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Mini Review

Glutamine Synthetase in Higher Plants: Regulation of Gene and Protein Expression from the Organ to the Cell

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Compared to other enzymatic systems, the regulation of *GS* isoenzyme expression shows a unique diversity. Considering that *GS* is one of the oldest existing and functioning genes found in all extant life forms, we can hypothesise that the evolution of metabolic pathways from primitive pre-procaryotes to lower and then higher plants might have gradually refined the function of GS to provide reduced nitrogen forms for the rest of the metabolism (Kumada et al. 1993). This refinement might explain the genetic and biological diversity encountered in the various modes of expression and regulation of higher plant GS isoenzymes both at the cellular and intracellular levels (Fig. 1). Although model plants are valuable sources of information helping to decipher fine regulatory control mechanisms (Lam et al. 1996), the study of this genetic diversity appears to be one of the most promising areas of research, necessary to better understand ammonia assimilation in plants and more generally improve nitrogen use efficiency.

Key words: Ammonium assimilation — Cytosolic glutamine synthetase — Organ-specific expression — Plastidic glutamine synthetase.

In the last two decades, an increasing number of studies have been undertaken on the regulation of inorganic nitrogen uptake and its subsequent incorporation into organic molecules. The two main reasons for this interest were because the regulation of nitrogen assimilation and management during plant development is an exciting area for fundamental research both at the molecular and whole plant level, and for socio-economic reasons: many crops grown for protein content and yield require large quantities of nitrogenous fertilisers to attain their maximal yields. Although adding fertilisers generally results in enhanced yield, the efficiency of the uptake decreases with the level of fertilisation. Therefore, the resulting yield increases the amount of unused fertilisers, leading to an environmental hazard (pollution, nitrate accumulation in leafy vegetables and forages) and an economic loss.

In higher plants, recent advances in plant molecular biotechnology combined with modern physiological and biochemical studies have expanded our understanding of the regulatory mechanisms controlling primary steps of inorganic nitrogen assimilation and the subsequent biochemical pathways involved in nitrogen supply for higher plant metabolism. Nitrate is the principal nitrogen source for most crops. Following its uptake by means of specific transporters located in the root cell membrane (Crawford and Glass 1998), the assimilation of nitrate is a two-step process. First, the enzyme nitrate reductase (NR, EC 1.6.6.1) catalyses the reduction of nitrate to nitrite. Sub-

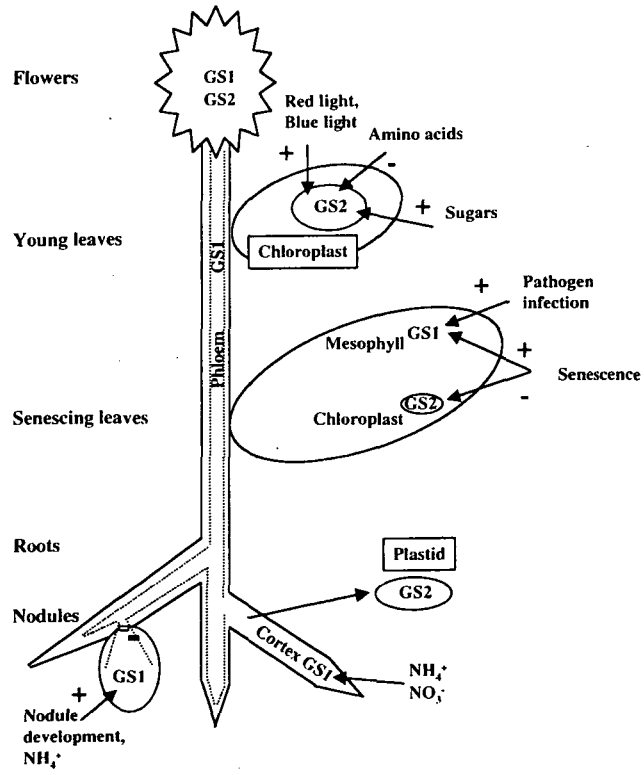

Fig. 1 Schematic representation depicting higher plant GS expression from the organ to the cell. + indicates a positive control and − a negative control. GS1, cytosolic glutamine synthetase; GS2, plastidic glutamine synthetase.

sequently, the enzyme nitrite reductase (NiR, EC 1.7.7.1) mediates the reduction of nitrite to ammonium. In addition to nitrate reduction, ammonium can be generated inside the plant by a variety of metabolic pathways such as photorespiration, phenylpropanoid metabolism, utilisation of nitrogen transport compounds, amino acid catabolism and symbiotic nitrogen fixation (Joy 1988).

Ammonia is then incorporated into an organic molecule by the enzyme glutamine synthetase (GS, EC 6.3.1.2). GS catalyses the ATP-dependent conversion of glutamate to glutamine utilising ammonia as a substrate. This reaction is now considered to be the major route facilitating the incorporation of inorganic nitrogen into organic molecules. GS functions together with ferredoxin-dependent glutamate synthase (GOGAT, EC 1.4.7.1), an enzyme which recycles glutamate and incorporates carbon skeletons into the cycle for the transfer of amino groups to keto-acids or other amino acids utilised for protein formation. Amino groups are also transferred to nucleotides used as basic molecules for RNA and DNA synthesis (Miflin and Lea 1980).

In the early seventies, GS activity was shown to be present in roots and photosynthetic organs in a limited number of higher plant species (Kanamori and Matsumoto 1972, O'Neal and Joy 1974). Later on, it was discovered that there exist two forms of GS, cytosolic GS (GS1), occurring in the cytoplasm of leaf and non-photosynthetic organ cells, and chloroplastic GS (GS2), present in the chloroplasts of photosynthetic tissue cells (Guiz et al. 1979, Mann et al. 1979). This new concept gave rise to the idea of a compartmentalisation of ammonia assimilation within the plant (McNally et al. 1983). Furthermore, it was found that the relative proportions of the cytosolic and plastidic GS activity are variable within different organs of the same plant or within different plant species depending on either their photosynthetic type or their environmental growth conditions (McNally and Hirel 1983). All together, these observations led to the proposal that each GS isoenzyme had a specific function in assimilating or reassimilating ammonia derived from a variety of processes such as nitrate reduction, photorespiration or nitrogen recycling (Hirel et al. 1993). Most of these hypotheses, originally based on biochemical studies, have now been largely confirmed and extensively refined by using a variety of new tools such as cytology, molecular genetics and transgenic technology (Harrison et al. 1999) which allow the study of the regulation of GS isoenzyme gene and protein expression under various developmental and environmental conditions (Ireland and Lea 1999).

By the means of selected examples, we will present a general view on the recent findings showing that glutamine synthesis in higher plants is compartmentalised and is controlled both at the transcriptional and post-transcriptional level in various organs and tissues.

Does cytosolic GS plays a specific role in different organs or plant tissues?—In C3 plants, the majority of cytosolic GS activity is confined to the roots whether the plant is a root or a shoot nitrate assimilator and regardless of the presence of a minor plastidic GS activity in roots of a few species (Woodall et al. 1996). In C4 plants, the situation is different since a large proportion of GS1 is also detected in the shoots (Becker et al. 1992). Root cytosolic GS is an octameric enzyme composed of either one or two polypeptides (M_r ranging from 39 to 45 kDa) depending on the plant species examined. When present, the relative proportions of the two GS polypeptides may vary depending on the developmental stage of the roots or the source of inorganic nitrogen (ammonium or nitrate) supplied to the plant (Ireland and Lea 1999). The synthesis of the root GS polypeptides and the expression of the corresponding genes have been thoroughly investigated in maize, demonstrating that root GS1 is encoded by a multigene family composed of five members and that there are major differences in their relative expression within the tissues of the root (Li et al. 1993). Transcripts of the $GS_{1\text{-}2}$ gene are expressed mostly in the vascular tissue while $GS_{1\text{-}1}$ and $GS_{1\text{-}5}$ gene products are detected in the cortical tissues. In contrast, $GS_{1\text{-}3}$ and $GS_{1\text{-}4}$ are constitutively expressed in all tissues. Immunocytochemical studies, performed on wheat and tobacco, confirmed that GS1 protein is present in both the cortical parenchyma and vascular stele (Peat and Tobin 1996, Brugière et al. 1999). In addition to a tissue-specific expression, $GS_{1\text{-}1}$ and $GS_{1\text{-}5}$ genes (named *GS1c* and *GS1d* in this study) are up-regulated following the application of external ammonia whereas $GS_{1\text{-}3}$ and $GS_{1\text{-}4}$ (named *GS1a* and *GS1b* in this study) are down-regulated (Sakakibara et al. 1996).

Despite these detailed investigations, the role of cytosolic GS in the different root cell types of plants assimilating reduced nitrogen in the shoots is still not clearly defined. In tobacco, the expression of a gene encoding GS1 (*Gln1-5*) is restricted to the vascular tissue of both roots and shoots (Dubois et al. 1996) suggesting that the corresponding enzyme activity plays a redundant role in both organs in the synthesis of glutamine, a mobile form of nitrogen exported to sink organs such as developing or reproductive organs (Carvalho et al. 1992, Lam et al. 1996). However, a recent study using transgenic plants impaired for GS activity in the phloem showed that the enzyme is rather involved in the production of proline (Brugière et al. 1999), an organic molecule used to temporarily store nitrogen in young developing leaves (Vansuyt et al. 1979) or under conditions of water stress (Delauney and Verma 1993). It has been proposed that cytosolic GS activity in the root cortex of maize may be important in the assimilation of external ammonia (Sukanya et al. 1994). It is also evident that in barley mutants lacking chloroplastic GS activity grown under non-photorespiratory conditions, the

remaining root enzyme activity is able to assimilate ammonia derived from nitrate reduction (Blackwell et al. 1988). However, further experiments will be required to make the bridge between the expression of the cognate gene and the corresponding enzyme activity in the different root cell types.

Compared to many other plant species, most legumes have a greater potential to assimilate major amounts of nitrogen in their root system whether or not they are under atmospheric nitrogen fixing conditions. However, in these species, the efficiency of root nitrogen assimilation has been questioned because of a possible competition with shoot inorganic nitrogen assimilation (Oaks 1992). This particularity is likely to be the result of an evolutionary adaptation to the symbiosis with *Rhizobium* bacteria. The establishment of this symbiosis leads to the formation of specialised organs called root nodules in which massive amounts of ammonia need to be efficiently assimilated, as the result of the bacterial nitrogenase activity (Oaks 1992). Therefore, considering both the economic importance of atmospheric nitrogen fixing symbiosis (Pawlowski 1997, Shantharam and Mattoo 1997) and the unique developmental and molecular events associated with nodule development, a large number of studies have been performed to unravel the regulatory mechanisms controlling ammonia assimilation both in the bacterium and the host (Waters et al. 1998). In early studies, a massive increase in nodule cytosolic GS activity was observed following the establishment of the *Rhizobium*-legume symbiosis (Robertson et al. 1975). Later on, a detailed analysis of the biochemical and molecular events associated with the induction of the enzyme was performed using *Phaseolus vulgaris* as a model plant.

Studies on the enzyme subunit composition showed that root GS consists of two polypeptides, α and β, whereas the nodule enzyme is formed by two polypeptides, β and γ. The increased root nodule GS activity measured after inoculation by *Rhizobium* corresponds to the appearance of a novel form of GS, GS_{n1}, composed of an hetero-octameric protein in which γ polypeptides are predominant (Forde and Cullimore 1989). Expression studies of the corresponding genes, performed following the introduction of promoter-reporter gene fusion constructs in transgenic *Lotus* nodules, showed that *gln-γ* is only expressed in the infected cells. In contrast, expression of *gln-β* is restricted to the vascular system in mature nodules (Forde et al. 1989). However, the lack of effect of the absence of a nodule enhanced GS_{n1} isoenzyme in a number of common bean genotypes (Gao and Wong 1994) raises the question of the physiological significance of such temporal and spatial distribution of nodule GS gene and protein expression. Intriguingly, in *Phaseolus*, the onset of nitrogen fixation is a positive factor regulating *gln-γ* gene expression, but ammonia per se does not seem to operate as a signal molecule. This situation is in contrast to soybean (a closely related species in terms of nodule structure and nitrogen metabolism) in which enhanced expression of at least one of the genes encoding GS1(*GS15*) is controlled by the availability of ammonia provided either externally to the roots or as the result of nitrogen fixation in the infected cells of the nodules (Hirel et al. 1987, Miao et al. 1991). Progressive deletions of the *GS15* promoter allowed the isolation of separate *cis*-acting elements likely to interact in a cooperative manner to stimulate the expression of the gene in the presence of ammonia (Tercé-Laforgue et al. 1999). Most unexpected was the finding that *GS15* is also expressed in the anthers and pulvini (Marsolier et al. 1993) although the function of the gene in these two organs is still unknown. This result suggests, however, that a single member of the GS multigene family may play the same function in structurally unrelated organs or tissues.

A similar situation is found during leaf senescence when protein nitrogen is remobilised and exported to sink organs such as developing reproductive or storage organs. At least in two *Solanacae* species (tomato and tobacco), a gene encoding root cytosolic GS was found to be induced during leaf ageing (Perez-Rodriguez and Valpuesta 1996, Masclaux et al. unpublished data). This finding strongly suggests that the promoter contains putative regulatory elements able to direct the expression of the gene in totally different cellular and physiological contexts.

An interesting model is the developmental regulation of GS gene and protein expression in the pedicel during maize seed development. The activity of a specific isoform, GS_{p1}, distinct from the leaf and root isoenzymes, is increased during kernel development. This increase appears to be the result of an enhanced transcription of a *GS* gene specifically expressed in the pedicel (Rastogi et al. 1998). Together with other older reports (Gallardo et al. 1988, Pereira et al. 1996), this work suggests that even in storage organs such as fruits, tubers or seeds, ammonia assimilation is regulated both in an organ-specific and developmental manner.

The role and regulation of plastidic glutamine synthetase in photosynthetic and non-photosynthetic tissues— In the majority of plant species, plastidic GS isoenzyme (GS2) is encoded by one nuclear gene per haploid genome. This gene encodes a polypeptide exhibiting a M_r of 43 to 45 kDa (depending on the plant species examined) which combines into an octameric complex leading to the formation of the native GS enzyme (Forde and Cullimore 1989). In all the GS2 subunits, a N-terminal signal peptide of almost 50 amino acids is found which targets the protein to the chloroplastic compartment (Lightfoot et al. 1988). In addition, a 16 amino acid conserved region, characteristic of the GS2 protein, is present at the C-terminal part of the subunit.

In a number of species, plastidic GS can represent

around 5% of total root GS activity. This finding could be explained by the capacity of certain plants, such as temperate legumes, to reduce nitrite in the plastids of the root cells. Thus, a physiological adaptation to root nitrate assimilation due to the nitrate-rich composition of the temperate soils compared to tropical areas has been suggested (Woodall and Forde 1996). In the root cells of these plants, applications of nitrate increase the level of plastidic GS polypeptides and mRNA (Sakakibara et al. 1992, Vézina and Langlois 1989). However, the presence of plastidic GS in roots is limited to a few plant species and is not a prerequisite for ammonia assimilation arising from nitrite reduction (Woodall et al. 1996). In legumes, GS2 was also detected in the plastids of root nodules (Brangeon et al. 1989); however, the lack of nitrate reduction in this organ argues against the role of GS2 in assimilation of ammonia.

In C3 plants, GS2 is predominant in all photosynthetic tissues (leaf mesophyll in particular), whereas in C4 plants almost equal proportions of GS2 and GS1 are detected (McNally et al. 1983, Becker et al. 1992). In C3 and C4 plants, light plays a fundamental role in the regulation of *GS2* both at the transcriptional and post-transcriptional level (Ireland and Lea 1999). Following illumination of etiolated leaves and cotyledons, an increase in both GS2 transcript and protein have been observed in a majority of plant species. This increase was found to be more rapid during a transition from dark to light than during the illumination of etiolated leaves, since in the first instance chloroplasts are already fully differentiated (Edwards and Coruzzi 1989, Galvez et al. 1990). The role of light-dependent factors on the *GS2* expression was confirmed when etiolated plants were exposed to different wavelengths of the spectrum. Experiments with white, red, far-red or blue light show that phytochrome and the blue-light photoreceptor are involved in the positive response to light (Edwards and Coruzzi 1989, Becker et al. 1992, Migge et al. 1998). More detailed studies on *Pinus sylvestris* showed that light regulation of *GS2* expression occurs coarsely at the transcriptional level and more finely at the post-translational level (Elmlinger et al. 1994) involving modifications of the subunit composition, as has also been shown in tomato seedlings (Migge et al. 1998). However, the biological role of the post-translational modification of GS2 subunit composition is still unknown.

Compared to the large number of other studies describing the light perception and the subsequent signal transduction pathway regulating the expression of genes encoding proteins and enzymes implicated in the photosynthetic process (Bowler and Chua 1994), very little is known about the mechanism controlling *GS2* gene transcription, likely because additional environmental and developmental factors are also involved. In order to identify light-responsive elements in the *GS2* promoter, transgenic plants expressing promoter-reporter gene fusion constructs have been produced. A 323 bp promoter fragment from pea contains *cis*-acting elements responsible for the light regulation of the *GUS* reporter gene in the leaf mesophyll cells of mature transgenic tobacco or *Arabidopsis* (Tjaden et al. 1995). As a basal level of *GUS* expression was detected in etiolated cotyledons, it was suggested that promoter elements other than the light-responsive one may be involved in *GS2* gene expression in non-photosynthetic tissues. Similarly, it was shown that a 460 bp fragment of the *Phaseolus vulgaris GS2* promoter was sufficient for light-regulation and specific photosynthetic tissue expression of the *GUS* reporter gene in transgenic tobacco (Cock et al. 1992).

In conjunction with light, nitrate may also play a regulatory role in controlling the production of GS2 in the leaves (Migge et al. 1996). In the presence of a nitrogen source and illumination with red- or far red-light, etiolated tomato seedlings synthesise two types of chloroplastic GS polypeptides while only one is detected in the presence of ammonium. Thus, specific wavelengths (via phytochrome) and nitrate can modify the GS subunit composition of tomato at the post-translational level (Migge et al. 1998). In the majority of plant species examined so far, ammonium does not seem to have any effect on chloroplastic GS activity. However, in both rice and tobacco leaves, chloroplastic *GS2* gene transcription is enhanced following the addition of ammonia to the growth medium (Kozaki et al. 1992, Lancien et al. 1999).

There is increasing evidence suggesting that in addition to light and nitrogen metabolites, the integrity of the plastids is prerequisite for optimal GS2 activity. For example, infection of tomato plants by *Pseudomonas syringae* or herbicide treatment with phosphinothricin (PPT), an inhibitor of GS, leads to rapid chlorosis. Following these two treatments, a decrease of both *GS2* gene expression and protein content concomitant to an increase in *GS1* expression was observed when plants were exposed to light (Perez-Garcia et al. 1995). In contrast, in non-photosynthetic conditions, these modifications were not observed, leading to the conclusion that light-dependent factors are involved in controlling the expression of the two GS isoenzymes (Perez-Garcia et al. 1998). In particular, the authors hypothesised that the decrease in chloroplastic GS following PPT treatment is the result of chloroplast degeneration due to a down-regulation of photosynthetic genes by the GS inhibitor. A similar situation seems to occur during natural senescence during which a rapid decrease in chloroplastic GS activity is associated with the degeneration of chloroplasts and the concomitant loss of photosynthetic functions (Kamachi et al. 1991, Perez-Rodriguez and Valpuesta 1996).

In dark-adapted *Arabidopsis* seedlings, sucrose enhances the expression of the chloroplastic *GS* gene, thus mimicking the effect of light. This result suggests that light

exerts an indirect effect on *GS2* gene expression and that an efficient photosynthetic activity producing sucrose and/or another metabolisable sugar is required to control *GS2* gene expression (Melo-Oliveira et al. 1996). In addition, Oliveira and Coruzzi (1999) have shown that GS expression is controlled by the relative abundance of carbon squeletons versus amino acids. It is also well known that temperature is an important environmental factor controlling positively or negatively the expression of several genes involved in the photosynthetic process. In pea and barley plants grown at 15°C instead of 25°C, a 50% reduction in GS2 activity was observed after 2 d, while the activity of GS1 was unaffected (Woodall et al. 1996), again indicating that an optimal photosynthetic activity is required to attain full GS activity in the chloroplast.

From the analysis of barley mutants deficient in GS2 activity, it became evident that the main function of the GS2 enzyme was to reassimilate photorespiratory ammonia (Blackwell et al. 1988), although its contribution in the nitrate assimilatory pathway is far from negligible (Keys et al. 1978) particularly in C4 plants where photorespiration is low (Oaks 1994). This raises the question as to whether in addition to photosynthesis, photorespiration is able to control the expression of *GS2*. A decrease in chloroplastic *GS* gene expression and activity is observed when pea or French bean plants are placed for 14 d under non-photorespiratory conditions (Edwards and Coruzzi 1989, Cock et al. 1991). In contrast, after 14 d under photorespiratory conditions, *GS2* gene transcription is enhanced (Edwards and Coruzzi 1989). The length of the response time suggests the involvement of metabolic acclimation to high CO_2 concentration which results in a decline of photosynthetic functions. Chloroplastic GS activity would therefore be affected by this progressive loss of photosynthetic functions rather than as a result of a direct effect of photorespiration (i.e. ammonium flux). In *Arabidopsis thaliana* and tobacco, rapid shifts from photorespiratory to non-photorespiratory conditions have no effect on GS2 protein and gene expression, thereby excluding a metabolic acclimation (Beckmann et al. 1997, Migge et al. 1997).

All together, these various reports strongly suggest that in addition to a variety of environmental factors such as light and the plant nutritional status, the integrity of the chloroplast machinery is essential for an optimal regulation of GS2 gene and protein expression. This is most likely due to the specific role of plastidic GS in photosynthetically active cells and tissues.

The physiological significance of cytosolic GS gene expression variability amongst the plant kingdom remains an enigma—Between three and five different genes encode both GS1 and GS2 depending on the plant species examined. The variability of GS gene and protein expression is still not fully understood. In addition, GS1 and GS2 holoenzyme subunit composition is very different from one plant to another and may be subjected to post-translational modifications in the case of both the plastidic and the cytosolic isoenzymes (Ireland and Lea 1999). There is speculation that the diversity of GS gene and protein expression is a means by which the plant can adapt to particular environmental conditions (Marsolier and Hirel 1993).

Attempts to modify the enzyme structure and catalytic properties in heterologous bacterial systems have been very often successful (Carvalho et al. 1997, Clemente and Marquez 1999); however, further work is required to establish whether kinetic differences between the isoenzymes are physiologically relevant. The available transgenic technologies and the eventual possibility of homologous recombination in plants will certainly be of great help in elucidating structure/function relations of the different GS forms.

One of the most outstanding features in comparing GS gene and protein expression patterns between various species, is the lack of a general model depicting the regulatory mechanisms involved. Although the regulation of plastidic *GS* expression appears to be conserved between different species and the presence of cytosolic GS in the phloem seems to be a common characteristic in angiosperms, the great variability between the other GS isoenzymes renders biochemical and physiological interpretation very difficult. One of the most glaring examples is the variability encountered in the leaf *GS1* expression pattern when C3, C4 plants or certain tree species are examined. Although in all plant types, leaf cytosolic GS is present in the phloem during the vegetative phase, large amounts of GS are detected in the leaf cytosol of C4 species as compared to C3 plants (Becker et al. 1992), whereas in gymnosperm seedlings the enzyme is only cytosolic (Garcia-Gutierrez et al. 1998). In C4 plants, the presence of high cytosolic GS content may be related to increased nitrogen use efficiency as the result of both low photorespiratory activity and separation of nitrate reduction and photorespiration in mesophyll and bundle sheath cells respectively (Oaks 1994). However, there is still no definitive proof demonstrating that high leaf cytosolic GS content and increased nitrogen use efficiency are interrelated. More basic physiological studies combined with emerging genomic technologies (Pennisi 1998) will be required. In gymnosperms, it was proposed that an adaptation to dark habitats and etiolation of the seedlings could explain the high GS1 content, similar to the case of heterotrophic achlorophyllous parasites which exhibit only cytosolic GS activity in the shoots (McNally et al. 1983). Evolutionary comparisons of *GS* gene sequences strongly suggest that the modification of gene function is tightly dependent upon biological function and not on origin. This obviously implies that the acquisition or modification of genes must be caused by the changes that take place among promoter and regulatory elements of the already existing paralogous genes (Biesiad-

ka and Legocki 1997). Since up to now very little homology was found between *GS* promoter sequences, this could partly explain the "biological diversity" of *GS* gene expression patterns.

These few selected examples and a number of other previous reports (Ireland and Lea 1999) suggest that control of *GS1* expression occurs at the transcriptional level because in many cases a fairly good correlation was observed between the expression of a single member of the *GS* multigene family and the corresponding translation product. However, it seems that a perfect coordination between the transcriptional activity and the assembly and turnover of the holoenzyme is a prerequisite condition to obtain an optimal enzyme activity in a given cellular environment. This hypothesis is supported by the observation that in a number of transgenic plants overexpressing *GS* genes, the amount of mRNA originating from transcription of the transgene is always largely in excess when compared to the amount of corresponding protein (Hirel et al. 1992, Vincent et al. 1997). Whether this regulatory control mechanism is organ- or tissue-specific and occurs at a post-transcriptional or post-translational level is still largely unknown, although there are good indications that substrate availability (Ortega et al. 1999) or phosphorylation (Moorhead et al. 1999) may be an important factor controlling the enzyme turnover and activity respectively.

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