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Glutamine synthetase in higher plants: molecular biology meets plant physiology

Glutamine synthetase (GS; EC 6.3.1.2) is a key enzyme of plant N metabolism, occurring in most or all plant tissues and contributing to a wide variety of physiological processes. This chapter looks at some recent developments in the molecular biology of the enzyme, and how these are leading to new insights into the participation of individual isoenzymes in different metabolic pathways.

Glutamine synthetase catalyses the incorporation of ammonium into glutamine, using glutamate as substrate. In higher plants it is the major enzyme responsible for the assimilation of ammonium (Miflin & Lea, 1980), acting in conjunction with glutamate synthase (GOGAT) to synthesize both glutamine and glutamate, the precursors of all other organic nitrogenous compounds in the plant. Not only is GS involved in primary N assimilation (using ammonium absorbed from the soil or generated by either NO_3^- reduction or symbiotic N fixation), but also in the reassimilation of the N released endogenously by a variety of ammonium-evolving processes, such as photorespiration, phenylpropanoid metabolism and the transamination of amino acids (Fig. 1; for review see Joy, 1988).

The complexity of the role that GS plays in plant N metabolism is compounded by the spatial and temporal diversity of the pathways of primary and secondary N assimilation. Some ammonium-generating processes (for example, N fixation in legumes) are restricted to a single cell type (infected cells of root or stem nodules). Others, such as photorespiration, are temporally regulated or, in the case of NO_3^- or ammonium assimilation in roots, are subject to unpredictable fluctuations in the external N source.

Given the above considerations, it is perhaps not surprising that molecular studies are revealing a complex system for regulating the biosynthesis of GS in higher plants. It now appears that, in most plant species, GS is encoded by a multigene family consisting of a minimum of four functional genes which encode one plastidic and at least three

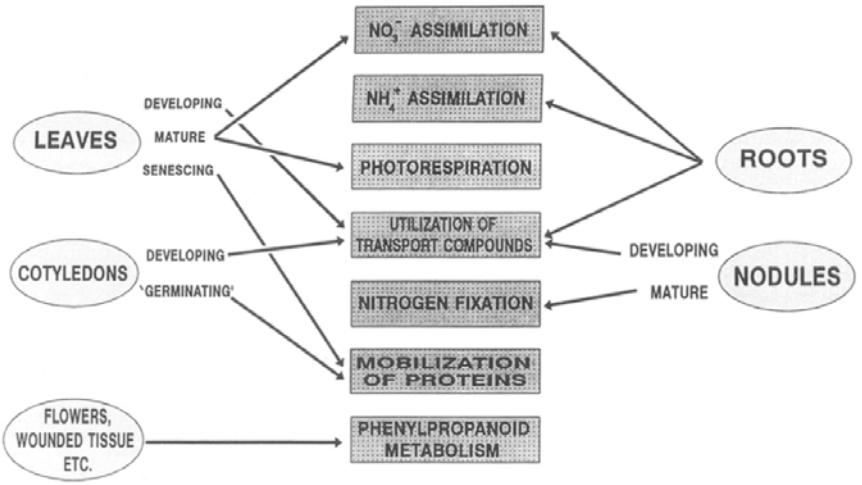

Fig. 1. Metabolic pathways involving GS in higher plants and their occurrence in different parts of the plant at different stages of development.

cytosolic GS polypeptides. This holds true in pea (Tingey, Walker & Coruzzi, 1987; Tingey *et al.*, 1988), French bean (Cullimore *et al.*, 1984; Gebhardt *et al.*, 1986; Lightfoot, Green & Cullimore, 1988), soybean (Miao *et al.*, 1991; Roche, Temple & Sengupta-Gopalan, 1993), Arabidopsis (Peterman & Goodman, 1991) and maize (Sakakibara *et al.*, 1992*b*; Li *et al.*, 1993). A possible exception is mustard, which may only have a single (plastidic) GS gene (Höpfner, Ochs & Wild, 1991). Initial studies indicated that, apart from the induction of the chloroplast enzyme by light (Lightfoot *et al.*, 1988; Tingey *et al.*, 1988), expression of individual GS genes was primarily under developmental control, with each organ displaying a different complement of GS mRNAs, polypeptides and isoenzymes (Forde & Cullimore, 1989). Since 1989, however, it has become evident that this is an over-simplification. In this review we will examine recent developments relating to the spatial distribution of GS isoenzymes in plant organs and the nutritional regulation of GS gene expression. In the final section we will discuss the prospects for genetic manipulation of GS in higher plants and review current progress in this area.

Spatial distribution of GS isoenzymes in plant tissues

Root nodules

When the promoters of two cytosolic GS genes from French bean (*gln*-β and *gln*-γ) were fused to the GUS reporter gene (*uidA*) and

introduced into *Lotus corniculatus*, it was possible to use a histochemical GUS assay to compare the spatial expression patterns of the two genes (Forde *et al.*, 1989). These studies confirmed that the nodule-enhanced expression of *gln*-γ and the root-enhanced expression of *gln*-β, which had been established from mRNA studies in French bean (Gebhardt *et al.*, 1986; Bennett, Lightfoot & Cullimore, 1989), are conferred by the transcriptional properties of their promoters. However, more significantly, the histochemical analysis showed that although both genes are expressed in nodules, they display quite different spatial patterns of expression. Thus *gln*-γ expression was localized to the central infected zone of the nodule and was strongest in the infected cells themselves. Expression of *gln*-β, on the other hand, occurred throughout the nodule in the early stages of nodulation, but in mature nodules its expression became restricted to the vascular strands.

Subsequent cytological studies using *in situ* hybridization and gene-specific probes (Teverson, 1990), looked at the distribution of GS mRNAs in French bean nodules at different stages of development. Confirming the results obtained with transgenic *Lotus*, it was found that the *gln*-γ mRNA was abundant in infected cells, but significant amounts could also be detected in the non-infected cells of the central zone and in the inner cortex. The *gln*-β mRNA was present throughout the nodule in the early stages of nodulation and its abundance rapidly declined with nodule maturity, particularly in the infected cells. In the mature and late nodule, *gln*-β mRNA was mainly confined to the cortex (inner, mid- and outer) and particularly to the vascular endodermis. Consistent with the distribution of the mRNAs, Chen & Cullimore (1989) found when they dissected French bean nodules, that GS in the cortex was mainly composed of the β subunit, while in the central tissue the γ subunit predominated.

The spatial separation of the two GS subunits within the nodule (although not complete) explains why there are two distinct cytosolic isoenzymes in French bean nodules, one (GS_{n1}) nodule-specific and composed of γ with some β, and the other (GS_{n2}) closely related to the root form and almost entirely a homooctamer of β (Lara *et al.*, 1984; Bennett & Cullimore, 1989). Thus GS_{n1} and GS_{n2}, which differ in their kinetic properties and their ratios of transferase to synthetase activities (Cullimore *et al.*, 1983), are also located in different regions of the nodule.

What does the spatial distribution of GS_{n1} and GS_{n2} tell us about their likely metabolic roles? The localization of the nodule-specific GS_{n1} isoenzyme in the central infected zone of the nodule is consistent with it having the major role in the assimilation of the ammonium released by the N-fixing bacteroids. However, the role of GS_{n2} is less clear.

One possibility is that it too is involved in primary ammonium assimilation, despite its physical separation from the infected cells. This could happen if excess ammonium, unassimilated by GS_{n1}, diffuses into the cortical region. Perhaps more likely, given its association with the nodule's vascular system, is that GS_{n2} has a different role that is in some way related to transport of nitrogenous compounds. If it is not assimilating the products of primary N assimilation, it must be assimilating ammonium released from other N-containing compounds. Possibilities include the deamination of amino acids or the catabolism of ureides. In alfalfa and *L. corniculatus*, ^{14}C-labelling studies have found that while little radiolabelled glutamine was present in nodules, glutamine was the major labelled amino acid in root extracts and was the third most abundant amino acid in xylem sap (Maxwell *et al.*, 1984). These observations suggest that there is significant recycling of nitrogenous compounds during their movement through the xylem to the shoot, involving lateral abstraction of N solutes from the xylem sap and reloading of the xylem with glutamine and other amino acids. Although the function of this recycling is unclear, it is possible that it begins even in the nodule vascular system and that the vascular-associated GS_{n2} could play a key role in the process.

An alternative source of amino acids for recycling through GS_{n2} is the phloem sap supplying root nodules. Although the primary function of phloem transport to the nodule is to provide carbon for growth, respiration and amino acid biosynthesis, the phloem sap also contains significant amounts of N (Pate *et al.*, 1979; Layzell *et al.*, 1981; Jeschke, Atkins & Pate, 1984). For white lupin and soybean, it has been estimated that as much as 15% of the N exported through the xylem to the shoot is returned to the nodule via the phloem (Walsh, 1990; Parsons *et al.*, 1993). The returning amino acids and amides may well undergo some transformations in the nodule while in transit between phloem and xylem, and again this is likely to require GS activity in the vicinity of the vascular tissue.

Parsons *et al.* (1993) have proposed a model for the regulation of nodule growth and activity in which the concentration of reduced N compounds flowing into the nodule from the phloem is used as a signal indicating the nitrogen status of the shoot. It is suggested that changes in phloem N content, in rates of C and oxygen supply, or in amide or ureide accumulation, are integrated into an effect on the concentration of a key compound (such as asparagine or glutamine) in the nodule cortex. In the model, the concentration of this key compound then regulates the short-term and long-term oxygen diffusion barriers. It may also have a feedback effect on N fixation and N assimilation.

Such a model may explain the unexpected finding that growth and N fixation are markedly stimulated in alfalfa plants in which root GS and the root form of GS in nodules (equivalent to GS_{n2}) are inhibited by a bacterial toxin (tabtoxin), that has no effect on the nodule-specific isoform (Knight & Langston-Unkefer, 1988). If, as in French bean, the root isoform of GS in nodules is associated with the vascular tissue, its inhibition would be expected to have a severe effect on N cycling, perhaps reducing the concentration of the key N compound and signalling a low N status in the shoot.

Other plant organs

The GUS reporter gene was also used to compare the expression patterns of the pea plastidic (GS2) and cytosolic (GS3A) GS genes in transgenic tobacco plants (Edwards, Walker & Coruzzi, 1990). It was found that the GS2 promoter conferred light-regulated expression on the GUS gene in photosynthetic cells of leaves, stems and cotyledons. In contrast, the GS3A promoter directed expression specifically in the phloem of leaves, stems and roots of mature plants, and vascular-specificity was also seen in the cotyledons, hypocotyl and roots of germinating seedlings. Similar results were obtained for the GS3A promoter in transgenic alfalfa plants (Brears, Walker & Coruzzi, 1991).

The GS_2 promoter from French bean has been shown to direct expression in palisade and mesophyll parenchyma of transgenic tobacco leaves, and, at much lower levels, in the leaf epidermis, but not in the pith parenchyma or the vascular tissue (Cock, Hémon & Cullimore, 1992). Three recent studies using immunocytological methods have confirmed the vascular localization of GS_1 and the mesophyll localization of GS_2 in leaves of rice (Kamachi *et al.*, 1992), potato (Pereira *et al.*, 1992) and tobacco (Carvalho *et al.*, 1992). In potato and tobacco the cytosolic GS protein was confined to the phloem companion cells.

There is therefore an accumulating body of evidence suggesting that the cytosolic and plastidic GS isoenzymes are spatially separated in the leaves of many plant species. However, these findings are at variance with earlier results obtained with protoplasts isolated from leaves of barley and pea, which indicated that a significant proportion of GS activity in mesophyll cells (from 15 to 50%) is in the cytosolic fraction (Wallsgrove, Lea & Miflin, 1979; Wallsgrove *et al.*, 1980). This discrepancy will need to be resolved by further experiments.

The physiological implications of a differential distribution of GS isoenzymes within the leaf were discussed by Edwards *et al.* (1990). These authors concluded that the spatial separation of GS_1 and GS_2

indicated they had non-overlapping roles: GS_2 being primarily involved in assimilating ammonium released in photosynthetic cells by photorespiration and nitrate/nitrite reduction, and GS_1 in the phloem companion cells having a role in intercellular glutamine transport.

Regulation of GS by N nutrition

A number of recent studies have addressed the question of whether GS expression in plants is regulated by either ammonium or NO_3^-.

Ammonium

The ability of externally applied ammonium to induce the expression of a soybean GS gene in roots has been demonstrated (Hirel *et al.*, 1987; Miao *et al.*, 1991). The promoter of the soybean GS15 gene, which encodes a cytosolic GS polypeptide in roots and nodules, conferred ammonium-inducible expression on the GUS gene in transgenic *L. corniculatus* plants: expression was increased more than three-fold by 10 mM ammonium sulphate, while application of KNO_3, asparagine or glutamine had no significant effect (Miao *et al.*, 1991). Earlier studies had shown that the ammonium effect could be seen in soybean roots at the mRNA level within 2 h of treatment (Hirel *et al.*, 1987). Progressive 5′ deletion of the GS15 promoter located the ammonium-responsive elements to the region between 3.5 and 1.3 kb upstream of the transcription start site (Marsolier, Carrayol & Hirel, 1993).

However it is clear that not all GS genes are ammonium inducible. There are many instances in the literature where ammonium treatments had little or no effect on GS activity (for example, Mann, Fentem & Stewart, 1980; Loyola-Vargas & Sánchéz de Jiménez, 1986; Vézina & Langlois, 1989; Cock *et al.*, 1990; Shen, 1991). In both pea and French bean, when photorespiratory ammonium production was suppressed by growth at increased CO_2 concentrations, there was a long-term effect that resulted in reduced levels of GS_2 mRNA (but not GS_1 mRNA) in leaves (Edwards & Coruzzi, 1989; Cock *et al.*, 1991). However, as this effect was not seen in short-term experiments (Cock *et al.*, 1991), it is likely to be due to an indirect effect on leaf metabolite concentrations that develops on long-term exposure to high CO_2, rather than to a direct effect of the absence of photorespiratory ammonium production.

There can be numerous reasons for the conflicting evidence concerning the ammonium inducibility of GS genes in plants. One of these is certainly that not all GS genes possess the ammonium-responsive *cis*-acting elements found in the soybean GS15 gene. However there are other important factors which relate to the experimental protocols that

are used to assess ammonium inducibility. The first of these arises from the use of alternative assay methods to monitor GS induction, that is mRNA, protein or enzyme activity. For example, in French bean, Hoelzle *et al.* (1992) found that a 28 h ammonium treatment led to a five-fold induction of root GS_1 activity, but in agreement with the mRNA studies of Cock *et al.* (1990) this occurred without any increase in GS_1 protein. These results emphasize the importance of following gene expression at all levels, since it seems that changes in GS activity can occur without changes in GS protein or mRNA, and it is very likely that the converse is also true.

The second important consideration is our lack of knowledge about the nature of the plant's response to ammonium treatment. There are many factors (genetic, physiological, developmental and environmental) that will determine how adding ammonium will affect the cellular ammonium concentration and the distribution of ammonium between the vacuole and the cytosol (Lee & Ratcliffe, 1991). Because the cytosolic fraction makes only a small contribution to the cellular ammonium concentration, measurements of tissue ammonium content really reflect the vacuolar concentration (Lee & Ratcliffe, 1991). The rate of assimilation of ammonium by GS has been shown to be an important factor in determining the extent to which ammonium accumulates in the cytoplasm (Lee & Ratcliffe, 1991), so that cells with a low basal level of GS activity are more likely than those with high basal levels of GS activity to experience a surge in cytoplasmic ammonium after ammonium is added to the external medium. We cannot even be certain that it is ammonium itself that is directly responsible for the induction of GS genes when this occurs: it is quite possible that the response is to fluctuations in other metabolites that are affected by the ammonium treatment. Thus, when the GS genes in a particular species or tissue are found not to be inducible by ammonium, this does not necessarily imply the lack of 'ammonium-responsive' *cis*-acting elements, but may reflect the nature of the physiological response to ammonium in that species/tissue. This conclusion is supported by the finding that the ammonium-inducible soybean GS15 promoter is unresponsive to ammonium treatment in transgenic tobacco, even though the same construct functions as expected in *L. corniculatus* (Miao *et al.*, 1991).

Nitrate

Nitrate is well known to be able to elicit the rapid induction (<2 h) of nitrate reductase (NR) and nitrite reductase (NiR), a response that is regulated at the transcriptional level (Pelsy & Caboche, 1992). The

first report that GS activity was also subject to NO_3^- regulation came from pea roots, where a 2.5-fold increase in plastidic GS activity was observed after 8 h (Emes & Fowler, 1983). Subsequently it was shown that this induction was specific to the plastidic form of the enzyme, occurred at the protein level and was specific to NO_3^- (Vézina & Langlois, 1989). Similar results have been obtained with *L. corniculatus*, as shown in Fig. 2. A NO_3^--specific induction of GS_2 mRNA and protein in maize roots has also been reported (Sakakibara *et al.*, 1992*a*; Redinbaugh & Campbell, 1993) and has been shown to involve an

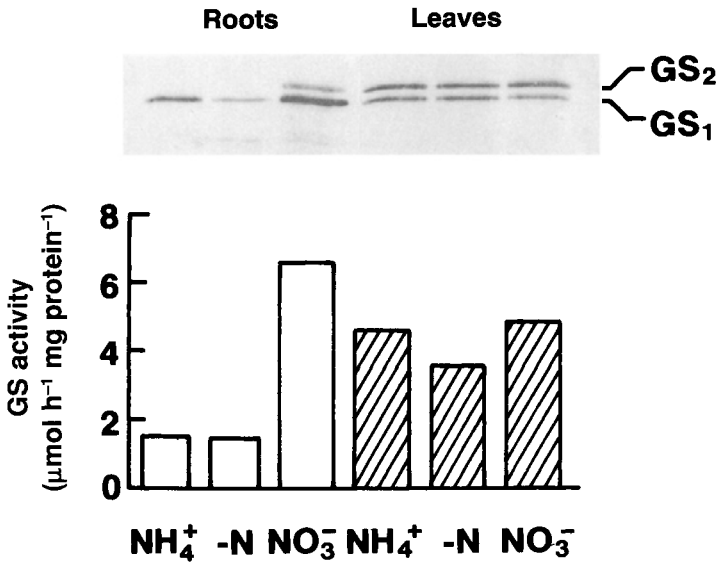

Fig. 2. Nitrate-inducibility of GS_2 in roots of *L. corniculatus*. Plants were grown under sterile conditions for 47 d in culture on N-free medium or medium containing ammonium (5 mM) or NO_3^- (5 mM) as indicated. GS activity was assayed by the semi-biosynthetic method and extracts of roots or leaves containing 2 μg soluble protein were electrophoresed and western blotted. GS protein was detected using rabbit antiserum raised against nodule GS from French bean (Cullimore & Miflin, 1984) and an anti-rabbit ExtrAvidin-Peroxidase kit (Sigma Chemical Co. Ltd).

accumulation of the GS_2 mRNA and to be accompanied by an induction of mRNA for ferredoxin-dependent glutamate synthase (Fd-GOGAT) (Redinbaugh & Campbell, 1993). Two proteins that provide reducing power for nitrite reduction and Fd-GOGAT activity, ferredoxin-$NADP^+$ oxidoreductase and ferredoxin, are also induced by NO_3^- in pea root plastids (Bowsher, Hucklesby & Emes, 1993).

Analysis of the kinetics of mRNA induction in maize roots showed that induction of the GS_2 gene occurs coordinately (within 30 min of treatment) with that of the NR gene and that, like the induction of NR and NiR, it is insensitive to cycloheximide and is thus part of the root's primary response to environmental NO_3^- (Redinbaugh & Campbell, 1991, 1993). In contrast, in maize leaves, the GS_2 and Fd-GOGAT genes are expressed constitutively and are not subject to NO_3^- inducibility (Redinbaugh & Campbell, 1993).

These observations imply a specific role for GS_2 in some species in the assimilation of ammonium generated endogenously by the NO_3^- assimilatory pathway in roots, but not in the assimilation of ammonium taken up directly from the soil. The latter function must be adequately covered by the cytosolic isoenzyme(s). A corollary of this hypothesis is that there should be a distinction between species that assimilate NO_3^- in their roots and those that are shoot assimilators in the occurrence of plastidic GS in their roots. It has been proposed that, in general, temperate legume species growing in low external NO_3^- concentrations are primarily root assimilators, while tropical and sub-tropical species are primarily shoot assimilators (Andrews, 1986*a*).

In a survey of 43 species of the Papilionoideae (grown in 1 mM NO_3^-; J.W. and B.G.F, unpublished observations), a GS_2-like polypeptide was found in the roots of each of 30 temperate species (representing 26 genera and encompassing 15 tribes), but was undetectable in the roots of any of 17 tropical or sub-tropical species (14 genera and 6 tribes) (Fig. 3). This strong correlation amongst such a phylogenetically diverse group of legumes suggests that, in the papilionoid legumes at least, the possession of a root plastidic GS isoenzyme is part of some form of adaptation to a temperate climate. Since many of the temperate legume species are primarily root assimilators of NO_3^-, it is likely that this isoform of GS does play a role in the NO_3^- assimilatory pathway. However, based on *in vivo* and *in vitro* NR assays, many of the temperate legumes resembled the tropical and sub-tropical legumes in having more than 50% of their NR activity in the shoot. Although NR assays are not a definitive method for determining the proportion of nitrate reduced in the root (Andrews, 1986*b*), it is possible that the occurrence of plastidic GS in the root is a more distinctive feature of temperate legumes than is root NO_3^- assimilation.

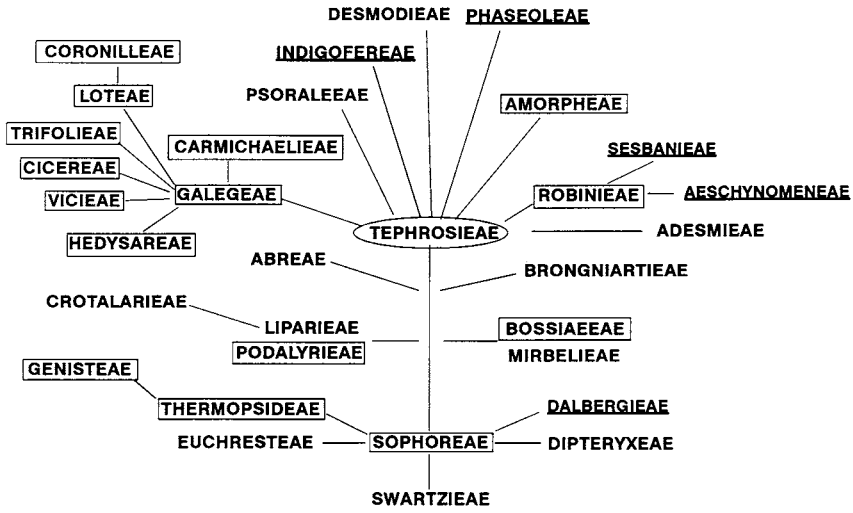

Fig. 3. Distribution amongst the tribes of the Papilionoideae of a GS_2-like polypeptide in roots. The phylogenetic tree is taken from Corby, Polhill & Sprent (1983). Boxes indicate those tribes in which all species tested by western blotting (see Fig. 2) have a GS_2-like polypeptide in the roots when grown on NO_3^-. Tribes in which no species with a root GS_2 have been found are underlined. In all cases, temperate species had a GS_2-like polypeptide in their roots, while tropical and sub-tropical species did not. The Tephrosieae (circled) include both temperate and tropical species, and when one of each type was tested the temperate species (*Wisteria floribunda)* had a GS_2 polypeptide, while the tropical species (*Tephrosia vogelii*) did not.

Genetic manipulation of glutamine synthetase

The key role that GS plays in plant N metabolism, the multiplicity of GS isoenzymes and the diversity of pathways in which they are potentially involved, combine to make GS a particularly attractive target for genetic manipulation. The identification of GS_2-deficient mutants of barley was important in establishing the role of this isoenzyme in the photorespiratory nitrogen cycle (Blackwell, Murray & Lea, 1987; Wallsgrove *et al.*, 1987), but selection methods that would allow the isolation of other types of GS mutant have not been devised. The availability of additional mutants that are deficient in a specific GS isoenzyme, or that have a particular organ or cell type that is deficient in that isoform, would be invaluable in establishing the role(s) that