Research Article

Role of *nod*D gene product and flavonoid interactions in induction of nodulation genes in *Mesorhizobium ciceri*

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ABSTRACT

Mesorhizobium ciceri is a host specific bacterium which nodulates the genus, *Cicer*. Host specificity is regulated at first step by induction of nodulation (*nod*) genes in the presence of NodD protein and inducers (flavonoids) of plant origin. The inducer specificity of *M. ciceri nod*D gene was studied in NodD$^-$ mutant strain HN-9 carrying heterologous *nod*D genes and *nod*A-*lac*Z fusion. The induction profile of *nod* promoter in *M. ciceri* revealed that *nod*D gene product of *M. ciceri* is specifically activated by chickpea root exudates only. *M. ciceri* HN-9 (*nod*A-*lac*Z) containing heterologous *nod*D genes from *Rhizobium leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii* and *Sinorhizobium meliloti* was induced in presence of a number of flavonoids. On the other hand, induction profile of *nod* promoter showed that heterologous *nod*D gene products were activated to different levels in NodD$^-$ mutant of *M. ciceri* in presence of root exudates from homologous as well as heterologous legume hosts. The transfer of FITA (Flavonoid independent transcription activation) *nod*D gene in NodD$^-$ mutant, *M. ciceri* HN-9, was able to break the inducer specificity barrier and *nod* promoter was induced to maximum level irrespective of the presence or absence of inducer. It is concluded from the results that host specificity in *M. ciceri* - chickpea (*Cicer arietinum*) symbiosis is regulated at first step by the host specific interaction of *nod*D gene product of *M. ciceri* and inducers present in the root exudates of chickpea. **[Physiol. Mol. Biol. Plants 2010; 16(1) : 69-77]** *E-mail : kamboj_dev@yahoo.com*

Keywords : *Mesorhizobium ciceri, nodD, flavonoids, host specificity, Cicer arietinium*

INTRODUCTION

Bacteria belonging to genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Azorhizobium* infect roots of leguminous hosts inducing nodule formation wherein nitrogen fixation takes place. The nodulation process is tightly regulated by exchange of molecular signals between the host and the symbiont. Several nodulation genes (designated as *nod*, *nol*, *noe*, *noi*) have been identified in *Rhizobium* and control the early steps in nodule morphogenesis (Sharma *et al*., 1993). Most nodulation genes (except *nod*D) require constitutively expressed *nod*D protein which is activated by inducers present in root and seed exudates (Schlaman *et al*., 1992). The interaction of *nod* gene inducers with constitutively expressed *nod*D gene product regulates the first host-specific step in legume-*Rhizobium* symbiosis. The *nod* gene encoded proteins are involved in the synthesis of host-specific Nod factors which initiate nodule morphogenesis (Denarie *et al*., 1996).

NodD regulators play an important role in the establishment of *Rhizobium*-legume symbiosis and the control of its specificity. NodD proteins belong to the LysR family of transcriptional regulators. Like most members of this family, they are activated by small molecules, specific flavonoids released in the rhizosphere by host plants. Flavonoids do not affect the binding affinity of NodDs for target promoters but instead enhance promoter activity by modifying DNA bending on conserved *nod*-boxes (Feng *et al*., 2003; Kobayashi *et al*., 2004; Suominen *et al*., 2003). Known targets of NodDs are the nodulation genes which encode

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for proteins which are responsible for the synthesis and export of lipo-chito-oligosaccharides, the Nod factors, which trigger nodule organogenesis, root infection and determine the host range (Machado and Krishnan, 2003; Tak *et al.,* 2004; Kobayashi and Broughton, 2008).

Although *nod*D genes are ubiquitous in rhizobia, their symbiotic characteristics vary from one species to another. Some rhizobia, such as *R. leguminosarum* bv. *trifolii*, have only one *nod*D gene while *B. japonicum*, *Rhizobium* sp. strain NGR234, *S. meliloti*, and *R. tropici* possess two to five copies of *nod*D (Bassam *et al*., 1988; Barnett and Long, 1990; van Rhijn *et al*., 1993; Lindstrom *et al*., 1995). Moreover, NodD homologues from the same strain may have different flavonoid preferences (Davis and Johnston, 1990; Gyorgypal *et al*., 1991). The genetic data and NodD-*nod* box (a 47 base pair sequence present in front of inducible *nod* genes) binding studies support the direct binding of flavonoids to the NodD proteins and suggest transcriptional activation of inducible *nod* operons by the flavonoid activated NodD. Earlier *nod*D gene was considered as a common nodulation gene but studies have shown that it is a host specific gene because there are instances where mutations in *nod*D were not complemented by heterologous *nod*D gene and transfer of *nod*D gene has been seen to extend the host range of nodulation (Horvath *et al*., 1987). Host specificity for nodulation can be altered by changing flavonoid specificity (Cardenas *et al*., 1995). The present investigations were conducted to study the role of *nod*D gene of *M. ciceri* in host specific induction of nodulation genes.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains and mutants used in the present study are listed in Table 1. Construction of plasmids pMP154 (containing promoter *nod*A of *R. leguminosarum* bv. v*iciae* fused with *lac*Z), pMP280 (containing *nod*D of *R. leguminosarum* bv. *viciae*), pMP283 (containing *nod*D of *R. leguminosarum* bv. *trifolii*), pMP284 (containing *nod*D1 of *S. meliloti*) and pMP604 (containing flavonoid independent transcription activator or FITA *nod*D gene) has been described by Spaink *et al*. (1987a,b).

Media and growth conditions

Rhizobium strains were grown in tryptone yeast extract mannitol (TYM) medium. Induction of *nod*A-*lac*Z fusion

Table 1. Strains of *M. ciceri* used in the present study

Strain/Mutant	Relevant characteristics	Source/Reference
HS-1	*M. ciceri* (NodD$^+$); Sm^r Nx^r	Sharma *et al*. (1991)
HSL-1V	HS-1 (NodD$^+$) containing pMP154 (*nod*A promoter of *R. leguminosarum* bv. *viciae* fused with *lac*Z); Nx^r, Cm^r	Kamboj *et al*. (2003)
HN-9	*M. ciceri* NodD$^-$ mutant of HS-1; Nx^r Nm^r	Kamboj *et al*. (2003)
HNL-9V	HN-9 (NodD$^-$) containing pMP154 (*nod*A promoter of *R. leguminosarum* bv. *viciae* fused with *lac*Z); Nx^r, Nm^r, Cm^r	Kamboj *et al*. (2003)
HNL-9DV	HN-9 (NodD$^-$) containing pMP154 (*nod*A promoter of *R. leguminosarum* bv. *viciae* fused with *lac*Z) and pMP280 (*nod*D of *R. leguminosarum* bv. *viciae*); Nx^r, Nm^r, Cm^r, Tc^r	Kamboj *et al*. (2003)
HNL-9DT	HN-9 (NodD$^-$) containing pMP154 (*nod*A promoter of *R. leguminosarum* bv. *viciae* fused with *lac*Z) and pMP283 (*nod*D of *R. leguminosarum* bv. *trifolii*); Nx^r, Nm^r, Cm^r, Tc^r	Kamboj *et al*. (2003)
HNL-9DM	HN-9 (NodD$^-$) containing pMP154 (*nod*A promoter of *R. leguminosarum* bv. *viciae* fused with *lac*Z) and pMP284 (*nod*D1 of *S. meliloti*); Nx^r, Nm^r, Cm^r, Tc^r	Kamboj *et al*. (2003)
HNL-9DF	HN-9 (NodD$^-$) containing pMP154 (*nod*A promoter of *R. leguminosarum* bv. *viciae* fused with *lac*Z) and pMP604 (FITA *nod*D); Nx^r, Nm^r, Cm^r, Tc^r	Kamboj *et al*. (2003)

was studied in the induction medium (Zaat *et al*., 1987) which consisted of *Rhizobium* minimal medium (RMM) supplemented with thiamine free vitamin solution. The stock solutions of antibiotics, i.e., streptomycin (100μgml^{-1}), kanamycin (50μgml^{-1}), neomycin (50μg ml^{-1}), tetracycline (10μgml^{-1}), chloramphenicol (50μg ml^{-1}) and nalidixic acid (50μgml^{-1}) were filter sterilized and added to pre-cooled liquid media.

Flavonoid solutions

Stock solutions of standard flavonoid compounds used in the present study were prepared in absolute alcohol and added at a final concentration of 100 nmol L^{-1} for induction studies.

Collection of root exudates

Root exudates of chickpea, pea, clover and alfalfa were collected as described by Vincent (1970). Surface sterilized seeds (10g) were germinated on agar (1 %) at 25 °C for 48-72 hours to get approximately 2.5cm seedlings. Seedlings were transferred to wire nets placed on 15cm long tubes containing 10ml minimal medium for root exudates collection. Tubes were incubated in dark at 25 °C in plant growth chamber. After one week, plants were removed from tubes and root exudates were collected and filter sterilized through 0.45μm nitrocellulose membrane filter.

Assay of *nod* genes induction using *nod*A-*lac*Z fusion

Induction of *nod* genes was studied in *M. ciceri* by measuring β-galactosidase activity in triplicates in the presence of different flavonoids, as described by Srivastava *et al*. (1999). Briefly, *M. ciceri* strains containing homologous or heterologous *nod*D genes were grown in 5ml thiamine free induction medium (RMM) at 30 °C for 24 hours on shaker. Inducers (root exudates, 100μl or standard flavonoids, 100nmol L^{-1}) were added and cultures were further incubated for another 24 hours at 30 °C on shaker followed by the estimation of β-galactosidase activity as described by Miller (1972). Induction of *nod*A promoter was quantified on the basis of β-galactosidase activity expressed from *nod*A promoter fused with *lac*Z gene.

Statistical analysis

Statistical analysis was performed using MS-EXCEL Analysis Toolpak using ONE WAY ANOVA. Data were represented as mean of triplicates ± standard deviation. Data analysis was done at $P< 0.01$.

RESULTS

Induction profile of inducible *nod* promoter by different NodD protein products in *M. ciceri* NodD$^-$ background in presence of root exudates

Four *nod*D genes cloned in plasmids pMP280 (containing *nod*D of *R. leguminosarum* bv. *viciae*), pMP283 (containing *nod*D of *R. leguminosarum* bv. *trifolii*), pMP284 (containing *nod*D1 of *S. meliloti*) and pMP604 (containing flavonoid independent transcription activator or FITA *nod*D gene) were transferred to NodD$^-$ mutant of *M. ciceri*, HNL-9V (containing promoter *nod*A of *R. leguminosarum* bv. v*iciae* fused with *lac*Z). The resulting strains were designated as HNL-9DV, HNL-9DT, HNL-9DM and HNL-9DF, respectively. The induction profile of these four strains of *M. ciceri* containing heterologous *nod*D genes was compared with *M. ciceri* strains HS-1 and HSL-1V (containing promoter *nod*A of *R. leguminosarum* bv. v*iciae* fused with *lac*Z) containing functional homologous *nod*D gene in presence and absence of root exudates of chickpea, pea, clover and alfalfa.

Results showed that only chickpea root exudates were able to induce the inducible *nod* promoter in the strain *M. ciceri* HSL-1V containing functional homologous *nod*D gene to about three folds (48.6 Miller's units) as compared to its uninduced control (Table 2), while pea, clover and alfalfa root exudates did not induce the inducible *nod*A promoter. The *nod*D$^-$ background of strain HNL-9V also failed to induce the inducible *nod*A promoter even in the presence of different inducers. This indicated that the specific interaction of NodD with appropriate inducer present in root exudates determines the transcriptional activation of inducible *nod* genes in *M. ciceri nod*D background.

In case of HNL-9DV (containing *nod*D gene from *R. leguminosarum* bv. *viciae*), the induction of *nod* promoter was about two folds in presence of chickpea root exudates, while pea, clover and alfalfa root exudates induced the nod promoter upto 18.7, 8.5 and 12.1 folds respectively, over uninduced control (Table 2). In case of HNL-9DT (containing *nod*D gene from *R. leguminosarum* bv. *trifolii*), clover root exudates induced the maximum expression of *nod* promoter (16.8 folds) followed by pea root exudates (7.2 folds) and alfalfa root exudates (5.4 folds). Chickpea root exudates failed to induce the *nod* promoter in *M. ciceri* HNL-9DT. In case of HNL-9DM (containing *nod*D1 gene from *S. meliloti*), alfalfa root exudates induced the *nod* promoter to the maximum (12.8 folds) followed by clover root

Table 2. Induction profile of *nod*A promoter in NodD$^-$ mutant of *M. ciceri* containing heterologous *nod*D genes in presence of different root exudates

Strain/ Mutant	Source of *nod*D gene	β-galactosidase activity (in Miller's Units)				
		Without inducer	Chickpea root exudates	Pea root exudates	Clover root exudates	Alfalfa root exudates
HS-1	*M. ciceri*	0.22±0.03	0.22±0.02	0.20±0.02	0.21±0.03	0.23±0.02
HSL-1V	*M. ciceri*	16.36±0.40	48.59±0.37 (2.97)	13.74±0.22 (0.84)	9.82±0.15 (0.60)	10.47±0.02 (0.64)
HNL-9V	-	0.86±0.05	0.89±0.19	0.86±0.05	0.83±0.02	0.88±0.02
HNL-9DV	*R. leguminosarum* bv. *viciae*	2.08±0.08	4.30±0.05 (2.06)	38.94±0.12 (18.72)	17.72±0.21 (8.52)	25.17±0.05 (12.10)
HNL-9DT	*R. leguminosarum* bv. *trifolii*	1.89±0.11	1.93±0.04 (1.02)	13.68±0.29 (7.24)	31.96±0.31 (16.8)	10.17±0.04 (5.38)
HNL-9DM	*S. meliloti*	1.36±0.04	2.26±0.04 (1.66)	2.49±0.12 (1.83)	5.70±0.19 (4.19)	17.45±0.22 (12.83)
HNL-9DF	FITA *nod*D	98.42±0.76	97.54±0.38	98.36±0.39	97.75±0.12	97.68±0.13

Values in parentheses represent number of folds increase in activity over uninduced control.
$P<0.01$

exudates (4.2 folds). On the other hand, chickpea and pea root exudates could induce the *nod* promoter to very low levels (1.6 and 1.8 folds, respectively) in HNL-9DM. These results indicate that the NodD protein in NodD$^-$ background of *M. ciceri* can be activated for maximum induction of *nod* promoter by root exudates from homologous host plant only. The induction profile studies in HNL-9DF (containing FITA *nod*D gene) showed that the *nod* promoter was not at all affected either by the presence or absence of any of the root exudates. The induction of *nod* promoter remained to the maximum level (97.5 to 98.4 Miller's units) irrespective of the presence or absence of root exudates (Table 2).

Induction profile of *nod*A promoter by different NodD protein products in *M. ciceri* NodD$^-$ background in presence of standard flavonoids:

The *M. ciceri* strains, viz., HNL-9DV, HNL-9DT, HNL-9DM and HNL-9DF along with HSL-1V were studied for interaction of different *nod*D gene products with standard flavonoid compounds for the induction of *nod*A-*lac*Z promoter. Results showed that naringenin was the most potent inducer of *nod* promoter (5.6 folds) followed by daidzein (2.0 folds), while luteolin, quercetin, chrysin and 7-hydroxy coumarin appeared to inhibit *nod*A induction in case of *M. ciceri* HSL-1V containing functional homologous *nod*D gene (Table 3). On the other hand, hesperetin, biochanin, genistein and flavone showed very low level of induction of *nod* promoter (less than two folds) in *M. ciceri* HSL-1V. In case of *M. ciceri* HNL-9DV containing heterologous *nod*D gene from *R. leguminosarium* bv. *viciae*, naringenin showed the maximum induction of *nod* promoter (22.9 folds) followed by luteolin (17.7 folds), chrysin (8.7 folds), hesperetin (6.0 folds) and 7-hydroxy coumarin (2.0 folds), while daidzein and genistein did not show any effect on *nod* promoter induction. Biochanin, flavone and quercetin could induce the expression of *nod* promoter to very low levels (less than two folds). In case of *M. ciceri* HNL-9DT containing heterologous *nod*D gene from *R. leguminosarum* bv. *trifolii*, hesperetin showed the maximum induction of *nod* promoter (24.7 folds) followed by naringenin (21.7 folds), luteolin (14.5 folds) and flavone (10.0 folds), while biochanin (0.9 folds) and genistein (0.8 folds) appeared to inhibit the *nod* promoter. On the other hand, daidzein, quercetin, chrysin and 7-hydroxy coumarin did not show visible induction of *nod* promoter (less than two folds). In case of HNL-

Table 3. Induction profile of *nod*A promoter in NodD$^-$ mutants of *M. ciceri* containing heterologous and FITA *nod*D genes by standard flavonoids

Strain/ Mutant	β-galactosidase activity (in Miller's Units)										
	Without inducer	Narin-genin	Hespere-tin	Daidzein	Biochanin	Genistein	Flavone	Luteolin	Quercetin	Chrysin	7-OH coumarin
HS-1	0.2±0.02	0.2±0.02	0.2±0.03	0.2±0.02	0.2±0.01	0.2±0.01	0.2±0.03	0.2±0.02	0.2±0.02	0.2±0.01	0.2±0.03
HSL-1V	16.4±0.19	91.9±0.22 (5.6)	21.0±0.46 (1.3)	32.7±0.53 (2.0)	26.2±0.30 (1.6)	20.9±0.38 (1.3)	18.9±0.32 (1.2)	12.3±0.30 (0.8)	12.7±0.61 (0.8)	13.6±0.47 (0.8)	15.1±0.29 (0.9)
HNL-9V	0.9±0.02	1.0±0.07	0.9±0.08	0.9±0.08	0.9±0.10	0.9±0.06	0.9±0.15	0.9±0.08	0.9±0.06	1.0±0.08	0.9±0.08
HNL-9DV	2.1±0.12	48.1±0.23 (22.9)	12.7±0.22 (6.0)	2.0±0.16 (1.0)	3.1±0.14 (1.5)	2.1±0.11 (1.0)	3.2±0.28 (1.5)	37.2±0.92 (17.7)	3.1±0.54 (1.5)	18.2±0.34 (8.7)	4.2±0.14 (2.0)
HNL-9DT	1.9±0.05	41.2±0.10 (21.7)	46.9±0.40 (24.7)	2.3±0.11 (1.2)	1.7±0.14 (0.9)	1.6±0.06 (0.8)	19.0±0.89 (10.0)	27.5±0.40 (14.5)	3.5±0.49 (1.8)	3.1±0.37 (1.6)	2.7±0.22 (1.4)
HNL-9DM	1.4±0.06	2.7±0.14 (1.9)	4.2±0.09 (3.0)	1.1±0.08 (0.8)	13.8±0.27 (9.9)	1.2±0.15 (0.9)	37.0±0.99 (26.4)	55.4±0.71 (39.6)	1.3±0.12 (0.9)	1.8±0.07 (1.3)	1.4±0.17 (1.0)
HNL-9DF	98.4±0.54	99.0±0.83	98.1±0.61	98.3±0.60	98.2±0.75	97.6±0.58	98.4±1.34	97.3±0.76	100.1±2.29	97.9±0.44	96.9±0.81

Values in parentheses represent number of folds increase in activity over uninduced control
$P<0.01$

9DM containing heterologous *nod*D1 gene from *S. meliloti*, luteolin showed maximum induction of *nod* promoter (39.6 folds) followed by flavone (26.4 folds), biochanin (9.9 folds) and hesperetin (3.0 folds), while daidzein (0.8 folds), genistein (0.9 folds) and quercetin (0.9 folds) appeared to inhibit the *nod* promoter. Rest of the flavonoids, i.e., naringenin, chrysin and 7-hydroxy coumarin induced the *nod* promoter to less than two folds. In case of HNL-9DF containing FITA *nod*D gene, it was observed that there was neither induction nor inhibition of *nod* promoter in the absence or presence of any flavonoid compound and the *nod* promoter was induced constitutively to the maximum level. It appears from the results that a specific *nod*D gene product interacts with a specific set of flavonoids to induce the inducible *nod*A promoter in *M. ciceri*. Hence, it can be concluded that the source of *nod*D gene along with the inducing compound determines the induction of the *nod* promoter in *M. ciceri*.

DISCUSSION

The *nod*D gene product is known to interact with inducers (flavonoids) exuded by plant roots (Gyorgypal *et al*., 1991; van Rhijn *et al*., 1993; van Rhijn and Vanderleyden, 1995). In general, inducers of *nod* genes in fast growing rhizobia are flavones and flavonones, while in slow growing rhizobia these are the isoflavones. Inducers of *nod* genes have been identified in a number of rhizobial species. For example, methylchalcone, luteolin, trigonelline and stachydrine in *S. meliloti* (Hartwig *et al*., 1989; Phillips *et al*., 1994); naringenin and hesperetin in *R. leguminosarum* bv. *viciae* (Zaat *et al*., 1987); 7,4´-dihydroxyflavone in *R. leguminosarum* bv. *trifolii* (Redmond *et al*., 1986); daidzein and genistein in *B. japonicum* (Kape *et al*., 1992); and naringenin and daidzein in *M. ciceri* (Srivastava *et al*., 1999). The interaction of inducers with NodD protein results in the activation of NodD protein which then binds to conserved *nod* box sequences present upstream of inducible *nod* promoters (Fisher and Long, 1993). This binding constitutes the first host specific step in legume- *Rhizobium* symbiosis (van Rhijn and Vanderleyden, 1995; Long, 1996). The spectrum of flavonoid specificity of NodD protein is correlated with the broadness of the host range of *Rhizobium* (Le Strange *et al*., 1990; Gyorgypal *et al*., 1991). *Mesorhizobium ciceri* is a narrow- host range bacterium which nodulates the genus *Cicer* only (Sharma *et al.*, 1994). Molecular mechanism of host specificity of nodulation has not been worked out in this bacterium. Host specificity is regulated at the level of induction of *nod* gene, synthesis of host specific Nod factor or infection by a specific *Rhizobium* (D'Haeze and Holsters, 2002; Geurts and Bisseling, 2002).

In this study we explored the specificity of *M. ciceri*-*Cicer* symbiosis at the level of *nod* gene induction. To study the role of *nod*D gene of *M. ciceri* in inducer specificity and host specificity of nodulation, mutants of *M. ciceri* defective in *nod*D gene were isolated by random *Tn*5 mutagenesis (Kamboj *et al*., 2003). Earlier mutants defective in *nod*D gene have been isolated using *Tn*5 mutagenesis in *R. leguminosarum* bv. *viciae* (Downie *et al*., 1985; Rossen *et al*., 1985; Wijffelman *et al*., 1985), *R. leguminosarum* bv. *trifolii* (Djordjevic *et al*., 1985b), *S. meliloti* (Honma and Ausubel, 1987), and *Rhizobium* sp. NGR234 (Djordjevic *et al*., 1985a). The mutants which are defective in *nod*D gene fail to induce *nod* genes. The functional homology among different *nod*D gene products of *R. leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii* and *S. meliloti* was tested by complementation. The NodD protein of *M. ciceri* induced the expression of *nod* promoter in the presence of chickpea root exudates, while root exudates from heterologous hosts, pea, clover and alfalfa, inhibited the expression of inducible *nod* promoters. On the other hand, NodD protein of *R. leguminosarum* bv. *viciae* was able to induce the expression of *nod* promoter to different levels in presence of root exudates from homologous and heterologous legume hosts. The NodD protein of *R. leguminosarum* bv. *trifolii* induced the expression of *nod* promoter in NodD$^-$ mutant of *M. ciceri* in presence of all the root exudates, except chickpea, while NodD1 of *S. meliloti* induced the expression of *nod* promoter only in presence of alfalfa and clover root exudates. Although the most potent inducers are different in different *Rhizobium* species, yet the root exudates of heterologous hosts may contain a mixture of different inducers which are able to induce the transcription of *nod* genes in different *Rhizobium* species. The induction of *nod* promoters of *R. leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii* and *S. meliloti* in presence of root exudates of homologous as well as heterologous hosts have earlier been reported (Spaink *et al*., 1987b; Spaink *et al*., 1989b). The induction of *nod* genes by NodD protein of *M. ciceri* only in the presence of root exudates from chickpea and not by root exudates from heterologous hosts indicates that interaction of NodD protein with specific flavonoids is the first host- specific step in host specificity of nodulation in *M. ciceri*- chickpea symbiosis. Induction profile of *nod* promoter shows that

NodD protein of *M. ciceri* is specifically activated by naringenin and daidzein only, while that of *R. leguminosarum* bv. *viciae* by a number of flavonoids, viz., naringenin, luteolin, chrysin, hesperetin and 7-hydroxy coumarin. *Rhizobium leguminosarum* bv. *trifolii* and *S. meliloti* NodD proteins are also activated by a large number of flavonoids, viz., naringenin, hesperetin, flavone and luteolin. All these flavonoid compounds are present in chickpea, pea, clover and alfalfa root exudates (Peters *et al*., 1986; Redmond *et al*., 1986; Zaat *et al*., 1987; Zaat *et al*., 1989; Recourt *et al*., 1991; Phillips *et al*., 1994; Dixon and Palva, 1995; Srivastava *et al.,* 1999). On the other hand, luteolin, quercetin, chrysin and 7- hydroxy coumarin acted as inhibitors of NodD protein of *M. ciceri*, while biochanin and genistein; and daidzein, genistein and quercetin acted as inhibitors of NodD proteins of *R. leguminosarum* bv. *trifolii* and *S. meliloti*, respectively. Anti-inducer activities of these compounds have also been reported in *R. leguminosarum* bv. *viciae* (Kosslak *et al*., 1987), *R. leguminosarum* bv. *trifolii* (Djordjevic *et al*., 1987) and *S. meliloti* (Peters and Long, 1988). This indicates that activation of NodD protein depends on the combination and composition of inducers and anti-inducers in root exudates, and *nod*D gene product of *M. ciceri* is highly specific for chickpea root exudates in inducing the inducible *nod* promoter. The barrier of inducer specificity has been bypassed by constructing or cloning of flavonoid-independent transcription activator (FITA) *nod*D genes in different *Rhizobium* species (Burn *et al*., 1989; Spaink *et al*., 1989a; Spanik *et al*., 1989b). In order to test whether the inducer specificity of *M. ciceri* can also be bypassed, FITA *nod*D gene was transferred to NodD$^-$ mutant of *M. ciceri*, designated as HNL-9DF. It was found that the presence or absence of flavonoids or root exudates (homologous or heterologous) did not alter the ability of HNL-9DF to induce the inducible *nod* promoter, which remained at maximum level. Therefore, in the presence of heterologous *nod*D genes from different *Rhizobium* species and FITA *nod*D gene, it was possible to induce *nod* genes in *M. ciceri* NodD$^-$ mutants.

On the basis of results, it is concluded that *nod* genes in *M. ciceri* can be induced in the presence of heterologous *nod*D genes but *nod*D gene of *M. ciceri* is a host specific gene, which can induce other *nod* genes only in the presence of host specific (chickpea) root exudates. Therefore, the interaction of NodD protein with specific flavonoids is the first host- specific step in host specificity of nodulation in *M. ciceri*-chickpea symbiosis.

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