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# Possible role of glutamine synthetase of the prokaryotic type (GSI-like) in nitrogen signaling in *Medicago truncatula*

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ABSTRACT

Genes containing domains related to glutamine synthetase of the prokaryotic type (GSI-like) are widespread in higher plants, but their function is currently unknown. To gain insights into the possible role of GSI-like proteins, we characterized the *GSI-like* gene family of *Medicago truncatula* and investigated the functionality of the encoded proteins. *M. truncatula* contains two-expressed *GSI-like* genes, *MtGSIa* and *MtGSIb*, encoding polypeptides of 454 and 453 amino acids, respectively. Heterologous complementation assays of a bacterial *glnA* mutant indicate that the proteins are not catalytically functional for glutamine synthesis. Gene expression was investigated by qRT-PCR and western blot analysis in different organs of the plant and under different nitrogen (N) regimes, revealing that both genes are preferentially expressed in roots and root nodules, and that their expression is influenced by the N-status of the plant. Analysis of transgenic plants expressing *MtGSI-like*-promoter-*gusA* fusion, indicate that the two genes are strongly expressed in the root pericycle, and interestingly, the expression is enhanced at the sites of nodule emergence being particularly strong in specific cells located in front of the protoxylem poles. Taken together, the results presented here support a role of GSI-like proteins in N sensing and/or signaling, probably operating at the interface between perception of the N-status and the developmental processes underlying both root nodule and lateral root formation. This study indicates that *GSI-like* genes may represent a novel class of molecular players of the N-mediated signaling events.



## 1. Introduction

Nitrogen (N) is a key element for plant growth and development and also a metabolic signal that is sensed and transduced by plants. While it is well known that N and the N-status can be sensed by plants to regulate their development, physiology and metabolism, the mechanisms underlying the N signaling pathways are still far from being fully understood. One of the grand challenges of our time is to improve plant nitrogen use efficiency (NUE), but achieving this aim requires a better understanding of nitrogen metabolism and of the signaling pathways elicited by N sources, for which the identification of the molecular players involved is a major breakthrough.

Legumes benefit from a privileged source of N, the atmospheric $N_2$, as these plants can form nitrogen-fixing nodules through symbiotic interaction with specialized soil bacteria known as rhizobia. Root nodule formation requires a constant fine-tuned signal exchange between plants and bacteria and only occurs under N-limitation [1]. Thus, the internal N status of the plant needs to be sensed and transduced into a developmental program leading to the formation of a novel organ, the root nodule. In order to achieve a successful symbiosis, root nodule organogenesis must occur at the site of bacterial infection, implying that these events must be spatially and temporally coordinated. Essential in this coordination is the induction of plant genes known as nodulins, which are characterized by having an enhanced, in some cases exclusive, expression in root nodules. They can be classified as early or late nodulins depending on their kinetics of expression [2,3].

Glutamine is the primary product of nitrogen assimilation from all inorganic nitrogen sources (nitrate, direct ammonium uptake and nitrogen fixation in the case of legumes) and also a central metabolite in nitrogen metabolism in plants. Glutamine is formed by the ATP-dependent condensation of ammonium with glutamate catalyzed by glutamine synthetase (GS; EC 6.3.1.2), a crucial

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enzyme in the network of N metabolism. Besides its key role in primary N assimilation, GS is also involved in N recycling, being responsible for the assimilation of ammonium released by a number of metabolic processes in the plant such as photorespiration and amino acid catabolism [4–6]. The enzyme is thus involved in all aspects of nitrogen metabolism. To achieve its multiple roles, GS exists in plants as a number of isoenzymes located in the cytosol (GS1) and in the plastids (GS2), which are encoded by a small family of genes.

The GS protein superfamily can be divided into three main classes, distinguishable by sequence, molecular mass and quaternary structure. GSI, the best-characterized class, includes enzymes typically found in prokaryotes, that display a homododecameric arrangement of subunits with masses ranging from 44 to 60 kDa [7]. Plant GS belong to class II, which represents the typical eukaryotic type GS, and forms decameric assemblies of subunits of 39–42 kDa [8,9]. The most recently discovered and least characterized class of GS enzymes GSIII, includes larger molecules formed by subunits of 75–83 kDa with a dodecameric architecture, but showing significant structural differences from GSI [10]. While at first GS classes were being associated with particular taxonomical domains, it is now evident that some organisms encode multiple enzymes of each type [11–16].

In the model legume *M. truncatula*, the GSII gene family consists of four expressed genes. *MtGS1a* and *MtGS1b* encoding cytosolic polypeptides of 39 kDa [17–19], and *MtGS2a* [20] and *MtGS2b*, encoding plastid located polypeptides of 42 kDa, the latter of which is seed specific and unique to *M. truncatula* and closely related species [21]. The other three GS genes are expressed in almost all organs of the plant, but in a cell-specific manner. *MtGS1a* is highly up regulated in the central infected cells of root nodules and encodes the isoenzyme responsible for the assimilation of the ammonia released by nitrogen fixation [18]. Intriguingly, in addition to the functional GS enzymes of the eukaryotic type GSII, *M. truncatula* contains genes encoding GS of the prokaryotic type I, referred as GSI-like [14]. These genes encode proteins with less than 25% amino acid similarity to GSII proteins, but with between 36% and 46% similarity to prokaryotic GS proteins and a similar molecular mass. Genes encoding proteins containing GSI-like domains have also been described in other plants and in fungus but they usually possess an N-terminal domain similar to nodulin 6 [11,12,14,22–24]. To date, *M. truncatula* is the only species in which genes encoding uniquely the GSI-like domain were identified [14].

The first eukaryotic protein containing a GSI-like domain to be described was FluG, characterized in the fungus *Aspergillus nidulans*. Mutating *FluG* results in the disruption of the programmed induction of asexual sporulation, a phenotype that can be rescued by growth next to wild-type colonies [22]. FluG is, therefore, required for the production of a diffusible signal necessary for conidiogenesis, yet the same authors have shown that this requirement occurs in conditions of N stress or increased osmolarity, but not under carbon stress [25]. Interestingly, it was shown that the GSI-like domain is the one responsible for the phenotype raising the hypothesis that FluG could play a novel enzymatic function related to the reaction catalyzed by glutamine synthetase. Though a diffusible signal was recently identified [26], the signaling cascade is still not comprehensively characterized.

Another example is *Arabidopsis* NodGS, which, in the same way as FluG, has a GSI-like C-terminal domain and an N-terminal domain that is similar to nodulin 6. It has been shown, through an RNA interference strategy, that the downregulation of AtNodGS results in plants with shorter main roots, disrupted development of the root cap and reduced meristematic activity. Additionally, it was shown that flagellin enhances the expression of AtNodGS, thus associating this protein with microbial elicitation. While the authors were unable to prove which domain was responsible for the phenotype, they show that, similarly to FluG, AtNodGS does not function as a GS enzyme [12]. Recently, the existence of a NodGS gene has also been reported in *Hordeum vulgare*, the gene was found to be repressed during leaf senescence but no functional studies were performed [11]. In legumes, however, nodulin and GSI-like domains can be found in independent proteins. The *M. truncatula MtN6* is an early nodulin that was associated with the onset of infection by rhizobia and its function is still unknown [27]. Nevertheless, in a transcriptomic study, this gene was found to respond to N signals, using the split-root system to compare N-sufficient with N-limited roots [28]. In soybean, on the other hand, GmN6L, whose amino acid sequence has a very strong homology with MtN6, is a late nodulin, and therefore, it is not expected that the two proteins play the same role [29]. However, both proteins are associated with the bacterial symbionts and could thus take part in the communication that is established between plant and bacteria.

To date, only one *GSI-like* gene has been described in legumes, *MtGSIa*, and its discovery in higher plants supports the paralogous evolution of GSI and GSII genes [14]. In this study, we show that GSI is encoded by a small gene family in *M. truncatula,* comprising genes encoding both the GSI domain alone and the composite protein NodGS. The genes encoding uniquely the GSI domain and their protein products are characterized providing information necessary for the understanding of the function of these proteins in the context of the legume-rhizobia symbiosis.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Plants of *M. truncatula* Gaertn. (cv. Jemalong J5) were grown in aeroponic conditions under 16 h light (22 °C)/8 h dark (19 °C) cycles and under a light intensity of 150–200 μmol $m^{-2}$ $s^{-1}$, in a nutrient solution supplemented with 5 mM ammonium nitrate as described by [30]. For nodule induction, the growth medium was replaced with fresh medium lacking a nitrogen source three days before inoculation with the wild-type *Sinorhizobium meliloti* effective wild-type strain Rm1021 pXLGD4 RCR 2011(GMI 151). Nodules were harvested at 14 and 21 days after infection. For the studies of N response, seven-day old seedlings grown on soft agar growth media supplemented with 5 mM $NH_4NO_3$, were transferred to a solution containing 0.5 mM, 5 mM and 25 mM glutamate, glutamine or asparagine in 5 mM potassium–phosphate buffer, pH 7. Controls were maintained in buffer solution, without any nitrogen added. Seven-day old seedlings showed on average a radicle length of 2.5 cm and hypocotyls of approximately 1.5 cm. The cotyledons were fully open and green, and the first leaf primordium could be seen. After 3 and 12 h of incubation, the seedlings were separated into shoots and radicles. Three pools of 5–10 seedlings were collected from 3 independent seed batches. All plant material was immediately frozen in liquid nitrogen and stored at −80 °C. Plants used for *in vitro* culture were maintained in an environmental cabinet at a temperature of 25 °C by day and 19 °C by night, 16 h day length and light intensity of 150–200 μmol $m^{-2}$ $s^{-1}$.

### 2.2. DNA and RNA extraction and cDNA synthesis

Genomic DNA was extracted and purified from young leaves of *M. truncatula* as described [31]. Total RNA was isolated from 100 mg of plant tissue, using the InviTrap® Spin Plant RNA Mini Kit (STRATEC Molecular), 1 μg of total RNA was reverse transcribed, with random hexamers, using NZY Reverse Transcriptase (Nzytech, Lda) according to the manufacturer's instructions. Concentration and purity of all nucleic acids were determined spectophometri-