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

Liliana S. Silva[a,b,1], Ana R. Seabra[a,1], José N. Leitão[a,2], Helena G. Carvalho[a,*]

[a] *Instituto de Biologia Molecular e Celular da Universidade do Porto, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal*
[b] *Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Portugal*

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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

* Corresponding author.
*E-mail address:* mhcarval@ibmc.up.pt (H.G. Carvalho).
[1] These authors contributed equally to this work.
[2] Present address: Department of Cell and Developmental Biology, John Innes Centre, Norwich, UK.

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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-

cally using a Nanodrop spectrophotometer (Thermo Scientific), and the integrity was confirmed by gel electrophoresis.

### 2.3. Quantitative real time PCR

Quantitative real-time PCR (qPCR) was performed using an iCycler Thermal Cycler (BIO-RAD) detection system with iQ™ SYBR® Green Supermix (BIO-RAD). Two replicate PCR amplifications were performed for each primer and sample combination using 50 ng of cDNA and 5 pmol of each primer: 5′ATTACAACTCACATGATAGAAC 3′ and 5′ATATTATTACATCCGCAAGAAG 3′ for *MtGSIa*; 5′CCCAAGAGAAGCCTTTAGAAGAG 3′ and 5′GCGGACGAGCAACCATAAG 3′ for *MtGSIb*; 5′CCACCAACCTTGACTGGTAC 3′ and 5′CCACGCTTGAGATCCTTCAC 3′ for *MtElf1α*. The qPCR conditions were the following: initial denaturation (3 min at 95 °C), followed by 40 cycles of amplification and quantification (10 s at 95 °C; 30 s at 55 °C and 30 s at 72 °C, with a single fluorescence measurement). A melting curve was generated to verify the specificity of the amplification reaction (55–95 °C, with a fluorescence measurement every 0.5 °C). Calculation of $C_q$ was performed using the iQ™ 5 Optical System Software (Version 2.1). The expression level was normalized using *MtElf1α* as a reference gene [32].

### 2.4. Expression of MtGSI recombinant proteins in E. coli

The complete coding region of *MtGSIa* and *MtGSIb* was amplified from a root nodule cDNA library using specific primers: 5′ CATTGAGAGA**CCATGG**ATTTGATTG 3′ and 5′CATGACATT**CTGCAG**TTCAATAACG 3′ for *MtGSIa* and 5′CATTGAGACCATGGATTTGATTG 3′ and 5′CATTAATTCGTC**GATATC**AATAGCG 3′ for *MtGSIb*. Primers were designed to introduce *NcoI*, *PstI* and *EcoRV* restriction sites (bold), the underlined bases in the primers correspond to substitutions with respect to the reference sequence. The resulting 1365 bp (*MtGSIa*) and 1388 bp (*MtGSIb*) PCR amplified products were further digested with *NcoI-PstI* (*MtGSIa*) or *NcoI-EcoRV* (*MtGSIb*) and inserted in the *NcoI-PstI* (GSIa) or *NcoI-SmaI* (GSIb) sites of pTrc99A expression vector (GE healthcare, LifeSciences). The resulting constructs GSIa:pTrc99A and GSIb:pTrc99A were sequenced (Stab Vida, Portugal) and transformed into the *Escherichia coli* glnA mutant ET8894 [33] for complementation assays and protein expression, as described in [34].

### 2.5. Protein extraction from plant tissues and from E. coli and determination of GS activity

Plant material was homogenized at 4 °C in a mortar and pestle in extraction buffer 50 mM Hepes-NaOH (Sigma) pH 7.5, 150 mM NaCl (Sigma), 1 mM $MgCl_2$ (Sigma), 1 mM EGTA (Sigma), 1 mM DTT (BioVectra, Inc.), 0.01% (v/v) TritonX-100 (Sigma) and 0.5% (v/v) protease inhibitor cocktail for plant cell extracts (Sigma). *E. coli* was collected by centrifugation (2800 × *g* for 10 min) after protein induction. The pellets were resuspended in extraction buffer and disrupted by sonication. The homogenates were centrifuged at 10,000 × *g* for 10 min at 4 °C and subsequently at 25,000 × *g* for 30 min at 4 °C. Soluble protein concentration was measured by the Coomassie dye-binding assay (Bio Rad) using bovine serum albumin as the protein standard. GS activity was measured using the transferase reaction as described in [34].

### 2.6. Protein determination, SDS-PAGE and western blot analysis

Soluble protein extracts were separated by 10% (w/v) SDS-polyacryamide gel electrophoresis (SDS-PAGE), and electroblotted onto nitrocellulose or PVDF membranes (Whatman) using a Criterion Blotter from BioRad. Immunodetection of GSI-like proteins was performed using an anti-NodGS from *Arabidopsis* (kindly provided by Pavla Binarová, Institute of Microbiology, Academy of Sciences of the Czech Republic), which specifically recognizes C-terminal sequences KNPDAYKQLIHRY of the *Arabidopsis* NodGS molecule [12]. GSII polypeptides were detected using the specific anti-GSII antibody AS08 295 (Agrisera). Goat anti-rabbit peroxidase conjugated antibody (Santa Cruz Biotechnology) was used as a secondary antibody. The immunocomplexes were detected in a photographic film, by chemiluminescence, using the ECL™ (GE Healthcare, Lifesciences) detection system. Representative blots of at least 3 independent experiments are shown in each of the figures.

### 2.7. Construction of transcriptional MtGSIa and MtGSIb promoter-gusA fusion

The promoter regions of *MtGSIa* (2.0 kb) and *MtGSIb* (1.8 kb) were amplified by PCR from *M. truncatula* genomic DNA. The 5′ flanking regions were isolated using primers (*MtGSIa*R 5'CAACTCAATCAAAT**CCATGG**TCTCTC 3′ and *MtGSIb*R 5′ CCATTGAGA**CCATGG**ATTTGATTG 3′) to create a *NcoI* site (bold) overlapping the start codon, in combination with a primer designed to add a *XhoI* site 2 kb upstream of the *MtGSIa* start codon (*MtGSIa*F 5′ GAGAAAGA**CTCGAG**GTTCAACTGAC 3′) or with a specific primer corresponding to a region 1.8 kb upstream of the *MtGSIb* start codon, which contains a *NcoI* restriction site (*MtGSIb*F 5′ CGTGTTTTGTAT**CCATGG-**AAATCTAATG 3′). The underlined bases in the primers correspond to substitutions with respect to the reference sequence. After digestion with the restriction enzymes, the *MtGSIa* and *MtGSIb* promoter regions were inserted upstream the GUS gene in pGreenI0029:GUS, as a 2.0 kb and a 1.8 kb PCR fragment, respectively, to obtain the plasmids p*MtGSIa*:pGreenI0029:GUS and p*MtGSIb*:pGreenI0029:GUS. The integrity of promoter-GUS plasmid sequences was confirmed by DNA sequencing. To generate pGreenI0029:GUS, an *EcoRI-PstI* fragment containing a GUS coding sequence and a CaMV 35S polyadenylation signal was inserted into the corresponding sites of pGreenI0029 [35].

### 2.8. Transformation of M. truncatula and recovery of transgenic plants

The binary vector p*MtGSIa*:pGreen0029:GUS was introduced into *Agrobacterium rhizogenes* strain ARqua1 [36] and used for *M. truncatula* root transformation by the method described in [37]. The transgenic hairy roots were obtained after kanamycin-selection and at approximately 3 weeks after inoculation by *A. rhizogenes*, composite *M. truncatula* plants were transferred to aeroponic growth chambers for nodulation. p*MtGSIb*:pGreen0029:GUS was introduced into *Agrobacterium tumefaciens* strain AGL1 and used to transform leaf segments of *M. truncatula* cv. Jemalong (genotype 2HA). Kanamycin resistant plants were regenerated *via* somatic embryogenesis as described [38] and propagated *in vitro*. The empty binary vector was used as control in all the transformation experiments.

### 2.9. Histochemical localization of GUS activity

Histochemical staining for GUS activity was performed according to [18]. Samples were mounted in glass microscope slides in water and observed with an Olympus BX50 light microscope. Photographs were taken using an Olympus DP21 digital camera.

## 3. Results

### 3.1. *The* GSI-like *gene family of* M. truncatula

A cDNA encoding a GSI-like protein (*MtGSIa*, genebank ID AJ238212) was previously identified in *M. truncatula* [14]. This gene contains the information to encode a 454 amino acid polypeptide with a predicted molecular mass of around 50 kDa. To characterize the *M. truncatula* GSI-like gene family, a blast search of the *M. truncatula* genome was performed using *MtGSIa* as query. This search identified two additional *GSI-like* genes, which we designate *MtGSIb* (Medtr3g035970) and *MtGSIc* (Medtr1g062470) and two genes encoding FluG-like proteins, here designated as *MtNodGSa* (Medtr1g062620) and *MtNodGSb* (Medtr1g062650) (Table 1). The *MtNodGS* genes contain the information to encode proteins with a predicted molecular mass of around 93 kDa comprising a N-terminal domain highly similar to MtN6 and a C-terminal GSI-like domain.

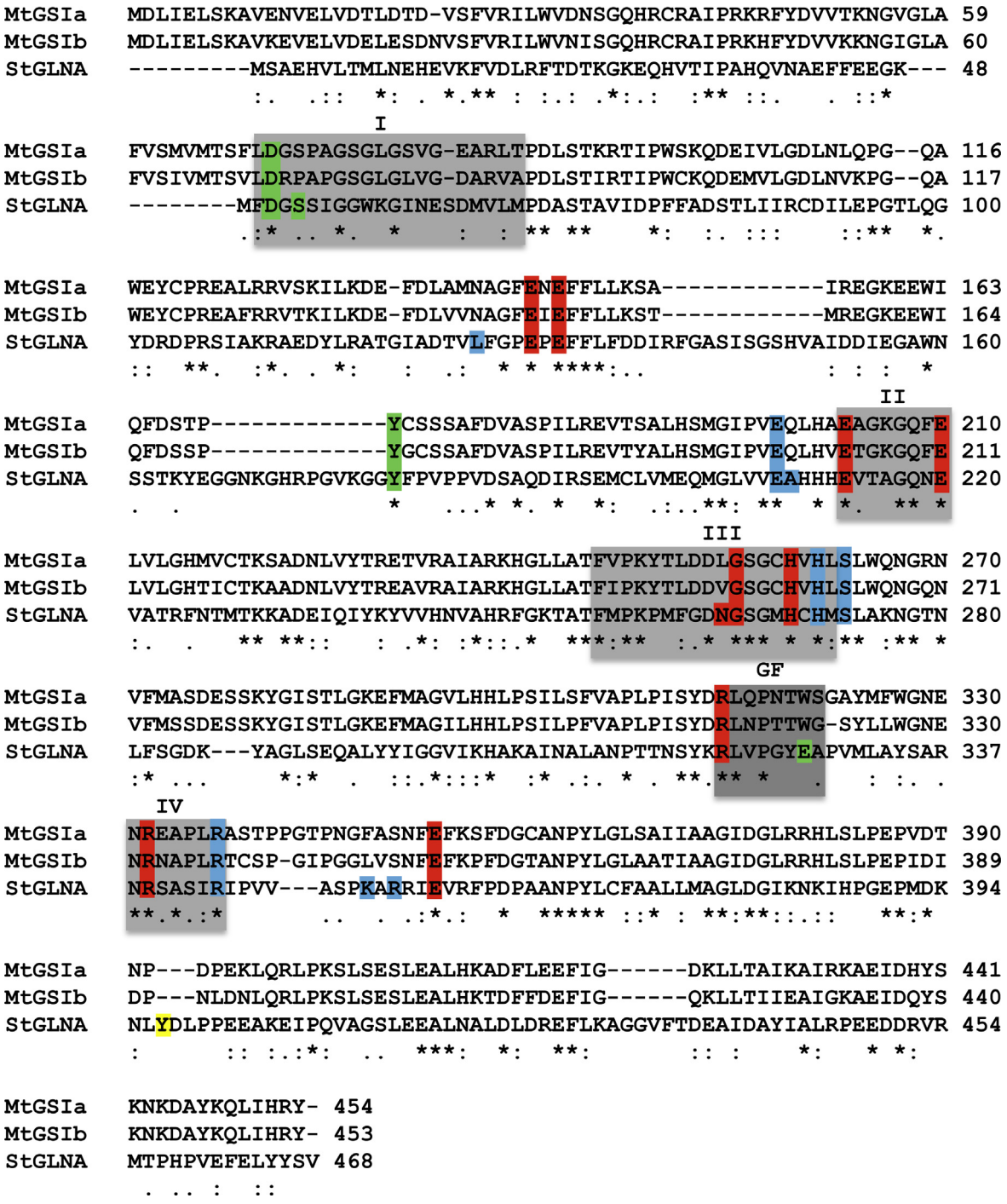


**Fig. 1.** Alignment of the deduced amino acid sequences of MtGSIa, MtGSIb and the functional GSI enzyme from *Salmonella typhimurium* (StGlnA). Residues known to be involved in binding of glutamate (red), ATP (blue) and ammonia (green) are indicated. The adenylylation site (yellow), characteristic of prokaryotic GSs is also labelled. Shaded fragments I–IV represent the GS conserved regions defined by [56]. The shaded box GF represents the conserved "Glutamate-flap". (*) Residues fully conserved; (:) highly conserved; (.) poorly conserved and () and not conserved. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 1**
*Medicago truncatula* GSI-like gene family.

| Gene name | Locus ID (Mt4.0) | Predicted MW (kDa)[a] |
|---|---|---|
| *MtGSIa* | Medtr0417s0020 [a] | 50.45 |
| *MtGSIb* | Medtr3g035970 | 50.49 |
| *MtGSIc* | Medtr1g062470 | 49.76 |
| *MtNodGSa* | Medtr1g062620 | 92.94 |
| *MtNodGSb* | Medtr1g062650 | 93.91 |

The GSI-like family in *Medicago truncatula* comprises 5 genes (Table 1): three genes encoding GSI-like proteins, *MtGSIa*, *MtGSIb* and *MtGSIc*; and two genes encoding nodulin/GSI-like fusions designated as *MtNodGSa* and *MtNodGSb* after the *Arabidopsis* ortholog *AtNodGS* [11]. There is no evidence, either experimental or *in silico* for the expression of *MtGSIc* and *MtNodGSb*.

[a] *MtGSIa* is placed in an unanchored scaffold and hence a chromosome location has not been assign yet [55].

*MtGSIb* is located on chromosome 3 and contains a open reading frame (ORF) spanning 10 exons, containing the information to encode a polypeptide of 453 amino acids, with 88.08% amino acid similarity to MtGSIa and similar molecular mass. The third gene, *MtGSIc* is located on chromosome 1, has an ORF also spanning 10 exons, encoding a polypeptide of 448 aminoacids, with 80.18% amino acid similarity to MtGSIa and a predicted molecular mass of 49.76 kDa. *MtGSIc* and *MtNodGSb* do not seem to be expressed *in planta* as we were unable to find any evidence, either experimental or *in silico* for the expression of these two genes. The present study concentrates on *MtGSIa* and *MtGSIb* because these two genes appear to be exclusive of legumes and are most highly expressed in the roots, according to the Medicago Gene Expression Atlas server (http://mtgea.noble.org/v3), which are crucial organs for the legume-rhizobium symbiosis.

An alignment of the deduced amino acid sequences of MtGSIa and MtGSIb with the functional enzyme from *Salmonella typhimurium* (Fig. 1) reveals a high degree of similarity, along their entire length, to the bacterial enzyme. However, only 15 out of the 23 residues identified as important for GS activity [7] are present in both *M. truncatula* GSI-like proteins, which suggest that the proteins are not functional GS enzymes.

### 3.2. Evaluation of the functionality of MtGSIa and MtGSIb

To evaluate whether the proteins encoded by the two *MtGSI* genes are catalytically functional for glutamine synthesis, we performed heterologous complementation assays. The plant *MtGSIa* and *MtGSIb* cDNAs were cloned into the *E. coli* expression vector pTrc99A and independently transformed into the *E. coli glnA* mutant strain ET8894 [33]. The previously described MtGS1a recombinant protein [34] was used as positive control, whereas the vector alone served as a negative control. Complementation, enabling growth on minimal media containing ammonium as the nitrogen source, was clearly observed for the bacteria expressing the functional GSII isoenzyme MtGS1a, whereas the bacteria transformed with *MtGSIa*, *MtGSIb* or the vector alone, could only grow in the presence of a glutamine supplement (Fig. 2a), indicating that MtGSIa and MtGSIb are not catalytically and physiologically active. It is noteworthy that the bacteria expressing either MtGSIa or MtGSIb were growing poorly, even in the presence of a glutamine supplement, when compared to MtGS1a or the bacteria expressing the vector alone, denoting that the two GSI-like proteins interfere with the bacterial metabolism.

Total soluble protein extracts from the different bacterial strains were analyzed by western blot using an anti-NodGS antibody [12], raised against the C-terminal sequence of AtNodGS (amino acids 833–845), which is highly conserved in both *M. truncatula* GSI-like polypeptides (Fig. 2b). This antibody recognizes the two GSI-like proteins, which were expressed in the bacterial host with the expected molecular mass of 50 kDa, but does not recognize the eukaryotic type GS MtGS1a of 39 kDa, which is only recognized by the specific anti-GSII antibody (Fig. 2b). GS activity was determined in triplicate independent experiments in soluble protein extracts from the different bacterial cultures using the colorimetric GS transferase assay (Fig. 2c). No measurable GS activity above the negative control (pTrc99a) was detected in the bacterial extracts expressing either MtGSIa or MtGSIb (<0.08 μmol/min/mg protein),

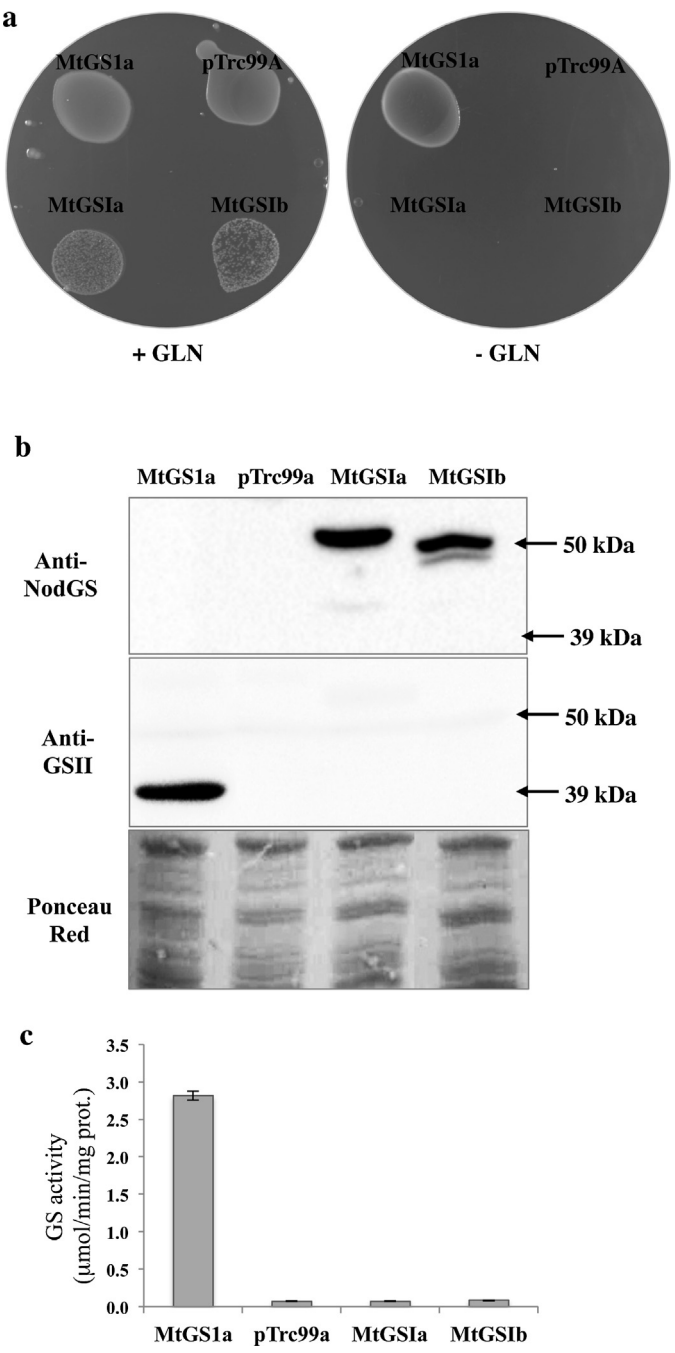


**Fig. 2.** Analysis of the catalytic functionality of GSI proteins. (a) Complementation analysis of an *E. coli glnA* mutant expressing either *MtGSIa* or *MtGSIb* plant cDNAs. Controls were transformed with the vector (pTrc99A) or with the cDNA of the functional enzyme MtGS1a in pTrc99A. Bacteria were grown on Minimal Medium (M9) with and without a glutamine supplement (0.25 mg ml $^{-1}$). (b) Western blot analysis of *E. coli glnA* extracts expressing recombinant MtGS1a, MtGSIa or MtGSIb proteins probed with anti-NodGS antibody [12] or a specific anti-GSII antibody AS08 297 (Agrisera), 10 μg of total proteins were loaded on each lane. Ponceau red staining demonstrates equal loading. (c) Quantification of GS activity by the transferase reaction in soluble protein extracts from the different bacterial cultures.

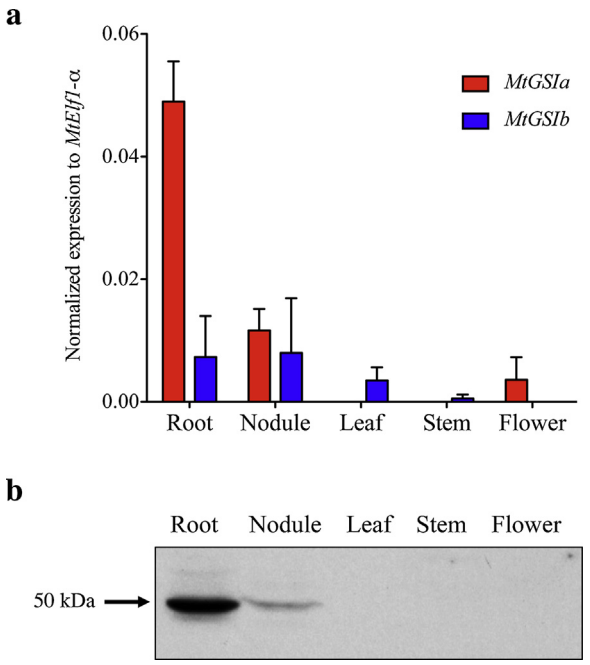


**Fig. 3.** Quantification of the expression of *MtGSI* genes. (a) Quantification, by qPCR, of *MtGSIa* and *MtGSIb* transcripts in roots of plants grown on nutrient medium containing 5 mM $NH_4NO_3$, 14 day old nodules, leaves, stems and flowers, nodules, leaves, flowers and stems. The expression was normalized using *MtElf1α* as a reference gene. (b) Western blot analysis of GSI proteins, probed with anti-NodGS antibody [12]. 20 μg of total proteins were loaded on each lane.

contrasting with the activity of 2.81 μmol/min/mg protein quantified in the bacterial extracts expressing MtGS1a, further confirming that the GSI-like proteins of *M. truncatula* do not contain GS activity.

### 3.3. *Expression analysis of* MtGSI *genes in several organs of* M. truncatula

The expression of the two members of the GSI-like gene family of *M. truncatula* was evaluated by qRT-PCR and western blot analysis in roots, root nodules, leaves, stems and flowers (Fig. 3a). Interestingly, both *MtGSIa* and *MtGSIb* were most abundantly expressed in roots and root nodules, the expression was very low to negligible in all other organs of the plant. This expression pattern is consistent with the information available in *M. truncatula* gene expression atlas (MtGEA). To investigate whether the levels of GSI-like transcripts correlate with the polypeptide content, total soluble proteins were extracted from the same organs and analysed by western blot (Fig. 3b). The anti-NodGS antibody [12] recognized GSI-like polypeptides of 50 kDa, compatible with the size of both MtGSIa and MtGSIb, most abundantly in roots and root nodules; thus, indicating that the mRNAs are translated into GSI-like proteins *in planta*.

### 3.4. *Localization of the expression of MtGSIa and MtGSIb by promoter-GUS fusions in roots and root nodules*

To determine the sites of expression of the two GSI-like genes, the 5′ upstream regions of *MtGSIa* and *MtGSIb* were fused to the *E. coli* reporter gene β-glucuronidase and transformed into the homologous transgenic system. The 2.0 kb and 1.8 kb lying immediately upstream of the translation initiation codon of *MtGSIa* and *MtGSIb*, respectively, were fused to *gusA* in a way to generate precise transcriptional fusions. These chimeric constructions were introduced into *M. truncatula* by means of *A. tumefaciens* in the case of *MtGSIb* and *A. rhizogenes* in the case of *MtGSIa*. A minimum of 4 independent transgenic plants or transgenic roots, were analyzed for each construct and the GUS activity patterns were found to be qualitatively identical within a construct. The following general features relating to the histological expression patterns of the gene fusions have been deduced from the observation of a large number of root samples, collected at several developmental stages from independent transgenic lines.

The two promoters were found to drive an overlapping expression (Figs. 4 and 5). Both genes were most strongly expressed in the vascular bundles of both roots and root nodules, a strong GUS staining was also observed at the lateral root (Figs. 4 a and 5 f) and root nodule primordia (Figs. 4 b and 5 a) and in growing root apices (Figs. 4 c and 5 b). Transversal sections trough the roots, either non-inoculated (Figs. 4 d–e and 5 c) or inoculated with Rhizobium (Figs. 4 f and g and 5 d–f) revealed that both genes are expressed at very localized regions within the vascular cylinder, more precisely in front of the protoxylem poles and interestingly, this localization was found to change in relation to developmental cues. In young roots (Figs. 4 d and 5 c), the genes are expressed in all the cell types internal to the endodermis, except the xylem and phloem vessels, whereas in mature roots it was restricted to the cell layer immediately adjacent to the endodermis and particularly strong in front of the protoxylem poles (Figs. 4 e–g and 5 d–f), which interestingly, is the exact position of root nodule emergence. A stronger blue staining was always observed at the dividing cells at the initial stages of both nodule and lateral root formation (Fig. 5f), suggesting a relationship between GSI-like gene expression and the development of both root lateral organs.

Transversal and longitudinal sections through GUS stained root nodules revealed that the expression of both genes was highest at the nodule vascular bundles. In the central tissues of the nodule, the staining was restricted to the uninfected cells of both the infection (zone II) and the nitrogen fixation (zone III) zones (Figs. 4 h–i and 5 g). Clearly none of the two expressed GSI-like genes is expressed at the infected cells.

### 3.5. *Evaluation of GSI-like response to exogenously fed amino acids*

To investigate whether the *MtGSI-like* genes could respond to the N-status of the plant, we evaluated the expression of both GSI-like genes in response to organic N, supplied in the form of amino acids. Seedlings of *M. truncatula* were grown for 7 days in a nutrient solution containing 5 mM $NH_4NO_3$ and then incubated for 3 and 12 h in the presence of 0.5, 5 and 25 mM glutamate, glutamine and asparagine (Fig. 6). The expression of *MtGSIa* and *MtGSIb* was determined in the roots by qRT-PCR and the GSI-like polypeptide content was analyzed by western blot. Interestingly, under these experimental conditions all the exogenously fed amino acids down regulated the expression of both *MtGSIa* and *MtGSIb*. The reduction rates could be generally directly correlated with increasing concentration of the amino acid, and this effect was more evident 3 h after incubation. The differences in gene expression are not detected at the level of GSI-like polypeptides, which were maintained at relatively constant levels in all conditions tested. This lack of correlation is probably due to a high half-life of the proteins.

## 4. Discussion

The existence of genes encoding domains of glutamine synthetase of the prokaryotic type in plants is intriguing due to a number of curious features. First, the proteins encoded by these genes are expressed in an organism that already accounts for several functional GS isoenzymes of the eukaryotic type. Its continued selection through time therefore, suggests an important function

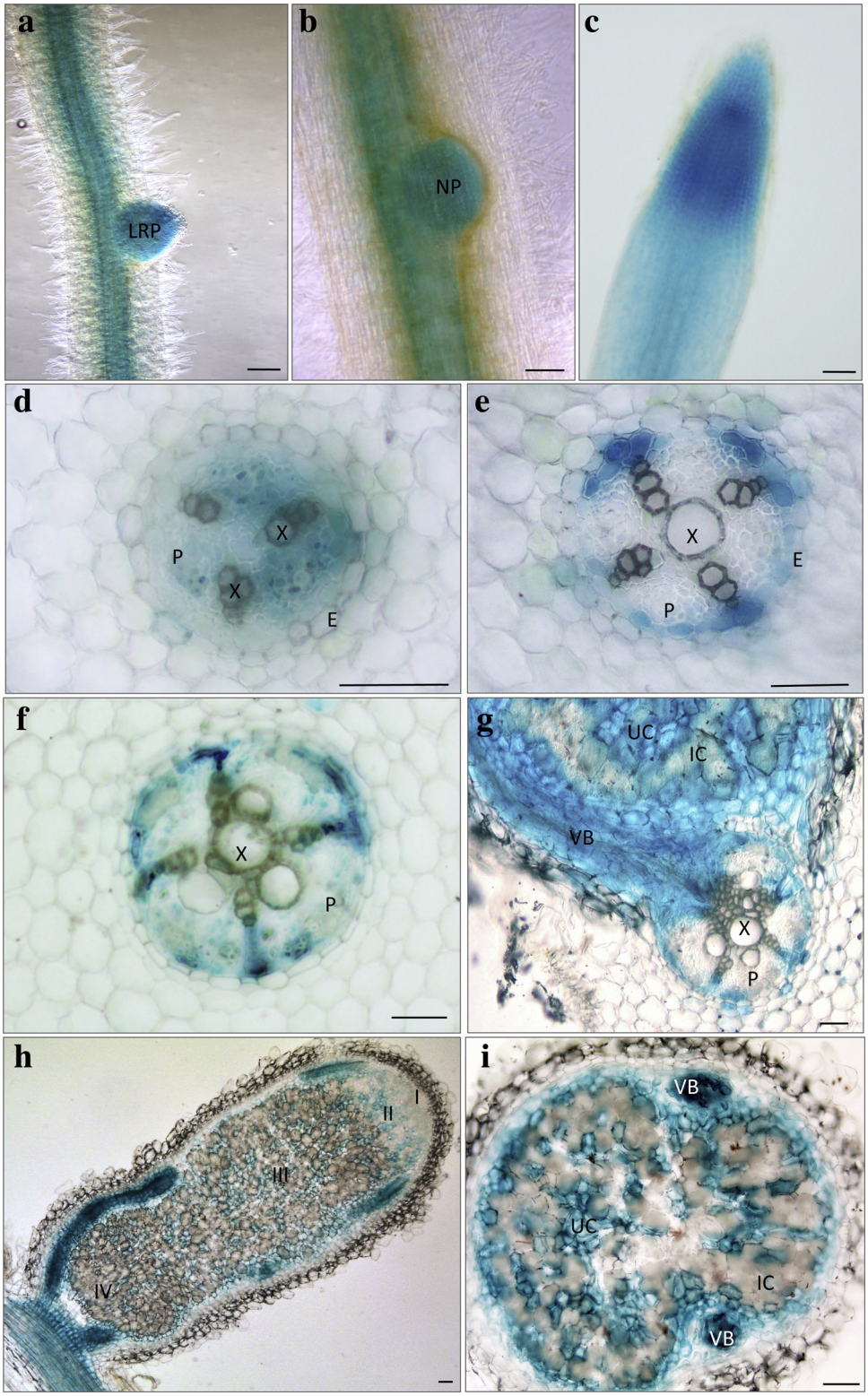


**Fig. 4.** Histochemical localization of GUS activity in transgenic *M. truncatula* roots expressing the *gusA* reporter gene under the control of a 2.0 kb *MtGSIa* promoter fragment. (a) Lateral root primordium. (b) Root nodule primordium. (c) Root tip. (d) Transversal section of a root (non-inoculated), about 1 cm from the root tip. (e) Transversal section of a root (non-inoculated), about 7 cm from the root tip. (f) Transversal section of a root in vicinity of a 14-day-old root nodule. (g) Transversal section of a root at the site of emergence of a 14-day-old root nodule. (h) Longitudinal section of a 21-day-old root nodule. (i) Transversal section through the nitrogen fixing zone III, of a 21 day old root nodule. LRP—lateral root primordium. NP—nodule primordium. X—xylem. P—phloem. VB—vascular bundle. UC—uninfected cells. IC—infected cells. I to IV—Nodule differentiation zones. Bars(d–i) = 50 μm. Bars (a–c) = 200 μm.

and not necessarily one of glutamine synthesis. Second, the eukaryotic homologues of these prokaryotic genes emerge as the product of gene fusion events, with a N-terminal domain encoding an amidohydrolase similar to nodulin 6. It has been reported that this fusion event occurred in *Arabidopsis* and other plants [12], but not in legumes, where nodulin 6 and GSI-like genes can be found

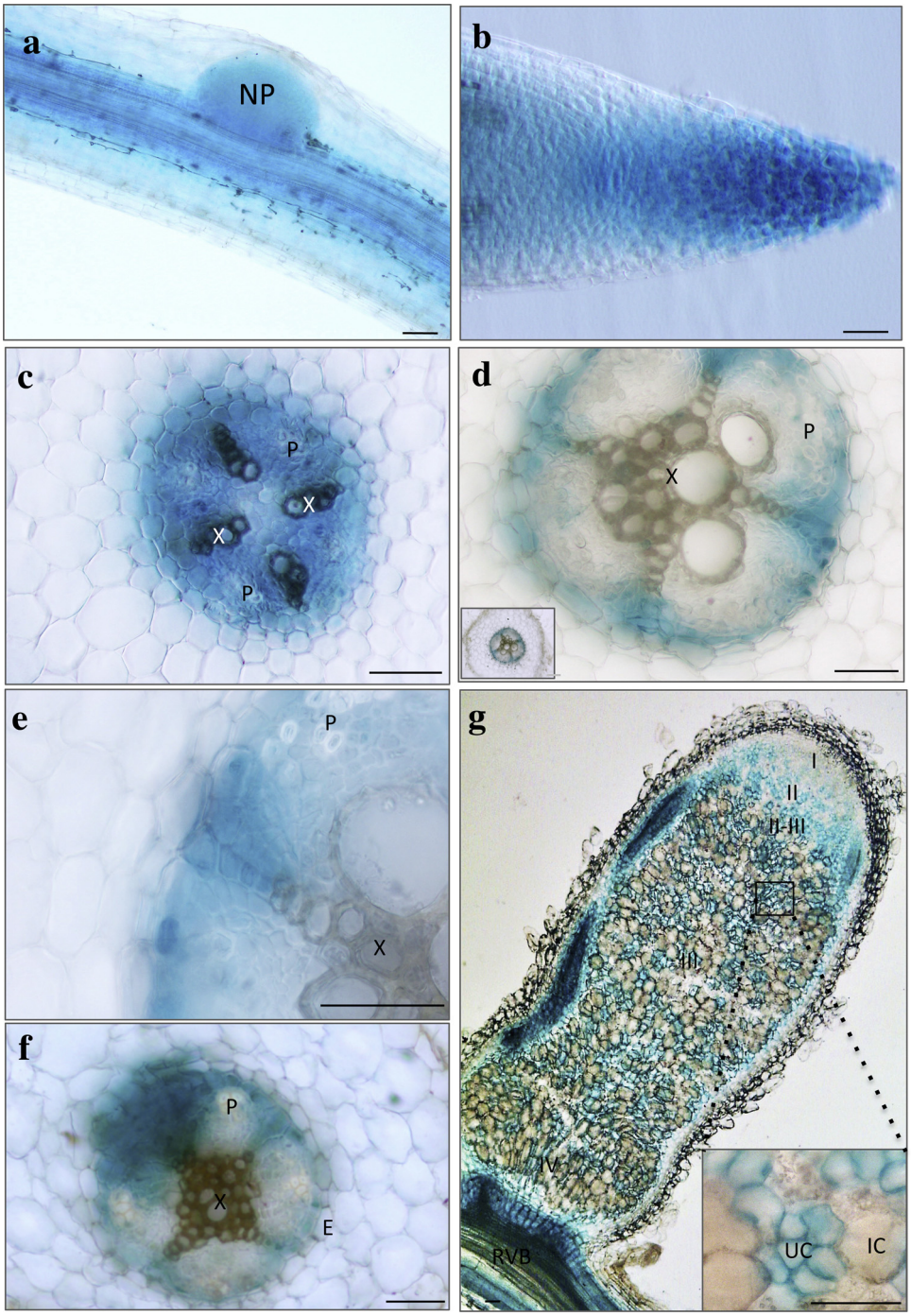


**Fig. 5.** Histochemical localization of GUS activity in transgenic *M. truncatula* roots expressing the *gusA* reporter gene under the control of a 1.8 kb *MtGSIb* promoter fragment. (a) Root nodule primordium. (b) Root tip. (c) Transversal section of a root from a one-week-old seedling, grown in the presence of 5 mM $NH_4NO_3$. (d) Transversal section through a root, collected 14 days after infection and located in the vicinity of a root nodule (e) Magnification of d showing GUS expression in front of the protoxylem poles. (f) Transversal section of a root showing intense GUS staining at the site of emergence of a lateral root. (g) Longitudinal section of a 21-day-old root nodule. NP—nodule primordium. X—xylem. P—phloem. VB—vascular bundle. UC—uninfected cells. IC—infected cells. I to IV—nodule differentiation zones. Bars (a–b)= 200 μm, bars (d–g) = 50 μm.

separately [14], suggesting that the separate domains could perform a function specific to legumes. Here, we report the existence of genes encoding both the composite protein and the separate domains in *M. truncatula*, we fully characterized the functionality and expression of the genes encoding the separate GSI-like domain, and provide evidence in favor of a role of GSI-like proteins in N sensing and/or signaling in *M. truncatula*.

The *M. truncatula* GSI-like gene family comprises 5 genes (Table 1). Three encode the GSI like domain and include the previously identified gene *MtGSIa* [14], the gene *MtGSIb* described in this paper and *MtGSIc*, which does not seem to be expressed. Two additional genes, *MtNodGSa* and *MtNodGSb*, encode a composite protein containing the GSI-like domain C-terminal to a domain highly similar to MtN6. From the available *in silico* information it seems that at least one of the *MtNodGS* genes, *MtNodGSa*, is expressed in planta, suggesting that *NodGS* and *GSI-like* genes probably play different roles in *M. truncatula*. NodGS has been previously characterized in *Arabidopsis* [12]. This study concentrates on *MtGSIa* and *MtGSIb*.

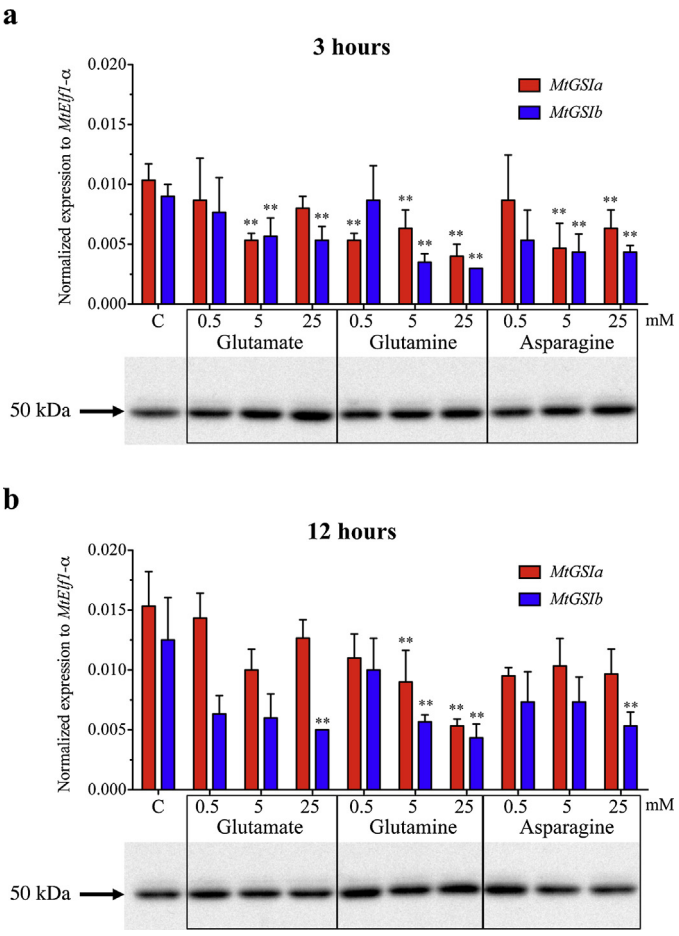


**Fig. 6.** Response of *MtGSI-like* genes to exogenously fed amino acids. Seedlings of *M. truncatula* were grown in a nutrient solution containing 5 mM $NH_4NO_3$ for 7 days and incubated for 3 h (a) and 12 h (b) in the presence of 0.5 mM, 5 mM and 25 mM glutamate, glutamine and asparagine. *MtGSIa* and *MtGSIb* expression was normalized using *MtElf1α* as a reference gene. Results are given as the mean of biological triplicates of pools of five to ten seedlings, with standard deviation. $^{**}t$-test $p < 0.05$ treatment vs control. GSI polypeptide content was analysed by western blot (10 μg load) using the anti-NodGS antibody [12].

A comparison of the *M. truncatula* GSI-like deduced amino acid sequences with the functional prokaryotic GS enzymes, reveals a conservation of the substrate binding sites, but a lack of essential amino acid residues known to be required for glutamine synthesis, suggesting that the proteins are unable to perform this enzymatic function (Fig. 1). Indeed, we have shown that their expression in the *E. coli glnA* mutant strain is unable to complement the glutamine-dependency phenotype (Fig. 2) and no GS activity could be detected using the GS colorimetric assay. Similarly, the GSI-like proteins FluG and AtNodGS do not contain GS activity [12,22]. This points to a function, which despite being distinct from glutamine synthesis, could still be related to the binding of ammonia, glutamate or glutamine, which are considered important N-status reporters [39]. This way, GSI-like proteins would be provided with an adequate mechanism for sensing the internal N status of the plant. It is noteworthy that the expression of the *M. truncatula* GSI-like proteins in *E. coli* negatively affects bacterial growth, even in the presence of glutamine, indicating a metabolic effect of the proteins in the bacteria. It seems, thus, reasonable to anticipate a metabolic effect of GSI-like also in plants.

Both *MtGSIa* and *MtGSIb* are preferentially expressed in roots and nodules and are barely detected in the remaining organs (Fig. 3). This root/nodule expression is of particular interest under the symbiotic context, wherein a tight regulation of the metabolic exchanges between the plant and its symbiotic partner is established. The localization of *MtGSI-like* expression by promoter-*gusA* fusions, revealed a pattern highly suggestive of a function related to N-signaling processes. The two genes were expressed in cells strategically placed for signaling, namely the vascular bundles of both roots and nodules and the uninfected cells of root nodules (Figs. 4 and 5). Particularly interesting is the specific expression observed in the dividing cells of the pericycle, located in front of the protoxylem poles and the association of this particular pattern of expression with lateral root and root nodule emergence. This specific pattern was not observed in young roots and was considerably enhanced at the initial stages of lateral root and root nodule formation. Normally, legume nodules arise from cell divisions in both cortical and pericycle cells in a radial sector opposite to xylem poles [40,41]. Lateral roots are also formed in front of the protoxylem poles. The specific expression of the MtGSI genes at this exact position, together with the increased expression at the initial stages of lateral organ formation, suggests an involvement of the encoded proteins at the interface between perception of the N-status and the developmental processes underlying both root nodule and lateral root formation. This pattern of expression also suggests that the genes are under the control of hormones, particularly auxin, which is a key regulator of both lateral root and nodule development [42–44].

A function related to developmental processes associated with N-metabolism has also been appointed for the composite GSI-like containing proteins FluG and NodGS from *Aspergillus* and *Arabidopsis*, respectively. FluG appears to participate in a cascade of transcriptional control for the onset of conidiogenesis, under conditions of N starvation, but not under carbon starvation [25–45]. It has been shown that the GSI-like domain of FluG is the one responsible for the phenotype and not the N-terminal domain, which shares homology with MtN6. Also, in *Arabidopsis*, knocking-down the homologue *AtNodGS* affects root morphogenesis [12] a process that can be modulated by the external concentration of nitrate and L-glutamate [46].

The N-mediated regulation of root architecture is influenced both by local and systemic controls. Downstream products of nitrogen assimilation such as glutamate or glutamine are known to function as systemic signals of organic N status [39]. In the present study, it is shown that the expression of both *MtGSIa* and *MtGSIb* is repressed by exogenously fed amino acids (Fig. 6), indicating that the expression of these genes can be adapted in response to the N-status of the plant. In bacteria, glutamine is the main nitrogen status reporter [47] being perceived by the PII signaling system, which subsequently regulates GS [48,49]. PII proteins were recently identified as glutamine sensors in different plant species, but unlike their prokaryotic homolog, an association to GS has not been demonstrated [50,51]. Nevertheless, glutamine feedback repression of the eukaryotic GS gene family [52,53] has been reported, indicating that the internal levels of glutamine must be perceived and transduced to control nitrogen assimilation by a mechanism that may or may not involve PII. The two *GSI-like* genes from *M. truncatula* are also repressed by glutamine albeit the lack of catalytic activity of the encoded proteins. Thus, although the function of GSI-like proteins is clearly not directly related to glutamine synthesis it appears that they operate within the nitrogen metabolic pathways. As the signature domains of the GSI-like proteins show an overall high identity to functional GS enzymes, it is likely that their function relies on a retained ability to bind glutamate, glutamine or ammonium. However, an enzymatic activity toward unknown compounds cannot be ruled out. Recently, it was shown that GS from *Microbacterium* has an N acetylation activity toward norfluoxacin, and it appears that norfluoxacin does not bind to the pocket for substrates and products but rather to the ATP/ADP binding site [54]. Similarly to the GS from *Microbacterium*, the *M. truncatula*

GSI-like proteins lack four of the amino acid residues known to be involved in the ATP binding site, it is thus conceivable that GSI-like could perform a catalytic function, different from glutamine synthesis, which could eventually result in the production of a signal molecule, as it seems to be the case of FluG in *Aspergillus* [22].

In conclusion, this study characterizes the *MtGSI* gene family of *M. truncatula* and shows that the GSI-like proteins are not catalytically active for glutamine synthesis, but rather involved in N-signaling. It is demonstrated that *MtGSIa* and *MtGSIb* are preferentially expressed in the vascular tissues of roots and root nodules and that its expression is enhanced in the dividing cells in front of the protoxylem poles, at the site of nodule emergence. It is also shown that the expression is influenced by exogenously fed nitrogen in the form of amino acids, indicating that the expression of the genes could be adapted in response to the N-status of the plant. Taken together, these observations provide evidence in favor of a role of GSI-like proteins at the interface between N signaling pathways and root architecture. This evidence should stimulate further studies on the function of GSI-like proteins and its significance in the complex network of N metabolism, growth, and development.

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