Differential expression of the two cytosolic glutamine synthetase genes in various organs of *Medicago truncatula*

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Abstract

In order to clarify the physiological roles of the cytosolic forms of glutamine synthetase (GS) in *Medicago truncatula*, we have performed a detailed analysis of the expression of the two functional cytosolic GS genes, *MtGSa* and *MtGSb* in several organs of the plant. Transcriptional fusions were made between the 2.6 or 3.1 kbp 5' upstream regions of *MtGSa* or *MtGSb*, respectively, and the reporter gene gusA encoding β-glucuronidase and introduced into the homologous transgenic system. *MtGSa* and *MtGSb* were found to be differentially expressed in most of the organs, both temporally and spatially. The presence of GS proteins at the sites where the promoters were active was confirmed by immunocytochemistry, providing the means to correlate gene expression with the protein products. These studies have shown that the putative *MtGSa* and *MtGSb* promoter fragments were sufficient to drive GUS expression in all the tissues and cell types where cytosolic GS proteins were located. This result indicates that the cis acting regulatory elements responsible for conferring the contrasting expression patterns are located within the region upstream of the coding sequences. *MtGSa* was preferentially expressed in the vascular tissues of almost all the organs examined, whereas *MtGSb* was preferentially expressed in the root cortex and in leaf pulvini. The location and high abundance of GS in the vascular tissues of almost all the organs analysed suggest that the enzyme encoded by *MtGSa* plays an important role in the production of nitrogen transport compounds. The enzyme synthesised by *MtGSb* appears to have more ubiquitous functions for ammonium assimilation and detoxification in a variety of organs. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

In plants, nitrogen is incorporated into an organic form mainly through the reaction catalysed by glutamine synthetase (GS). GS brings nitrogen into metabolism by condensing ammonium with glutamate, with the aid of ATP, to form glutamine, which then provides nitrogen groups, directly or via glutamate, for essentially all nitrogenous cell compounds [1]. Nitrogen metabolism in plants involves the uptake and reduction of inorganic nitrogen (nitrate, ammonium and, in legumes, fixation of atmospheric nitrogen), and reassimilation of ammonium released from metabolic processes such as photorespiration, phenylpropanoid metabolism, amino acid catabolism and utilisation of nitrogen transport compounds [2]. Several of these pathways have the potential to produce very large amounts of ammonium and they may be temporally, as well as spatially separated. This is reflected by the fact that GS exists as a number of different isoenzymes located in both the cytosol and the plastids, which occur in varying extents in different organs and tissues of the plant, as a result of the differential expression of a small multigene family [3]. The plastid-located GS is generally encoded by a single nuclear gene, whereas the cytosolic forms are encoded by complex multigene families [3–6].
The GS genes encode subunits of 38–43 kDa which assemble to form the active octameric isoenzymes within the plant. *Medicago truncatula* contains the smallest GS gene family identified to date in a higher plant, consisting of only three expressed genes, one encoding a plastid located polypeptide of 43 kDa (*MtGSd*) and two encoding cytosolic polypeptides of 39 kDa (*MtGSa* and *MtGSb*). The cytosolic polypeptides may assemble as either homo or hetero-octamers [7]. There is an additional cytosolic GS gene, *MtGSc*, that appears not to be expressed [8].

The differential expression of GS genes observed in several different plant species [5,6,8–14], together with biochemical studies that have shown that the GS isoenzymes have different catalytic properties, is consistent with the idea that the distinct GS isoenzymes perform different roles in particular organs/tissues of the plant [15]. However, most of the work assigning the differential roles of GS isoenzymes has been devoted to chloroplastic versus cytosolic isoenzymes and only a few studies have distinguished between the different members of the cytosolic GS gene family. There is good genetic and physiological evidence that the plastidial GS assimilates the ammonium released from phororespiration and probably from nitrate reduction [16]. Cytosolic GS plays a central role in ammonium assimilation in roots and root nodules and is probably involved in the re-mobilisation of nitrogen during germination, senescence and pathogen attack. Increased activity of cytosolic GS has been observed in all these situations [16,17]. The localisation of significant amounts of cytosolic GS in vascular elements has been interpreted as reflecting a role in providing glutamine for intercellular transport. But recently, by the use of an antisense RNA strategy, the phloem located GS was found to play a major role in proline synthesis [18].

If we are to understand the physiological meaning of the existence of different cytosolic enzymes, it is essential to know the specific functions of all the GS gene products within a single plant. *Medicago truncatula* offers an excellent model for this study for two main reasons. First because it has only two expressed genes encoding cytosolic GS [7,8] which greatly simplifies the study and second because it is amenable to genetic transformation via *Agrobacterium* allowing the analysis of transgenes in the homologous system. All the other legumes studied so far possess at least three genes encoding cytosolic GS, in addition multiple alleles of each gene may occur in the case of tetraploid species such as alfalfa and soybean [19–21].

In an earlier work we have studied the localisation of expression of the two functional cytosolic GS genes in root nodules of *M. truncatula* [22]. We have shown that *MtGSa* and *MtGSb* are differentially expressed in distinct tissues of the nodules, suggesting that their encoded products are involved in different aspects of the nodule metabolism. The present investigation is devoted to the localisation of expression of these two genes in other organs of *M. truncatula* using promoter-GUS fusions in transgenic plants. The presence of GS proteins in these organs was confirmed by immunocytochemistry, providing the means to correlate gene expression with the protein products. The results presented here provide new insights concerning the relative functions of cytosolic isoenzymes in plant nitrogen metabolism.

2. Materials and methods

2.1. Plant material and growth conditions

Plants of *M. truncatula* Gaertn. cv Jemalong were grown from surface sterilised seeds under 13-h light (23°C)/11-h dark (19°C) in vermiculite watered with the growth medium described by Lullien et al. [23]. Plants used for in vitro culture (genotype H39) were maintained in an environmental cabinet at a temperature of 23°C by day and 19°C by night, 13 h day length and light intensity of 150–200 μmol m⁻² s⁻¹.

2.2. Immunolocalisation

Plant material was fixed and embedded in paraffin as described in [22]. Immunolocalisation was carried out essentially as described [22] using a specific anti-GS antibody [24] as primary antibody and biotinylated swine anti-rabbit immunoglobulin-G (Dako, Denmark) as a secondary antibody. Development of the label was carried out using the Vectastain ABC reagent kit (Vector Lab, USA). The sections were observed using an Olympus BX50 light microscope and photographs were taken using a digital camera. Control sections
were treated as experimental sections, except that the primary antibody was replaced with rabbit non-immune serum.

2.3. Construction of transcriptional M. truncatula GS gene promoter-gusA fusions

Transcriptional fusions between 2.6 kbp of MtGSa and 3.1 kbp of MtGSb gene promoter fragments and the Escherichia coli gusA gene were constructed as described in [22]. The two gene fusions were cloned into the binary vector pBin19 [25] to obtain plasmids pBin-prMtGSa-GUS and pBin-prMtGSb-GUS.

2.4. Transformation of M. truncatula and recovery of transgenic plants

The binary vectors pBin-prMtGSa-GUS and pBin-prMtGSb-GUS were introduced into the Agrobacterium tumefaciens strain LBA4404 and used to transform leaf segments of M. truncatula H39. Kanamycin-resistant plants were regenerated by somatic embryogenesis as described by [26] and propagated in vitro.

2.5. Histochemical localisation of GUS activity

Histochemical staining for GUS activity was performed according to Jefferson et al. [27]. Whole plant fragments were fixed by vacuum infiltration for 5 min in an ice cold solution of 0.5% p-formaldehyde in 0.1 M phosphate buffer at pH 7.0, followed by incubation on ice for 1 h and two washes in phosphate buffer. The material was immersed in the GUS substrate solution containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucoronide cyclohexylammonium; Biosynth, Switzerland), 5 mM EDTA; 0.5 mM potassium ferrocyanide; 0.5 mM potassium ferricyanide and 0.1 M potassium phosphate buffer, pH 7.0. The immersed tissues were incubated in the dark at 37°C for 1–16 h, depending on the intensity of the coloration. Tissue sections (80–150 μm thick) were obtained in a vibratome (Leica VT 1000). Samples were mounted in water between a slide and a coverslip and observed by bright field or dark field microscopy using an Olympus BX50 light microscope. Whole tissues were photographed using an Olympus SZX9 stereomicroscope. Photographs were taken using a digital camera.

3. Results

3.1. Analysis of the expression driven by the 5’-upstream regions of MtGSa and MtGSb in transgenic M. truncatula plants

To analyse the expression directed by the MtGSa and MtGSb promoters and upstream regions, we have produced transgenic plants expressing the E. coli reporter gene gusA encoding β-glucuronidase, under the direction of each cytosolic GS promoter. The 2.6 kbp and 3.1 kbp of DNA lying immediately upstream of the translation initiation codon of MtGSa and MtGSb, respectively, were fused to gusA in a way to generate precise transcriptional fusions. These chimeric constructions were then introduced into M. truncatula by means of an Agrobacterium tumefaciens leaf-disc transformation protocol [26]. Eleven GUS-expressing plants were analysed for each and the GUS activity patterns were found to be qualitatively identical within a construct.

For the comparative analysis, special care was taken to collect the organs from different plants and transgenic lines at about the same stage of development. All the GUS assays were carried out in similar conditions and the incubation times were noted in order to obtain comparative data on the expression levels. To try to uncover different physiological and developmental stages, in this study we have chosen to analyse seedlings, mature and senescing plants of both MtGSa-gusA and MtGSb-gusA transformed plants.

Reporter gene expression driven by the MtGSa promoter was found to be mainly located in the vascular bundles of all the organs examined. In roots, the blue staining was located in the vascular cylinder throughout the whole root system (Fig. 1A). Generally the GUS staining was more intense at the site of emergence of a lateral root and was also present in growing root apices (Fig. 1A). Transversal and longitudinal sections through GUS stained roots revealed that the reporter gene expression was restricted to all the cell types internal to the endodermis, except the xylem and phloem vessels (Fig. 1B).

The MtGSa promoter was also found to direct GUS expression in the vascular elements of leaves, stems and petioles (Fig. 1C–G and I). Examination of a large number of these organs from different plants and transgenic lines revealed that
Fig. 1.
this vascular expression was stronger in senescent leaves (Fig. 1C) and cotyledons (Fig. 1I), two situations where translocation of nitrogen compounds are known to occur. The cellular localisation of reporter gene expression was visualised in transversal sections of petioles and stems (Fig. 1E-G). Such sections revealed the presence of the blue precipitate in phloem and xylem parenchyma cells (Fig. 1E-G) as exemplified by the magnification of the petiole vascular bundle (Fig. 1F). A weak GUS activity was also found in the starch sheath at the base of each vascular bundle (Fig. 1F). In flowers the MtGSa gene was expressed in the anther theca (Fig. 1H) and the pollen (not shown). GUS activity was found to be particularly intense in the stomatal guard cells, as evidenced in the epidermis of the cotyledons of germinating seedlings (Fig. 1J and K).

Histochemical staining of whole roots from plants carrying the MtGSb-gusA construct showed that this promoter is active throughout the whole root system (Fig. 2A). Similar to the MtGSa promoter, the expression was more intense at the site of emergence of the lateral roots and at the root tips (Fig. 2A). However, the reporter gene expression was not concentrated in the vascular cylinder as was the case of MtGSa-gusA transformed plants, but was also found in the root cortex and epidermis as can be clearly observed in a transverse section (Fig. 2B).

In leaves of MtGSb-gusA transformed plants a strong reporter gene activity was detected in the pulvinus of young, mature and senescing leaves (Fig. 2C–E). Staining of whole leaves revealed a strong expression of MtGSb throughout the leaf blade of both very young (Fig. 2D) and senescing leaves (Fig. 2C), and in the green cotyledons (Fig. 2J) whereas in mature leaves the expression was almost restricted to the pulvini (Fig. 2E). In a transverse section of a pulvinus it appears that the GUS staining is evenly distributed between flexor and extensor cells (Fig. 2F). In stems the MtGSb promoter appears to be less active than MtGSa, however, a weak blue staining was detected in the starch sheath that exists in the base of each vascular trace and the angular colenchyma of the stem (Fig. 2G). In the epidermal cells the reporter gene activity was quite strong with lower levels of GUS activity in the stomatal guard cells (Fig. 2H) in contrast to the expression of MtGSa. An intense blue staining was also found in the glandular trichomes (Fig. 2I). In flowers the MtGSb promoter expressed the reporter gene in the anthers and filaments of the stamen and in the pollen grains (Fig. 2K).

### 3.2. Immunocytochemical localisation of GS polypeptides

To correlate gene expression with GS protein, we have performed an immunolocalisation of GS in roots, leaves, stems and petioles. For all the immunolocalisation work we used an anti-GS antibody raised against a cytosolic form of GS from Phaseolus vulgaris [24], which recognises all three forms of GS in M. truncatula GsAs, Gsb and the plastidial Gsd [7]. Control sections were treated as experimental sections except that primary antibody was replaced with rabbit non-immune serum. In roots GS was found to be present in the vascular cylinder, the epidermis and underlying cortical cells, with weaker staining observed in the main cortex (Fig. 3B). Within the vascular cylinder, cytosolic GS was found to be distributed in all cell types except for the xylem and phloem vessels (Fig. 3C).

Leaf sections, which have been treated with anti-GS antibody, showed that GS is present in the cytosol and the chloroplasts. An overall view of a leaf section showed immunostaining clearly...
Fig. 2.
associated with the chloroplasts of leaf mesophyll cells, while in the vascular bundles the labelling was present in the cytosol of phloem cells and also in some xylem parenchyma cells in both the central bundle (Fig. 3E) and in lateral vascular bundles (Fig. 3F and G). Immunostaining was also evident in the stomatal guard cells (Fig. 3H). As differences were detected in expression of the cytosolic GS genes during leaf development, we have compared the protein distribution in mature and senescing leaves. In the mesophyll of senescing leaves, the immunostaining in the chloroplasts was found to be dramatically reduced when compared with a mature leaf (Fig. 3F and G), whereas the staining in the cytosol appears to be stronger in senescing leaves. In Fig. 3F and G, the abundance of cytosolic GS in phloem companion cells of a lateral vascular bundle is also evident.

In petioles (Fig. 3I–K) and stems (Fig. 3L–N), cytosolic GS was particularly abundant in phloem and xylem parenchyma cells. In stems, a weak staining was also detected in the starch sheath located at the base of the vascular bundles (Fig. 3L and N). GS was also evident in the plastids particularly abundant in the more external cortical layers of the petiole (Fig. 3I and K).

4. Discussion

The existence of cytosolic GS gene families raises the question of their functional significance. Distinct cytosolic GS genes appear to be differentially expressed in several plant species, which suggests that different cytosolic isoforms perform diverse physiological functions. However, the nature of these functions is still not clear, particularly in relation to different cytosolic forms within a single plant. As *M. truncatula* contains only two expressed cytosolic GS genes, and is transformable, we have chosen this model plant to investigate the cell-specific expression of the cytosolic GS genes in different organs using promoter-GUS fusions transformed into the homologous plant. The complexity of GS gene expression using heterologous hosts has clearly been pointed out for the soybean GS21 gene, where the gene was found to behave differently in soybean compared to three heterologous hosts, *Lotus corniculatus*, *Nicotiana tabacum*, and alfalfa [21,28,29].

The expression of the different members of the cytosolic GS gene family within the different organs of *M. truncatula* has earlier been investigated by northern blot analysis, using gene specific probes [8]. However, northern blot analysis is limited by the fact that gene expression can only be monitored at the organ level which might not evaluate differences between cell types comprising a small proportion of the total organ cell population but involved in specific aspects of nitrogen metabolism. In the present study, the promoter fragments of *MtGSa* and *MtGSb* were found to confer a differential and cell/tissue specific expression in most of the organs analysed. In roots, leaves, stems and petioles this expression was found to be largely non-overlapping strongly suggesting that the encoded enzymes perform distinct physiological functions in these organs.

In roots, earlier studies using promoter-GUS fusions has shown that the soybean ammonium-induced GS15 gene and the pea GS3A gene are expressed in the root vascular system and root tips of transgenic heterologous plants [21,28–31]. The only report considering localisation of different members of the cytosolic GS gene family in different tissues was performed in maize by measuring the levels of GS transcripts in dissected root tissues: some of the genes were preferentially expressed in the root cortex and others in the vascular cylinder and four out of five of the genes were also expressed in the root tips [6]. In this study we have shown that the *MtGSb* gene, which
is expressed about 10-fold higher than \textit{MtGSa} in roots [8], showed expression throughout the whole root system (the cortex, vascular cylinder and also in the tips) whereas the expression of \textit{MtGSa} is restricted to the root vascular cylinder and growing apices. The high level of GS in root apices of all the plants studied is probably required to maintain the high metabolic activity of the tissues. The localisation of \textit{MtGSa} expression in the vascular cylinder of the root is consistent with the putative
Fig. 3. Immunocytochemical localisation of GS polypeptides in sections of M. truncatula roots (A–C), mature leaves (D, E, F, H), senescing leaves (G) petioles (I–K) and stems (L–N). Sections were incubated with anti-GS antibodies and detected with peroxidase conjugated anti-rabbit IgG. Control sections (A, D, J, M) were treated as experimental sections except that primary antibody was replaced with rabbit non-immune serum. (A) Control root section. (B) Transversal section through a root. (C) Magnification of B, showing GS distribution in the vascular cylinder. (D) Control leaf section. (E) Leaf cross section showing the central vein. (F) Mature leaf blade cross section showing the abundance of plastidial GS in mesophyll chloroplasts and cytosolic GS in phloem companion cells (*) of the lateral veins (boxed). (G) Senescent leaf blade cross section, showing abundant cytosolic GS in mesophyll cells and companion cells (*) of a lateral vein (boxed). (H) Detail of E showing immunolabelling in stomatal guard cells (SGC). (I) Petiole cross section. (J) Control petiole section. (K) Magnification of one vascular bundle shown in J. (L) Stem cross section. (M) Stem control section. (N) Magnification of one vascular bundle shown in L. CP, central pith; P, phloem; SGC, stomatal guard cells, SS, starch sheath, XP, xylem parenchyma, XV, xylem vessels. Bars (A–E, I–N) = 100 μm. Bars (F–H) = 10 μm.

function of the encoded isoenzymes in supplying glutamine for transport to the aerial parts of the plant whereas the function of GSb may be in assimilating nitrogen, taken up directly from the soil.

In leaves, the MtGSa promoter was found to drive GUS expression in the vasculature whereas MtGSb was strongly expressed in the pulvini and also in the leaf blade at certain developmental stages. Both the genes were expressed in stomatal guard cells and in trichomes. Immunolocalisation confirmed these localisations and showed that in mesophyll cells the chloroplast GS is abundantly present.

In common with work on leaves from other species [30,31], the tissue-specific expression of GS in M. truncatula varied depending on the developmental stage of the organ. In mesophyll cells MtGSb was more highly expressed in young leaves than in mature ones where the chloroplast GS predominates. Major changes were seen in the expression of the three GS forms during senescence, as the nitrogen metabolism changes from assimilation to re-mobilisation. During this process chloroplast and other proteins are degraded, and the nitrogen from this source is transported to sinks within the plant. Several other studies have reported an increase in cytosolic GS during senescence [13,32,33] and in rice leaves this increase was found to be associated with the vascular elements and accompanied by a reduction in the plastid GS [32–34]. From the results presented here it appears that the expression of both cytosolic GS forms increase during senescence of M. truncatula leaves but in different tissues: GSa in vascular elements and GSb in mesophyll cells, whereas the abundance of the chloroplast GS decreases. These results suggest that during leaf senescence GSb is responsible for the re-assimilation of ammonia released from protein catabolism in mesophyll cells whereas GSa might be involved in the production of nitrogen compounds for long distance transport.

The idea that vascular GS is involved in transport of nitrogenous compounds is supported by the localisation of cytosolic GS in specialised cells with specific functions in the transport of solutes. Cytosolic GS is abundantly present in phloem companion cells of Nicotiana tabacum [14,35], Solanum tuberosum [36] and rice [34] and as shown here, is localised in the phloem and xylem parenchyma of M. truncatula. Companion cells are believed to provide metabolic support for phloem loading and are interconnected by plasmodesmata with the vascular parenchyma cells. Many xylem and phloem parenchyma cells are considered as transfer cells and are thought to be involved in collecting and passing solutes produced in nearby mesophyll cells [37]. The observation that the expression of MtGSa in these specialised cells was stronger in situations where mobilisation of nitrogen is taking place, such as senescing leaves, young leaves and germinating cotyledons, provides further support for its involvement in long-distance transport. It is noteworthy that the pea orthologue of MtGSa was also found to be highly expressed in the phloem tissues of cotyledons in germinating seedlings [31].

Some other interesting features of the expression were revealed by the transgenic studies presented here. MtGSb was found to be expressed in collenchyma cells and both MtGSa and MtGSb are expressed, but less strongly, in the starch sheath at the base of the vascular bundles. The specific expression of GS in collenchyma cells and in the starch sheath is quite surprising and suggests that these cells play some unknown role in nitrogen metabolism.
Strong expression of cytosolic GS was detected in two cell-types and tissues subject to turgor changes. Both the genes, but particularly MtGSa, are expressed in stomatal guard cells, where cytosolic GS protein was also detected. Cytosolic GS was also found in rice leaf stomatal guard cells [32]. The expression of MtGSb in the pulvinus, another rhythmic, turgor-mediated movement system, supports an involvement of cytosolic GS in osmotic regulation. Pulvini are structures capable of bringing about movements of leaves or leaflets through the expansion and contraction of their dorsal, extensor and ventral, flexor cells [38]. Although we did not detect any significant difference in GUS expression between the two types of cells in M. truncatula, a soybean GS15-GUS gene construct was found to be more highly expressed in the flexor than the extensor cells of transgenic Lotus corniculatus, but no relationship between GS expression and the rhythmic movements of the pulvinus was observed [21,39]. The physiological explanation for the high expression of GS in pulvini could be that cytosolic GS activity is needed in all plant cells having a reduced number of plastids and a high metabolic activity, as suggested by Marsolier et al. [39], although alternatively GS may have some special physiological role in pulvini perhaps associated with the metabolism of stored reserves during leaf movement (see [38]).

MtGSa and MtGSb were found to be highly expressed in the glandular trichomes, as shown previously for cytosolic GS in N. tabacum [14]. Glandular trichomes are known to be involved in the secretion of various substances including organic solutes [38] and it is possible that the role of GS in trichomes might be related to the synthesis of such secreted substances.

In flowers of M. truncatula, both cytosolic GS genes were found to be expressed at similar levels, consistently with the results obtained by northern analysis [8]. Both genes were expressed in anther theca and pollen grains at a late stage of flower development, when anthers and pollen were already fully differentiated. Expression of cytosolic GS in anther theca and pollen grains has previously been reported for the soybean GS15 gene [39] and two cytosolic GS genes in tobacco [14]. Flowers are sinks for nitrogen compounds and the enzyme may play a central role during flower development in providing glutamine for reproductive organ metabolism.

In conclusion, these studies have shown that the putative MtGSa and MtGSb promoter fragments were sufficient to drive GUS expression to almost all the tissues and cell types where GS proteins were located. These results suggest firstly that the unique expression patterns of the MtGSa and MtGSb genes are largely due to transcriptional regulation and secondly that the cis-acting regulatory elements responsible for conferring these contrasting expression patterns in most of the organs are located within the approximate 3 kbp region upstream of the coding sequences. A comparison between MtGSa and MtGSb sequences showed a relatively high conservation between coding sequences (82%) but low homology in 5′ and 3′ non-coding sequences. This 5′ sequence divergence, together with the differential patterns of expression may reflect the acquisition or evolution by each gene of distinct cis-acting elements that confer responsiveness to different regulatory signals. In this respect it is noteworthy that the two genes, like their orthologues in pea [40] are located on different chromosomes (T. Huguet, pers. commun.). In spite of the high homology between the two polypeptides (89% identity), slightly different kinetic properties were found between the isoenzymes composed of identical or non-identical subunits and studies of complementation in E. coli suggest that these differences may be of physiological significance [7]. Further work is required to establish whether the differences detected in the kinetic properties are sufficient to make certain enzymes more suited to their metabolic roles or whether the differential regulation of the two genes is more important in allowing a flexibility of regulation at the transcriptional level.

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