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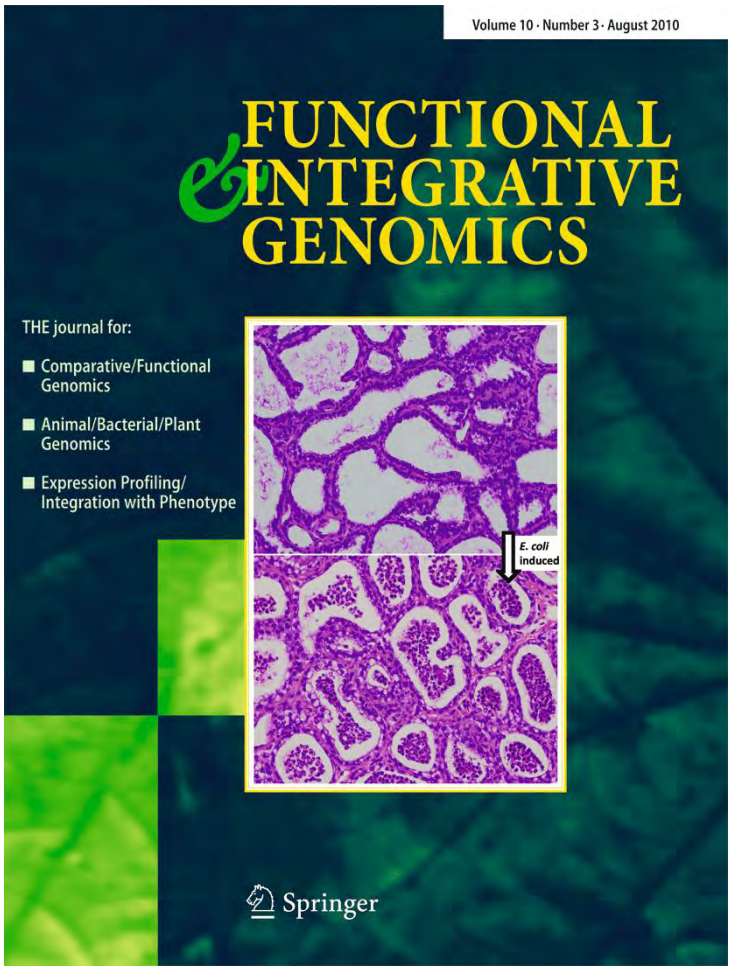

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SHORT COMMUNICATION

# The *nodC*, *nodG*, and *glgX* genes of *Rhizobium tropici* strain PRF 81

**Luciana Ruano Oliveira · Francismar Corrêa Marcelino · Fernando Gomes Barcellos · Elisete Pains Rodrigues · Manuel Megías · Mariangela Hungria**

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**Abstract** *Rhizobium tropici* is a diazotrophic microsymbiont of common bean (*Phaseolus vulgaris* L.) that encompasses important but still poorly studied tropical strains, and a recent significant contribution to the knowledge of the species was the publication of a genomic draft of strain PRF 81, which revealed several novel genes [Pinto et al. Funct Int Gen 9:263–270, 2009]. In this study, we investigated the transcription of *nodC*, *nodG*, and *glgX* genes, located in the *nod* operon of PRF 81 strain, by reverse-transcription quantitative PCR. All three genes showed low levels of transcription when the cells were grown until exponential growth phase in the presence of common-bean-seed exudates or of the root *nod*-gene inducer naringenin. However, when cells at the exponential phase of growth were incubated with seed exudates, transcription occurred after only 5 min, and *nodC*, *nodG*, and *glgX* were transcribed 121.97-, 14.86-, and 50.29-fold more than the control, respectively, followed by a rapid decrease in gene transcription. Much lower levels of transcription were observed in the presence of naringenin; furthermore, maximum transcription required 8 h of incubation for all three genes. In light of these results, the mechanisms of induction of the nodulation genes by flavonoids are discussed.

**Keywords** Biological nitrogen fixation · Gene transcription · Nodulation genes · *Phaseolus vulgaris* · Reverse transcription quantitative PCR · *Rhizobium tropici* · RT-qPCR · Symbiosis

L. R. Oliveira · F. C. Marcelino · F. G. Barcellos · E. P. Rodrigues · M. Hungria (✉)
Embrapa Soja,
Cx. Postal 231, CEP 86001-970 Londrina, Paraná, Brazil
e-mail: hungria@cnpso.embrapa.br
e-mail: hungria@pq.cnpq.br

L. R. Oliveira
e-mail: lu_ruano@hotmail.com

F. C. Marcelino
e-mail: francm@cnpso.embrapa.br

F. G. Barcellos
e-mail: fgbarcel@yahoo.com.br

E. P. Rodrigues
e-mail: bioliza@yahoo.com.br

L. R. Oliveira · M. Hungria
Department of Microbiology, Universidade Estadual de Londrina, Londrina, Brazil

L. R. Oliveira · E. P. Rodrigues · M. Hungria
Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-MCT),
Brasília, Brazil

M. Megías
Facultad de Farmacia, Universidad de Sevilla,
Apdo. Postal 874, 41080 Sevilla, Spain
e-mail: megiasg@us.es

## Introduction

Many species of the family Leguminosae are capable of establishing symbioses with a group of bacteria collectively called rhizobia, of which the most important feature is the capacity of forming specific structures in the roots of the host plant—the nodules—where the biological process of fixing atmospheric nitrogen ($N_2$) takes place (Allen and Allen 1981). Root-nodule formation requires the expression of nodulation genes (*nod*, *nol*, and *noe*) in the bacteria: in the great majority of the rhizobia-legume symbioses, natural nodulation (*nod*)-gene-inducing factors, mainly flavonoids, are released from the host legume and in the

presence of an appropriate product from the regulatory *nodD* gene induce the transcription of common *nod* genes (*nodABC*) in the bacteria. In response to the inducers released by the host plant, rhizobia synthesize lipo-chito-oligosaccharides (LCOs), also known as Nod factors, which at very low concentrations are responsible for a variety of root phenotypes essential for nodulation and that can even trigger nodule formation. Common and host-specific *nod* genes contribute to the synthesis of Nod factors, the basic structure of which consists of a backbone of four to five glucosamine residues *N*-acylated at the non-reducing end, with several O-substituents (e.g., reviews by Long 1989; Hungria and Stacey 1997; Debellé et al. 2001; Brencic and Winans 2005).

Grains of the legume common bean (*Phaseolus vulgaris* L.) represent the most important source of protein for poor populations in Central and South America and in parts of sub-Saharan Africa. The legume is considered "promiscuous" as it is capable of nodulating and fixing $N_2$ with a broad range of rhizobial species (e.g., Hungria et al. 2000; Grange and Hungria 2004; Pinto et al. 2007). This promiscuity is far from being understood, but it may be associated with the variety of flavonoid *nod*-gene inducers released by the legume (Hungria et al. 1991a, b; Bolanõs-Vásquez and Werner 1997). The variety of Nod factors released by the rhizobial microsymbionts of common bean (Morón et al. 2005; Estévez et al. 2009) may also be relevant.

Despite its global economic and social importance, few studies have been reported on *Rhizobium tropici*, an important symbiont of common bean in tropical acid soils (Martínez-Romero et al. 1991) which is abundantly present in all Brazilian biomes (e.g., Grange and Hungria 2004; Pinto et al. 2007). Several strains belonging to the *Rhizobium tropici* species—and one major example is strain PRF 81 (SEMIA 4080)—have been recognized as very effective in fixing $N_2$, competitive against indigenous rhizobial populations, genetically stable, and adapted to stressful tropical environments, all of which are important properties for their use as inoculants (Hungria et al. 2000, 2003; Pinto et al. 2009). Several strains belonging to the *R. tropici* species (one major example is strain PRF 81, SEMIA 4080) have been recognized as very effective in fixing N2, competitive against indigenous rhizobial populations, genetically stable, and adapted to stressful tropical environments, all of which are important properties for their use as inoculants (Hungria et al. 2000, 2003; Pinto et al. 2009). Studies of gene transcription in *R. tropici* carried out in our research indicate, for the first time, the effects of flavonoids on the transcription of *nod* genes using reverse-transcription quantitative PCR (RT-qPCR). The transcription patterns of *nodC*, *nodG*, and *glgX* genes are shown to be consistent with their key function in the establishment of nodulation.

## Materials and methods

### Strains, media, and growth conditions

*R. tropici* strain PRF 81 (SEMIA 4080) was obtained from the Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soybean and transferred to Petri plates containing yeast-mannitol medium (Vincent 1970) with Congo red (0.025%) and incubated at 28°C for 48 h. Cells were cultured in 10 mL of liquid tryptone-yeast (TY) broth (Beringer 1974) and shaken at 120 rpm until an optical density (O.D.) at 600 nm of 0.9 was reached to obtain the pre-inoculum.

### Assays for induction of *nod* genes

In the first assay, 1 mL of the pre-inoculum was added to 200 mL of TY broth (Beringer 1974) and incubated for 48 h in the presence of either the inducer naringenin or exudates from common-bean seeds, prepared as described by Hungria et al. (1991a). The final concentration of naringenin was 1.5 µM and for the seed exudates, we used the O.D. registered at 289 nm and the extinction coefficient of the naringenin (log ∈=4.23 at 289 nm) to estimate an approximate concentration of 1.5 µM. After 48 h (O.D. 0.4–0.8) gene expression/transcription was evaluated.

In the second assay, the cells were cultured until they reached the exponential growth phase (O.D. 0.4–0.8) before induction by naringenin or exudates and the gene expression was determined after various periods of time: 5 ($T_5$ min), 15 ($T_{15}$ min) min and 1 ($T_1$ h), 4 ($T_4$ h), and 8 ($T_8$ h) h after induction. Water and methanol (the same amount used as seed exudates and naringenin, respectively) were used as the control treatment for gene expression/transcription variation in relation to the exudates and narigenin inducers, respectively.

The assays were conducted in a completely randomized block design with three biological replicates, represented by three independent experiments, of cultures grown in different times, and each experiment was performed with three replicates.

### RNA extraction

Total RNA from these bacteria was prepared as described by Farrell (1998). Briefly, after reaching the desired O.D., cells were harvested by centrifugation at 10,000×*g* for 10 min at 4°C. The cell pellet was resuspended in lysing buffer, consisting of 0.75% Nodenit P40, 250 mM sucrose, and 20 mM EDTA and potassium acetate buffer (3 M $KCH_3O_2$ and 100 g of guanidinium thiocyanate per 100 mL), and kept on ice for 10 min. The sample was centrifuged and the supernatant extracted with chloroform/isoamyl alcohol (24:1). The upper phase was precipitated

with 0.6 volumes (*v*) of ice-cold isopropanol. The RNA was collected by centrifugation at 10,000×*g* for 10 min at 4°C. Contaminating DNA was removed from RNA preparations by using DNase I and Qiagen columns (clean-up procedure). RNA was quantified by NanoDrop ND-1000 (NanoDrop Technologies, Inc.), and the integrity of the RNA was assessed on a 1% (*w*/*v*) agarose gel. The first strand of cDNA was obtained using 1.0 µg of RNA and the TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, Foster, CA, USA), following the manufacturer's instructions.

### Primers design

Primers were designed using PrimerExpress3.0 (Applied Biosystems) targeting an amplicon size of 50–150 bp. The partial genome of *R. tropici* (Pinto et al. 2009, http://www.bnf.lncc.br/webbie/final/main.html) was used to design primers specific for *nodC, nodG*, and *glgX* genes. The primers used are listed in Table 1.

### RT-qPCR

Samples were amplified with primers for the *nodC, nodG*, and *glgX* genes (Table 1) and also with primers for the 16S rRNA gene (Table 1), used as the endogenous control, and the SYBR Green Master Mix Kit (Applied Biosystems). Reactions were performed on a 7500 RT-qPCR thermocycler (Applied Biosystems), following the manufacturer's instructions. After the initial steps at 50°C for 2 min (UNG activity) and at 95°C for 10 min (activation of the AmpliTaq Gold polymerase), a two-step program of 95°C for 15 s and 62°C for 1 min was conducted for 45 cycles. The dissociation curves were obtained to guarantee the absence of unspecific amplifications. The data were collected in the last phase (extension phase), and the results were analyzed by the Sequence Detection program (Perkin Elmer, Massachusetts, CA, USA) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The $E = \left[10^{-1/\text{slope}}\right] - 1$ formula was used to calculate the reaction efficiency. The calibration curve was established by the Ct and the log of the cDNA dilutions.

The relative quantification (RQ) was estimated applying the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The mean values of the Ct of the target and endogenous control genes were estimated. The Ct of the target gene was then subtracted from the Ct of the endogenous control, resulting in the ΔCt. The ΔCt of the target gene was then subtracted from the ΔCt of the calibrator (non-treated control), resulting in the ΔΔCt. The RQ was estimated by the equation of $1,95^{\Delta\Delta Ct}$, adjusted by the efficiencies obtained between the target gene and the endogenous control. A final RQ was estimated considering the RQ obtained in each biological replicate, represented by each independent experiment, each with three replicates. Significant differences were determined by estimates of standard deviation (SD) and also with the REST software version 2.0.7 ($p<0.05$) (Pfaffl et al. 2002; http://www.gene-quantification.info).

**Table 1** Sequences of the primers used in the RT-qPCR and sizes of the PCR products obtained

| Gene | Primers sequence | Amplicon size |
|---|---|---|
| *nodC* | F 5′ CAAGCTGCGCCCTTATCTG 3′<br>R 5′ CAAGCAACGTGTCACGGAAA 3′ | 68 bp |
| *nodG* | F 5′ GAGCTGACCCATCCGATGA 3′<br>R 5′ CGACGACCGAGGTGATGTT 3′ | 64 bp |
| *glgX* | F 5′ CCGGTGAGAGCATCATCCTT 3′<br>R 5′ AACTGCGATCAGTCTTCCAGAAG 3′ | 64 bp |
| *16S rRNA* | F 5′ CAAGGCGACGATCCATAGCT 3′<br>R 5′ AGGAGTTTGGGCCGTGTCT 3′ | 72 bp |

## Results and discussion

Few studies have been performed on nodulation genes of *R. tropici*, although new information has been revealed in the genome draft of strain PRF 81 (Pinto et al. 2009), which is used in commercial inoculants applied to common-bean crops in Brazil (Hungria et al. 2000, 2003). In this study, we investigated the transcription of *nodC*, *nodG*, and *glgX* genes in PRF 81, after induction by common-bean seed exudates or by the flavonoid naringenin—a major inducer released by common bean roots (Hungria et al. 1991b). Naringenin was used at a concentration of 1.5 µM, as in a previous study with PRF 81, incubation with 2.0 µM resulted in abundant Nod-factor production (Hungria et al. 2000). To have a base of comparison, the concentration of seed exudates was estimated to be similar to that of naringenin; however, it is noteworthy that seed exudates comprise many compounds with strong *nod*-gene-inducing activity (Hungria et al. 1991a). Gene transcription was studied in cells in the exponential growth phase (O.D. 0.4–0.8) after induction by seed exudates and by naringenin and, for the first time, the transcription of *nod* genes in *R. tropici* was evaluated by RT-qPCR.

As in other fast-growing rhizobia, in *R. tropici* type strain CIAT $899^T$ the common nodulation genes *nodABC*, preceded by *nodD* and a *nod*-box, are localized in the symbiotic plasmid (pSym); however, there are still uncertainties about the number of *nodD* copies (Vargas et al. 1990; van Rhijn et al. 1993). It has been demonstrated that NodC plays a role in the biosynthesis of the basic structure of the chito-oligosaccharides (Geremia et al. 1994; Spaink et al. 1994), more specifically it is an important determinant

of the LCO chain length (Kamst et al. 1997). The putative NodC of PRF 81 (Pinto et al. 2009) is highly similar in BLASTP to that reported for CIAT 899$^T$ (Folch-Mallol et al. 1996), and might be also similar in several other *R. tropici* strains (Pinto et al. 2007). When grown for 48 h in the presence of the inducer naringenin, no transcription of *nodC* gene of PRF 81 was observed, however, an up-regulation of 1.88-fold was observed when induced by seed exudates (Table 2). In the second experiment, bacteria were grown in regular culture medium for 48 h and then exposed to the inducers for different periods of time, ranging from 5 min to 8 h, and remarkably, the highest level of gene transcription was observed after only 5 min of incubation with seed exudates, with 121.97-fold induction in comparison to the control (Fig. 1), statistically significant ($p \leq 0.001$) considering the REST program (data not shown). Meanwhile, in the presence of narigenin, a far more modest rate of *nodC* transcription—1.89-fold—was observed only after 8 h of incubation (Fig. 1).

Studies of expression/transcription of *nodC* in rhizobial species have been performed using different methods. Adopting reporter plasmids carrying *lacZ*, Mulligan and Long (1985) have detected that in presence of exudates of the host legume *Medicago sativa*, *nodC* gene of *Sinorhizobium meliloti* strain 1021 had a 30-fold induction; in addition, 0.5 μM of naringenin induced *nodABC* genes of *R. leguminosarum* bv. viciae strain D923 by approximately 37-fold (Tsvetkova et al. 2006), and of *R. leguminosarum* bv. phaseoli by 1,490-fold (Hungria et al. 1991b). In another study using microarrays, a 35.1-fold increase of *nodC* expression in *Bradyrhizobium japonicum* strain USDA 110 was observed, when cells with O.D. of 0.4–0.6 were incubated for 8 h with 1 μM of genistein (Lang et al. 2008), while by using RT-qPCR a 218.1-fold up-regulation was verified in the same strain after 6 h of incubation of cells at early exponential phase (O.D of 0.1) with 5 μM of genistein (Wei et al. 2008). What should be highlighted in our study is the extremely fast transcription in the presence of inducers composing the seed exudates, with a maximum value observed after only 5 min of incubation.

The genome draft of PRF 81 revealed a putative *nodG* and the sequence obtained shows high BLASTP similarity (92%) with the gene described in *R. leguminosarum*, which encodes a 3-oxoacyl-(acyl) carrier protein reductase involved in the biosynthesis and transfer of common fatty acids, with the same function of FabG (López-Lara and Geiger 2001). *nodG* is also present in other rhizobia (*S. meliloti*, *Rhizobium* sp. strain N33, and *Mesorhizobium* sp. strain 7653R), being localized immediately downstream *nodFE* (López-Lara and Geiger 2001). As previously discussed (Pinto et al. 2009), phenotypes reported for *nodG* mutants go from lack or slight effects to a reduction in nodule formation and size. In our study, when grown for 48 h in the presence of inducers, differential transcriptions of *nodG* of 1.40- and 1.46-fold for seed exudates and narigenin, respectively, in comparison to the control, were observed (Table 2). In the second experiment, an increase of 14.86-fold occurred in the presence of seed exudates (Fig. 1) and the results were statistically different considering the REST program (data not shown); this is the first report of *nodG* transcription in *R. tropici*. In the presence of narigenin, *nodG* transcription increased by 1.64-fold after 5 min and by 1.56-fold after 8 h, although due to the high variability of the method this last value was not statistically different from the control; no further increase was observed after 8 h.

*glgX* is a gene involved in the transformation of glycogen into 1,4-glycan (Preiss and Romeo 1989, 1994). In *R. tropici* CIAT 899$^T$, *glgX* has been located immediately upstream of orfN (Marroqui et al. 2001), showing high BLASTP similarity with the putative *nodN* described in PRF 81 (Pinto et al. 2009). Analyzing completely sequenced rhizobial genomes, we have seen that *glgX* precedes *nodN* in *Agrobacterium* (*Rhizobium*) *radiobacter*, *A. vitis*, *A. tumefaciens*, *R. etli*, *S. meliloti* and in *R. leguminosarum*, but not in *M. loti* or *Bradyrhizobium*; therefore, apparently the evolution of *nodN* in the first group of bacteria—and also in *R. tropici*—occurred together with the glycogen operon. In our study, a 1.48-fold increased transcription of *glgX* was observed in cells grown for 48 h with seed inducers, but not with naringenin (Table 2). In the second experiment, as observed with the two other genes from this study, maximum gene transcription was observed after 5 min of incubation with seed exudates—50.29-fold—whereas in the presence of naringenin a 2.88-fold increase was observed after 8 h of incubation (Fig. 1).

The extremely rapid transcription of *R. tropici nodC, nodG*, and *glgX* genes obtained in this study, especially in the presence of seed exudates, indicates that signal

**Table 2** Transcription of *nodC*, *nodG*, and *glgX* genes of *Rhizobium tropici* strain PRF 81 grown for 48 h in the presence of seed exudates or naringenin

| Gene | Relative expression (fold)[a] | |
|---|---|---|
| | Seed exudates[b] | Naringenin[b] |
| *nodC* | 1.88±0.30 | 0.85±0.15 |
| *nodG* | 1.40±0.20 | 1.46±0.40 |
| *glgX* | 1.48±0.21 | 1.04±0.10 |

[a] Means and standard deviation of three biological replicates, each with three replicates

[b] Expression in relation to the control with water for seed exudates and control with methanol for the naringenin. Relative quantification was estimated by the equation $1,95^{\Delta\Delta Ct}$, as described in the "Materials and methods" section

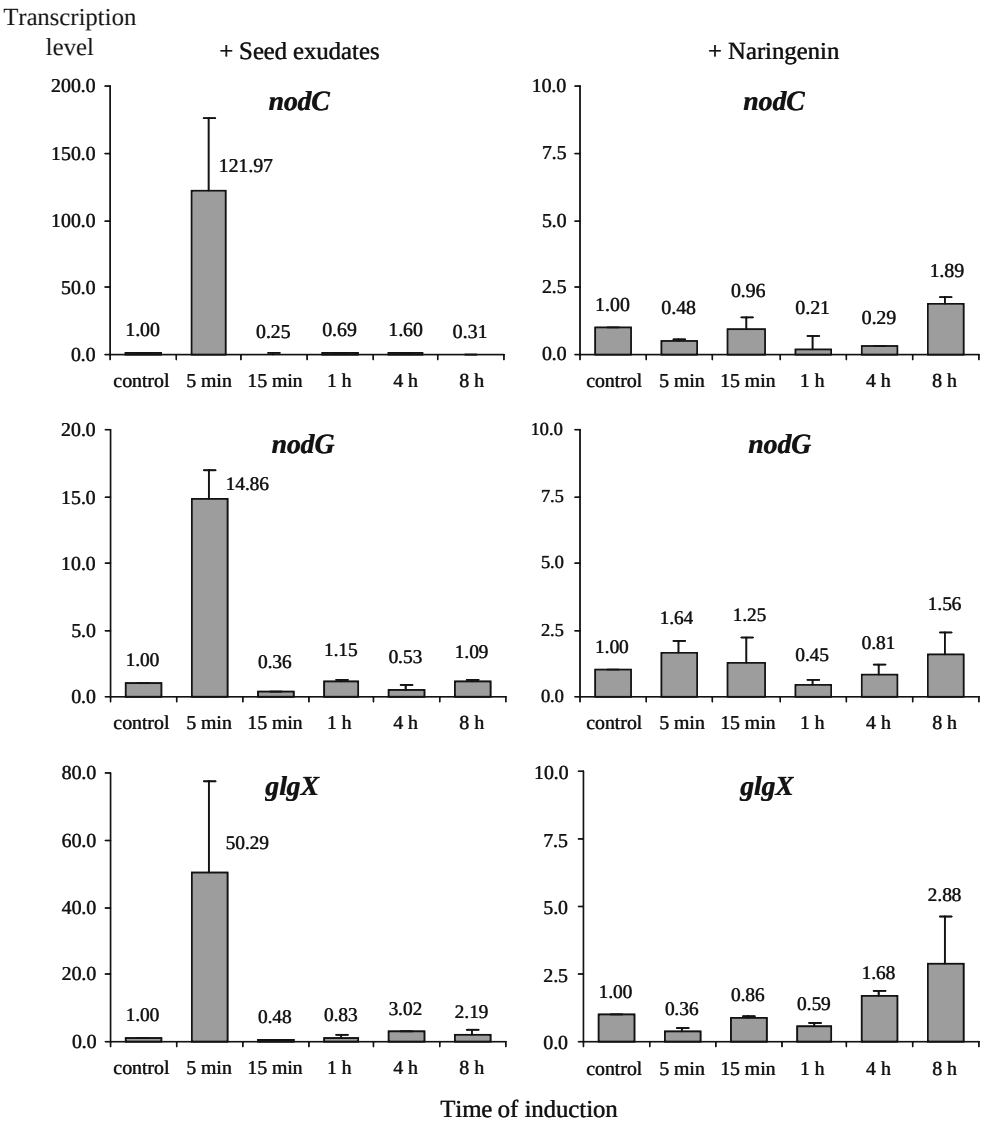


**Fig. 1** Transcription of *nodC*, *nodG*, and *glgX* genes of *Rhizobium tropici* strain PRF 81 grown until exponential phase and then incubated with seed exudates or naringenin for various periods of time. Data represent the means of three biological replicates, each with three replicates ±SD. Relative quantification was estimated by the equation $1,95^{\Delta\Delta Ct}$, as described in the "Materials and methods" section

exchanges between host legumes and rhizobia may occur faster than previously thought. In addition, our results show that maximum gene transcription, activated by one inducer present in root exudates, occurred later and at a much lower level than with seed exudates, indicating a need for rapid gene transcription simultaneously with seed germination. There is, indeed, strong evidence from other studies that legume-rhizobia signaling may take place promptly: (1) flavonoids with high *nod*-gene-inducing activity in *R. leguminosarum* bv. *phaseoli* were released by common-bean seeds within 6 h of contact with water (Hungria et al. 1991a); (2) Fellay et al. (1995), using competitive hybridization, and Perret et al. (1999), with radioactively labeled RNA, showed that the genes involved in the synthesis of Nod factors of *Rhizobium* sp. strain NGR 234 were induced rapidly, i.e., within a few minutes of the addition of root exudates or flavonoids to the growth medium; (3) sharp oscillations of cytoplasmic calcium-ion concentration (calcium spiking) in *M. sativa* root hairs, which occurred after minutes of exposure to Nod factors (Ehrhardt et al. 1996), were confirmed when *M. truncatula* was exposed to *S. meliloti* (Wais et al. 2002), and in other symbioses (e.g., Harris et al. 2003).

On the other hand, decreased gene transcription after 5 min of incubation with seed exudates can be explained in terms of prokaryote mRNAs being transcribed and translated in a single cellular compartment with both processes directly related and occurring almost simultaneously. Furthermore, mRNAs in prokaryotes are unstable, with half lives of only a few minutes (on average, approximately 2 min); translation into proteins occurs even before transcription is complete, and degradation of mRNA occurs as soon as translation finishes (e.g., Lewin 2004; Condon 2007). Therefore, after maximum gene expression, mRNA degradation may explain the decreases in gene transcription observed in our study. It remains to be determined whether

*nod*-boxes precede *nodG* and *glgX* and are directly related to the transcription of these two genes, or if the transcription is dependent exclusively on the *nod*-box related to NodD, initiating a rapid transcription of the three genes investigated in this study. Interestingly, 19 *nod*-boxes, 18 of which were induced by the flavonoid daidzein, were identified in the symbiotic plasmid of *Rhizobium* sp. NGR 234 (Kobayashi et al. 2004), and, when the first complete genome of strain PRF 81 becomes available, it will be possible to verify how many *nod*-boxes it carries.

In this study the remarkable high values verified after 5 min of incubation with seed exudates, followed by a considerable decrease soon after may suggest some mechanisms controlling nodulation. The results could be interpreted as leading to an initial high production of Nod factor synthesis enzymes, which, very rapidly, is reduced to much lower levels and one hypothesis is that this mechanism could prevent inhibition of nodulation by a too high concentration of Nod factors. The results also indicate that there are compounds in seed exudates still to be determined, that might be responsible for nodulation gene induction in much faster periods of time.

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