Viewpoints

Is plastidic glutamine synthetase essential for C_3 plants? A tale of photorespiratory mutants, ammonium tolerance and conifers

Summary

Agriculture faces the considerable challenge of having to adapt to a progressively changing climate (including the increase in CO_2 levels and temperatures); environmental impact must be reduced while at the same time crop yields need to be maintained or increased to ensure food security. Under this scenario, increasing plants' nitrogen (N) use efficiency and minimizing the energy losses associated with photorespiration are two goals of crop breeding that are long sought after. The plastidic glutamine synthetase (GS2) enzyme stands at the crossroads of N assimilation and photorespiration, and is therefore a key candidate for the improvement of crop performance. The GS2 enzyme has long been considered essential for angiosperm survival under photorespiratory conditions. Surprisingly, in Arabidopsis GS2 is not essential for plant survival, and its absence confers tolerance towards ammonium stress, which is in conflict with the idea that NH_4^+ accumulation is one of the main causes of ammonium stress. Altogether, it appears that the 'textbook' view of this enzyme must be revisited, especially regarding the degree to which it is essential for plant growth under photorespiratory conditions, and the role of NH_4^+ assimilation during ammonium stress. In this article we open the debate on whether more or less GS2 is a desirable trait for plant productivity.

Introduction

The glutamine synthetase/glutamate synthetase (GS/GOGAT) cycle is the pathway for the incorporation of inorganic nitrogen (N) into organic molecules. Glutamine synthetase catalyzes the conversion of glutamate (Glu) and ammonium (NH_4^+) into glutamine (Gln). Then, GOGAT produces two molecules of Glu from Gln and 2-oxoglutarate (Fig. 1). In seed plants, the GS family is composed of the cytosol-localized GS1 and the plastid-localized GS2. In a recent study, it was proposed that GS1 is divided into two evolutionary lineages, named according to their sequence and functional similarity to the gymnosperm GS1s: GS1a-like and GS1b-like (Valderrama-Martín *et al.*, 2022). GS1b is found in all seed plants and GS1a is present exclusively in gymnosperms and basal angiosperms. GS2 is present in all seed plants except conifers and gnetales (Valderrama-Martín *et al.*, 2022). In general, GS1 is encoded by multiple genes, whereas GS2 is, in diploid species, generally encoded by a single gene. For instance, the *Arabidopsis thaliana* genome harbors five GS1 genes (*GLN1-5*) and one gene for GS2 (*GLN2*). Interestingly, some diploid species, such as *Medicago truncatula*, possess a second GS2 gene that is exclusively expressed in seeds (Seabra *et al.*, 2010). Although different GS isozymes exhibit specific functions, GS1 is generally considered to govern primary NH_4^+ assimilation and its reassimilation during N remobilization and translocation. For GS2, its main role is the reassimilation of NH_4^+ released during photorespiration and the assimilation of NH_4^+ derived from nitrite reduction in the plastids (Bernard & Habash, 2009; Thomsen *et al.*, 2014; Hirel & Krapp, 2021). Nitrogen is the major nutrient which limits crop productivity, and research on GS has therefore been extensive, with the aim of improving plants' N use efficiency (NUE) via GS overexpression strategies (James *et al.*, 2018a; Amiour *et al.*, 2021). Among the different isozymes of GS, the genetic manipulation of GS2 has provided contrasting results that have always been interpreted in reference to its function in photorespiration (Table 1). In light of recent results obtained with GS2 mutants, the best strategy for the biotechnological use of GS2 (i.e. increasing or decreasing its expression to improve crop productivity) is a topic of ongoing debate.

Is GS2 necessary for plant survival? A tale of photorespiration and photorespiratory mutants

Mutants lacking GS2 were first isolated in barley by screening a large ethyl methanesulfonate (EMS)-mutagenized population in search of photorespiratory mutants (Blackwell *et al.*, 1987, 1988; Wallsgrove *et al.*, 1987). GS2 mutants showed severe stress symptoms, such as chlorosis and necrosis of the leaves, and finally died when grown under normal air conditions. Indeed, these mutants lacked the ability to reassimilate the NH_4^+ lost during photorespiration and apparently died not because of the toxic buildup of NH_4^+ but rather because of the drain on the organic nitrogen pool (Wallsgrove *et al.*, 1987). In agreement with this explanation, the mutants grew normally under a CO_2-enriched atmosphere, where photorespiration is suppressed, leading the authors to conclude that GS2 is only necessary for plant survival when photorespiration is active. The finding that a plastidial enzyme was responsible for the reassimilation of NH_4^+ produced in the mitochondria by the breakdown of glycine was puzzling. Indeed, due to the high flux of the photorespiratory route, a transporter that permits the import of photorespiratory NH_4^+ into

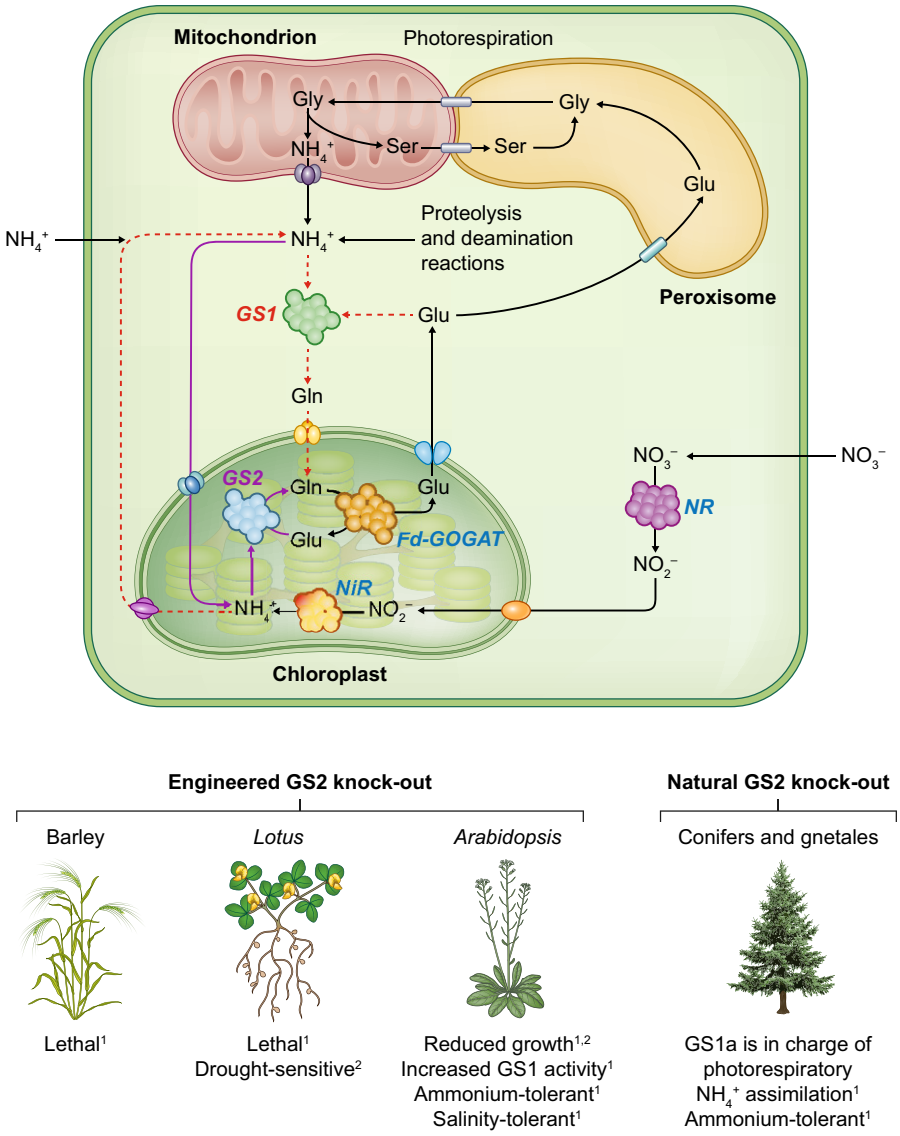

Fig. 1 Schematic illustrating the basics of ammonium assimilation in photosynthetic cells and a summary of the reported effects of the absence of GS2 (in mutants/conifers). Purple solid lines show pathways related to GS2 activity. Red dashed lines show pathways related to GS1 activity. Black solid lines show common pathways. 1 and 2 refer to photorespiratory and nonphotorespiratory conditions, respectively.

the chloroplast is likely needed. However, in spite of many efforts to identify such a transporter, it has not yet been found (Kuhnert *et al.*, 2021).

Extensive screening for photorespiratory mutants was also carried out in Arabidopsis, but mutants lacking GS2 were not isolated (Somerville & Ogren, 1982). Indeed, while mutants from other Arabidopsis photorespiratory enzymes, like Fd-GOGAT, were identified, Arabidopsis *GLN2* mutants were missing for > 35 years after the original screenings. This was especially surprising since GS2 mutants with a severe photorespiratory phenotype were also isolated in the model legume *Lotus japonicus* (Orea *et al.*, 2002). The hypotheses that have been put forward in various attempts to explain the Arabidopsis GS2 enigma included, among others, a possible lethal phenotype of this mutation, and the presence of compensatory cytosolic GS activity (reviewed by Lam *et al.*, 1996). Finally, an Arabidopsis *GLN2* mutant was described by Ferreira *et al.* (2019). The mutant showed lower growth under normal air (photorespiratory active conditions), but surprisingly did not show the strong and finally lethal phenotype observed in other species, and was even able to complete its life cycle. These results were also confirmed by Hachiya *et al.* (2021). Altogether, these observations break a long-standing paradigm and demonstrate that GS2 function is not essential for plant survival in normal air, since, unlike *L. japonicus* and barley, GS2 absence in Arabidopsis is not lethal.

Photorespiration is an energetically expensive process that may have a strong impact on crop yields. At present, photorespiration is estimated to reduce US soybean and wheat production by up to 36% and 20%, respectively (Walker *et al.*, 2016). In spite of the differences in the phenotypes reported for GS2 mutants from different species (Fig. 1; Table 1), there is no doubt that this enzyme has a central role in photorespiration. In a future climate change scenario, elevated CO_2 will probably reduce

Table 1 Catalogue of mutant and transgenic plant phenotypes engineered for higher or lower GS2 expression.

Species	Transgenic plant/mutant	Growth conditions	Phenotype	Reference
Hordeum vulgare cv Maris Mink	KO (azide)	Control conditions NPC	Lethal Similar to WT	Wallsgrove *et al*. (1987) Blackwell *et al*. (1988)
H. vulgare cv Maris Mink	KO (GS2) × KO (Fd-GOGAT) (azide)	Control conditions NPC	Lethal Similar to WT	Blackwell *et al*. (1988)
Nicotiana tabacum	Overexpression 35S:NtGS2 Co-suppression 35S:NtGS2	High-intensity light	Increased tolerance Increased sensitivity	Kozaki & Takeba (1996)
Oryza sativa cv Kinuhikari	Overexpression 35S:OsGS2 Co-suppression 35S:OsGS2	Salinity Cold stress Salinity	Increased tolerance Increased tolerance Increased sensitivity	Hoshida *et al*. (2000)
N. tabacum line SR1	Overexpression rbcS:NtGS2	Control conditions	Increased growth	Migge *et al*. (2000)
Lotus japonicus	KO (EMS)	Control conditions NPC	Lethal Similar to WT	Orea *et al*. (2002)
Brassica napus	Reduced expression 35S: antisense BnGS2	Control conditions	Similar to WT	Husted *et al*. (2002)
N. tabacum line SR1	Co-suppression 35S:PsGS2	Control conditions	Reduced growth and chlorosis	Oliveira *et al*. (2002)
Arabidopsis thaliana Col-0	Overexpression 35S:DvGS2[1]	Control conditions Low N	Increased growth Increased growth	Zhu *et al*. (2014)
O. sativa cv Zhongua 11	Co-overexpression pOsAct1:PsGS1 + pZmUbi1:PsGS2	Phosphinothricin	Enhanced resistance	Sun *et al*. (2005a)
O. sativa cv. Zhongua 11	Co-overexpression pOsAct1:PsGS1 + pZmUbi1:PsGS2	N deficiency	Enhanced growth	Sun *et al*. (2005b)
Triticum aestivum	Co-overexpression pOsAct1:PsGS1 + pZmUbi1:PsGS2	Phosphinothricin	Enhanced resistance	Huang *et al*. (2005)
L. japonicus	KO (EMS)	Drought in NPC	Increased sensitivity	Díaz *et al*. (2010)
O. sativa cv Zhongua 11	Co-suppression 35S:OsGS2	Control conditions	Reduced growth and chlorosis	Cai *et al*. (2010)
N. tabacum cv Xanthi	Overexpression rbcS:AtGS2 Co-overexpression rbcS:AtGS2 + rbcS:Dof1;7	Low-N condition	Increased growth Increased growth relative to rbcS:AtGS2	Wang *et al*. (2013)
T. aestivum cv Ji5265	Expression of GS2 allele from Xiaoyan 54 pTaGS2-2Ab:TaGS2-2Ab	High-N field trial Low-N field trial	Increased growth and yield Increased growth and yield	Hu *et al*. (2018)
O. sativa cv Nipponbare	Co-overexpression with GS1;1 pOsAct1:OsGS2 + pOsAct2 OsGS1;1	Osmotic stress Salinity Drought Phosphinothricin	Increased tolerance Increased tolerance Increased tolerance Enhanced resistance	James *et al*. (2018b)
N. tabacum cv K326	Overexpression SP: TaGS2	Control conditions N starvation	Similar to WT Similar to WT	Wei *et al*. (2018)
A. thaliana Col-0	Knocked-out (T-DNA)	Normal air Salinity NPC	Reduced growth and chlorosis Increased tolerance Reduced growth	Ferreira *et al*. (2019)
N. tabacum cv K326	Overexpression SP: TaGS2	Drought	Increased tolerance	Yu *et al*. (2020)
A. thaliana Col-0	Co-suppressed 35S:AtGS2 Knocked-out (T-DNA)	Control conditions Control conditions Ammonium stress	Reduced growth, no chlorosis Reduced growth, no chlorosis Increased tolerance	Hachiya *et al*. (2021)

At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Dv, *Dunaliella viridis*; EMS, ethyl methanesulfonate mutagenesis; KO, knockout; NPC, non-photorespiratory condition; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Ps, *Pisum sativum*; SP, super promoter; Ta, *Triticum aestivum*; WT, wild-type; Zm, *Zea mays*.
[1]*DvGS2* is a homologue of the *GLN2* gene from the green algae *Chlamydomonas reinhardtii* and thus corresponds to a different evolutionary lineage than GS2 from seed plants (Valderrama-Martín *et al*., 2022).

photorespiratory rates (Walker *et al*., 2016), and it is possible that high levels of GS2 might not be strictly necessary. The introduction of different photorespiratory bypasses that avoid NH_4^+ release from glycine breakdown have been successful in increasing plant yield (Shen *et al*., 2019; Cavanagh *et al*., 2022). Although bypassing GS2 will certainly reduce photorespiratory ATP losses, it will also prevent the protective role of this cycle under stress conditions. How photorespiration will evolve in the future and whether more or less GS2 is a desirable trait for plant productivity are still open questions.

Arabidopsis GLN2 mutants reveal that plastidic shoot NH_4^+ assimilation may be detrimental to plants' ammonium tolerance

Although NH_4^+ is an essential intermediate for N incorporation into biomolecules, when plants are exposed to high soil NH_4^+ concentrations they often display stress symptoms that include growth retardation and leaf chlorosis. When severe, these symptoms may even lead to plant death (Britto & Kronzucker, 2002). Obtaining crops that exhibit optimal performance under

ammonium nutrition is of great interest, since boosting ammonium-based nutrition, as opposed to nitrate-based nutrition, has the benefit of mitigating NO_3^- leaching and N_2O greenhouse gas emission (Subbarao & Searchinger, 2021). The cause of ammonium stress-derived symptoms is multifactorial and includes, among other factors, oxidative stress, pH alterations, energetic trade-offs and cation imbalance. In general, the excessive NH_4^+ accumulation in tissues has been conventionally accepted to be the main trigger underlying plants' sensitivity to ammonium stress. In agreement with this idea, the promotion of NH_4^+ assimilation has been generally shown to act as a tolerance-promoting mechanism, and mutants defective in NH_4^+ assimilation showed enhanced sensitivity towards ammonium nutrition, such as the Arabidopsis *gln1;1:gln1;2, gln1;2,* and *gln1;2:gln1;3* GS1 mutants (Guan *et al.*, 2016; Konishi *et al.*, 2017), the rice *OsGS1;1* knockout mutant (Kusano *et al.*, 2011), and *Arabidopsis* mutants defective in *NADH-GOGAT* (Konishi *et al.*, 2014). Surprisingly, Arabidopsis *GLN2* mutant plants, in addition to being able to survive under normal air conditions, were also more tolerant to ammonium stress than wild-type plants (Hachiya *et al.*, 2021). In agreement with our understanding of GS2 function, *GLN2* mutant plants accumulated very high quantities of NH_4^+. Obviously, these observations are in conflict with the idea that NH_4^+ is a toxic molecule when present at high concentrations, and they indicate that NH_4^+ assimilation, rather than NH_4^+ accumulation, may be responsible for the sensitivity of Arabidopsis to ammonium nutrition. Likewise, Poucet *et al.* (2021) also reported that NH_4^+ accumulation in the leaves of tomato plants grown under ammonium nutrition was dependent on leaf phenological stage and was not correlated to their reduced growth compared to leaves of plants grown with nitrate (NO_3^-). Hachiya *et al.* (2021) described the phenotype of *GLN2* mutants in relation to the prevention of shoot acidification associated with plastidic proton (H^+) release during excessive NH_4^+ assimilation by GS2. Indeed, this acidification did not occur in *gln1;2:gln1;3* mutant plants (Hachiya *et al.*, 2021). These observations lead to a number of key questions which need to be answered in order to advance our understanding of plants' metabolic adaptation to ammonium stress. First, it appears that the localization of NH_4^+ assimilation – plastidic in the shoot vs cytosolic in the root – is associated with different functions, in terms of coping with ammonium stress. Among other observations, the contrasting response between *GLN1* and *GLN2* mutants (i.e. *GLN1* mutants are sensitive to ammonium stress) implies the existence of a root-specific mechanism to efficiently deal with H^+ release that is yet to be elucidated. In addition, it might be possible that the toxic effect of NH_4^+ overaccumulation is dependent on its subcellular localization, a hypothesis that needs to be explored. Finally, it should not be forgotten that NO_3^- assimilation, but not NH_4^+, depends on the reducing power exported from the chloroplast through the malate valve driven by the flux through the photorespiratory pathway (Shi & Bloom, 2021). If photorespiratory levels are reduced under a future climate scenario, plants that use NH_4^+ as a primary N source might have an advantage over plants that depend on NO_3^-. Should this be the case, a reduction in GS2 levels (less need for photorespiratory capacity and probably increased ammonium tolerance) potentially appears to be a win–win approach.

Can gymnosperms shed light on the need for GS2?

Conifers and gnetales lack GS2 (Valderrama-Martín *et al.*, 2022) but they have high photorespiratory rates (Hanawa *et al.*, 2017). As an alternative to GS2, gymnosperms possess GS1a, which, although cytosolic, performs a GS2-like function, namely photorespiratory NH_4^+ reassimilation (Cánovas *et al.*, 2007). Indeed, as is the case with GS2, GS1a is mainly expressed in photosynthetic tissues, and its gene expression is light-dependent (Cantón *et al.*, 1999; Valderrama-Martín *et al.*, 2022). This fact, together with the findings regarding *GLN2* mutants in Arabidopsis (Ferreira *et al.*, 2019; Hachiya *et al.*, 2021), demonstrate that GS subcellular location is not essential for plant survival, but probably for the coordination of GS gene expression with photosynthesis and photorespiration, as is the case for GS1a in conifers. In fact, Arabidopsis *GLN2* mutants exhibit increased expression of *GLN1;2* and *GLN1;3* (Ferreira *et al.*, 2019).

Primary forest soils are generally acidic, with low nitrification rates, and NH_4^+ is therefore the dominant form of N. In agreement, most conifers take up NH_4^+ preferentially and are tolerant to ammonium nutrition (Cui & Song, 2007; Kronzucker *et al.*, 1997). However, recent findings have demonstrated that mature conifer trees can also assimilate NO_3^- efficiently in natural conditions (Zhou *et al.*, 2021). As described in the previous section, the absence of GS2 confers upon Arabidopsis tolerance to ammonium stress. Since the reduction of NO_3^- takes place in the plastids, we can hypothesize that when NO_3^- reduction is low, a plastidic GS2 is not essential. This hypothesis is in agreement with the enhanced ammonium tolerance observed in Arabidopsis *GLN2* mutants (Hachiya *et al.*, 2021). Thus, the absence of GS2 in gymnosperms might be an evolutionary mechanism to promote growth in NH_4^+-rich habitats. This hypothesis must be studied through the analysis of chloroplast pH control in conifers and the production of conifer transgenic lines overexpressing a plastidic GS in different photorespiratory and nutritional conditions.

Is GS2 overexpression a strategy to increase yield?

Increasing plastidic GS expression has been shown to generally be beneficial for plant growth (Table 1) by improving, among other traits, those related to N use (Zhu *et al.*, 2014; Hu *et al.*, 2018). In addition, GS activity and expression are known to be enhanced by a number of abiotic stresses (Bernard & Habash, 2009; James *et al.*, 2018b). In particular, GS2 overexpression lines have generally shown increased tolerance to abiotic stresses (Table 1). One hypothesis that may explain the beneficial effect of GS2 overexpression is related to photorespiration acting as an electron-sink that would dissipate excessive reducing power, thus protecting against the production of reactive oxygen species and conferring photoprotection (Kozaki & Takeba, 1996; Betti *et al.*, 2016; James *et al.*, 2018b). Alternatively, abiotic stresses are known to increase processes that promote NH_4^+ release, such as protein degradation.

Thus, a higher GS activity would be beneficial for the synthesis of osmolytes such as proline or polyamines. In agreement with this idea, *L. japonicus* GS2 mutant plants showed low proline synthesis and drought sensitivity (Díaz *et al.*, 2010), and rice plants co-overexpressing *OsGS1;1/OsGS2* accumulated osmolytes and showed enhanced tolerance to drought and salinity (James *et al.*, 2018b). Again, Arabidopsis appears to be an exception, and in a study by Ferreira *et al.* (2019), Arabidopsis *GLN2* mutants displayed enhanced tolerance to salinity, but the metabolic adjustment associated with GS2 absence was not studied in this work. However, Hachiya *et al.* (2021) observed proline accumulation in Arabidopsis GS2 mutants under ammonium stress, which might explain the phenotypes reported by Ferreira *et al.* (2019). However, it remains unclear why proline levels in the context of GS2 expression levels are species-dependent.

What to breed for? More or less GS2 activity?

Climate-resilient crops are needed to maintain agricultural productivity, meaning that crops adapted to constantly increasing atmospheric CO_2 conditions that are also able to deal with higher temperatures and water scarcity are required. In addition, the impact of agriculture on the environment should be minimized, and one aim should be a reduction in the loss of N, which is often > 50% of the amount of applied N (Socolow, 1999; Coskun *et al.*, 2017). Thus, breeding crops with a higher NUE is desirable. In addition, the use of ammonium-based nutrition combined with nitrification inhibition is of great interest, but this approach demands that crop plants are better adapted to the use of NH_4^+ as their main source of N (Coskun *et al.*, 2017; Marino & Moran, 2019; Subbarao & Searchinger, 2021). GS2 stands at a crossroads of different processes, such as photorespiration, N assimilation and stress tolerance; therefore, breeding to alter GS2 expression levels represents a promising but also debatable strategy, especially in light of the conflicting data obtained for GS2 mutants and overexpression lines that have been summarized in this article (Table 1). Indeed, GS2 overexpression has generally been considered to be a means by which higher NUE can be achieved (Zhu *et al.*, 2014; Hu *et al.*, 2018). However, recent reports in Arabidopsis showed enhanced growth of GS2 mutants under ammonium nutrition, and this finding, together with the fact that ammonium-tolerant conifers lack GS2, suggests that plastidic GS2 absence may represent a benefit for plant performance when grown under ammonium nutrition. The future rise in atmospheric CO_2 concentrations should reduce photorespiration, which is assumed to be positive for plant productivity (Walker *et al.*, 2016). However, elevated temperatures and increasingly frequent episodes of drought will also influence photorespiration (Betti *et al.*, 2016), making it difficult to predict how photorespiratory activity will change in the future. These puzzling results open a debate regarding the interest of breeding for more or for less GS2 in order to optimize crop yield and quality. Interestingly, it appears that in conifers and Arabidopsis photorespiratory NH_4^+ could be assimilated by alternative enzymes, such as cytosolic GS. In this sense, engineering crops with slightly modified levels of this enzyme may be a promising approach. Besides, another promising strategy to be explored is breeding for increased GS1 expression while minimizing GS2 expression. Altogether, more research is needed to fully understand GS2 function in different plant species. For example, the role of GS2 under ammonium nutrition must be studied in species other than Arabidopsis in order to observe whether this phenotype is exclusive to Arabidopsis or conserved in other species from the Brassicaceae and in other plant families. Obviously, a rapid approach could be to grow the available GS2 mutants or co-suppressed lines under nonphotorespiratory conditions with NH_4^+ as the main source of N. While it is evident that there are several layers of complexity to the GS2 puzzle due to the involvement of this enzyme in several key processes, we believe that the recent advances pave the way to newly promising strategies for crop improvement by engineering GS2 expression.

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Author contributions

DM conceived the article; DM, RAC and MB wrote the manuscript.

ORCID

Marco Betti https://orcid.org/0000-0002-7334-5734
Rafael A. Cañas https://orcid.org/0000-0001-9727-5585
Daniel Marino https://orcid.org/0000-0002-8788-6646

Daniel Marino[1,2]*, Rafael A. Cañas[3] and Marco Betti[4]

[1]Department of Plant Biology and Ecology, University of the Basque Country (UPV/EHU), E-48940 Leioa, Spain;
[2]Ikerbasque, Basque Foundation for Science, E-48011 Bilbao, Spain;
[3]Integrative Molecular Biology Lab, Universidad de Málaga, Campus Universitario de Teatinos, 29071 Málaga, Spain;
[4]Departamento de Bioquímica Vegetal y Biología Molecular, Facultad de Química, Universidad de Sevilla, 41012 Sevilla, Spain
(*Author for correspondence: email daniel.marino@ehu.eus)

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