Is plastidic glutamine synthetase essential for C₃ 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 CO₂ 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 NH₄⁺ 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 NH₄⁺ 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 (NH₄⁺) 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 NH₄⁺ assimilation and its reassimilation during N remobilization and translocation. For GS2, its main role is the reassimilation of NH₄⁺ released during photorespiration and the assimilation of NH₄⁺ 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 NH₄⁺ lost during photorespiration and apparently died not because of the toxic buildup of NH₄⁺ 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 CO₂-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 NH₄⁺ 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 NH₄⁺ into...
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 CO₂ will probably reduce
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.

1DsG2 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 NH4+ 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.

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

| Species                  | Transgenic plant/mutant                        | Growth conditions | Phenotype                     | Reference                      |
|--------------------------|-----------------------------------------------|------------------|-------------------------------|--------------------------------|
| *Hordum vulgare* cv Maris Mink | KO (azide)                                    | Control conditions NPC | Lethal | Wallsgrove et al. (1987) |
| *N. tabacum* cv Maris Mink | KO (GS2) × KO (Fd-GOGAT) (azide)              | Control conditions NPC | Lethal | Blackwell et al. (1988)  |
| *Nicotiana tabacum*      | Overexpression 35S:NtGS2                       | High-intensity light | Increased tolerance | Kozaki & Takeba (1996) |
| *Oryza sativa* cv Kinuhikari | Overexpression 35S:OsGS2                      | Cold stress | Increased tolerance | Hoshida et al. (2000) |
| *N. tabacum* line SR1    | Overexpression rbcS:NtGS2                     | Control conditions NPC | Increased growth | Migge et al. (2000) |
| *Lotus japonicus*        | KO (EMS)                                       | Control conditions NPC | Lethal | Orea et al. (2002)   |
| *Brassica napus*         | Reduced expression 35S: antisense BnGS2       | Control conditions | Reduced growth, no chlorosis | 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                      | Control conditions | Increased growth | Zhu et al. (2014) |
| *O. sativa* cv Zhongua 11 | Co-overexpression pOsAct1:PsGS1 + pZmUbi1:PsGS2 | Low N | Phosphinothricin | 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:OsGS1 + pZmUbi1:PsGS2 | KO (EMS) | Enhanced resistance | Huang et al. (2005) |
| *L. japonicus*           | Co-suppression 35S:OsGS2                      | Drought in NPC | Increased sensitivity | Diaz et al. (2010)  |
| *N. tabacum* Xanthi      | Overexpression rbcS:AtGS2                     | Control conditions | Reduced growth and chlorosis | Cai et al. (2010)  |
| *N. tabacum* cv Xanthi   | Co-overexpression rbcS:AtGS2 + rbcS:Dof1;7    | Low-N condition | Increased growth | Wang et al. (2013)  |
| *T. aestivum* cv JI5265  | Expression of GS2 allele from Xiaoyan 54      | High-N field trial | Increased growth and yield | Hu et al. (2018)   |
| *O. sativa* cv Niiponbare | Co-overexpression with GS1;1                  | Low-N field trial | Increased growth and yield | James et al. (2018b) |
| *N. tabacum* cv JI5265  | Overexpression SP: TaGS2                     | Osmotic stress | Increased tolerance | Wei et al. (2018)  |
| *A. thaliana* Col-0      | Knocked-out (T-DNA)                           | Salinity          | Increased tolerance | Ferreira et al. (2019) |
| *N. tabacum* cv JI5265  | Overexpression SP: TaGS2                     | Control conditions | Reduced growth and chlorosis | Yu et al. (2020)   |
| *A. thaliana* Col-0      | Knocked-out (T-DNA)                           | Control conditions | Reduced growth, no chlorosis | Hachiya et al. (2021) |

*Arabidopsis GLN2* mutants reveal that plastidic shoot NH4+ assimilation may be detrimental to plants’ ammonium tolerance

Although NH4+ is an essential intermediate for N incorporation into biomolecules, when plants are exposed to high soil NH4+ 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$_2$O 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, Pouget 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 photosynthetic 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 (Diaz 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 CO2 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 greater interest, but this approach demands that crop plants are better adapted to the use of NH4+ 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 CO2 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 NH4+ 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 NH4+ 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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