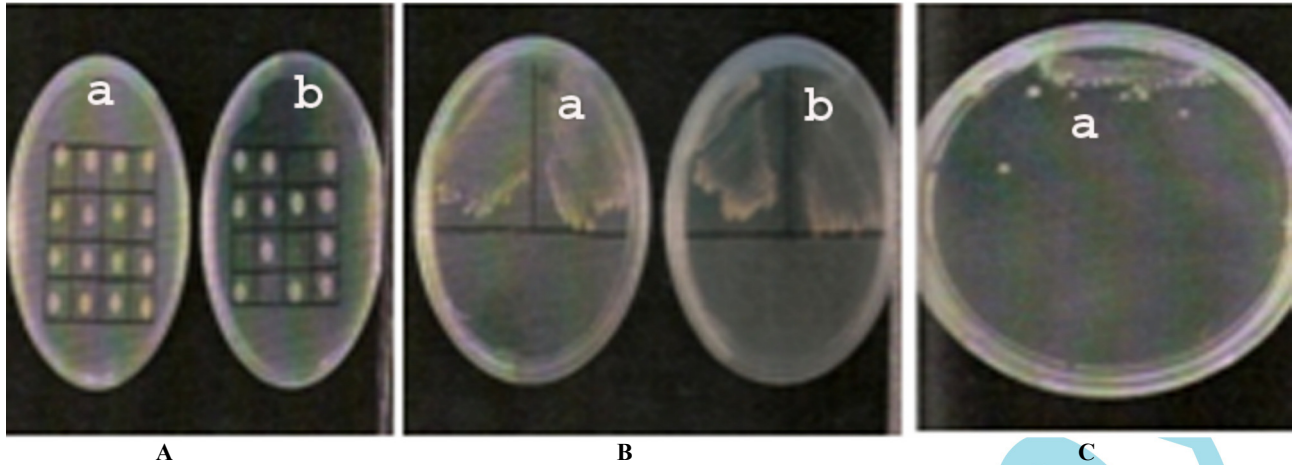


Fig. 1 : Growth pattern of auxotrophs mutant in RMM supplement medium showing: A- a. Growth of auxotroph in rhizobial complete media; b. Growth of auxotroph rhizobial minimal media; B- a. Growth of auxotrophs in RMM+Cytosine+Uracil+Guanine+Riboflavin; b. Growth of auxotroph in RMM+Cytosine+Uracil medium; C- a. Growth of auxotrophs in RMM+Serine

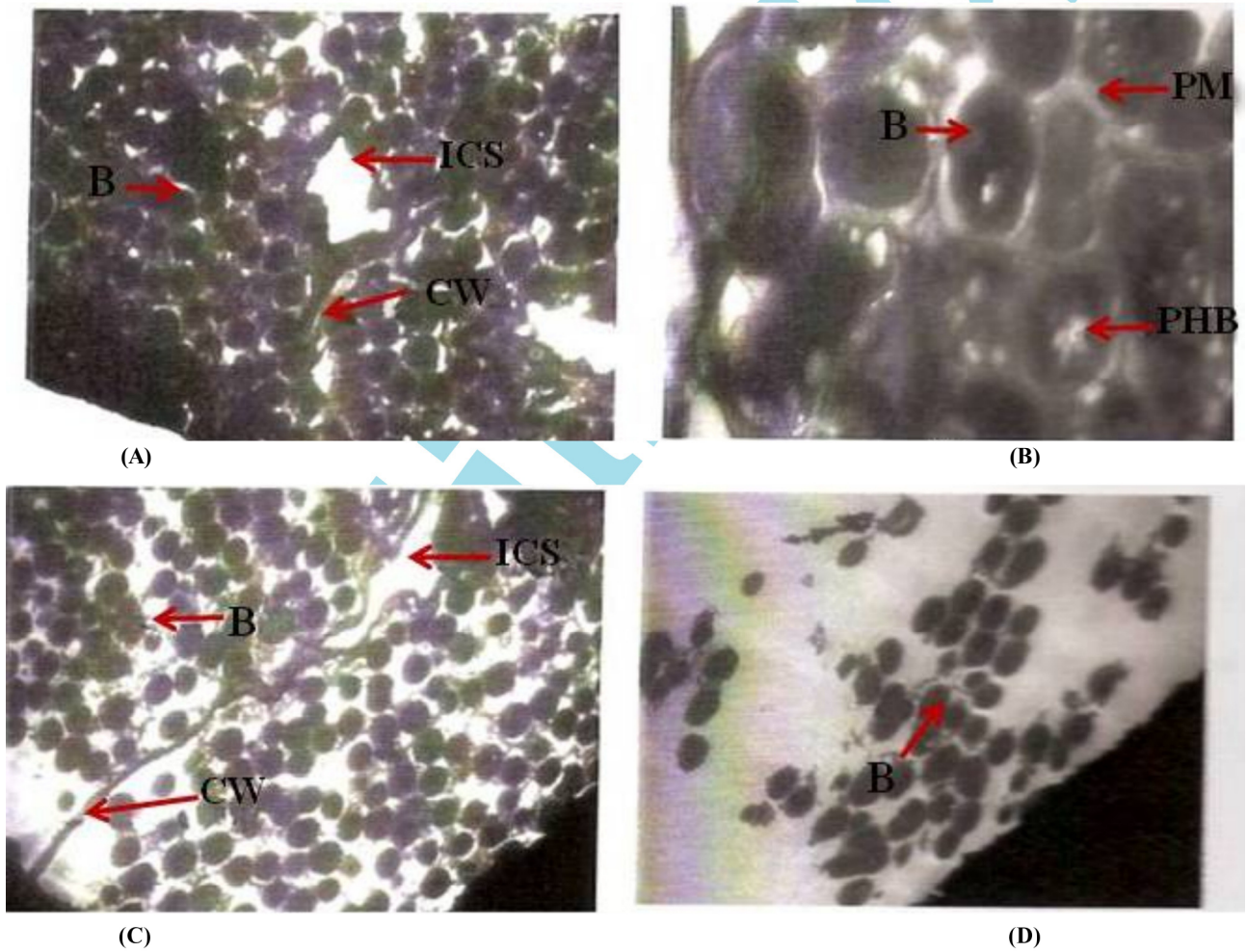


Fig. 2 : TEM transverse sections of nodules infected by wild and defective strain (A) TAL 620 B-Bacteroid, ICS-Intercellular space, CW-Cell wall (B) TAL 620 PM- Peribacteroid membrane, PBH-Polyhydroxy butarate (C) TL130 ICS- Intercellular space, CW-Cell wall (D) TL 141 CY- Cytoplasm

following reasons (i) a reduced bacterial capacity to invade developing nodules, thus resulting in empty or partially empty nodules; (ii) the inability of SBs to induce normal levels of a functional nitrogenase complex, perhaps because of problems in carbon exchange and O₂ availability; (iii) a reduced or altered SB maintenance, or a premature nodule senescence, or both. On the other hand Arcondeguy *et al.* (1997) reported that, a high level of ARA activity was not indicative of a correct nodule function, since it showed that ARA activity in nodules induced by some mutants was normal, while N fixed was not efficiently exported to the plant. Thus overall study of this experiment showed the importance of amino acid, vitamins and nucleotides for their effective role in symbiosis.

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