A Dual-Targeted Soybean Protein Is Involved in *Bradyrhizobium japonicum* Infection of Soybean Root Hair and Cortical Cells

Marc Libault,¹ Manjula Govindarajulu,² R. Howard Berg,² Yee Tsuey Ong,¹ Kari Puricelli,¹ Christopher G. Taylor,² Dong Xu,³ and Gary Stacey¹

¹Division of Plant Sciences, National Center for Soybean Biotechnology, C.S. Bond Life Sciences Center, University of Missouri, Columbia 65211 U.S.A.; ²Donald Danforth Plant Science Center, 975 North Warson Road, St. Louis 63132 U.S.A.; ³Computer Science Department, C.S. Bond Life Sciences Center, University of Missouri, Columbia 65211, U.S.A.

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The symbiotic interaction between legumes and soil bacteria (e.g., soybean [*Glycine max* L.] and *Bradyrhizobium japonicum*) leads to the development of a new root organ, the nodule, where bacteria differentiate into bacteroids that fix atmospheric nitrogen for assimilation by the plant host. In exchange, the host plant provides a steady carbon supply to the bacteroids. This carbon can be stored within the bacteroids in the form of poly-3-hydroxybutyrate granules. The formation of this symbiosis requires communication between both partners to regulate the balance between nitrogen fixation and carbon utilization. In the present study, we describe the soybean gene *GmNMNa* that is specifically expressed during the infection of soybean cells by *B. japonicum*. *GmNMNa* encodes a protein of unknown function. The *GmNMNa* protein was localized to the nucleolus and also to the mitochondria. Silencing of *GmNMNa* expression resulted in reduced nodulation, a reduction in the number of bacteroids per infected cell in the nodule, and a clear reduction in the accumulation of poly-3-hydroxybutyrate in the bacteroids. Our results highlight the role of the soybean *GmNMNa* gene in regulating symbiotic bacterial infection, potentially through the regulation of the accumulation of carbon reserves.

Legumes interact symbiotically with soil bacteria (rhizobia), leading to the development of a new root organ, the nodule. This symbiosis is initiated by the exchange of chemical signals between both partners, which determines the specificity of the host–symbiont interaction. More specifically, in response to flavonoids secreted by the plant, the bacteria synthesize a lipo-chitoooligosaccharide known as a Nod factor (Lerouge et al. 1990). The perception of the Nod fac-

tor by the plant leads to the deformation of root hair cells and to the activation of root cortical cell divisions leading to the formation of the nodule primordium. Inside the nodule, bacteria differentiate into bacteroids, which are able to fix atmospheric nitrogen for assimilation by the plant. In exchange, the plant provides a steady carbon supply to the bacteria, which can store this carbon in the form of poly-3-hydroxybutyrate (PHB) granules. Bacteroid PHB can subsequently be utilized as a carbon source or may also have other functions. For example, PHB controls the redox potential in the bacteroid; a consequence of the microaerobic conditions existing in the nodule that are necessary for optimal fixation of atmospheric nitrogen (Trainer and Charles 2006).

In past years, the functions of a limited number of legume genes involved in root hair cell infection by symbiotic bacteria have been defined based on forward and reverse genetic studies. These studies have relied heavily on the use of mutants derived from the two model legumes, *Medicago truncatula* and *Lotus japonicus* (Oldroyd and Downie 2008). However, as highlighted by Ferguson and Gresshoff (2009), soybean is now reemerging as a model in legume biology due to the release of the sequenced soybean genome (Schmutz et al. 2010), the development of routine methods for gene silencing (Govindarajulu et al. 2009; Libault et al. 2009, 2010c), and the application of a variety of functional genomic methods (Libault et al. 2010a and b). By way of example, the soybean *NFR1* and *NFR5* genes, encoding the receptor kinases recognizing the Nod factor, were recently identified through the screening of a soybean mutant population (Indrasumunar et al. 2010, 2011). The analysis of the soybean transcriptome led to the identification of 233 genes consistently regulated at various times during root hair infection by *Bradyrhizobium japonicum* (Libault et al. 2010a). Among these genes, sequence comparisons failed to predict functions for 73, due to the lack of sequence similarity to proteins with known functions. In this study, we investigated the role of one of these “proteins of unknown function”, which we have named *GmNMNa* (nucleolar/mitochondrial protein involved in nodulation a). Our initial transcriptional analysis clearly established *GmNMNa* as one of the most strongly induced root hair genes in response to *B. japonicum* inoculation (Libault et al. 2010a). Our study confirmed the strong induction of *GmNMNa* expression during soybean root hair infection and localized expression using promoter reporter-gene fusions. RNA-mediated gene silencing of *GmNMNa* expression led to various phenotypes, including a decrease in nodule number, a

Current address for M. Govindarajulu: Genome Center and Department of Plant Sciences, University of California, Davis, 95616 U.S.A.

Current address for K. Puricelli: Midwest Research Institute, 425 Volker Blvd, Kansas City, MO 64110 U.S.A.

Current address for C.G. Taylor: Departments of Plant Pathology, Ohio State University, Wooster 44691 U.S.A.

Corresponding author: M. Libault; E-mail: libaultm@missouri.edu

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reduction in the number of bacteroids per infected cells, and a decrease in the density of PHB granules in each bacteroid. Expression of a *GmNMNa*-green fluorescent protein (GFP) fusion in tobacco cells localized the protein to both the nucleolus and the mitochondria. Hence, one possibility is that *GmNMNa* plays an important role in mitochondrial function in the nodule, which may be manifested by effects on carbon metabolism reflected in fewer bacteroids and lower accumulation of carbon reserves (i.e., PHB). Indeed, earlier work showed that mitochondria accumulate at the periphery of legume infected cells to presumably maintain plant cell function under the low oxygen conditions of the nodule interior (Cermola et al. 2000; Day et al. 1986; Newcomb et al. 1985). Our data clearly suggest a role for *GmNMNa* in regulating symbiotic bacterial invasion and survival during the early steps of soybean nodulation.

RESULTS

GmNMNa expression is induced in root hair cells in response to *B. japonicum* inoculation and during the early stages of soybean nodule development.

GmNMNa (Glyma01g45280) was initially identified as one of the most strongly upregulated genes during soybean root hair cell infection by *B. japonicum* (Libault et al. 2010a). These results suggest that *GmNMNa* might play a critical role in the early infection of soybean root hair cells by *B. japonicum*.

Transcriptional induction of *GmNMNa* expression in response to *B. japonicum* inoculation was confirmed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) using mRNA isolated from inoculated and mock-inoculated (i.e., water-treated only) root hair cells isolated 0 to 72 h after inoculation (HAI) (Fig. 1A; Supplementary Table 1). *GmNMNa*

Fig. 1. Gene expression analysis of *GmNMNa* gene during the different steps of nodulation and in different soybean organs. The expression of the *GmNMNa* gene was quantified by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (black). *ENOD40* (gray) was used as a positive control for the nodulation response. Previous to any analysis, the expression levels were normalized against the geometric mean of the soybean reference genes *cons4*, *cons6*, and *cons7*. **A**, Fold change in gene expression during root hair infection between *Bradyrhizobium japonicum*-inoculated root hairs versus mock-inoculated root hairs. **B**, Fold change in gene expression during nodule development between *B. japonicum*-inoculated roots versus mock-inoculated roots. **C**, Gene expression levels were quantified in 14 different soybean tissues, including isolated nodules (10, 16, 24, and 32 days after inoculation [DAI]). At 24 DAI, *GmNMNa* expression was not detected by qRT-PCR. The different time points and tissues tested are shown on the x axis, while the y axis presents the fold changes (A and B) or the normalized expression levels (C) for each gene analyzed. Average relative expression levels of each gene across three replicates in each tissue are shown on the y axis. Standard error bars are shown. Asterisk indicates Student's *t* test < 0.05 when comparing inoculated/nodulated versus mock-inoculated/nodule-free tissues.

expression was also measured in nodulated and mock-inoculated roots (0 to 32 days after inoculation [DAI]) (Fig. 1B). In both analyses, *GmENOD40* was used as a positive control for the infection response (Minami et al. 1996) and gene expression levels were normalized against the geometric mean of the soybean reference genes *cons4*, *cons6*, and *cons7* (Libault et al. 2008). Consistent with our previous results (Libault et al. 2010a), *GmNMNa* expression was strongly induced during root hair infection, with the highest expression occurring between 6 and 18 HAI (>100-fold changes), and during nodule development, with the highest expression occurring at 4 DAI (approximately 700-fold change). A strong decrease in *GmNMNa* expression occurred at later time points (24- and 15-fold changes at 24 and 32 DAI, respectively). Compared with the *GmENOD40* expression profile where maximum induction occurred during later nodule development, the maximum expression of *GmNMNa* was restricted to the earliest stages of soybean nodulation (Fig. 1A and B).

To investigate the possible expression of *GmNMNa* in other parts of the plant, we consulted the two independent soybean transcriptome atlases (Libault et al. 2010b; Severin et al. 2010), which present gene expression results from 28 different tissues and conditions as measured by high-throughput cDNA sequencing. This analysis showed that *GmNMNa* expression was exclusively restricted to soybean root hair cells independent of *B. japonicum* inoculation (Supplementary Table 2). The absence of *GmNMNa* expression in mature nodules, which were used during generation of the soybean expression atlases, is consistent with the drop of expression observed during nodule development (Fig. 1B). To confirm the *GmNMNa* expression pattern across different tissues and to better understand the regulation of its expression in soybean nodules, we performed qRT-PCR in 14 different soybean tissues, including nodules isolated at different days after *B. japonicum* inoculation (i.e., root tip, stripped root [root devoid of root hairs], root hair, root, stem, leaves, shoot apical meristem, flower, green seed, young pods, and 10-, 16-, 24-, and 32-DAI nodules). With the exception of the root hair cells and the mature root, *GmNMNa* was expressed at very low levels in all soybean tissues; including nodules (Fig. 1C). Interestingly, *GmNMNa* was expressed at a higher level in 10-DAI nodules compared with mature nodules (16-, 24-, and 32-DAI nodules) (Fig. 1C). We assumed that the detection of *GmNMNa* transcripts in the mature root reflects its overall expression in root hair cells rather than the entire root system, based on the fact *GmNMNa* transcripts were barely detected in stripped roots (i.e., roots devoid of root hair cells). Similarly, induction of *GmNMNa* expression in nodulated roots (Fig. 1B) likely reflects the strong expression of *GmNMNa* in infected root hair cells (Fig. 1A) and emerging nodules. Altogether, our data suggest that *GmNMNa* expression is restricted to the root hair cell and is specifically induced during root hair cell infection by *B. japonicum* and, at a lower level, during the earliest stages of soybean nodule development.

To further localize the site of *GmNMNa* expression, transgenic soybean roots expressing GFP and β -glucuronidase (GUS) under the control of the *GmNMNa* promoter (i.e., approximately 2,000 bp located upstream of the *GmNMNa* ATG codon) were monitored during *B. japonicum* inoculation. GFP signal and GUS staining were not observed in mock-inoculated transgenic roots. However, the GFP signal was clearly seen in a subset of the root hair cells in response to *B. japonicum* inoculation, confirming the induction of *GmNMNa* in these cells (Supplementary Fig. 1). Interestingly, these GFP-positive root hair cells appeared to be deformed or curled, perhaps indicative of the response to *B. japonicum* infection (Fig. 2A). During nodule development, the *GmNMNa* expression pattern changed considerably. In immature nodules, *GmNMNa*

expression was restricted to the center of the emerging nodule, which is the location of the cells newly infected by *B. japonicum* (Fig. 2B). Later, in mature nodules, *GmNMNa* expression was observed in the uninfected cortical cells but at a very low level (Fig. 2C). Altogether, *GmNMNa* expression was stronger in developing nodules than in mature nodules (Fig. 2B and C), confirming the qRT-PCR results described above.

The NMN family is conserved in legumes.

The recent release of the soybean genome sequence (Schmutz et al. 2010) and the nearly complete barrel medic and lotus genome sequences are useful tools to better understand legume biology and evolution. These sequences were mined to identify potential *GmNMNa* orthologs, as well as possible paralogs in the soybean genome. For further comparison, we also sought *GmNMNa* homologous genes in the *Arabidopsis thaliana* genome.

Comparison of the *GmNMNa* protein sequence against the soybean genome identified one putative homolog on chromosome 11 (from 118,735 to 119,219 bp; tblastn, score: 276, *e* value: $4.8e^{-72}$). Based on the published genome annotation, no gene was predicted at this location. However, this is not surprising because several expressed but currently unannotated genes were identified through high-throughput sequencing of soybean cDNA (Libault et al. 2010a and b). Indeed, examination of the soybean transcriptome atlas (Libault et al. 2010b) identified a transcript corresponding to the *GmNMNa* homolog (i.e., *GmNMNb*) located on the chromosome 11 (from 118,691 to 119,156 bp), confirming the transcriptional activity of this gene. Similarly to *GmNMNa*, *GmNMNb* was also expressed in inoculated root hair cells but at a significantly lower level compared with *GmNMNa*. Using FGENESH (Salamov and Solovyev 2000), a protein composed of 234 amino acids lacking known protein domains was predicted to be encoded by *GmNMNb*. *GmNMNa* and *GmNMNb* appear to lie within regions of clear homology based on a comparison of syntenic regions found on soybean chromosome 1 and 11 (Libault et al. 2010b) (Supplementary Table 3; Supplementary Fig. 2).

The *GmNMNa* protein sequence was also compared against the *L. japonicus*, *M. truncatula*, and *A. thaliana* coding sequences. In *A. thaliana*, no *GmNMNa* homolog was identified (i.e. blastp; best hit: At05g65925, score = 33, *e* value = 0.067). In *L. japonicus*, a putative ortholog encoding a protein of 132 amino acids was identified (LjCM0200-133; tblastn, score = 60, *e* value = $4e^{-09}$). This gene was not represented on the array used to establish the transcriptome atlas of *L. japonicus* (Høgsund et al. 2009). One *M. truncatula* predicted gene (i.e., MedTr05g005450), encoding a protein of 172 amino acids, shared significant similarity to *GmNMNa* (blastp, *e* value = $3e^{-19}$). Based on synteny analyses, this gene is a likely orthologous to *GmNMNa*. Indeed, consistent with this hypothesis, expression of MedTr05g005450 (i.e., *MtNMN*, Affymetrix probe set Mtr.26415.1.S1_at), as shown in the *M. truncatula* gene expression atlases (Benedito et al. 2008; He et al. 2009), was elevated during nodulation and in mycorrhizal roots, suggesting a role for this gene in plant-microbe symbiosis (Supplementary Fig. 3).

The *GmNMNa* protein sequence was also compared against the National Center for Biotechnology Information nonredundant protein sequence (blastp) and DNA sequence (tblastn) databases. Several putative homologs were identified but only in plant species, suggesting that the NMN family may be a plant-specific gene family (Supplementary Table 4). However, the overall poor scores and *e* values that resulted from this analysis suggest that the NMN-like protein sequences have rapidly diverged during plant evolution.

Given the lack of functional annotation for *GmNMNa*, we used MaliP software to identify conserved domains. The N-ter-

minimal amino acid sequence located on the N-terminal end of NMN proteins was poorly conserved. However, the 40 amino acids located at the NMN C-terminal end were strongly conserved and enriched in charged amino acids (e.g., aspartate, glutamate, lysine, and arginine) and tyrosine (Supplementary Fig. 4). Altogether, these amino acids represented 55% of the 40 conserved amino acids on the C terminal of NMN proteins. The function of this conserved domain is unknown.

Multiple subcellular localization of GmNMNa.

GmNMNa is predicted to encode a protein of 147 amino acids. We assume that the *GmNMNa* protein sequence is the full-length protein for two reasons: i) the *GmNMNa* transcript was predicted with high confidence based on the identification of its full-length cDNA sequence (Schmutz et al. 2010) and ii) the similarities between the size and the amino acid sequences between *GmNMNa*, *MtNMN*, and *LjNMN* proteins strongly support the idea that these proteins are full-length proteins.

Analysis of the *GmNMNa* amino acid sequence revealed the presence of a putative mitochondrial targeting signal (MTS) at the N terminus, as identified using iPSORT (⁹KQSKLKH¹⁵)

(Bannai et al. 2002) and PSORT software (²¹MRILKK²⁶). Interestingly, the ⁹KQSKLKH¹⁵ MTS is strongly conserved between *GmNMNa*, *GmNMNb*, *MedTr05g005450*, and *LjCM0200-133* proteins.

To confirm these bioinformatic predictions, we expressed the fusions between the *GmNMNa* and the GFP cDNAs under the control of the dual 35S promoter in tobacco leaf cells by infiltrating *Agrobacterium tumefaciens* strains carrying the plasmids of interest. As a control, GFP alone was expressed in tobacco leaves.

The GFP alone was localized in the cytoplasm and in the nucleus of tobacco cells, with the exception of the nucleolus (Fig. 3A) (Libault et al. 2010c). When fused to *GmNMNa*, the GFP signal was specifically detected in the nucleolus (Fig. 3B through D; Supplementary Videos 1 and 2). We hypothesized that the nuclear localization of the GFP-*GmNMNa* protein is probably due to its passive diffusion through the nuclear membrane (i.e., the predicted 47-kDa size of GFP-*GmNMNa* chimeric protein is below the exclusion limit for plant nuclei [i.e., 40 to 60 kDa]) (Berg and Beachy 2008). However, the nucleolar localization of

Fig. 2. Expression pattern of *GmNMNa* during root hair infection by *Bradyrhizobium japonicum* and soybean nodule development. *GmNMNa* promoter sequence was fused upstream to the green fluorescent protein (GFP) and β -glucuronidase (GUS) reporter genes. **A**, Representative expression patterns of *GmNMNa* promoter-GFP in the soybean whole root 7 days after *B. japonicum* inoculation. At this time, soybean transgenic root hair cells are infected by *B. japonicum*. **B** and **C**, Representative expression patterns of *GmNMNa* promoter-GUS in nodule cross sections 28 days following *B. japonicum* inoculation. At this stage, transgenic roots showed a mixture of immature (**B**) and mature nodules (**C**). Confocal (**A**) and light microscope (**B** and **C**) images are shown. Bars = 50 μ m (**A**) and 250 μ m (**B** and **C**).

GmNMNa could reflect either its true biochemical role, because GFP alone was excluded from the nucleolus (Fig. 3A), or the nonspecific binding of GmNMNa protein to the charged (basic) nucleolar compartment.

In addition to its localization in the nucleolus, GFP fluorescence was also seen in discrete cytosolic dots when GFP was fused to the C terminus of GmNMNa (Fig. 3B). In comparison, this localization was not seen when the GFP was fused to the N terminus of GmNMNa, presumably due to blocking of the putative mitochondrial targeting signal (Fig. 3C). These spots appeared to co-localize with mitochondria. To further investigate this localization, we co-expressed the GmNMNa-GFP fusion with the mt-rk plasmid (Nelson et al. 2007) carrying a translational fusion between an MTS and mCherry in tobacco leaves. We verified the absence of cross contamination between the GFP and mCherry fluorescent signals with the set of filters used on the confocal microscope (Supplementary Fig. 5). A clear overlap of the GFP and mCherry signals was observed, supporting the idea that the GmNMNa protein was targeted to the mitochondria (Fig. 3E).

Silencing of *GmNMNa* reduces nodule number, bacteroid density, and the accumulation of polyhydroxybutyrate.

To characterize the role of *GmNMNa* during nodulation, we generated soybean transgenic roots expressing a *GmNMNa* RNA interference (RNAi) construct using the hairy root transformation method (Govindarajulu et al. 2009; Libault et al. 2009, 2010c). The RNAi construct consisted of a DNA fragment derived from the 3' untranslated region (UTR) of *GmNMNa* designed to form a double-stranded RNA. The 3' UTR region of *GmNMNa* was selected in order to maximize the specificity of the RNAi construct. Comparison of this sequence against the soybean genome confirmed its specificity against *GmNMNa* (score = 299.8, e value = $7.3e^{-80}$; second-best hit: *GmNMNb*, score = 69.9, e value = $1.2e^{-10}$). Based on the overall very low expression of *GmNMNb*, we assume that our RNAi construct will predominantly affect *GmNMNa* mRNA abundance.

The number of nodules on 45 RNAi-*GmNMNa* and 75 GUS-RNAi control transgenic roots was counted 42 DAI using three independent biological replicates. Past experience indi-

Fig. 3. In vivo observation of the mitochondrial and nucleolar localization of GmNMNa. Epidermal cells of tobacco leaves expressing GmNMNa protein fused to the green fluorescent protein (GFP) were observed with a confocal microscope. **A**, As a control, tobacco leaves expressing the GFP alone (green) were also stained with propidium iodide (red). Independently of the direction of the fusions between GmNMNa and the GFP (**B**, GmNMNa-GFP; **C** and **D**, GFP-GmNMNa), GmNMNa was targeted to the nucleolus (white arrows). To confirm this nucleolar localization, the nucleolus was stained with propidium iodide (**C**, red). Depending on the orientation of the translational fusion, GmNMNa was targeted (**B**, GmNMNa-GFP) or not (**C**, GFP-GmNMNa) to plant cell organelles. **E**, Localization of GmNMNa to the mitochondria was confirmed (white and gray arrows) by co-transforming epidermal tobacco cells with the GmNMNa-GFP (green) and a mitochondrial targeting signal-mCherry fusions (red).

cates that transgenic hairy roots nodulate more slowly than wild-type roots and, as a consequence, 42-DAI soybean hairy roots contain a mixture of developing and mature nodules. On average, the RNAi-*GmNMNa* transgenic roots produced approximately 60% fewer nodules than the control GUS-RNAi transgenic roots. Silencing of *GmNMNa* appeared to block nodule development and not initiation because the total number of nodule primordia was not significantly different between the

silenced and control transgenic roots (Fig. 4A; Student's *t* test, *P* value = 0.47). However, there was a significant reduction in the number of developing and mature nodules formed on the RNAi-*GmNMNa* transgenic roots (Fig. 4A; developing nodules, *P* value < 0.002; mature nodules, *P* value < 0.02). Microscopic analysis of nodule sections showed that there was no change in the cellular organization of the nodule primordia or mature nodules as a result of *GmNMNa* silencing. In addition, *GmNMNa*-

Fig. 4. Silencing of *GmNMNa* reduces poly-3-hydroxybutyrate (PHB) granule accumulation, bacteroid per infected cell, and nodule number. **A**, Number of nodules formed on *GmNMNa*-RNAi (RNA interference) transgenic roots as a percentage of nodules formed on control RNAi- β -glucuronidase (GUS) roots. Nodules were categorized by their stage of development. Green fluorescent protein (GFP) was used as a reporter gene to identify the transgenic roots. Nodule numbers were counted on 45 and 75 *GmNMNa*-RNAi and GUS-RNAi transgenic roots, respectively. Standard error bars are shown. **B**, Representative transmission electron micrographs of infected cells of soybean mature GUS-RNAi (left picture) and *GmNMNa*-RNAi (right picture) nodules. Bacteroids (white arrows) were observed in both GUS-RNAi and *GmNMNa*-RNAi nodules but were found in lower abundance in *GmNMNa*-RNAi transgenic nodules. In addition, a significant decrease in PHB accumulation was observed in *GmNMNa*-RNAi bacteroids (black arrows). Bars = 0.5 μm . **C**, Average number of bacteroids per symbiosome in infected cells of *GmNMNa*-RNAi and GUS-RNAi transgenic nodules. A Student's *t* test = 0 was calculated when comparing both sets of infected cells. Standard error bars are shown. **D**, Average area of PHB particles per bacteroid in *GmNMNa*-RNAi- and GUS-RNAi-infected cells of transgenic nodules. A Student's *t* test (*P* value < 1×10^{-36}) was calculated when comparing both sets of bacteroids. Standard error bars are shown.

RNAi mature nodules exhibited a normal level of leghemoglobin expression, suggesting that they were fully functional.

Electron microscopy showed that both the RNAi-*GmNMNa* and GUS-RNAi root nodules were infected by *B. japonicum* (Fig. 4B). Infection threads could be easily seen in RNAi-*GmNMNa* nodules; again, suggesting no significant effect on the infection process (Supplementary Fig. 6A). However, the number of bacteroids per symbiosome in infected cells was strongly reduced in RNAi-*GmNMNa* nodules (Fig. 4B and C; bacteroid number from 1,915 and 1,302 RNAi-*GmNMNa* and GUS-RNAi infected cells, respectively, were counted; Student's *t* test = 0). These results were confirmed through observation of sections of RNAi-*GmNMNa* and GUS-RNAi nodules stained with SYTO13 fluorescent dye. As previously described by Veereshlingam and associates (2004), SYTO13 dye preferentially stains bacteroids, making this dye ideal to observe a potential defect in nodule infection as well as infection thread formation. In addition to the reduction in the number of bacteroids per symbiosome in infected cells, the number of PHB granules per bacteroid was also significantly reduced in bacteroids seen in the RNAi-*GmNMNa* nodules (Fig. 4B and D; PHB area [in square micrometers] from 201 and 635 RNAi-*GmNMNa* and GUS-RNAi bacteroids, respectively, was measured; Student's *t* test < $1.e^{-36}$).

To confirm the decrease of *GmNMNa* transcripts in silenced roots, we quantified *GmNMNa* expression by using qRT-PCR in RNAi-*GmNMNa* and GUS-RNAi control roots. As expected based on our previous qRT-PCR experiments, *GmNMNa* expression was detected at a very low level in the 42-DAI transgenic roots. However, we clearly quantified a lower expression of *GmNMNa* in the RNAi-*GmNMNa* transgenic roots compared with the control roots: *GmNMNa* expression was detected in a higher number of RNAi-GUS control roots compared with the RNAi-*GmNMNa* roots. When detected, *GmNMNa* transcripts were 2.4 times less abundant in RNAi-*GmNMNa* roots compared with the RNAi-GUS control roots (Supplementary Fig. 7). To address the question of the specificity of the RNAi construct, we quantified the expression of *GmNMNb* in the RNAi-*GmNMNa* and GUS-RNAi transgenic roots by performing qRT-PCR. Independent of the roots analyzed, we were unable to detect expression of the *GmNMNb* transcript. To better characterize the expression profile of *GmNMNb*, we also quantified *GmNMNb* expression across various soybean tissues, including inoculated and mock-inoculated root hair cells. The lack of expression of *GmNMNb* across soybean tissues strongly supports the soybean transcriptome atlas dataset, showing that *GmNMNb* expression was consistently very low. Altogether, the lack of expression of *GmNMNb* indicates that the nodule phenotype of RNAi-*GmNMNa* roots was most likely due to the specific silencing of *GmNMNa* expression.

DISCUSSION

As is the case for all sequenced genomes, a significant percentage (17%) of the predicted 46,430 protein-encoding genes in the soybean genome lacks functional annotation or even identified protein domains of unknown function. Previous transcriptome analysis identified 73 genes of unknown function, from a total of 233 soybean root hair genes that specifically responded to *B. japonicum* inoculation (Libault et al. 2010a). Defining a mechanistic role for these genes in the nodulation process is difficult because there is no obvious starting point for such an analysis.

In the case of *GmNMNa*, we used a variety of molecular approaches to define the cellular location of the protein and its expression pattern and phenotype after RNAi silencing to deduce its possible role in soybean nodulation.

Silencing of *GmNMNa* reduces nodulation and impacts bacteroid development.

In plant-bacteria symbiosis, differentiated bacteria fix and assimilate atmospheric nitrogen for the plant. In return, the plant provides a carbon source to the bacteroid. The sucrose molecules produced by plant photosynthesis are transported to the nodule and degraded through the glycolysis pathway and the TCA cycle leading to the synthesis of dicarboxylic acids (e.g., malate), which are thought to serve as the primary carbon source for the bacteroid (Benedito et al. 2010; Ronson et al. 1981; Udvardi and Day 1997). Excess carbon is stored within the bacteroids in the form of PHB particles. In soybean, PHB is not used as a source of energy to maintain nitrogenase activity (Wong and Evans 1971). Previously, Ludwig and associates (2005) hypothesized that PHB accumulated in the rhizobial symbiont to be used during infection of the plant root hair cells and differentiation into bacteroids. This last hypothesis is consistent with the reduction in nodule and bacteroid development seen upon silencing of *GmNMNa* expression. Indeed, the localization and timing of *GmNMNa* expression is consistent with a role during the early events of nodulation. The impact of *GmNMNa* silencing on PHB accumulation may be the result of defective carbon metabolism (perhaps through disruption of mitochondrial function) or some other cause. However, the reduction of PHB accumulation in the bacteroids of the *GmNMNa* silencing nodules may be sufficient to explain the nodulation phenotype. For example, Wang and associates (2007) reported a significant delay in nodulation and nitrogen fixation when alfalfa roots were inoculated with a *Sinorhizobium meliloti phbC* mutant defective in PHB biosynthesis.

GmNMNa is targeted to two distinct cellular compartments.

Only a few examples of multiple targeted plant proteins have been confirmed in vivo (Carrie et al. 2009; Mitschke et al. 2009; Schwacke et al. 2007). Carrie and associates (2009) reported a total of 49 dual-targeted *Arabidopsis thaliana* proteins. Among them, 42 of the 49 were localized in the mitochondria and in the chloroplast due to the redundancy of biochemical pathways between the two organelles. Bioinformatic analysis based on possible targeting signals suggests that a significant number of dual-targeted proteins might be present in plants. For example, analysis of the predicted *Arabidopsis* proteome suggested that 523 *Arabidopsis* proteins are targeted to more than one subcellular compartment (Mitschke et al. 2009). Localization of *GmNMNa*-GFP translational fusions supports the targeting of this protein to both the nucleolus and mitochondria. The latter is supported by the prediction of two putative MTS at the N terminus of *GmNMNa*. The interpretation of the nucleolar localization of *GmNMNa* remains unclear: it could reflect the true biochemical role of *GmNMNa* because GFP alone was excluded from the nucleolus (Fig. 3A) or it could reflect nonspecific binding to the charged nucleolar compartments. This latter interpretation would suggest that the major site of *GmNMNa* function is in the mitochondria.

Dual targeting to both the mitochondria and nucleus is not well characterized in plants. For example, only one *Arabidopsis* gene (At1g79430), encoding the ALTERED PHLOEM DEVELOPMENT (APL)-like MYB transcription factor, was reported to be targeted to the mitochondria (Carrie et al. 2009) and to the nucleus (Bonke et al. 2003). This differential localization was dependent on which transcript variant of APL was fused to the GFP. When the GFP was fused to the N terminus of the longest APL transcript (358 amino acids), the translational protein was exclusively localized in the nucleus (Bonke et al. 2003). In contrast, when GFP was fused to the C terminus of the shortest APL transcript variant (293 amino acids),

the chimeric protein was localized exclusively to the mitochondria (Carrie et al. 2009). According to Carrie and associates (2009), the differential subcellular localization of APL proteins is dependent of the change of the relative localization of an MTS at the N terminus of APL. In a previous study, de Castro Silva Filho and associates (1996) also noticed that the relative N-terminal location of MTS was important to properly target a protein to the mitochondria.

An examination of the non-plant literature identified the eukaryotic ribonucleoporin endoribonuclease RNase MRP protein as another example of a protein localized to the nucleolus and mitochondria. In the nucleus, this protein is involved in the cleavage of the ribosomal RNA (rRNA) precursor (Lindahl and Zengel 1995; Tollervy 1995). In the mitochondria, RNase MRP is involved in the formation of the primers necessary for the replication of the mitochondrial DNA (Chang and Clayton 1987; Gold et al. 1989).

Given the similar subcellular localization of GmNMNa and RNase MRP, we explored the possibility that GmNMNa might bind to RNA. Indeed, analysis of the GmNMNa protein sequence predicted three putative nucleic-acid-binding domains using three independent bioinformatic tools: DNABindR (Yan et al. 2006), BindN (Wang and Brown 2006), and DP-Bind (Hwang et al. 2007) (Supplementary Fig. 8). In addition, using another bioinformatic tool, SVM-Prot (Cai et al. 2003), GmNMNa was predicted to interact with rRNA. In order to test this directly, we purified the hemagglutinin-tagged GmNMNa protein from transgenic tobacco leaves and tested the ability of this protein to bind to rRNA (data not shown). However, these experiments failed to reveal any significant RNA binding and, therefore, the role of GmNMNa as an RNA-binding proteins remains hypothetical.

Conclusion.

It is common to identify a significant number of proteins of unknown function when conducting transcriptomic studies. These proteins are particularly difficult to characterize functionally due to the lack of any predicted starting point for such an analysis (e.g., a specific predicted enzymatic activity). GmNMNa is among the most strongly upregulated genes during the early events of root hair infection by *B. japonicum* and, therefore, was predicted to play an important role in symbiotic development. The nodulation phenotypes seen upon *GmNMNa* silencing supported this notion and also revealed a strong effect on the abundance of bacteroids in the nodule and their physiology (i.e., as revealed by a marked reduction in PHB content). Localization of a GmNMNa-GFP fusion indicates that this protein is targeted to both the nucleolus and mitochondria. The latter might suggest a direct role in nodule respiration, which could impact bacteroid carbon metabolism.

MATERIALS AND METHODS

Identification of *GmNMNa* homologous and orthologous genes.

To identify *GmNMNa* homolog and orthologs, the GmNMNa protein sequence was blasted against the *Glycine max* (tblastn), *M. truncatula* (blastp), *L. japonicus* (tblastn), and *A. thaliana* (blastp; The *Arabidopsis* Information Resource) genomes. Synteny analyses between *GmNMNa*, *GmNMNb*, and their *M. truncatula* orthologs was highlighted based on the order, content, and direction of genes located on more than 100,000 bp of genomic sequences. A P value $< e^{-20}$ was used as a cut-off in the Blast analyses between gene sequences. Microsynteny relationships were generated by using the CMTV software (Sawkins et al. 2004).

Bacterial cultures.

Escherichia coli, *Agrobacterium rhizogenes*, and *B. japonicum* USDA110 were grown and washed as described by Libault and associates (2009). *A. tumefaciens* (strain C58C1 [pCH32]) was grown as described by Libault and associates (2010c).

Cloning.

GmNMNa promoter cloning (i.e., 2 kb of nucleotide sequences located upstream of the first codon encoding GmNMNa protein) upstream of the GFP and GUS cDNAs was performed as described by Libault and associates (2010c). Primers used to amplify the *GmNMNa* promoter with the Gateway compatible AttB boxes using soybean ‘Williams 82’ genomic DNA as template were *GmNMN*apromoAttB-for (AAAAAGCAGGCTGACAAGTGAATTTAATTTGATTTTCT) and *GmNMNa*-promoAttB-rev (AGAAAGCTGGGTACTGAAATGGTCGATGATGAAAG) first and the universal AttB primers (attB1-adaptor [GGGGACAAGTTTGTACAAAAAAGCAGGCT] and attB2-adaptor [GGGGACCACTTTGTACAAGAAAGCTGGGT]) second.

Using Gateway BP and LR Clonase II enzyme mixes (Invitrogen, Carlsbad, CA, U.S.A.), the *GmNMNa* promoter fragment was introduced into the pDONR-Zeo vector (Invitrogen), then into pYXT1 or pYXT2 destination vectors carrying the GUS and GFP reporter genes, respectively (Xiao et al. 2005).

Similarly, two-step PCR (i.e., *GmNMNacDNA*AttB-for [AAAAAGCAGGCTATATGAGAAAGGAATTAGCAAAGAA GAA] and *GmNMNacDNA*AttB-rev [AGAAAGCTGGGTACACAGGCACAGGGCGGCTGT] primers, and then, attB1-adaptor and attB2-adaptor primers were used to amplify *GmNMNa* cDNA with the AttB boxes) followed by the use of the Gateway system led to the creation of the translational fusion between *GmNMNa* and *GFP* cDNAs into pMDC43 and pMDC83 vectors (Curtis and Grossniklaus 2003).

To silence *GmNMNa*, we PCR-amplified a 244-bp fragment specific to the 3' UTR region of *GmNMNa* cDNA by using the primers *GmNMN*-RNAi-for (GAACGAAAGTCAATCAAGATATCAAA) and *GmNMN*-RNAi-rev (GAGCGCCATAGGAATGTCAC). This PCR product was cloned into the entry vector CGT 11050, then into the destination vector CGT 11017A (Libault et al. 2009) as described by Libault and associates (2010c). The resulting *GmNMNa*-RNAi vector and the RNAi control vector CGT 5200 (Govindarajulu et al. 2009) were electroporated into *A. rhizogenes* K599 and used for composite plant production.

pYXT1-*GmNMNa* promoter, pYXT2-*GmNMNa* promoter, CGT11017-*GmNMNa*-RNAi, and CGT5200 vectors were used to transform *A. rhizogenes* (strain K599) while *A. tumefaciens* [strain C58C1 (pCH32)] was transformed with *GmNMNacDNA*-pMDC43, *GmNMNacDNA*-pMDC83, and pMDC43 vectors.

Plant culture and treatment.

Isolated root hair cells inoculated with *B. japonicum* and mock-inoculated, nodulated, and nodule-free roots and the different soybean tissues were produced as described by Libault and associates (2009, 2010a and b). At least three independent biological replicates per condition were harvested to ensure reproducibility of the plant tissues analyzed.

Plant transformation and microscopy.

A. tumefaciens strains carrying *GmNMNacDNA*-pMDC43, *GmNMNacDNA*-pMDC83, and the negative control pMDC43 vectors were used to transform *Nicotiana benthamiana* leaves. The GFP localization was observed with a Zeiss LSM 510 META confocal microscope 3 days after leaf infiltration (488-nm excitation wavelength and 500- to 550-nm emission filter).

Co-localization experiments between GmNMNa and mitochondria were performed by co-transforming tobacco leaves with the GmNMNa-GFP constructs described above and CD3-992 vector carrying a translational fusion between a mitochondria targeting signal and the mCherry red fluorescent protein (Nelson et al. 2007). In addition, when needed, nuclei were stained by using propidium iodide (5- μ g/ml final concentration, 543-nm excitation wavelength, and 565- to 615-nm emission filter).

Soybean hairy root transformation was performed as described by Libault and associates (2009) using *A. rhizogenes* strains carrying the *GmNMNa* promoter-GUS and -GFP binary vectors or the RNAi constructs. Two-week-old soybean shoots cut between the first true leaves and the first trifoliolate were placed into rock-wall cubes (Fibrgro, Sarnia, Canada) and inoculated with 4 ml of *A. rhizogenes* carrying the construct of interest (optical density at 600 nm [OD₆₀₀] = 0.3). After 3 days of drying (23°C, 50% humidity, long-day conditions), the plants were watered with deionized water. One week later, the plants were transferred to vermiculite/perlite mix (3:1) wetted with nitrogen free-plant nutrient solution (Lullien et al. 1987). After 7 days, the shoots were transferred to the greenhouse (27°C, 20% humidity, long-day conditions). Finally, the shoots were inoculated with *B. japonicum* (10 ml, OD₆₀₀ = 0.08) 7 days later. Roots were observed 7 and 28 days after *B. japonicum* inoculation. Approximately 15% of the roots were transgenic based on the expression of the reporter genes (i.e., GFP or GUS). The nontransgenic roots were not removed during our experiments to avoid a wounding stress of the composite plants. However, the phenotypes reported were measured only from those roots showing GFP or GUS expression.

GmNMNa promoter-GUS roots were fixed and stained as described by Govindarajulu and associates (2008). Roots with GUS-stained nodules were sectioned and observed as described by Libault and associates (2010c). *GmNMNa* promoter-GFP roots were observed as described by Libault and associates (2010c).

GmNMNa-RNAi and GUS-RNAi transgenic roots expressing the GFP were isolated 6 weeks after inoculation with *B. japonicum* and nodule numbers were counted using a Leica MZFLIII stereomicroscope equipped with GFP Long Pass filter (500 nm Long Pass, number 41018; Chroma Technology Corp., Rockingham, VT, U.S.A.). SYTO13 staining of *GmNMNa*-RNAi and GUS-RNAi nodule sections was performed as described by Libault and associates (2009).

Transmission electron microscopy of freeze-substituted mature nodules was performed as described by Libault and associates (2010c).

RNA extraction, DNase treatment, reverse transcription, quantitative PCR primer design, quantitative PCR conditions, and data analysis.

Total RNA isolations, DNase treatments, reverse transcription, and qRT-PCR primer design were performed as described by Libault and associates (2008). The primers used in this study were designed with primer3 software using the criteria described by Libault and associates (2010a). The primer list is available in Supplementary Table 5.

The qRT-PCR tests were performed as described by Libault and associates (2010c) using the geometrical average of *Cons4*, *Cons6*, and *Cons7* expression (Libault et al. 2008) to normalize the expression levels of *GmNMNa* and *GmENOD40* genes. Fold changes of gene expression between inoculated and mock-inoculated samples were calculated at the indicated time point during the time course for the three different biological replicates. The average of these fold changes is represented. To statistically validate the differences, a Student's *t*

test between the three inoculated and the three mock-inoculated expression levels was calculated at each time point of the time course.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The *Arabidopsis* Information Resource database:
<http://www.arabidopsis.org/Blast/index.jsp>
 iPSORT web server: ipsort.hgc.jp
 The Joint Genome Institute Soybean (*Glycine max*) website:
www.phytozome.net/soybean.php
 Kazusa DNA Research *Lotus japonicus* webpage www.kazusa.or.jp/lotus
 Medicago genome assembly website:
www.medicagohapmap.org/?genome
 Primer3 software: biotools.umassmed.edu/bioapps/primer3_www.cgi
 PSORT prediction server: psort.hgc.jp/form.html
 Softberry MaliP software: linux1.softberry.com/berry.phtml
 SVM-Prot Protein Functional Family Prediction server:
jmg.ccz3.nus.edu.sg/cgi-bin/svmprot.cgi