

The Importance of Cytosolic Glutamine Synthetase in Nitrogen Assimilation and Recycling

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Summary

Glutamine synthetase assimilates ammonium into amino acids, thus it is a key enzyme for nitrogen metabolism. The cytosolic isoenzymes of glutamine synthetase assimilate ammonium derived from primary nitrogen uptake and from various internal nitrogen recycling pathways. In this way, cytosolic glutamine synthetase is crucial for the remobilization of protein-derived nitrogen. Cytosolic glutamine synthetase is encoded by a small family of genes that are well conserved across plant species. Members of the cytosolic glutamine synthetase gene family are regulated in response to plant nitrogen status, as well as to environmental cues, such as nitrogen availability and biotic/abiotic stresses. The complex regulation of cytosolic glutamine synthetase at the transcriptional to post-translational levels is key to the establishment of a specific physiological role for each isoenzyme. The diverse physiological roles of cytosolic glutamine synthetase isoenzymes are important in relation to current agricultural and ecological issues.

Abbreviations: ATP, adenosine triphosphate; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; PAL, phenylalanine ammonia lyase; QTL, quantitative trait locus.

Key words: abiotic stress, biotic stress, glutamine synthetase (GS), nitrogen metabolism, quantitative trait locus (QTL), regulation, remobilization, seed.

Introduction

Nitrogen, a key element for plant growth and reproduction, is an essential building block of nucleic acids and proteins. Plants can store nitrogen in large amounts within enzymes involved in carbon fixation, such as leaf Rubisco (Stitt & Schulze, 1994). However, nitrogen is also often a limiting nutrient in many natural environments, and different plant species have evolved specific strategies to acquire nitrogen from their environments and assimilate it into organic compounds. Glutamine synthetase (GS, E.C. 6.3.1.2) plays a major role in fixing ammonium (NH_4^+) to form the amino acid glutamine. The cytosolic isoform of GS is particularly important for assimilating

ammonium from different sources, for both primary nitrogen assimilation and recycling. Recent studies have led to a better understanding of the specific roles of this isoform of GS in flowering plants and conifers. Here, we focus on the current knowledge of cytosolic GS and its central role in nitrogen assimilation and recycling.

Nitrogen is the most abundant element in the atmosphere. In some systems, biological nitrogen fixation of atmospheric N_2 contributes to much of the primary nitrogen source available to plants, mostly through symbiosis with diazotrophic bacteria (Oldroyd & Downie, 2008). Symbiosis

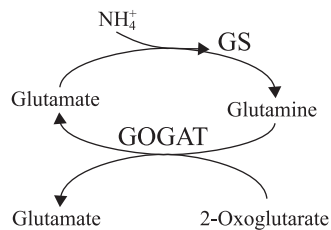


Fig. 1 The glutamine synthetase–glutamate synthase (GS–GOGAT) cycle.

with arbuscular mycorrhizal fungi and ectomycorrhiza has also been shown to improve the capacity of plants to take up nutrients, including nitrogen (Buscot *et al.*, 2000; He *et al.*, 2003). The nitrogen sources directly available to plants include inorganic nitrogen compounds, such as nitrate and ammonium, as well as organic compounds, such as amino acids (e.g. glycine, alanine, glutamic acid, aspartic acid) and small peptides (Schimel & Bennett, 2004; Bardgett, 2005). In nonfertilized ecosystems, the majority of mineral nitrogen is produced by microbial mineralization of organic nitrogen (Paul & Clark, 1996). The nature of nitrogen sources varies between soil types rich in ammonium, such as boreal forest soil, and others rich in nitrate, such as agricultural fields (Britto & Kronzucker, 2005). In addition, the soil concentration of inorganic nitrogen can range from micromolar to molar (Britto & Kronzucker, 2005). Mechanisms for optimizing nitrogen capture in a variety of environments have evolved in plants. The uptake of soil nitrate and ammonium by plant roots has been well characterized at the physiological and molecular level, and several multigene families of low- and high-affinity nitrate and ammonium transporters have been described and studied in detail (Glass *et al.*, 2002; Miller *et al.*, 2007).

The main pathways of inorganic nitrogen assimilation and remobilization in plants have been established at the biochemical level. GS is a key enzyme, because it catalyses the adenosine triphosphate (ATP)-dependent fixation of ammonium to the δ -carboxyl group of glutamate to form glutamine (Fig. 1). The enzyme glutamate synthase (GOGAT; E.C. 1.4.7.1) catalyses the conversion of glutamine and 2-oxoglutarate to two molecules of glutamate, thus providing glutamate for ammonium assimilation. The net outcome of the GS–GOGAT cycle is the production of glutamate, which can then be incorporated into other amino acids through the action of aminotransferases or transaminases (Forde & Lea, 2007). Specific amino acids can subsequently become precursors for all nitrogen-containing organic molecules, such as proteins, chlorophyll, cytochrome/phytochrome, secondary metabolites and nucleic acids. The product of GS, glutamine, is itself the main form of organic nitrogen for transport in the phloem of rice (Hayashi & Chino, 1990; Yamaya & Oaks, 2004) and in the xylem of poplar (Sauter & van Cleve, 1992). Arginine and asparagine are also commonly involved in nitrogen transport, asparagine in particular being an efficient compound for

nitrogen transport and storage as a result of its high molecular nitrogen to carbon ratio (for a review, see Lea *et al.*, 2007).

In plant cells, nitrogen assimilation is compartmentalized between the cytosol and chloroplast, in relation to the different sources of ammonium. Primary sources of ammonium include direct ammonium uptake from the soil and the reduction of nitrate and atmospheric N_2 , whereas secondary sources consist of amino acid catabolism following protein degradation, photorespiratory nitrogen cycling and the production of ammonium by phenylalanine ammonia lyase (PAL; E.C. 4.3.1.5) and asparaginase (E.C. 3.5.1.1). Nitrate is first reduced into nitrite via nitrate reductase (E.C. 1.6.1.1), which is cytosolic, after which the chloroplast-located nitrite reductase (E.C. 1.7.7.1) catalyses the reduction of nitrite into ammonium. Photorespiratory nitrogen cycling produces large amounts of ammonium in the mitochondria (Keys *et al.*, 1978). During leaf senescence, although some amino acids generated through protein breakdown may be converted to alternative amino acids and/or exported via the phloem to developing organs (Hortensteiner & Feller, 2002), other amino acids are degraded further, leading to the production of free ammonium. For example, in tobacco and ryegrass leaves, protein degradation during senescence coincides with increased levels of ammonium (Schjoerring *et al.*, 1993; Masclaux *et al.*, 2000; Mattsson & Schjoerring, 2003).

Glutamate dehydrogenase (GDH; E.C. 1.4.1.2) and asparaginase have been described as sources of ammonium deriving from the catabolism of amino acids. GDH specifically catalyses the reversible amination/deamination between 2-oxoglutarate and glutamate; its physiological role in nitrogen metabolism has been the subject of controversy (Lea & Mifflin, 2003; Forde & Lea, 2007). Although some evidence suggests a role for GDH in ammonium assimilation, most studies indicate that GDH functions primarily in the deamination direction, thereby producing ammonium in the mitochondria (Lea & Mifflin, 2003). It is most likely that GDH functions as a shunt to divert some of the carbon skeleton from nitrogen metabolism to carbon metabolism and the tricarboxylic acid cycle (Mifflin & Habash, 2002; Lea & Mifflin, 2003). Asparaginase catalyses the hydrolysis of asparagine to form ammonium and aspartate, thereby constituting the major route for asparagine catabolism.

Focusing primarily on cytosolic GS, we discuss below the current knowledge of the enzyme structure, gene family and evolution. We then examine information regarding cytosolic GS regulation in the context of specific roles with regard to primary nitrogen assimilation and recycling.

Different GS isoforms have evolved

Three types of GS have been described in living organisms, based on molecular mass, quaternary structure and gene sequences. GSI and GSII are both present in eukaryotes and prokaryotes, with GSI being more abundant in prokaryotes

and GSII in eukaryotes. In addition, a third type (GSIII) has been described in *Bacteroides*, *Butyrivibrio* and some cyanobacteria (Hill *et al.*, 1989; García-Domínguez *et al.*, 1997). Kumada *et al.* (1993) estimated that GSI and GSII arose from gene duplication *c.* 3500 Mya, based on the phylogenetic tree calibrated by Dickerson's estimate that animal and plant kingdoms diverged 1200 Mya. This study suggests that GS genes are one of the oldest existing and functioning genes. In higher plants, the GSII type is the most abundant, although some recent studies have shown the presence of GSI-encoding genes in *Medicago truncatula*, *Arabidopsis thaliana* and sugarcane (*Saccharum* spp.) (Mathis *et al.*, 2000; Nogueira *et al.*, 2005). Thus far, the function of these GSI-type genes remains unknown in plants; GSII genes have been better characterized. The atomic structure of maize cytosolic GS has recently been elucidated at 2.63-, 3.50- and 3.80-Å resolutions, indicating that plant GS polypeptides (Type II) form decamers (Unno *et al.*, 2006), which differ from the dodecameric structure of bacterial GS (Type I) (Almassy *et al.*, 1986; Krajewski *et al.*, 2005). Cytosolic GS decamers, composed of two face-to-face pentameric rings of subunits, contain 10 active sites, with each site being formed between every two adjacent subunits in a pentamer (Unno *et al.*, 2006).

In higher plants, two isoforms of GS (Type II) were first resolved by ion exchange chromatography. The cytosolic (GS1) isoform was the first to be eluted, followed by the plastidic (GS2) form (McNally & Hirel, 1983), although the opposite elution pattern has been described in some species (Woodall *et al.*, 1996; Orea *et al.*, 2002). They also differ by molecular mass, with the plastidic isoform being larger (*c.* 44–45 kDa) than the cytosolic isoform (*c.* 38–40 kDa) (Forde & Cullimore, 1989). The relative abundance of GS isoforms varies within different organs of the same plant and within different plant species, depending on either their photosynthetic type or environmental growth conditions (McNally & Hirel, 1983). In the mature green leaves of C_4 plants, the relative amount of cytosolic to chloroplastic GS is much higher than in C_3 plants, and this relates to C_4 metabolism (Becker *et al.*, 1993). Cytosolic and chloroplastic isoenzymes are regulated differently within specific cell types and organs, and in response to different developmental, metabolic and environmental cues. This specialization ensures a rapid reassimilation of ammonium derived from multiple sources (Mifflin & Habash, 2002). Overall, these two major GS isoforms play primarily nonoverlapping roles in plant nitrogen assimilation, with chloroplastic GS holding a major role in ammonium assimilation within photorespiratory nitrogen cycling (Wallsgrave *et al.*, 1987; Leegood *et al.*, 1995). The roles and regulation of chloroplastic GS have been discussed elsewhere (Cren & Hirel, 1999; Zozaya-Hinchliffe *et al.*, 2005; Betti *et al.*, 2006).

In angiosperms, the chloroplastic isoenzyme is encoded by one gene, whereas two to five genes may encode the cytosolic form. In conifers, the functional expression of GS2 has so far not been demonstrated, suggesting that cytosolic GS is

involved in nitrogen assimilation related to photosynthetic metabolism (Cánovas *et al.*, 2007). Studies of GS gene families were first carried out in legume species, particularly *Phaseolus vulgaris* (Lara *et al.*, 1983), *Pisum sativum* (Tingey *et al.*, 1987) and *Medicago truncatula* (Stanford *et al.*, 1993). More recently, GS gene families have been described in nonlegume plant species, such as *Zea mays* (Li *et al.*, 1993), *Arabidopsis thaliana* (Ishiyama *et al.*, 2004c), rice (*Oryza sativa*; Ishiyama *et al.*, 2004a), wheat (*Triticum aestivum*; Bernard *et al.*, 2008), potato (*Solanum tuberosum*; Teixeira *et al.*, 2005) and sugarcane (*Saccharum* spp.; Nogueira *et al.*, 2005). GS gene and protein sequences are well conserved both within and across species. For example, in wheat, members of the cytosolic GS gene family are *c.* 80% identical at the protein level (Bernard *et al.*, 2008). Some key domains of the GS protein have been characterized, such as ATP-binding and glutamate-binding sites. Site-directed mutagenesis studies have demonstrated the functionality of some highly conserved residues in cytosolic GS polypeptide. For example, the aspartic acid at position 56 on the glutamine α -polypeptide from *P. vulgaris* is essential for transferase activity, whereas the glutamic acid residue (position 297) is possibly involved in stabilizing the transition state for a reaction subsequent to ammonium binding (Clemente & Marquez, 1999a,b). Clemente & Marquez (2000) also demonstrated that the cysteine residue at position 92 is important for thermal stability. Further, recent point-mutation studies in rice and maize have identified particular residues that are critical in determining the kinetic characteristics of cytosolic GS isoenzymes and heat tolerance (Ishiyama *et al.*, 2004a,b; Unno *et al.*, 2006). The occurrence of isoenzymes with distinct biochemical characteristics, together with the heteromeric nature of the GS enzyme, could lead to many GS isoenzymes if these were expressed in the same cells and at the same time. However, the functional significance of these isoenzymes has not been studied *in vivo*.

Phylogenetic studies of GS nucleotide or amino acid sequences have shown that chloroplastic and cytosolic GSs form two sister groups (Doyle, 1991; Pesole *et al.*, 1991) and have evolved through gene duplication (Biesiadka & Legocki, 1997). Kumada *et al.* (1993) proposed that the common ancestor of the two genes diverged an estimated 300 Mya, based on a phylogenetic tree calibrated by Dickerson's estimate that animal and plant kingdoms diverged 1200 Mya. This analysis suggests that the duplication occurred earlier than the monocot/dicot divergence. Chloroplastic GS is also present in *Ginkgo biloba* (García-Gutiérrez *et al.*, 1998), but absent in pine species. In *Pinus sylvestris*, two cytosolic GS genes have been identified, suggesting that the duplication of an ancestral cytosolic GS occurred long before the divergence of gymnosperms/angiosperms (Sáez *et al.*, 2000). Doyle (1991) proposed that members of the cytosolic GS gene family in plants have evolved by duplication of an ancestral cytosolic GS gene and the evolution of separate gene copies to fulfil different metabolic cell requirements. Phylogenetic analysis

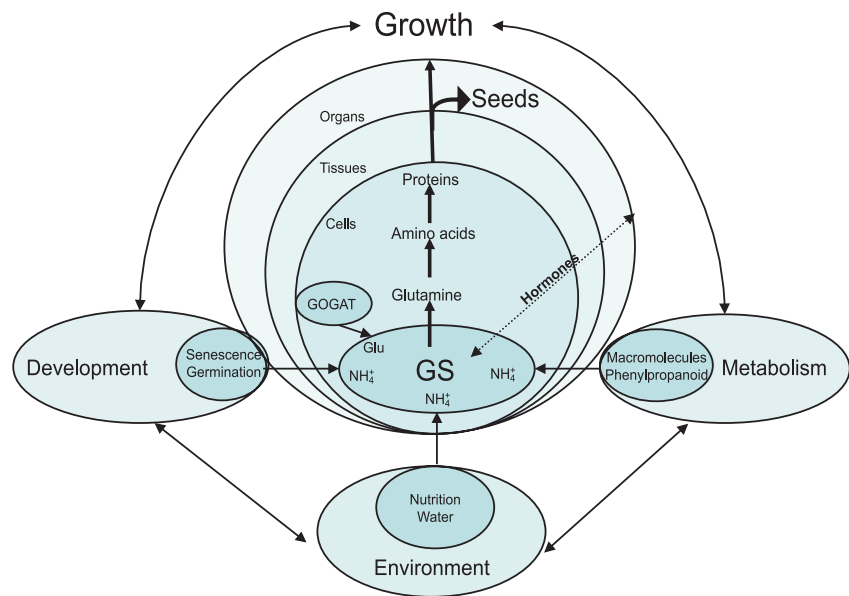


Fig. 2 The central role of cytosolic glutamine synthetase (GS) in plant growth and seed production. The central circle highlights the function of GS fixing inorganic nitrogen (N) into amino acids that can be transported to developing tissues and organs. Sources of ammonium are highlighted around the central circles. GOGAT, glutamate synthase.

has shown that cytosolic GS sequences from plant species that can establish symbiotic relationships with either *Rhizobia* or *Frankia* cluster in three groups (Biesiadka & Legocki, 1997; Kim *et al.*, 2004). These groups do not seem to correspond to functionally different subfamilies, as each contains one cytosolic GS isoform involved in assimilating ammonium from N_2 fixation. Cytosolic GS proteins from the Poaceae family also form three separate clades; however, these correspond to three functionally distinct subfamilies of cytosolic GS (Bernard, 2005; Hirel *et al.*, 2007a; Bernard *et al.*, 2008). Given the roles of some of the cytosolic GS isoforms in nitrogen assimilation and recycling, we believe that the evolution of these genes merits further attention.

In the three major cereal crops, studies have uncovered quantitative trait loci (QTLs) for cytosolic GS proteins and/or GS activity, demonstrating the existence of variation for GS in maize (Hirel *et al.*, 2001), rice (Obara *et al.*, 2001, 2004) and wheat cultivars (Habash *et al.*, 2007). In these studies, some of the QTLs were localized to the mapped structural GS genes, establishing allelic variation, whereas others did not co-localize with the mapped GS genes, demonstrating that other genetic elements are influencing the activity or protein abundance of GS isoenzymes. These studies support and beautifully illustrate the complexity of the regulation of GS isoenzymes, discussed below.

Cytosolic GS isoenzymes are regulated at transcriptional to post-translational levels and hold specific roles

Plant GSs may be regulated at several levels: (1) transcription of different gene family members; (2) processing and stabilization of the transcripts; (3) translation; (4) subcellular localization,

processing or modification of the GS polypeptide; (5) assembly of an active enzyme; (6) post-translational modification of the enzyme; and/or (7) enzyme degradation (Forde & Cullimore, 1989; Temple *et al.*, 1993; Cren & Hirel, 1999). Transcriptional controls are considered to be particularly important in determining GS polypeptide abundance and cellular localization patterns (Cren & Hirel, 1999). The complex regulation of cytosolic GS is key to the establishment of a specific physiological role for each isoenzyme (Fig. 2). The physiological roles for cytosolic GS are also closely related to the sources of ammonium discussed previously.

Cytosolic GS assimilates ammonium from primary sources in roots, nodules and leaves

Ammonium uptake from soil

The localization of cytosolic GS transcripts and polypeptides in roots has led to suggestions that it is involved in the assimilation of ammonium from primary nitrogen uptake (Sukanya *et al.*, 1994; Ishiyama *et al.*, 2004a,c). Furthermore, *in situ* hybridization and promoter–green fluorescent protein fusion studies on the localization of rice and *A. thaliana* GS gene expression have shown that the location of *OsGln1;2* transcripts is consistent with the site of ammonium uptake, as indicated by the localization of ammonium transporters (Sonoda *et al.*, 2003; Ishiyama *et al.*, 2004a; Tabuchi *et al.*, 2007). Studies in rice have suggested that *OsNADH-GOGAT1* provides glutamate for ammonium assimilation by cytosolic GS in the cell layers at the root surface (Ishiyama *et al.*, 2003; Tabuchi *et al.*, 2007). Ferredoxin-GOGAT may be involved when excess ammonium is present and reaches the central cylinder and root cortex (Tabuchi *et al.*, 2007).

The role of root cytosolic GS in total plant primary nitrogen assimilation is not well understood, especially for species primarily assimilating nitrogen in the shoot. Furthermore, a high level of GS activity in the root was negatively correlated with above-ground biomass in *Lotus japonicus* (Limami *et al.*, 1999). Pea plants overexpressing a soybean GS1 under the control of a root-specific promoter showed no consistent effect on total biomass (Fei *et al.*, 2003). Of the two lines showing high root GS activity, one had decreased biomass and the other showed no significant difference. Lian *et al.* (2005) found a QTL for root weight that was localized in the vicinity of the structural root cytosolic GS gene when rice plants were grown under low nitrogen. Further studies are necessary to firmly establish the contribution of root cytosolic GS in total nitrogen assimilation, especially considering the different nitrogen sources and levels of nitrogen availability. Cruz *et al.* (2006) showed that a high level of GS activity in the dark, in either roots or leaves, was necessary for plants to tolerate high levels of ammonium in their growth medium.

Furthermore, Finnemann & Schjoerring (1999) demonstrated that ammonium could be translocated from the root to the leaf through the xylem, contributing up to 11% of the total amount of nitrogen translocated in the stem xylem sap in oilseed rape (*Brassica napus*). Ammonium translocation in the xylem may provide ammonium for primary assimilation in the shoot, where it can be assimilated by GS.

Ammonium originating from N₂ reduction

The enzyme nitrogenase catalyses the reduction of N₂ to ammonium. This oxygen-sensitive enzyme is not present in plants, but associations between nitrogen-fixing microorganisms and plants can be established. The production of ammonium that occurs either within the plant's tissues or in specialized organs called nodules can significantly contribute to plant nitrogen nutrition. Cytosolic GS assimilates ammonium from the three major types of nitrogen-fixing symbiotic association involving plants and either *Rhizobium*, actinomycetes, such as *Frankia*, or cyanobacteria (Rai *et al.*, 2000). In some species, a specific cytosolic GS isoenzyme is induced in nitrogen-fixing root nodules (Lara *et al.*, 1983; Forde *et al.*, 1989), whereas, in others, cytosolic GS already present is involved (Carvalho *et al.*, 2000a). In alfalfa, the level of transcripts for a nodule-specific GS gene was dependent on the nodule nitrogen-fixing capacity (Vance *et al.*, 1994). In some species, cytosolic GS is present in infected plant cells within the nodule, where it assimilates ammonium that is locally released from the nitrogenase-mediated reduction of atmospheric N₂ (Oaks, 1992; Carvalho *et al.*, 2000b). By contrast, in *Frankia*-infected *Dactylis glomerata* plants, GS is highly abundant in uninfected nodule cortical tissues at a distance from the N₂ reduction site (Berry *et al.*, 2004). In the nodules, cytosolic GS is post-translationally regulated by phosphorylation in response to nitrogen fixation (Lima *et al.*, 2006). Cytosolic GS also

assimilates ammonium produced by endophytic diazotrophs colonizing the internal parts of some plants, such as sugarcane, without establishing symbiotic relationships (Nogueira *et al.*, 2005).

Ammonium from nitrate reduction

Some plant species show a preference for root nitrate assimilation, whereas, for many others, this occurs primarily in the leaf (Andrews, 1986; Wallace, 1986; Woodall & Forde, 1996). Together with the possibility of ammonium translocation to the shoot, this difference suggests that, depending on the plant species, primary nitrogen assimilation can occur in the roots and/or shoots. Nitrate reduction is compartmentalized between the cytosol, where nitrate reductase is active, and the plastid, where nitrite reductase produces ammonium. In barley roots, where cytosolic GS is the major form, GS activity was higher in nitrate-fed plants, without any corresponding increase in polypeptide abundance (measured by western blot and immunolabelling) (Peat & Tobin, 1996). This higher activity suggested that cytosolic GS is involved in the assimilation of ammonium originating from nitrate reduction in the roots (Peat & Tobin, 1996; Tobin & Yamaya, 2001), and that post-translational regulation of cytosolic GS occurs in response to nitrate treatment. GS could be detected by immunolocalization in the plastids of young barley root; however, plastidic GS is a minor form in barley root and could not be detected (by western blot) in whole-root protein extracts (Peat & Tobin, 1996). Woodall & Forde (1996) have suggested that the presence of plastidic GS in the roots could be the result of an adaptation of legume species to nitrate-rich temperate soils.

In young and mature photosynthesizing leaves, both chloroplastic and cytosolic GS proteins and activities have been measured (McNally *et al.*, 1983; McNally & Hirel, 1983; Yamaya & Oaks, 1988; Sakakibara *et al.*, 1992; Becker *et al.*, 2000; Habash *et al.*, 2001; Tobin & Yamaya, 2001), thus confirming that they are both assimilating ammonium. The proportion of chloroplastic to cytosolic isoforms differs, depending on the species studied (McNally *et al.*, 1983). Although the levels of cytosolic GS and GS2 were equal in maize bundle-sheath cells, the level of cytosolic GS protein was 1.8-fold higher than that of GS2 protein in mesophyll cells (Becker *et al.*, 1993). In these cells, the GS1-3 isoenzyme is expressed constitutively, suggesting that it assimilates ammonium originating from nitrate reduction (Martin *et al.*, 2006).

For annual C₃ grasses, such as wheat, rice or barley, cytosolic GS is less abundant in the leaf. Nevertheless, it is an important component, considering its vascular cell localization and specific physiological functions, as discussed below. A study of barley mutants deficient in GS2 showed a normal phenotype and no nitrogen deficiency when grown under nonphotorespiratory conditions (Wallsgrove *et al.*, 1987; Leegood *et al.*, 1995), suggesting that cytosolic GS is involved in primary nitrogen

assimilation. The cellular localization of cytosolic GS in vascular bundles, particularly in the phloem and xylem parenchyma cells of the leaves and stem, in C_3 species (Kamachi *et al.*, 1992; Carvalho *et al.*, 2000b) suggests that ammonium is also assimilated in these cells. Ammonium may originate from local sources or be transported from mesophyll cells, where nitrate is reduced, to the vascular cells, where most of the cytosolic GS is localized.

Cytosolic GS assimilates ammonium from nitrogen remobilization

Nitrogen remobilization from protein breakdown is the main source of nitrogen for grain filling in annual species, supplying c. 80% of the total nitrogen in rice grains (Mae & Ohira, 1981; Tabuchi *et al.*, 2007). A number of studies have suggested a role for cytosolic GS in the remobilization of the inorganic form of nitrogen from senescing leaves for grain filling in annual species. First, the localization of cytosolic GS in the vascular tissues and the developmental regulation of cytosolic GS suggest that it is important for nitrogen remobilization. Subsequently, rice and maize mutant studies, as well as QTL analyses, have shown that cytosolic GS is necessary for grain filling (Hirel *et al.*, 2001; Obara *et al.*, 2001, 2004; Habash *et al.*, 2007; for a recent review, see Hirel *et al.*, 2007b).

Cytosolic GS cellular localization indicates a role in the transport of nitrogen

In both C_3 and C_4 species, cytosolic GS is located in vascular bundles (Sakurai *et al.*, 1996; Kichey *et al.*, 2005; Martin *et al.*, 2006). Furthermore, recent studies have shown that cytosolic GS is located in intermediary cells, where nitrogen is exchanged between different cell types, such as the primary pit fields connecting the mesophyll sheath cells and the neighbouring parenchyma and vascular cells in wheat leaves (Kichey *et al.*, 2005), and the core linking the mesophyll and vascular bundle cells in sugarcane leaves (Nogueira *et al.*, 2005). Cytosolic GS protein accumulates in the mesophyll cells of tomato (*Lycopersicon esculentum*) plants in response to stress, such as pathogen attack (Pérez-García *et al.*, 1998b), or senescence (Brugière *et al.*, 1999, 2000; Kichey *et al.*, 2005), suggesting a role for this isoform in the reassimilation of the nitrogen released during the disassembly of the photosynthetic apparatus.

Cytosolic GS assimilates ammonium in a developmentally controlled manner

Leaf GS isoenzymes are regulated in a developmentally controlled manner during vegetative and reproductive plant growth stages (Tobin *et al.*, 1985; Kamachi *et al.*, 1991; Sakurai *et al.*, 1996; Pérez-Rodríguez & Valpuesta, 1996; Finnemann

& Schjoerring, 2000; Habash *et al.*, 2001; Bernard *et al.*, 2008), and particularly through senescence (Buchanan-Wollaston *et al.*, 2003). Studies in many species have noted a decline in abundance of mRNA, protein and activity of GS2 in senescing leaves, coinciding with an increase in abundance of cytosolic GS (Finnemann & Schjoerring, 2000; Masclaux *et al.*, 2000; Habash *et al.*, 2001; Bernard *et al.*, 2008). In wheat, two cytosolic GS genes are regulated differently during leaf senescence: *GS1* transcript abundance increases from anthesis, whereas *GSr* transcript levels remain steady until a late stage of development (Bernard *et al.*, 2008). In maize, GS2 is less abundant in the leaves, whereas cytosolic GS is present throughout leaf development and under varying nitrogen levels. Several GS isoenzymes are necessary to maintain a high level of cytosolic GS activity in the leaves, with *gln3* being up-regulated in senescing maize leaves (Hirel *et al.*, 2005). Finnemann & Schjoerring (2000) suggested that phosphorylation and binding to 14-3-3 proteins was also a possible means by which to regulate cytosolic GS during senescence, mainly by increasing enzyme stability.

Leaf senescence involves the highly coordinated recycling of nitrogen (Hortensteiner & Feller, 2002), with cytosolic GS assimilating ammonium from the catabolism of amino acids. Interestingly, GDH activity increases during senescence (Masclaux *et al.*, 2000), where it mostly functions in the deamination direction, thus producing ammonium in the mitochondria. This activity also provides carbon in the form of 2-oxoglutarate to the tricarboxylic acid cycle, and is especially important for carbon-starved plants (Miyashita & Good, 2008).

In perennial plants, seasonal nitrogen cycling is an important process of remobilization and transport of nitrogen compounds from senescing tissues towards overwintering storage tissues in the autumn, and reassimilation from the storage tissues during the spring flush (Cooke & Weih, 2005). A study carried out in poplar suggests that one cytosolic GS gene is up-regulated early on during autumn senescence, and that its transcript level subsequently declines (Andersson *et al.*, 2004). Thus far, the role of GS in the spring remobilization of nitrogen has not been investigated.

Cytosolic GS remobilizes nitrogen for grain filling

Significant correlations were obtained between grain number/size and a locus for cytosolic GS protein content in rice (*OsGln1;1*; Yamaya *et al.*, 2002; Obara *et al.*, 2004). In maize, coincidences of QTLs were found for GS activity, cytosolic GS locus (*gln1*) and grain yield (Hirel *et al.*, 2001, 2007b; Gallais & Hirel, 2004). In wheat, GS activity (chloroplastic and cytosolic isoforms) was shown to be positively correlated with grain and stem nitrogen content (Habash *et al.*, 2007). These studies highlight the importance of cytosolic GS genes in determining several aspects of nitrogen use traits in cereal crops, with potential implications for breeding and agriculture (for a review, see Andrews *et al.*, 2004; Hirel *et al.*, 2007b).

The most compelling evidence for the role of cytosolic GS in nitrogen remobilization for grain filling has come from recent studies of mutants deficient in leaf cytosolic GS in rice and maize (Tabuchi *et al.*, 2005; Martin *et al.*, 2006). In rice, the homozygous lines of three *OsGS1;1*-knockout mutants showed a decline in cytosolic GS enzyme activity (Tabuchi *et al.*, 2005). The phenotypes of these mutants, retardation in growth rate, reduced spikelet weight and number, and reduced fertility, strongly suggest a role for *OsGS1;1* in grain filling in rice. In maize, two mutants were identified with the maize Mutator (*Mu*) system, showing an insertion in either *Gln1-3* or *Gln1-4*, and a third double mutant was constructed with *Mu* inserted in both genes (Martin *et al.*, 2006). The two single mutants displayed specific phenotypes, with the *gln1-4* mutant exhibiting a reduced kernel size and the *gln1-3* mutant a reduced kernel number. As opposed to the phenotype seen in rice, the loss of cytosolic GS activity in the leaves had no effect on the shoot dry weight of the three maize mutants, including the double mutant.

In addition, cytosolic GS assimilates ammonium in sink tissues, as suggested by the strong expression of the cytosolic GS gene in developing maize seeds (Rastogi *et al.*, 1998), and the fact that it is the major GS isoform in maize immature tassels, cobs and husks (Muhitch, 2003). In these tissues, asparaginase is a possible source of ammonium because it catabolizes asparagine (for a review, see Lea *et al.*, 2007).

Nitrogen remobilization during germination

During germination, the growth of plantlets is dependent on the release of nutrients from the seed storage compounds. Cytosolic GS has been detected in different parts of the embryos of both starch-accumulating seeds, with a protein content of 10–15% of total grain dry weight (Shewry & Halford, 2002), and legumes, which tend to accumulate a larger amount of storage proteins (Duranti & Gius, 1997). Genetic studies in maize (Limami *et al.*, 2002) showed the co-localization of a QTL for a high rate of germination with the *Gln1-3* structural gene. This study also suggested that high levels of GS activity in the germinating seed could lead to faster germination. Several cytosolic GS genes are involved in germination and show specific cellular expression (Cantón *et al.*, 1999; Ávila *et al.*, 2001; Glevarec *et al.*, 2004; Rodriguez *et al.*, 2006). Gómez-Maldonado *et al.* (2004a) proposed that hormonal control, particularly by gibberellic acid, is part of the regulation of cytosolic GS in the early stages of pine development.

Ammonium assimilation during lignin synthesis

The enzyme PAL catalyses the first step of the phenylpropanoid pathway in deaminating phenylalanine, with the subsequent release of ammonium. To maintain an efficient nitrogen economy, this ammonium must be reassimilated back into

amino acids. This is performed by cytosolic GS, which has been shown to co-localize and co-express with PAL in vascular xylem cells (Suárez *et al.*, 2002; Cantón *et al.*, 2005). Studies in rice have also demonstrated the co-localization of cytosolic GS and PAL in the vascular cells of immature leaves (Sakurai *et al.*, 2001). Conifers invest large amounts of carbon in the phenylpropanoid pathway for the synthesis of lignin (Cánovas *et al.*, 2007). The *cis*-acting elements found in the promoter region of the pine *GS1b* gene showed a similarity to those of the promoter of the bean *PAL2* gene (Gómez-Maldonado *et al.*, 2004b). Further analyses demonstrated an interaction between MYB transcription factors and the *cis*-acting elements, suggesting that this was a possible way of co-regulating lignin synthesis and nitrogen reassimilation in pine.

The role of cytosolic GS in nitrogen assimilation and recycling is important for plant growth and seed production

The significance of cytosolic GS in plant growth and development has been emphasized in studies using forward and reverse genetic approaches. A variety of plant species have been modified to overexpress cytosolic GS, following different strategies in terms of promoter and transgene selection. Although the outcomes of these studies have been variable, they imply that cytosolic GS is important for efficient nitrogen assimilation, plant growth and biomass accumulation. Tobacco and poplar plants expressing a transgenic *GS1* under the control of a constitutive promoter showed a decrease in ammonium content in the leaves, indicating a higher rate of nitrogen assimilation (Oliveira *et al.*, 2002; Man *et al.*, 2005). An increase in total leaf soluble protein content was noted in *GS1*-overexpressing poplar plants (Gallardo *et al.*, 1999; Fu *et al.*, 2003; Jing *et al.*, 2004), tobacco (Temple *et al.*, 1993; Oliveira *et al.*, 2002) and *L. japonicus* (Ortega *et al.*, 2004). The effects of *GS1* overexpression were also noticed in transgenic tobacco plants, particularly as these were able to maintain high photosynthetic rates even under limiting nitrogen conditions (Fuentes *et al.*, 2001). The effects of increased cytosolic GS on plant growth were particularly dramatic in poplar plants overexpressing a pine *GS1* gene. In these plants, vegetative growth was increased, leading to an increase in height in both glasshouse and field experiments (Gallardo *et al.*, 1999; Jing *et al.*, 2004). A similar result was obtained with *L. corniculatus* plants overexpressing a soybean *GS1* gene, which senesced earlier than control plants (Vincent *et al.*, 1997).

The impact of GS overexpression on plant biomass is variable, depending on the species studied and the growth conditions. Tobacco and *L. japonicus* plants overexpressing a *GS1* gene under the control of a constitutive promoter showed an increase in total plant fresh and dry weight (Oliveira *et al.*, 2002; Ortega *et al.*, 2004). An increase in dry weight was observed in the roots and grains of wheat plants overexpressing

cytosolic GS, specifically in the leaves, ultimately leading to an increased grain nitrogen yield (Habash *et al.*, 2001). These studies thereby demonstrate that the manipulation of GS produces a range of phenotypes affecting growth, development and nitrogen assimilation. To focus and target the modulation more precisely necessitates cell-specific and developmentally specific promoters for transformation. This remains an open field for research.

Cytosolic GS response to varying environmental conditions and plant nitrogen status

Cytosolic GS responds to nitrogen availability in the external medium, plant nitrogen status (ratio of glutamine to glutamate), light/dark and the presence of abiotic and biotic stressors. Thus far, it has been difficult to establish a global model of GS response to variation in nitrogen availability in both roots and leaves. Although some cytosolic GS gene members are up-regulated by the addition of ammonium, some are down-regulated or do not respond (Sakakibara *et al.*, 1996; Finnemann & Schjoerring, 1999; Ishiyama *et al.*, 2004b; Hirel *et al.*, 2005). These discrepancies could be explained by differences between experimental procedures and/or by species variation in terms of response to nitrogen deficiency and variation in nitrogen sources. Differences in gene number among species could also lead to more complex response patterns in some species. Although most studies have focused on the response to severe nitrogen deficiency, few have examined the effect of steady-state nitrogen deficiency on nitrogen metabolism, which is more relevant to field-grown plants.

Variations in soil nitrogen availability, including the type of nitrogen source and amount, affect plant nitrogen status and the direct availability of ammonium for assimilation at both cellular and organ levels. In nitrogen-repleted oilseed rape, the accumulation of cytosolic GS gene transcripts declined in the roots, leading to enhanced translocation of ammonium to the shoots (Finnemann & Schjoerring, 1999). Ammonium itself regulates cytosolic GS genes at the transcriptional level (Hirel *et al.*, 1987; Kozaki *et al.*, 1992; Sukanya *et al.*, 1994). In soybean, the cooperation of three distinct promoter regions is necessary for ammonium-stimulated expression of the gene (Tercé-Laforgue *et al.*, 1999). The interaction among these three regions may be facilitated by an HMG A-like protein that binds to the proximal and distal promoter regions of the soybean *GS15* gene (Reisdorf-Cren *et al.*, 2002). Plant nitrogen status also alters the transcriptional regulation of cytosolic GS genes by ammonium in the roots (Sonoda *et al.*, 2003; Ishiyama *et al.*, 2004c).

Transcript stability is another means of cytosolic GS regulation in response to nitrogen nutrition (Ortega *et al.*, 2006). However, it is not clear whether plant nitrogen status or nitrate molecules interact with the *cis*-acting element at the 3' end of the *GS1* transcripts. An increased level of cytosolic

GS polypeptide has been measured in the leaves of *A. thaliana* *Wassileskija* accession plants grown under nitrogen-deficient conditions, probably to assimilate ammonium from the catabolism of protein (Lemaître *et al.*, 2008). The capacity of plants to tolerate low nitrogen availability may also be linked to their tolerance to other stresses. For example, *Thellungiella halophila*, a relative of *A. thaliana*, has been shown to be tolerant to salt stress and nitrogen limitation, because of its higher nitrate uptake and assimilation (Kant *et al.*, 2008).

In response to drought or salt stress, the abundance of the *GS2* polypeptide and its activity decline, whereas cytosolic GS tends to increase or maintain the same level in the leaves (Bauer *et al.*, 1997; Lutts *et al.*, 1999; Santos *et al.*, 2004; Martinelli *et al.*, 2007; D. Z. Habash *et al.*, unpublished). Transgenic poplar plants expressing a pine cytosolic GS gene show enhanced water stress resistance compared with controls (El-Khatib *et al.*, 2004). These results suggest that GS over-expression leads to an increased photorespiratory activity, thus providing a protective sink for electrons from photosynthetic reaction centres (although the photoprotective role of photorespiration remains controversial). The response of GS to drought or salt stress in the roots is less clear, with studies in rice seedlings and potatoes showing a decline in GS total activity in response to salt stress (Lutts *et al.*, 1999; Teixeira & Pereira, 2007), and studies in rice and sunflower showing an increased cytosolic GS polypeptide abundance and activity (Santos *et al.*, 2004; Yan *et al.*, 2005). The response of cytosolic GS to osmotic stress is related to the reassimilation of nitrogen from increased amino acid catabolism, and is necessary for the production of proline (Brugière *et al.*, 1999).

Some evidence exists for the induction of either cytosolic GS genes or novel forms of cytosolic GS proteins in infected leaf cells in plants under pathogen attack (Pérez-García *et al.*, 1995, 1998a,b; Oléa *et al.*, 2004; Pageau *et al.*, 2006; Tavernier *et al.*, 2007). Pageau *et al.* (2006) showed that *GS1* was up-regulated in tobacco leaves in response to viral and bacterial attacks, especially when an incompatible reaction was triggered. However, this induction did not depend on a hypersensitive response induction, suggesting that *GS1* shows the same pattern of expression as an early plant defence gene. Similarly, Tavernier *et al.* (2007) showed that *Gln-α* from *P. vulgaris* was up-regulated in response to pathogenic and nonpathogenic strains of fungus, again implying that it responds in the same manner as an early-response defence gene. In the latter study, Tavernier *et al.* (2007) also showed that the expression of *Gln-α* followed that of *PAL3*, perhaps indicating that cytosolic GS was there to reassimilate ammonium from PAL activity. This up-regulation of *Gln-α* was also accompanied by an increase in cytosolic GS polypeptide abundance, but an overall decline in activity, mainly resulting from the decline in chloroplastic GS. The response of cytosolic GS to abiotic and biotic stresses occurred mainly at the transcript abundance levels. However, it is unclear whether an increase in transcription occurs, or whether mRNA transcripts are more stable.

GS is key to adjusting nitrogen assimilation and recycling in response to varied environmental conditions

The coordination and optimal functioning of the metabolic pathways for nitrogen and carbon assimilation in plants are critical in determining plant growth and, ultimately, biomass accumulation (Krapp *et al.*, 2005). GS is a necessary enzyme for nitrogen metabolism, as it catalyses the assimilation of all inorganic nitrogen incorporated into organic compounds, such as proteins and nucleic acids. This reaction is coupled to the formation of glutamate by GOGAT as part of the GS/GOGAT cycle. Although Ferredoxin-GOGAT plays a critical part in the reassimilation of ammonium released by glycine decarboxylase during photorespiration, NADH-GOGAT assimilates ammonium from both primary and secondary sources during nitrogen remobilization (Lea & Mifflin, 2003).

The fundamental role of GS within nitrogen metabolism may explain its ancient origins and justify its presence in both eukaryotes and prokaryotes. In plants, different GS genes have evolved that assimilate ammonium from various sources in different cells and tissues. Cytosolic GS is of particular interest, because it is encoded by many genes and holds many functions within the plant. Genetic modification of a cytosolic GS gene via mutation or transgenesis has led to alterations in whole plant metabolism and phenotypes, particularly plant and seed biomass. However, these effects are not always consistent between studies, probably because of the complex regulation of GS. Continuous research demonstrates that cytosolic GS genes, transcripts and proteins are also responsive to both the plant status and environmental cues. Overall, this suggests that GS may constitute a regulatory point at which environmental signals are integrated and translated into a plant response in terms of growth and seed production.

Although our understanding of cytosolic GS has greatly increased since its discovery, many questions remain: in particular, certain aspects of its regulation are still not fully understood. However, a further understanding of the molecular basis for plant nitrogen uptake/assimilation and its regulation is critical for understanding plant response to fluctuating environmental conditions.

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