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Characterization of a *nodM/glmS* Homologous Gene in the Symbiotic Cyanobacterium *Nostoc* PCC 9229

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Abstract

A *nodM/glmS* homologue was identified by PCR in *Nostoc* PCC 9229, a cyanobacterial strain forming symbiosis with *Gunnera* plants, and the corresponding gene was isolated and sequenced. The predicted amino acid sequence shows significant homology (40% to 67% identity) to other bacterial glutamine-fructose-6-P-aminotransferases. The gene is present as a single copy in the genome of the *Nostoc* PCC 9229 as well as in other symbiotic and free-living cyanobacteria. Initial data indicate that the isolated gene is linked to the *psaC* gene and that there is no evidence for the presence of a *nod* genes cluster organized as in rhizobia. RNAse protection assays did not show any induction in the expression pattern of the gene by *Gunnera* plant mucilage, seed rinse extract or different flavonoids. These data suggest that the *nodM* homologue in *Nostoc* should be considered as a *glmS* housekeeping gene which does not play an immediate role in the *Nostoc-Gunnera* symbiosis. The nucleotide sequence data reported in this paper has been deposited in GenBank (accession number AFO28734).

Keywords: *glmS*, *Gunnera*, *psaC*

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1. Introduction

Cyanobacteria are nitrogen-fixing phototrophs some of which can form symbioses with eukaryotic organisms. The only intracellular interaction between a higher plant and a cyanobacterium is the *Gunnera-Nostoc* symbiosis (Bergman et al., 1992). The cyanobacterium provides nitrogenous compounds to the plant and receives back fixed carbon (Söderbäck et al., 1993). The infection process consists of several different stages, each characterized by specific modifications both in the host plant and the cyanobacterium (Johansson and Bergman, 1992). An acidic mucilage, mainly composed of sugars and putative arabinogalactan proteins (Rasmussen et al., 1996), is secreted from stem glands, the organs susceptible to *Nostoc* infection. The mucilage contains factors which induce both a rapid synthesis of *Nostoc* specific proteins and turn the vegetative *Nostoc* filaments into hormogonia, motile non-nitrogen-fixing filaments (Rasmussen et al., 1994). The hormogonia move into channels present in the glands and are taken up into cytoplasmatic plant cells lining the channel. Once inside, the *Nostoc* differentiates supernoumerous N_2-fixing cells, heterocysts, and fixes N_2 at a relatively higher rate as compared to the free-living stage (Söderbäck et al., 1993).

Little is known about the molecular communication in the *Gunnera-Nostoc* symbiosis. In symbiotic rhizobia, *nod* genes are involved in the production of Nod factors, chitin oligomers with an acyl chain at the non-reducing end, which have numerous effects on host and non-host plants (Heidstra and Bisseling, 1996). In fast-growing rhizobia most *nod* genes are localized as a cluster on large Sym-plasmids and are induced by compounds released by the host (Mercado-Blanco and Toro, 1996). Homologies to infection-related rhizobial and agrobacterial genes have been detected in the cyanobacterium *Anabaena azollae* (Plazinski et al., 1991) and in the actinomycete *Frankia* (Chen et al., 1991). Common and host specific *nod* genes have been detected also in the bacterium *Azospirillum* (Fogher et al., 1985; Vieille and Elmerich, 1990). In *Nostoc* PCC 9229, an isolate from *Gunnera monoika*, low stringency heterologous hybridization suggested that genes homologous to the rhizobial *nodEF*, *nodMN* and *exoY* genes, along with the *nod* box (the *nodD* binding part of the nod promoter), might be present. No homologies were found to the common *nod* genes, *nodABC*, *nodD1* and *nodD2*, using the same hybridization techniques (Rasmussen et al., 1996).

The *nodM* gene has been characterized in *Rhizobium meliloti* as a second copy of the *glmS* (glucosamine-fructose-6-phosphate aminotransferase, EC 2.6.1.16) gene (Baev et al., 1991). This enzyme is involved in the formation of N-acetylglucosamine, an essential building block of both bacterial and fungal cell walls as well as a constituent of the carbohydrate backbone of the host

interactive Nod factors released by rhizobia. In the present work we report the identification, cloning and expression analysis in the symbiotic cyanobacterium *Nostoc* PCC 9229 of a gene corresponding to the rhizobial *nodM/glmS* gene, with the aim to verify its possible involvment in the infection process of the *Gunnera-Nostoc* symbiosis.

2. Material and Methods

Cyanobacterium growth conditions

The cyanobacterium *Nostoc* PCC 9229 was cultivated in BG-11_0 medium (Stanier et al., 1971) under continuos light and with shaking as described previously (Johansson and Bergman, 1992). For induction experiments different inducers were tested: *Gunnera* mucilage (250 µl/ml), *Gunnera* seed rinse (see below) and the rhizobial *nod* gene inducers chrysin (10 µM), kaempferol (10 µM), naringenin (10 µM), and quercetin (10 µM). The different compounds were added for 6 or 16 hours to the *Nostoc* cultures, kept under the same growth conditions as above. The cultures were then harvested and frozen in liquid nitrogen.

The *Gunnera* mucilage was diluted (1:1) in water and then filter sterilized. Seeds of *G. manicata* were sterilized in 70% ethanol, washed in sterile water and then soaked overnight in sterile water (1 g seed/5 ml) and the rinse tested for induction of *nodM/glmS* expression in *Nostoc* PCC 9229.

PCR amplification

Amino acid sequences of *nodM* from different rhizobial strains and of *glmS* from *E. coli* were used to design degenerate oligonucleotide primers [coding for RWATHE (5') and GETADT (3')] for PCR in order to amplify the coding region of the corresponding *Nostoc* gene.

Library construction and screening

A *Nostoc* PCC 9229 library was constructed by using 15–22 Kb fractions of genomic DNA, partially digested with *Sau*3AI, and phage λ-EMBL3 arms cut with *BamH*I (Stratagene). The screening was performed according to the manufacturer's instructions using the PCR isolated partial clone as a probe.

The DNA sequence was determined for both strands with the T7 DNA polymerase sequencing kit (Pharmacia).

DNA-RNA extraction and hybridization

Total DNA was extracted from *Nostoc* PCC 9229 according to Dzelzkalns et al. (1988), separated on a 0.8% agarose gel and blotted onto a Zeta-Probe GT membrane (Bio-Rad). The DNA probes were obtained by labeling with [^{32}P] dCTP (Amersham) using the DECAprime kit (Ambion). Hybridizations were performed according to the manufacturer's instruction for the Zeta-Probe membrane at 42°C and washed up to 65°C in 0.1 x SSC, 0.1% SDS.

Total RNA was extracted from *Nostoc* PCC 9229 using a Qiagen kit according to the manufacturer's instructions. Riboprobes for RNAse protection assays (RPA) were generated from the 0.45 kb *Eco*RV/*Eco*RI fragment of *Nostoc* PCC 9229 *nodM/glmS* subcloned in pBluescript SK, linearized with *Bam*HI. RPA was performed with equal amounts (5 µg) of total *Nostoc* RNA, using RPAII reagents (Ambion). Equal loading was verified on ethidium bromide-stained gels.

3. Results

Isolation and characterization of the nodM/glmS gene homologue

The primer combination used for the PCR amplification of *Nostoc* PCC 9229 DNA resulted in the synthesis of a 800-bp product, corresponding to an open reading frame (ORF) of 260 amino acids homologous to the expected protein. Using the cloned PCR product as a probe, a genomic library of *Nostoc* PCC 9229, was screened to isolate a fragment containing the entire *nodM/glmS* gene. A *Hin*dIII fragment of 3 kb, a *Hin*dII fragment of 1.4 kb and a *Eco*RV/*Eco*RI fragment of 0.45 kb were subcloned (Fig. 1) and the complete sequence determined (GenBank AFO28734).

The cloned ORF predicted a protein of 625 amino acids coding for a glucosamine-fructose-6-P aminotransferase (*glmS*).

A computer alignment (ClustalW) of the obtained *Nostoc* PCC 9229 gene with other cyanobacterial, rhizobial and bacterial *nodM/glm*S genes shows that the amino acid identity between *Nostoc* and other glucosamine transferases genes varied between 40% (*E. coli*, *R. meliloti* and *R. leguminosarum*) to 67% (*Synechocycstis* PCC 6803), the N- and C-termini being the most conserved regions of the protein (data not shown). At the nucleotide level no significant homology can be found between the *Nostoc* gene and the rhizobial *nodM*.

Upstream of the *Nostoc* PCC 9229 *nodM/glmS* coding sequence 860-bp were also sequenced. A 520-bp sequence separates the *nodM* ORF from a *psa*C gene ORF, coding for an 81 amino acid protein of Photosystem I (subunit VII) (Fig. 2).

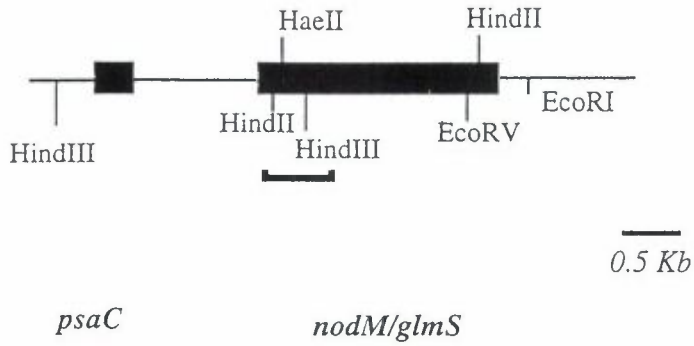

Figure 1. Partial restriction map of recombinant phage showing the location of the *psaC* and *nodM/glmS* homologue genes in *Nostoc* PCC 9229. The black bar indicate the position of the 800 bp *nodM/glmS* PCR isolated fragment.

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1     ccttttgattacggcctatcaagcactgcgtaaactatgcgttgattatgattctttcgc
61    agagaagaacactcgaaaggagccgttttttcaatgtctcataccgtaaaaatctacgat
                                      M  S  H  T  V  K  I  Y  D

121   acctgcattggctgcacccaatgcgtccgcgcttgccccactgacgtactggagatggtt
       T  C  I  G  C  T  Q  C  V  R  A  C  P  T  D  V  L  E  M  V
181   ccttgggatggctgcaaggctgctcaaattgcctcttcaccccgtacagaagactgtgta
       P  W  D  G  C  K  A  A  Q  I  A  S  S  P  R  T  E  D  C  V

241   ggctgtaagcgttgcgaaactgcttgccccacagacttttgagcattcgggtttacctg
       G  C  K  R  C  E  T  A  C  P  T  D  F  L  S  I  R  V  Y  L
301   ggtgctgaaacaactcgcagtatgggtctggcttactaaaaacctcgtgcagagtgctga
       G  A  E  T  T  R  S  M  G  L  A  Y  *
361   gtacctgtgctgagatacaaactaggaaattacaaaaataatttcttgattcctcactca
421   gtattcggaactcagccatcaagactctgtagtgtcatagcactacttacattcagagca
481   gtgcagtccttgtctgcaaagtgcaactgtgttggtactgcactggccttacaaaatcgt
541   aatttagaatggggataaaatctctgggtatttaaatcctataagcgcagcttttgtacc
601   gtccaatccaaacccagaatctatataacaatacctagtggcatagagggagcaaatagc
661   ccccttttttttggtaatttttaattagttgttgagtgttaaccgtcaacagttaacac
721   tcaacagtcaacgaaactaaaacttttgacttgtttcaccttcttagccgtttttgtatc
781   agccctatctttgcaaaacacccagtttgtggtaacaactatacggatgtaatcatcctg
841   aaaaaaaatggtgtgagcaatgtgtgggatcgttggatatataggcactcaagcggcgac
                            M  C  G  I  V  G  Y  I  G  T  Q  A  A  T
901   agacattttactagctgggctggaaaaactagagtacaggggctacgattcggctggaat
       D  I  L  L  A  G  L  E  K  L  E  Y  R  G  Y  D  S  A  G  I
961   tgccactgtttgggaaggtgagattaattgtgtgcgggcaaagggcaaactgcataacct
```

Figure 2. Nucleotide sequence of the *Nostoc* PCC 9229 *psaC* gene and upstream flanking region of the *nodM/glmS* homologue. The deduced amino acid sequences are shown below the respective open reading frames.

Table 1. Symbiotic (S) and free-living (F) cyanobacteria tested for the presence of the *nodM/glmS* homologue.

Strain	Source	Hybridization*
Nostoc PCC 9229	S**	+
Nostoc 268	F	+
Nostoc PCC 7107	F	+
Nostoc PCC 6720	F	+
Nostoc PCC 27896	F	+
Nostoc PCC 7120	F	+
Anabaena cylindrica	F	+
A. azollae	S***	+
Nostoc PCC 8002	S**	+
Nostoc PCC 7422	S****	+
Nostoc PCC 73102	S****	+

*All DNAs were digested with *Eco*RI. The blots were hybridized with a *Nostoc* PCC 9229 *nodM/glmS* subcloned 240-bp PCR fragment (a.a 1-80) at the conditions described in Materials and Methods. **Isolated from *G. monoika*, ***Isolated from the water-fern *Azolla*, ****Isolated from the cycads *Cycas* (sp.) and *Macrozamia* (sp.), respectively.

Alignment of the *Nostoc* PCC 9229 PsaC protein shows an identity of 97% to 100% to PsaC of other cyanobacterial strains such as *Anabaena* PCC 7120, *Synechocystis* PCC 6803 and *Nostoc* 8009 (data not shown). No typical bacterial trancriptional initiation region or rhizobial *nod* box region could be identified between the two ORFs in *Nostoc* PCC 9229.

The copy number of the *Nostoc* PCC 9229 *nodM/glmS* homologue was determined by Southern blot analysis of *Nostoc* genomic DNA digested with several enzymes (Fig. 3). The hybridization pattern obtained was characteristic for a single copy gene, since multiple hybridization signals were detected only for enzymes with restriction sites in the coding region of the gene (Fig. 1). Therefore, it was concluded that the *nodM/glmS* homologue occurs as a single copy in the genome of *Nostoc* PCC 9229.

The presence of *nodM/glmS* homologue was also tested by Southern hybridization in five other symbiotic and in six free-living cyanobacterial strains (Table 1). Positive hybridization was observed in all the cyanobacteria tested.

Expression experiments

The expression pattern of the *nodM/glmS* homologue gene in *Nostoc* PCC 9229 was investigated in order to examine a possible up-regulation by different

flavonoids, by *Gunnera* mucilage and by *Gunnera* seeds rinse (Fig. 4). Transcription of the *nodM/glmS* homologue was only detected using the RNAse protection assay (RPA). As shown in Fig. 4, none of the inducers was able to increase the constitutive expression of the *Nostoc* PCC 9229 *nodM/glmS* homologue gene.

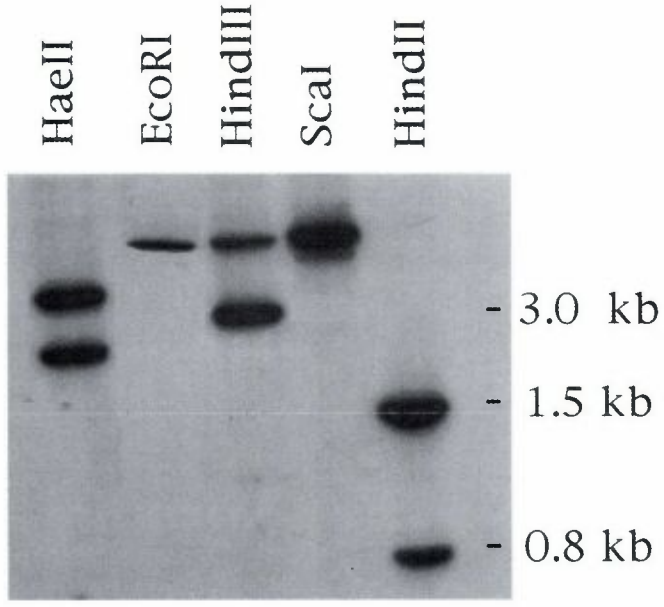

Figure 3. Autoradiograph of Southern blot containing genomic DNA of *Nostoc* PCC 9229 digested with restriction enzymes as indicated. The blot was hybridized with the 800-bp cloned *Nostoc* PCC 9229 *nodM/glmS* homologue PCR fragment.

Figure 4. RNAse protection assay on total RNA isolated from *Nostoc* PCC 9229 cultures after induction by different plant compounds. 1- control, 2- *Gunnera* mucilage (250 µl/ml - 6 hr), 3- *Gunnera* mucilage (250 µl/ml - 16 hr), 4- chrysin (10 µM), 5- kaempferol (10 µM), 6- naringenin (10 µM), 7- quercetin (10 µM), 8- *Gunnera* seed rinse (0.5 ml), 9- *Gunnera* seed rinse (1.5 ml), 10- non-homologous mRNA (from pond snail, *Lymnaea*). The arrow indicates the size of the band given by single strand cRNA transcribed from the *nodM/glmS* subclone (positive control).

4. Discussion

Due to the importance of the *nod* operon in rhizobial-plant relationships we examined the possible occurrence of a parallel system in the *Gunnera-Nostoc* symbiosis. A PCR approach enabled the isolation of a gene in *Nostoc* PCC 9229 homologous to the rhizobial *nodM/glmS*. Genetic analysis of the flanking region of the isolated gene did not reveal the presence of any other *nod* homologous genes or a *nod* box sequence typically present in rhizobia. The isolated *Nostoc* PCC 9229 DNA λ-clone was also hybridized with *nodL, E, F* and *N* probes from *R. leguminosarum* bv. viciae and *R. trifolii* (data not shown) but no positive signal was obtained. This does, however, not exclude their presence in other parts of the *Nostoc* PCC 9229 genome, as heterologous hybridizations previously suggested homologies to *nodEF, nodN* and the *nod* box but not to *nodDABC* (Rasmussen et al., 1996).

In addition, the isolated *Nostoc* PCC 9229 λ-clone was also hybridized with these genes from *R. galegae* (Räsänen et al., 1991) for a more sensitive hybridization than the one performed on the genomic DNA. Also in this case no signal was obtained at low stringency strengthening the conclusion that no such gene cluster is present in this genomic region of *Nostoc* PCC 9229.

The expression studies indicate a constitutive and very low level of expression of the gene in *Nostoc,* detectable only by RPA (Fig. 4). As previous data had demonstrated the involvement of stem mucilage in the establishment of the symbiosis between *Nostoc* PCC 9229 and *G. manicata* (Rasmussen et al., 1994) mucilage was tested as a possible inducer. Furthermore, *Gunnera* seed rinse was previously shown to be capable of inducing *nodD* gene expression in *R. meliloti* (Rasmussen et al., 1996). These two factors, as well as the flavonoids tested present in *Gunnera* plants (Patricia et al., 1989), did, however, not increase the expression levels of the *Nostoc* PCC 9229 *nodM/glmS* homologue at concentrations previously demonstrated to be active in the early infection process of the *Nostoc-Gunnera* symbiosis (Rasmussen et al., 1996).

nodM from *R. meliloti* complemented the *E. coli glmS* mutation, indicating that indeed *nodM* can be considered a second copy of the *glmS* gene, which provides glucosamine in sufficient amount for the synthesis of Nod factors crucial for the establishment of symbiosis between *Rhizobium* and legume (Heidstra and Bisseling, 1996; Baev et al., 1991).

In *Nostoc* PCC 9229 the *nodM/glmS* gene appears to be a single copy gene and is found in a locus downstream of a *psaC* ORF, devoid of any regulatory sequences characteristic for rhizobial *nod* operons. The homology and expression data presented above therefore strongly suggest that the isolated gene acts solely as a *glmS* house-keeping gene, most probably involved in cell wall biosynthesis of *Nostoc* PCC 9229 and other cyanobacterial symbiotic and

free-living isolates (Table 1). Consequently, the signalling systems underlying the *Nostoc-Gunnera* symbiosis may differ from those in the *Rhizobium*-legume system.

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