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REVIEW

Emerging insights into nitrogen assimilation in gymnosperms

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Abstract

Key message **The current status of molecular regulation of nitrogen assimilation and recent advances made in gymnosperms are reviewed.**

Abstract Gymnosperms are a heterogeneous and ancient group of seed plants that includes conifers, ginkgos, cycads and gnetophytes. Molecular studies on extant gymnosperms have been constrained by some discouraging features for experimental research such as their long life cycles, large sizes, complex megagenomes and abundant phenolic compounds in their woody tissues. However, the development of high-throughput sequencing and refined multiomics technologies in the last few years has allowed to explore the molecular basis of essential processes in this ancient lineage of plants. Nitrogen is one of the main limiting factors determining vascular development and biomass production in woody plants. Therefore, nitrogen uptake, metabolism, storage and recycling are essential processes for fundamental gymnosperm biology. Here, recent progress in the molecular regulation of nitrogen assimilation in gymnosperms is reviewed and some future perspectives on this topic are outlined.

Keywords Seed plants · Gymnosperms · Conifers · Nitrogen acquisition · Nitrate · Ammonium · Glutamine synthetase · GS isoenzymes

Introduction

Gymnosperms (*Gymnospermae*) include a wide range of vascular and seed-producing plants that diverged from angiosperms approximately 300 million years ago (Clarke et al. 2011). Currently, this ancient and widespread plant lineage comprises approximately 1000 different species (Wang and Ran 2014), a number considerably lower than the 370,000 species estimated for angiosperms (Kew 2016). It was initially thought that they represented a group of relict plants, however, recent findings indicate that gymnosperms have been subjected to pulses of extinction and expansion throughout their evolution and possibly still are occurring today (Davis and Schaefer 2011). In fact, it has been estimated that 40% of the species are threatened and at high risk of extinction, which is a great concern for conservation science (Forest et al. 2018). Gymnosperms comprise four main lineages of seed plants: conifers, cycads, ginkgos and gnetophytes (Crisp and Cook 2011).

Within gymnosperms, conifers constitute the most numerous and representative group both from an economic and ecological point of view, consisting of more than 600

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species with a wide distribution worldwide (Farjon 2017). Conifers cover vast extensions of land mainly in the Northern Hemisphere, where they are the main constituents of the forests of North America and Eurasia. Therefore, conifers are of extraordinary ecological relevance because they play an essential role in global carbon fixation and the maintenance of biodiversity. In contrast, the areas occupied by this group of seed-bearing land plants are much smaller in the Southern Hemisphere, although paradoxically, it is in these particular regions of the planet where the greatest biodiversity of coniferous species is found (Farjon 2017).

The distribution of conifers shows great versatility in terms of adaptation, with species located both at sea level (Burban and Petit 2003) and in the Himalayas (Farjon 2018). Conifers are very often present in suboptimal climates and/or poor soils for the growth of most plant species, mainly angiosperms, likely reflecting an environmental adaptation developed throughout their long evolutionary history (Farjon 2018). One important feature supporting their wide distribution is the symbiotic interaction established between the roots of conifers and fungi, forming ectomycorrhizal systems that can enhance the capacity to capture water and nutrients by up to ten thousand times (Taylor and Alexander 2005; Martin et al. 2016; Farjon 2018). From an economic point of view, conifers are the largest contributors of raw material for wood industries worldwide, as they provide higher yields due to better growth and more predictable wood shapes and sizes compared to angiosperms (Farjon 2018). It is also worth mentioning the use of conifers as ornamental plants, a practice initiated during the XIX century, and the demand for seeds of different types of conifers to decorate gardens and parks.

Most gymnosperms are woody plants with long lifespans and life cycles, large sizes, and the ability to produce large amounts of phenolic secondary metabolites (De la Torre et al. 2014; Cañas et al. 2019). All these characteristics have hampered molecular biology studies in members of this ancient lineage of plants. In addition, the enormous magnitude and the highly repetitive nature of their genomes (15–30 Gb) have largely limited the structural and functional genomics of gymnosperms (De La Torre et al. 2014; Cañas et al. 2019). The increasing capacity and lower costs of next generation sequencing (NGS) technologies after the completion of the human genome project have facilitated the characterization of the genomes of a growing number of plant species and have also paved the way for the genome assembly of several conifer species, such as *Picea abies* (Nystedt et al. 2013), *Pinus taeda* (Zimin et al. 2014), *Picea glauca* (Warren et al. 2015) and *Pinus lambertiana* (Stevens et al. 2016). The recent development of single-molecule sequencing technologies such as PacBio and Oxford Nanopore has provided much longer reads, overcoming the inherent difficulties for the characterization of highly repetitive gymnosperm megagenomes. These advanced techniques in combination with conventional NGS have permitted the genome assemblies of an increased number of gymnosperm species during the last few years covering representatives not only for conifers (Scott et al. 2020; Xiong et al. 2021; Niu et al. 2022; Neale et al. 2022) but also for cycads (Liu et al. 2022), ginkgo (Liu et al. 2021) and gnetophytes (Wan et al. 2021). All these genomic resources are of paramount significance for the development of functional genomics studies that will be able to provide new insights into fundamental plant biology from an evolutionary perspective. These new advances have recently been used to further study the molecular basis of nitrogen (N) assimilation and metabolism in gymnosperms. In this work, a review of studies in this research area is presented and discussed.

Nitrogen availability for plant nutrition

N is an essential element as constituent of the main biomolecules of paramount importance for life, such as nucleic acids and proteins as well as a wide range of primary and secondary metabolites (Kishorekumar et al. 2020). In natural environments, N is one of the main limiting factors for plant growth determining raw material production and crop yield (Hirel and Krapp 2021). Moreover, N is also involved in plant architecture and resistance against environmental stresses (Kishorekumar et al. 2020). Although N is the most abundant element in the atmosphere, only diazotrophic microorganisms, either free-living or associated with plants, can fix atmospheric dinitrogen (N_2) (Sharma et al. 2021). Therefore, the major sources of N for plants are found in soil (Shafreen et al. 2021), where a substantial part of the available N is indeed provided by microorganisms (Courty et al. 2015; Verzeaux et al. 2017).

In soils, N can be found in both organic and inorganic forms (Fig. 1). Plants have evolved N uptake mechanisms to adapt to their own environments, thus allowing the incorporation of organic N compounds such as peptides and amino acids and inorganic N molecules such as nitrate and ammonium (Hirel and Krapp 2021; Farzadfar et al. 2021). Furthermore, some of these mechanisms allow plants to uptake N from soils that are poor in nitrogenous compounds, while others protect them from toxicity due to eventual high N availabilities (Nacry et al. 2013). Several studies have shown that, especially under low levels of inorganic N, plants can efficiently take up organic N such as amino acids or peptides (Moran-Zuloaga et al. 2015). Indeed, the production rates of organic N compounds have been found to be higher than the rates from N mineralization in forest ecosystems (Näsholm et al. 2009). Nevertheless, the high competition for these organic N forms, mostly used by microorganisms, together

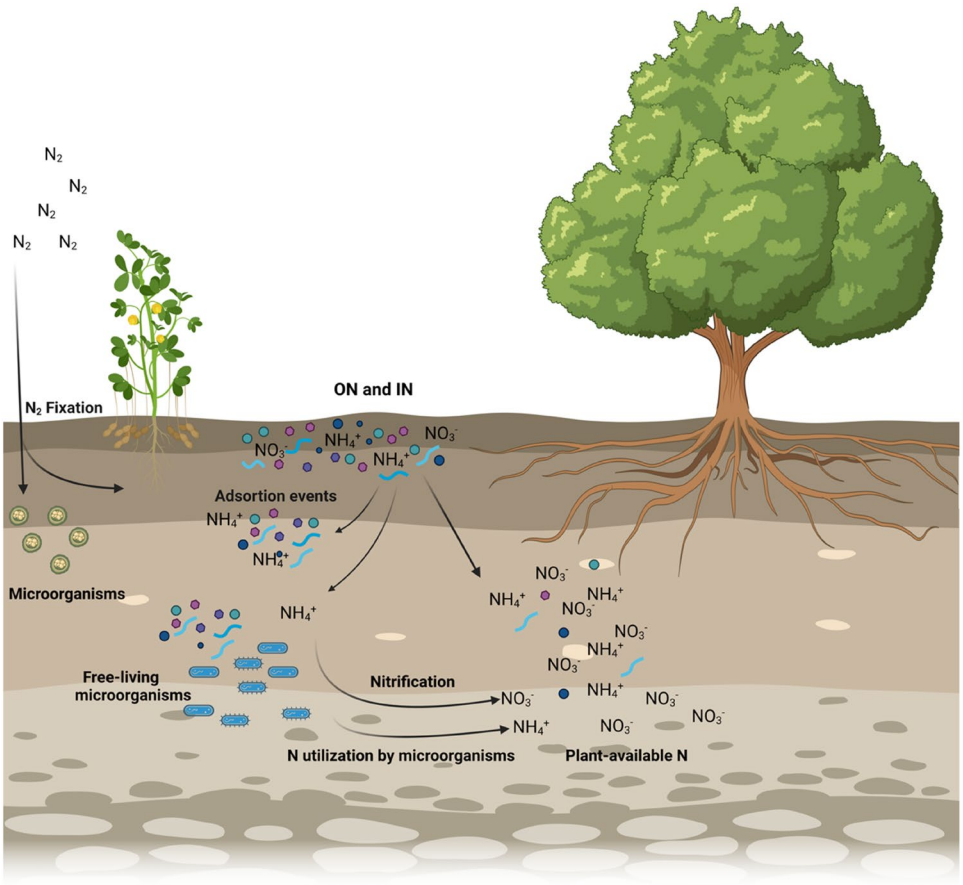

Fig. 1 Plant-available N sources. *ON* organic N, *IN* inorganic N

with the adsorption process by the soil, does not allow plants to fulfill their requirements, even though those compounds are a great source of N for different plant species in several ecosystems (Näsholm et al. 2009; Franklin et al. 2017; Lim et al. 2022).

Nitrate and ammonium are the two main forms of inorganic N in soils, mostly produced by microbial mineralization of organic compounds (Bernard and Habash 2009). The relative abundance of these substrates in soil depends on several factors, such as environmental conditions and the chemical nature of the soil (Esteban et al. 2016), thus producing a concentration range that goes from micromolar to molar (Britto and Kronzucker 2006).

The importance of N in crop production led to the "Green Revolution" after the Second World War, when the use of N fertilizer in agriculture suffered a huge increase to keep enough food incoming for a constantly growing population (Galloway et al. 2013; Nacry et al. 2013). The massive use of fertilizers has a negative impact on the biosphere and environment. Over the 50–75% of the N supplied by fertilizers is lost by leaching into the soil or released as N gases to the atmosphere (Hirel et al. 2011; Cameron et al. 2013). Excess N can also lead to eutrophication of aquatic and terrestrial habitats (Harding et al. 2019), an increase of N reactive species input into the environment (Canfield et al. 2010) and other environmental problems (Hirel et al. 2011), which have repercussions on the biodiversity of the ecosystems (de Graaf et al. 1998).

Therefore, the study of the mechanisms involved in plant N use efficiency (NUE) has gained increased importance in recent decades, particularly those focusing on crop yield improvement, as well as the attenuation of the environmental impact due to extensive agricultural activities (Liu et al. 2022). Biotechnological advances, together with crop and forest management strategies, are required for this purpose.

Assimilation of nitrate and ammonium

In plants, any form of inorganic N is first reduced to ammonium and then assimilated into organic molecules. Nitrate is first reduced to nitrite in the cytosol by nitrate reductase (NR, EC 1.7.1.1) using NADH as reductant. Afterward, nitrite is reduced to ammonium by nitrite reductase (NiR, EC 1.7.7.1), a plastid-located ferredoxin-dependent enzyme (Hirel and Krapp 2021). As nitrite and ammonium are toxic molecules for the plant, their assimilation must be well coordinated in response to N demand and supply (Wang et al.

2018). Ammonium is assimilated by glutamine synthetase (GS, EC 6.3.2.1) to produce glutamine in an ATP-dependent reaction (Heldt and Piechulla 2011). Finally, glutamate synthase (GOGAT, EC 1.4.1.14; EC 1.4.7.1) uses glutamine, along with 2-oxoglutarate and reducing power, in the form of either of ferredoxin (Fd) or NADH, to produce two molecules of glutamate. One of these two molecules will be used as a substrate of the GS/GOGAT cycle, and the other molecule is the net product of the reactions of these two enzymes during N assimilation (Fig. 2) (Bernard and Habash 2009; García-Gutiérrez et al. 2018).

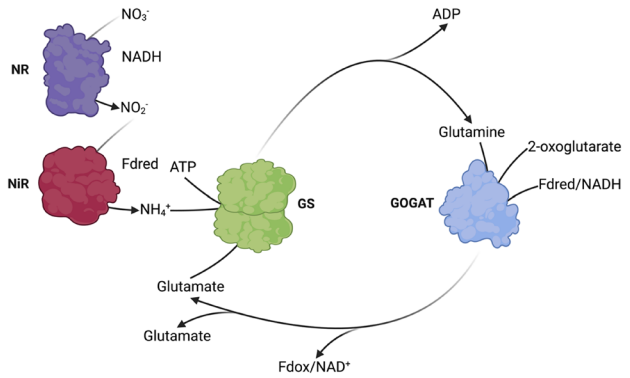

Fig. 2 Scheme of the nitrate reduction and ammonium assimilation by the glutamine synthetase/glutamate synthase cycle. Nitrate reductase (NR); nitrite reductase (NiR); glutamine synthetase (GS); glutamate synthase (GOGAT)

The GS/GOGAT cycle is the major pathway for the incorporation of inorganic N into organic N (Hirel and Krapp 2021). Some studies using ^{15}N and mutants deficient in GS and GOGAT have shown that 95% of ammonium in plants is assimilated via GS (Lea and Ireland 1999). This cycle is also one of the main links between carbon and N metabolism as it allows the assimilation of N into carbon skeletons using 2-oxoglutarate directly provided through the Krebs cycle (Hirel and Krapp 2021). Glutamine and glutamate are used as precursors for the biosynthesis of all N-containing molecules in plants, such as amino acids, proteins, nucleic acids, chlorophylls and secondary metabolites (Fig. 3) (Forde and Lea 2007; Bernard and Habash 2009). In addition, these amino acids are also used to transport organic N to developing and storage organs (Tegeder and Masclaux-Daubresse 2018). Therefore, the GS/GOGAT cycle has a main role in NUE, emphasizing GS activity, whose complex regulation and importance in N remobilization, yield, grain production and growth rate have been reiterated by some studies focused on quantitative trait loci (QTL) and using different types of crop plants exhibiting contrasting NUE (Gallais and Hirel 2004; Obara et al. 2004; Habash et al. 2007: Fontaine et al. 2009; Cañas et al. 2012; Kaminski et al. 2015). Consequently, GS probably remains the most studied enzyme in terms of NUE enhancement in monocot and dicot plants, but also in trees (Castro-Rodríguez et al. 2016a; Hirel and Krapp 2021) (Fig. 3).

In plants, there are different isoforms of both enzymes. Regarding GOGAT, two different plastid-located isoenzymes

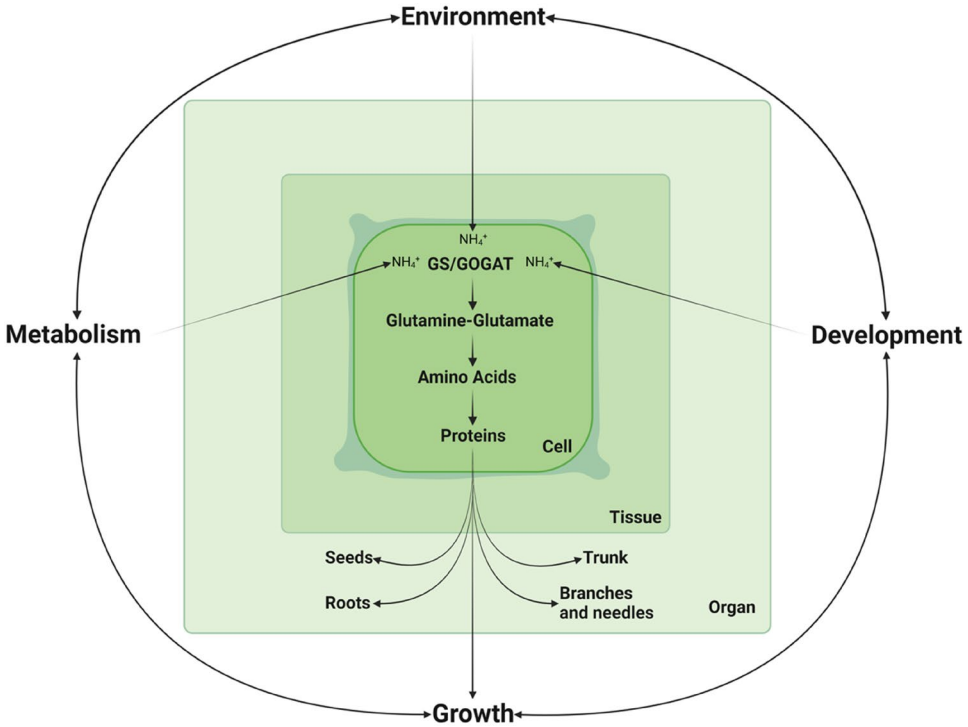

Fig. 3 Involvement of glutamine synthetase in different functional processes of trees. *GS* glutamine synthetase, *GOGAT* glutamate synthase

can be found, NADH-GOGAT and Fd-GOGAT. These enzymes play a key role in primary N assimilation and N recycling. Fd-GOGAT mainly acts in photosynthetic tissues, whereas NADH-GOGAT acts in nonphotosynthetic tissues (Bernard and Habash 2009; García-Gutiérrez et al. 2018). In conifers, independent of the N source taken up from soil, ammonium is the ultimate form to be incorporated into amino acids through GS1a and Fd GOGAT in photosynthetic tissues, and GS1b and NADH-GOGAT in nonphotosynthetic tissues (Avila et al. 2022a). The identification of a glutamine translocator in isolated chloroplasts from maritime pine (*P. pinaster*) supports the function of the compartmentalized GS/GOGAT cycle in conifer cells (Claros et al. 2010).

Glutamine synthetase: a key enzyme in nitrogen metabolism of land plants

Studies over the past two decades have provided a very deep understanding of the GS phylogeny. Three GS superfamilies have been identified thus far, namely, GSI, GSII and GSIII, and all these enzymes are differentiated by the number of subunits, molecular size and kingdom distribution (Goshroy et al. 2010). The GSI superfamily is predominantly present in prokaryotes, although its presence in mammals and plants has also been reported (Mathis et al. 2000; de Carvalho Fernandes et al. 2022). The GSII superfamily was described as a characteristic group of *Eukarya* and some *Bacteria*, such as *Proteobacteria* and *Actinobacteria* (James et al. 2018). However, this superfamily appears also to be present in *Euryarchaeota*, a phylum of the domain *Archea*, in public sequence databases. Finally, the GSIII superfamily has been found and described in bacteria, including cyanobacteria (James et al. 2018), and some eukaryotes such as diatoms and other heterokonts, thus suggesting the presence of GSIII gene in the nucleus of early eukaryotes (Robertson et al. 2006). Different works support that these GS superfamilies appeared prior to the divergence of prokaryotes and eukaryotes (Robertson et al. 2006).

In plants, GS activity is carried out by members of the GSII superfamily (James et al. 2018), which have been described to be holoenzymes with octameric and decameric tridimensional structures in different organisms (Eisenberg et al. 2000; Llorca et al. 2006; Unno et al. 2006; Krajewski et al. 2008; He et al. 2009). Two main GSII clades have been identified in the *Viridiplantae* group: eukaryotic origin GSII (GSIIe) and eubacterial origin GSII (GSIIb). It has been hypothesized that GSIIb arose as a result of horizontal gene transfer (HGT) that took place after the prokaryote and eukaryote divergence, which indeed constitutes a sister group with GSII from γ-proteobacteria (Ghoshroy et al. 2010). Regarding GSIIe, its importance in plant growth and development has led to extensive studies of these enzymes in vascular plants, particularly in crops including trees (Plett et al. 2017; Cánovas et al. 2018; Mondal et al. 2021).

It is generally indicated that angiosperms present two groups of nuclear *GSIIe* genes, one coding for cytosolic proteins (GS1) and a second coding for a plastidic GS (GS2), each playing nonredundant physiological roles within the plant (Ghoshroy et al. 2010; Hirel and Krapp 2021). Usually, there are different cytosolic isoforms encoded by a small multigene family, while only one nuclear gene encodes the plastid isoform (James et al. 2018) with some exceptions such as in *M. truncatula* and poplar (*Populus trichocarpa*) where multiple *GS2* genes have been detected (Seabra et al. 2010; Castro-Rodríguez et al. 2011).

Phylogenetic analyses suggested that *GS2* may have arisen as a result of a gene duplication of a *GS1* gene (Biesiadka and Legocki 1997) 300 million years ago, before the divergence between monocotyledons and dicotyledons (Bernard and Habash 2009). Interestingly, the presence of a *GS2* was reported in the gymnosperms *Ginkgo biloba* (García-Gutiérrez et al. 1998) and *Cycas revoluta* (Valderrama-Martín et al. 2022), but no biochemical, molecular or microscopic analysis has allowed the detection of plastid isoforms in conifers (Cánovas et al. 2007; Avila et al. 2022a) and no *GS2* genes have been found in their fully sequenced genomes (Nystedt et al. 2013; Zimin et al. 2014; Warren et al. 2015; Stevens et al. 2016; Neale et al. 2017; Scott et al. 2020). Instead, conifers present two well-differentiated families of cytosolic GS isoforms, GS1a and GS1b, with different molecular and kinetic properties (Ávila-Sáez et al. 2000; de la Torre et al. 2002; Avila et al. 2022a). These GS1 lineages are found in all gymnosperms and in basal groups of angiosperms and Magnoliidae, such as *Amborella trichopoda* and *Magnolia grandiflora* (Valderrama-Martín et al. 2022).

In gymnosperms and angiosperms, both the synthesis and relative activity of each GS isoenzyme are species specific, but its expression is also regulated according to nutritional status, tissue, developmental stages, and environmental conditions (Cánovas et al. 2007; Bernard and Habash 2009; Mondal et al. 2021) in which transcription factors play a key role (Thomsen et al. 2014). Many *cis*-acting elements have been identified as potential binding sites of distinct transcription factors regulating the expression of plant GS genes (Mondal et al. 2021). In pine *(Pinus pinaster)*, several members of the Myb family (Myb 1, Myb 4 and Myb8) are involved in the transcriptional regulation of the *GS1b* gene during lignification (Gómez-Maldonado et al. 2004b; Craven-Bartle et al. 2013). In this context, it is also interesting to mention that a single transcription factor, PpDof 5, differentially regulates the genes encoding GS1a and GS1b isoforms suggesting that the correct spatial distribution of the isoforms in the tree is transcriptionally controlled (Rueda-López et al. 2008).

A wide variety of regulatory mechanisms at different levels have been observed on GS (Fig. 4), thus showing the precise and complex regulation of this enzyme. The transcription rate of *GS* isogenes from angiosperms (Oliveira and Coruzzi 1999) and gymnosperms (Cantón et al. 1999; Gómez-Maldonado et al. 2004a) has been shown to be affected by light, and by carbon levels (Oliveira and Coruzzi 1999). N metabolites such as glutamate can directly upregulate GS1 gene expression (Masclaux-Daubresse et al. 2005) as well as the cellular ratio of glutamine/glutamate (Watanabe et al. 1997). Furthermore, nitrate positively regulates GS2 expression in the leaves of maize (*Zea mays*) (Sakakibara et al. 1997) and ammonium has been previously reported to upregulate GS1 expression in the roots of maritime pine (Ortigosa et al. 2022). At the same time, nitrate seems to reduce the accumulation of GS1 transcripts in leaves at the posttranscriptional level (Ortega et al. 2001). Indeed, the GS1 transcript accumulation in *Medicago sativa* by N/C metabolites has been shown to be mediated through the 3′UTR of these transcripts (Ortega et al. 2006). However, studies suggest that is probably glutamine or a product of glutamine metabolism, but not nitrate, the molecule mediating this 3′UTR-turnover (Simon and Sengupta-Gopalan 2010). In addition to regulation at the transcriptional level, GS has also been reported to be regulated by different protein modifications such as oxidation (Ortega et al. 1999), phosphorylation and binding to 14-3-3 proteins (Finnemann and Schjoerring 2000; Lima et al. 2006a, b) and tyrosine nitration (Melo et al. 2011), which produce different effects on GS isoenzymes (Fig. 4). Recently, it has been shown that GS2 phosphorylation specifically regulates plant growth and defense (Ding et al. 2022).

GS2 gene encodes a 44–45 kDa polypeptide, which is indeed larger than the GS1 polypeptide (38–40 kDa), mainly due to the presence of the plastid targeting peptide in the N-terminus and a C-terminal extension

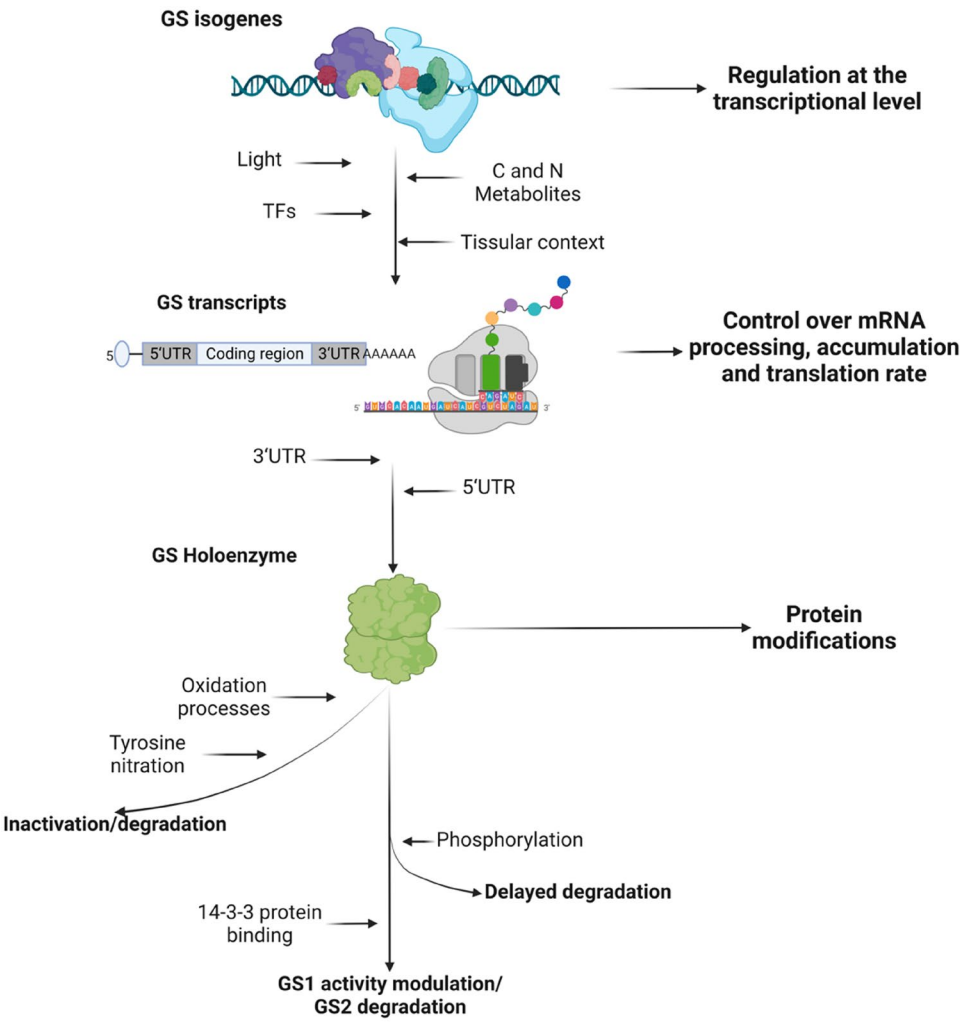

Fig. 4 GS regulatory mechanism. Factors such as light, metabolites, even the cellular type or the tissue could determine which *GS* gene is going to be transcribed and the rate of transcription. The 3′UTR is also responsible for the regulation of this enzyme at the post-transcriptional level. The 5′UTR of the mRNA determines the context of the start codon and it is believed to bind different mRNA, determining this way the translation rate. Post-translational protein modifications such as phosphorylation of serine residues, nitration of cysteine residues and nitration of tyrosine residues, determines de activity of the different isoforms or mark them for degradation

of approximately 16 amino acids. The function of this C-terminal extension remains unknown, but it has been described to be important for the GS2-glutamate interaction in *Medicago truncatula* (Ferreira et al. 2017). This isoform is mainly expressed in photosynthetic tissues of angiosperms associated with the chlorophyll-containing parenchyma (Castro-Rodríguez et al. 2015). This expression pattern suits his role in the assimilation of ammonium from photorespiration and nitrate reduction (Tegeder and Masclaux-Daubresse 2018).

On the other hand, although GS1 isoform expression is mainly related to vascular tissues, the expression patterns of the different GS1 isoenzymes cover the entire plant (Lea and Miflin 2018). GS1 isoforms are predominantly implicated in primary N assimilation in roots, remobilization, and recycling (Thomsen et al. 2014). These isoforms have also been described as a key component of plant NUE with roles in processes such as senescence (Thomsen et al. 2014), amino acid catabolism and different stress responses (Bernard and Habash 2009). Moreover, the direct implication of some GS1 isoenzymes in developmental processes such as grain and biomass production has been demonstrated in many plant species (Martin et al. 2006; Jing et al. 2004; Funayama et al. 2013; Bao et al. 2014; Guan et al. 2015; Urriola and Rathore 2015; Cánovas et al. 2018; Gao et al. 2019; Ji et al. 2019; Wei et al. 2021; Fujita et al. 2022).

Previous works on *GS1a* in conifers have determined a similar expression pattern of this gene to that exhibited by *GS2* in other plants. This cytosolic enzyme has also been found to be associated with the chlorophyll-containing parenchyma of photosynthetic organs (Ávila et al. 2001) and, as well as *GS2* from angiosperms, its expression is upregulated by light (Cantón et al. 1999; Gómez-Maldonado et al. 2004a). In this sense, *GS1a* has been proposed to fulfill the role of *GS2* in gymnosperms (Cantón et al. 1999; Valderrama-Martín et al. 2022). For its part, conifer *GS1b* is phylogenetically more related to cytosolic isoforms from angiosperms than to coniferous *GS1a* (Ávila Sáez et al. 2000). The GS1b isoform is ubiquitously expressed, and its expression is also related to vascular tissues (Ávila et al. 2001). GS1b has been proposed to play a role in N remobilization between source and sink organs during the active growth period (Suárez et al. 2002). Furthermore, studies in pine suggest a role of GS1b in the canalization of ammonium to glutamine during embryogenesis, seed germination and early developmental stages of seedlings (Ávila et al. 2001), a hypothesis that is supported by its expression patterns in different developmental stages of zygotic and somatic pine embryos (Pérez-Rodríguez et al. 2005; Avila et al. 2022b). The upregulated expression of *GS1b* in reaction wood, together with its association with vascular tissues, indicates that this gene is also involved in ammonium reassimilation during lignin biosynthesis (Cantón et al. 2005; Craven-Bartle et al. 2013). Recently, a new GS1b (GS1b.2) isoenzyme has been identified and characterized that appears to be restricted to the *Pinus* and *Picea* genera (Valderrama-Martin et al. 2023).

GS1b in angiosperms is encoded by a multigene family, while GS1b in conifers is usually encoded by a single gene (James et al. 2018). However, the identification of an additional variant in *Pinus* and *Picea* suggests that a diversification process of *GS1b* genes (*GS1b.1* and *GS1.b2*) is currently ongoing in this group of gymnosperms. In some angiosperms, the expansion of this family has led to different isoforms with overlapping functions within the plant (Castro-Rodríguez et al. 2015). In pine, *PpGS1b.2* probably arose via gene duplication of the already well-characterized *GS1b.1* gene (Valderrama-Martin et al. 2023). Based on localized *PpGS1b.2* expression, a role in developing tissues has been proposed for this enzyme, similar to some GS1b isoforms in other plants (Bao et al. 2014; Guan et al. 2015; Urriola and Rathore 2015; Gao et al. 2019; Ji et al. 2019; Wei et al. 2021; Fujita et al. 2022). Taken together, these findings imply evolutionary convergence since a new GS1b isoform arose in a process of neofunctionalization likely to meet different metabolic needs, independent of the GS1b from angiosperms.

Notably, even though these two GS1b paralogs (GS1b.1 and GS1.b2) are quite similar in protein sequence, they present differences in their molecular characteristics (Valderrama-Martin et al. 2023). Therefore, both enzymes have been shown to present differences in their thermostability, with GS1b.1 being more thermostable than GS1b.2. Although these isoenzymes showed optimal activity at 42 ºC, both reached their maximum activity at different pH levels (6.5 and 6 for GS1b.1 and GS1b.2, respectively). Moreover, regarding the behavior toward substrates and the kinetic properties, they have also been shown to present differences. Considering all the above results, two hypotheses have been suggested: (a) GS1b.2 could supplement GS1b.1 activity in developing tissues that exhibit a high demand for glutamine; (b) GS1b.2 could play a specific role, still unknown, in certain developing tissues. *In silico* studies over the promoter have also revealed differences in the regulatory region of *PpGS1b.1* and *PpGS1b.2* also supporting this idea (Valderrama-Martin et al. 2023). Nevertheless, additional comparative studies of both isogenes including the functional characterization of their regulatory regions will be required to shed light on this topic.

Evolution of glutamine biosynthesis in seed plants

GS is a key gene in N metabolism in the vast majority of organisms, as can be inferred by its wide distribution in all kingdoms (Ghoshroy et al. 2010). It is also one of the oldest known genes, which is why some authors have described it as a molecular clock with excellent potential for phylogenetic analyses (Pesole et al. 1991).

Numerous reports have been made about the evolution of glutamine synthetase, not only in plants but also regarding the evolution of this gene family within eukaryotes (Ghoshroy et al. 2010). Phylogenetic analyses of different protein and nucleic acid sequences have allowed a better understanding of the evolution of this family of genes in seed plants (Valderrama-Martín et al. 2022). The results of these analyses clearly suggest the occurrence of 3 well-differentiated GS families within seed plants: GS1a, GS1b, and GS2 (Valderrama-Martín et al. 2022). This hypothesis is also supported by phylogenetic analyses carried out including GS1b.2 sequences (Valderrama-Martín et al. 2023). The emergence of tracheophytes during the evolution of land plants likely led to the evolution of a cytosolic GS (GS1) located in vascular cells able to synthesize glutamine for N transport and N distribution to different tissues and organs. Another role of this GS1 enzyme should be the reassimilation of the ammonium released in lignin biosynthesis, quantitatively the most important metabolic fate of phenylalanine biosynthesis in trees (Craven-Bartle et al. 2013; Pascual et al. 2016; El-Azaz et al. 2022). A duplication of this ancestral gene and neofunctionalization of young duplicates could possibly lead to the appearance of GS1b and GS1a isoforms in gymnosperms (Fig. 5).

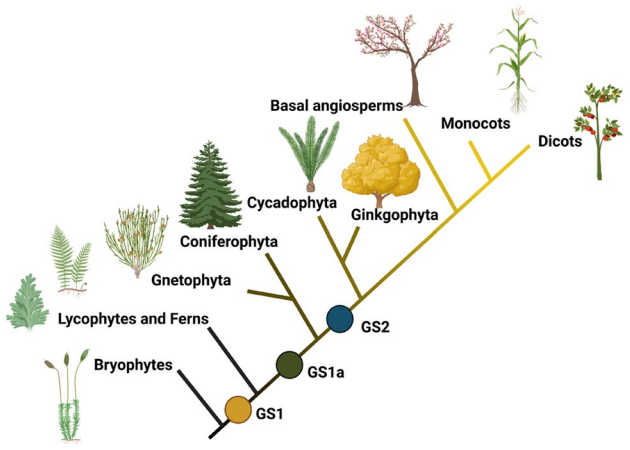

Fig. 5 Proposed scheme for the evolution of GS isoforms in land plants. *GS1* cytosolic GS, *GS1a* cytosolic GS located in photosynthetic cells, *GS2* chloroplastic GS

GS1a is specifically located in the cytosol of conifer photosynthetic cells (García-Gutiérrez et al. 1998; Ávila et al. 2001), and it was proposed to be involved in the reassimilation of ammonium released from photorespiration and the primary assimilation of ammonium from nitrate reduction (Cánovas et al. 2007; Avila et al. 2022a), therefore fulfilling the role of GS2 in angiosperms (Avila et al. 2022a; Valderrama-Martín et al. 2022). On the other hand, it is well established that enzymes in the GS1b group have a role in primary assimilation, reassimilation and remobilization of N, as well as in developmental processes, amino acid catabolism, senescence and stress response (Thomsen et al. 2014; Ji et al. 2019; Wei et al. 2021; Fujita et al. 2022).

Recent reports have noted that the groups of *Cycadopsida* and *Ginkgoopsida* may form a monophyletic clade in gymnosperms (Wu et al. 2013; One Thousand Plant Transcriptomes Initiative 2019). In line with this, the new insights into nitrogen assimilation reported here support this assumption, but in addition, these data could indicate that the monophyletic clade conformed by *Cycadopsida* and *Ginkgoopsida* may be more related to angiosperms than previously thought (Valderrama-Martín et al. 2022). The most parsimonious hypothesis resulting from phylogenetic analyses indicates that the *GS2* gene must have been absent in a common ancestor of gymnosperms and angiosperms and probably evolved, as a result of a duplication of the *GS1a* gene and the acquisition of a plastid transit peptide, in a common ancestor of *Cycadopsida*/*Ginkgoopsida* and angiosperms (Fig. 5). This hypothesis also fits well with the overlapping roles previously proposed for GS1a and GS2 (Cantón et al. 1999; Avila et al. 2022a).

Nitrogen assimilation and subcellular localization of glutamine biosynthesis

The presence of this enzyme in the chloroplast confers additional advantages to the plant since more efficient nitrate assimilation likely allowed angiosperms to colonize new ecological niches that were rich in nitrate, different from those ecosystems populated by conifers in which ammonium was the major N source. In concordance, although conifers are widely distributed throughout the world, these plants are mostly present in boreal zones where temperature hinders nitrification. Moreover, several studies have previously reported a preference of conifers for ammonium over nitrate (Warren and Adams 2002; Boczulak et al. 2014; Ortigosa et al. 2020). However, recent studies have shown that the concentrations of both nitrate and ammonium are equalized with depth in the soils, and adult trees of these species can use nitrate as well as ammonium (Zhou et al. 2021). In addition, there are gymnosperm species such as *P. pinaster*, that are autochthonous from warm places where nitrification

can take place and soils are probably richer in nitrate. Indeed, previous works have shown a strict regulation of nitrate uptake (Ortigosa et al. 2020), which is interesting considering the fact that the NRT3 (component of the nitrate high-affinity transport system) family has been expanded in this conifer species and that this phenomenon has not taken place in angiosperms (Castro-Rodríguez et al. 2017). Unfortunately, the above studies are restricted to conifers and no similar information is available for other gymnosperms. In this context, it would be of great interest to know the nutritional preference of cycads and ginkgo for nitrate and ammonium to determine how the assimilation of inorganic N is related to the appearance of GS in plastids. Likewise, the investigation of the NRT3 family in the different groups of gymnosperms deserves particular attention to further understand the molecular basis of N assimilation in this ancient lineage of plants.

Independent of whether the presence of a plastid GS will be an evolutionary advantage in plants or not, the following question arises: why do conifers and gnetophytes conserve GS1a and not present a plastid GS isoform? The recent report of toxicity phenotypes related to GS activity in the chloroplast may shed light on the above question. Studies carried out by Hachiya et al. (2021) in *Arabidopsis* suggested a toxic effect resulting from excessive ammonium assimilation in the chloroplast by GS2, probably linked to the acidification effect related to this enzyme activity. The homeostasis of the pH in the chloroplast is essential and excessive acidification of the stroma could lead to problems with the photosynthetic apparatus (Kuvykin et al. 2009; Tikhonov 2013), which is necessary to produce the reducing power required for N and carbon assimilation. Therefore, the assimilation of ammonium in the cytosol of conifer photosynthetic cells would prevent these toxic effects.

In line with this, a conflict between nitrate assimilation and CO_2 fixation has been previously discussed (Bloom 2015). Rubisco is the enzyme in charge of catalyzing the combination of CO_2 and ribulose-1,5-bisphosphate (RuBP) to produce two molecules of 3-phosphoglycerate (3-PGA), which are further used for the biosynthesis of fructose-6-phosphate. However, this enzyme also catalyzes a second reaction where O_2 is used instead of CO_2 producing one molecule of 3-PGA and one molecule of 2-phosphoglycolate (2-PG), thus leading to the restoration of RuBP from 2-PG in a process considered to be energy-wasting and releasing ammonium that should be efficiently reassimilated: photorespiration (Eisenhut et al. 2015, 2019) (Fig. 6). Although the increase in CO_2 concentration in the atmosphere produces a consequent decrease in photorespiration, which would be considered favorable, several works have reported that C3 plants are impaired in their ability to assimilate nitrate (Bloom et al. 2012). Different hypotheses stand up to this conflict, nevertheless, the hypothesis that best fits the decrease in sugar content under nitrate nutrition would be a competence for reducing power between the NR and NiR enzymes and the enzymes from the Calvin–Benson Cycle (Bloom 2015). Thus, the photorespiration process somehow acts as a regulator of the cellular redox

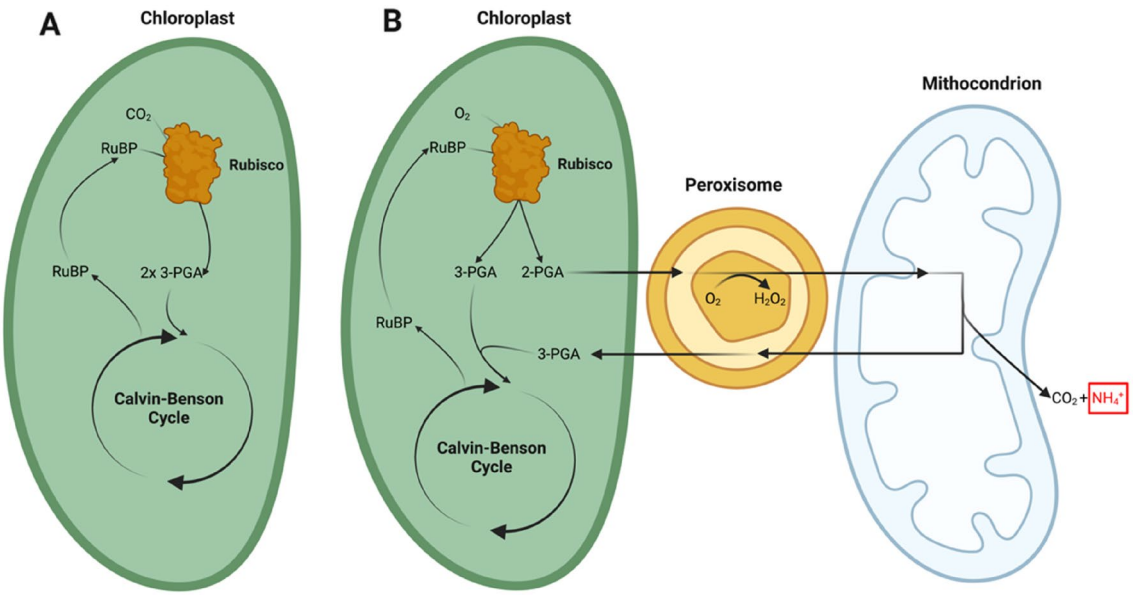

Fig. 6 Schematic representation of the carboxylase (**A**) and oxygenase activity of the rubisco enzyme and the photorespiratory process through the chloroplast, peroxisome and mitochondria (**B**). Ribulose-1,5-bisphosphate (RuBP); 3-phosphoglycerate (3-PGA); 2-phosphoglycolate (2-PG). Ammonium released in the mitochondria (red square) should be efficiently reassimilated by GS either in the cytosol (gymnosperms) or in the chloroplast (angiosperms). Otherwise nitrogen deficiency would occurs

homeostasis allowing nitrate assimilation to occur. This phenomenon takes place both in gymnosperms and angiosperms. In gymnosperms, ammonium released in photorespiration will be assimilated via GS1a in the cytosol, while in angiosperms, this ammonium needs to be transported to the chloroplast and assimilated via GS2. It is unknown whether ammonium exchange across the inner membrane envelope is a passive or active process (Eisenhut et al. 2015). However, the pine ammonium transporters (AMT) lack a plastid target peptide and are specifically located in the plasma membrane of both root and leaf cells (Castro-Rodríguez et al. 2016b). Similarly, it is also unclear how nitrite is transported into the chloroplast for NiR activity, an essential step for nitrate assimilation. The elucidations of the mechanisms involved in the transport of nitrite and ammonium are therefore potential areas deserving further investigation efforts.

Taken together, the above data suggest that the subcellular localization of the GS enzyme, one of the main links between carbon and N metabolism, may be relevant for the metabolic regulation of photorespiration and inorganic N assimilation. Bloom (2015) also showed that those plants irrigated with ammonium did not present any growth problems under high CO_2 or low O_2 conditions when compared with controls, thus supporting a conflict with nitrate assimilation. The high amounts of reducing power required in nitrate assimilation can also explain differences in biomass in pine seedlings irrigated with ammonium and with nitrate. Ortigosa et al. (2020) showed a higher accumulation of biomass in plants irrigated with ammonium than in those irrigated with nitrate. Metabolite analyses showed a decrease in the amount of carbohydrates in plants irrigated with nitrate when compared with plants irrigated with ammonium, which could be associated with a limited availability of carbon for such metabolic processes due to the higher requirement of reducing power by nitrate reduction. In contrast, pine seedlings irrigated with ammonium presented considerably higher concentrations of sugars such as sucrose, D-fructose and D-glucose (Ortigosa et al. 2020). Free sugars are required for N assimilation to provide carbon skeletons, thus explaining the occurrence of high levels of these monosaccharides and disaccharides when ammonium is the main N source since it is directly assimilated in roots. Nitrate is mainly stored long-term in the vacuoles and photoassimilated in smaller quantities at a time, avoiding the excessive consumption of reducing power and therefore requiring lower levels of carbon skeletons. In this respect, the preference of conifers for ammonium nutrition over nitrate nutrition may be due to a more efficient assimilation of ammonium itself, a preference that is caused by the higher tolerance of these plants for ammonium.

Author contribution statement JMVM, RAC and FMC have written the initial version of the manuscript; FO, FRC and CA made additional contributions and edited the text.

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Declarations

Conflict of interest The authors have no conflict of interest.

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References

Avila C, Suárez MF, Gómez-Maldonado J, Cánovas FM (2001) Spatial and temporal expression of two cytosolic glutamine synthetase genes in Scots pine: functional implications on nitrogen metabolism during early stages of conifer development. Plant J 25:93–102

Avila C, Cañas RA, de la Torre FN, Pascual MB, Castro-Rodríguez V, Cantón FR, Cánovas FM (2022a) Functional genomics of Mediterranean pines. In: De La Torre A (ed) The pine genomes. Compendium of plant genomes. Springer, Cham, Berlin, p 254

Avila C, Llebrés MT, Castro-Rodríguez V, Lobato-Fernández C, Trontin JF, Harvengt L, Cánovas FM (2022b) Identification of metabolic pathways differentially regulated in somatic and zygotic embryos of maritime pine. Front Plant Sci 13:877960

Avila Sáez C, Muñoz-Chapuli R, Plomion C, Frigerio J, Cánovas FM (2000) Two genes encoding distinct cytosolic glutamine synthetases are closely linked in the pine genome. FEBS Lett 477:237–243

Bao A, Zhao Z, Ding G, Shi L, Xu F, Cai H (2014) Accumulated expression level of cytosolic glutamine synthetase 1 gene (*OsGS1;1* or *OsGS1;2*) alter plant development and the carbon–nitrogen metabolic status in rice. PLoS One 9(4):e95581

Bernard SM, Habash DZ (2009) The importance of cytosolic glutamine synthetase in nitrogen assimilation and recycling. New Phytol 182:608–620

Biesiadka J, Legocki AB (1997) Evolution of the glutamine synthetase gene in plants. Plant Sci 128:51–58

Bloom AJ (2015) Photorespiration and nitrate assimilation: a major intersection between plant carbon and nitrogen. Photosynth Res 123:117–128
Bloom AJ, Asensio JS, Randall L, Rachmilevitch S, Cousins AB, Carlisle EA (2012) CO_2 enrichment inhibits shoot nitrate assimilation in C3 but not C4 plants and slows growth under nitrate in C3 plants. Ecology 93:355–367
Boczulak SA, Hawkins BJ, Roy R (2014) Temperature effects on nitrogen form uptake by seedling roots of three contrasting conifers. Tree Physiol 34:513–523
Britto DT, Kronzucker HJ (2006) Futile cycling at the plasma membrane: a hallmark of low-affinity nutrient transport. Trends Plant Sci 11:529–534
Burban C, Petit RJ (2003) Phylogeography of maritime pine inferred with organelle markers having contrasted inheritance. Mol Ecol 12:1487–1495
Cameron KC, Di HJ, Moir JL (2013) Nitrogen losses from the soil/plant system: a review. Ann Appl Biol 162:145–173
Cañas RA, Quilleré I, Gallais A, Hirel B (2012) Can genetic variability for nitrogen metabolism in the developing ear of maize be exploited to improve yield? New Phytol 194:440–452
Cañas RA, Pascual MB, de la Torre FN, Ávila C, Cánovas FM (2019) Resources for conifer functional genomics at the omics era. In: Cánovas FM (ed) Advances in botanical research, vol 89. Academic Press, Elsevier, pp 39–76
Canfield DE, Glazer AN, Falkowski PG (2010) The evolution and future of Earth's nitrogen cycle. Science 330:192–196
Cánovas FM, Avila C, Cantón FR, Cañas RA, de la Torre F (2007) Ammonium assimilation and amino acid metabolism in conifers. J Exp Bot 58:2307–2318
Cánovas FM, Cañas RA, de la Torre FN, Pascual MB, Avila C (2018) Nitrogen metabolism and biomass production in forest trees. Front Plant Sci 9:1449
Cantón FR, Suárez MF, Josè-Estanyol M, Cánovas FM (1999) Expression analysis of a cytosolic glutamine synthetase gene in cotyledons of Scots pine seedlings: developmental, light regulation and spatial distribution of specific transcripts. Plant Mol Biol 40:623–634
Cantón FR, Suárez MF, Cánovas FM (2005) Molecular aspects of nitrogen mobilization and recycling in trees. Photosynth Res 83:265–278
Castro-Rodríguez V, García-Gutiérrez A, Canales J, Avila C, Kirby EG, Cánovas FM (2011) The glutamine synthetase gene family in *Populus*. BMC Plant Biol 11:119
Castro-Rodríguez V, García-Gutiérrez A, Cañas RA, Pascual B, Avila C, Cánovas FM (2015) Redundancy and metabolic regulation of the glutamine synthetase gene family in poplar. BMC Plant Biol 15:20
Castro-Rodríguez V, García-Gutiérrez A, Canales J, Cañas RA, Kirby EG, Avila C, Cánovas FM (2016a) Poplar trees for phytoremediation of high levels of nitrate and applications in bioenergy. Plant Biotechnol J 14:299–312
Castro-Rodríguez V, Assaf-Casals I, López-Tienda J, Avila C, Miller AJ, Cánovas FM (2016b) Deciphering the molecular basis of ammonium uptake and transport in maritime pine. Plant Cell Environ 39:1669–1682
Castro-Rodríguez V, Cañas RA, de la Torre F, Pascual MB, Avila C, Cánovas FM (2017) Molecular fundamentals of nitrogen uptake and transport in trees. J Exp Bot 68:2489–2500
Clarke JT, Warnock RC, Donoghue PC (2011) Establishing a time-scale for plant evolution. New Phytol 192:266–301
Claros MG, Aguilar M, Cánovas FM (2010) Evidence for an operative glutamine translocator in chloroplasts from maritime pine (*Pinus pinaster* Ait.) Cotyledons. Plant Biol 12:717–723
Courty PE, Smith P, Koegel S, Redecker D, Wipf D (2015) Inorganic nitrogen uptake and transport in beneficial plant root-microbe interactions. CRC Crit Rev Plant Sci 34:4–16
Craven-Bartle B, Pascual MB, Cánovas FM, Avila C (2013) A myb transcription factor regulates genes of the phenylalanine pathway in maritime pine. Plant J 74:755–766
Crisp MD, Cook LG (2011) Cenozoic extinctions account for the low diversity of extant gymnosperms compared with angiosperms. New Phytol 192:997–1009
Davis CC, Schaefer H (2011) Plant evolution: pulses of extinction and speciation in gymnosperm diversity. Curr Biol 21:995–998
de Carvalho Fernandes G, Turchetto-Zolet AC, Pereira Passaglia LM (2022) Glutamine synthetase evolutionary history revisited: tracing back beyond the last universal common ancestor. Evolution 76:605–622
de Graaf MCC, Bobbink R, Roelofs JGM, Verbeek PJM (1998) Differential effects of ammonium and nitrate on three heathland species. Plant Ecol 135:185–196. https://doi.org/10.1023/A:1009717613380
de la Torre F, Cañas RA, Pascual MB, Avila C, Cánovas FM (2014) Plastidic aspartate aminotransferases and the biosynthesis of essential amino acids in plants. J Exp Bot 65:5527–5534
De la Torre A, Birol I, Bousquet J, Ingvarsson P, Jansson S, Jomes SJM, Keeling CI, MacKay J, Nilsson O, Ritland K, Street N, Yanchuk A, Zerbe P, Bohlmann J (2014) Insights into conifer giga-genomes. Plant Physiol 166:1724–1732
Ding S, Lv J, Hu Z, Wang J, Wang P, Yu J, Foyer CH, Shi K (2022) Phytosulfokine peptide optimizes plant growth and defense via glutamine synthetase GS2 phosphorylation in tomato. EMBO J e111858. https://doi.org/10.15252/embj.2022111858
Eisenberg D, Gill HS, Pfluegl GM, Rotstein SH (2000) Structure-function relationships of glutamine synthetases. Biochim Biophys Acta 1477:122–145
Eisenhut M, Hocken N, Weber APM (2015) Plastidial metabolite transporters integrate photorespiration with carbon, nitrogen, and sulfur metabolism. Cell Calcium 58:98–104
Eisenhut M, Roell MS, Weber APM (2019) Mechanistic understanding of photorespiration paves the way to a new green revolution. New Phytol 223:1762–1769
El-Azaz J, Cánovas FM, Barcelona B, Avila C, de la Torre FN (2022) Deregulation of phenylalanine biosynthesis evolved with the emergence of vascular plants. Plant Physiol 188:134–150
Esteban R, Ariz I, Cruz C, Moran JF (2016) Review: mechanisms of ammonium toxicity and the quest for tolerance. Plant Sci 248:92–101. https://doi.org/10.1016/j.plantsci.2016.04.008
Farjon A (2017) A handbook of the world's conifers 2 vols. Brill Academic Pub, Leiden, p 1154
Farjon A (2018) The Kew review: conifers of the world. Kew Bull 73:8
Farzadfar S, Knight JD, Congreves KA (2021) Soil organic nitrogen: an overlooked but potentially significant contribution to crop nutrition. Plant Soil 462:7–23
Ferreira MJ, Vale D, Cunha L, Melo P (2017) Role of the C-terminal extension peptide of plastid located glutamine synthetase from *Medicago truncatula*: crucial for enzyme activity and needless for protein import into the plastids. Plant Physiol Biochem 111:226–233
Finnemann J, Schjoerring JK (2000) Post-translational regulation of cytosolic glutamine synthetase by reversible phosphorylation and 14-3-3 protein interaction. Plant J 24:171–181
Fontaine JX, Ravel C, Pageau K, Heumez E, Dubois F, Hirel B, Le Gouis J (2009) A quantitative genetic study for elucidating the contribution of glutamine synthetase, glutamate dehydrogenase and other nitrogen-related physiological traits to the agronomic performance of common wheat. Theor Appl Genet 119:645–662
Forde BG, Lea PJ (2007) Glutamate in plants: metabolism, regulation, and signalling. J Exp Bot 58:2339–2358

Forest F, Moat J, Baloch E, Brummitt NA, Bachman SP, Ickert-Bond S, Hollingsworth PM, Liston A, Little DP, Mathews S, Rai H, Rydin C, Stevenson DW, Thomas P, Buerki S (2018) Gymnosperms on the EDGE. Sci Rep 8:6053

Franklin O, Cambui CA, Gruffman L, Palmroth S, Oren R, Näsholm T (2017) The carbon bonus of organic nitrogen enhances nitrogen use efficiency of plants. Plant Cell Environ 40:25–35

Fujita T, Beier MP, Tabuchi-Kobayashi M, Hayatsu Y, Nakamura H, Umetsu-Ohashi T, Sasaki K, Ishiyama K, Murozuka E, Kojima M, Sakakibara H, Sawa Y, Miyao A, Hayakawa T, Yamaya T, Kojima S (2022) Cytosolic glutamine synthetase GS1;3 is involved in rice grain ripening and germination. Front Plant Sci 13:835835

Funayama K, Kojima S, Tabuchi-Kobayashi M, Sawa Y, Nakayama Y, Hayakawa T, Yamaya T (2013) Cytosolic glutamine synthetase1;2 is responsible for the primary assimilation of ammonium in rice roots. Plant Cell Physiol 54:934–943

Gallais A, Hirel B (2004) An approach to the genetics of nitrogen use efficiency in maize. J Exp Bot 55:295–306

Galloway JN, Leach AM, Bleeker A, Erisman JW (2013) A chronology of human understanding of the nitrogen cycle. Philos Trans R Soc Lond B Biol Sci 368:20130120

Gao Y, de Bang TC, Schjoerring JK (2019) Cisgenic overexpression of cytosolic glutamine synthetase improves nitrogen utilization efficiency in barley and prevents grain protein decline under elevated CO_2. Plant Biotechnol J 17:1209–1221

García-Gutiérrez A, Cantón FR, Dubois F, Gallardo F, Sangwan RS, Cánovas FM (1998) Two different modes of early development and nitrogen assimilation in gymnosperm seedlings. Plant J 13:187–200

García-Gutiérrez A, Cánovas FM, Avila C (2018) Glutamate synthases from conifers: gene structure and phylogenetic studies. BMC Genomics 19:65

Ghoshroy S, Binder M, Tartar A, Robertson DL (2010) Molecular evolution of glutamine synthetase II: phylogenetic evidence of a non-endosymbiotic gene transfer event early in plant evolution. BMC Evol Biol 10:198. https://doi.org/10.1186/1471-2148-10-198

Gómez-Maldonado J, Ávila C, Barnestein P, Crespillo R, Cánovas FM (2004a) Interaction of cist-acting elements in the expression of a gene encoding cytosolic glutamine synthetase in pine seedlings. Physiol Plant 121:537–545. https://doi.org/10.1111/j.1399-3054.2004.00353.x¡

Gómez-Maldonado J, Avila C, de la Torre F, Cañas R, Cánovas FM, Campbell MM (2004b) Functional interactions between a glutamine synthetase promoter and MYB proteins. Plant J 39:513–526

Guan M, Møller IS, Schjoerring JK (2015) Two cytosolic glutamine synthetase isoforms play specific roles for seed germination and seed yield structure in *Arabidopsis*. J Exp Bot 66:203–212

Habash DZ, Bernard S, Schondelmaier J, Weyen J, Quarrie SA (2007) The genetics of nitrogen use in hexaploid wheat: N utilisation, development and yield. Theor Appl Genet 114:403–419

Hachiya T, Inaba J, Wakazaki M, Sato M, Toyooka K, Miyagi A, Kawai-Yamada M, Sugiura D, Nakagawa T, Kiba T, Gojon A, Sakakibara H (2021) Excessive ammonium assimilation by plastidic glutamine synthetase causes ammonium toxicity in Arabidopsis thaliana. Nat Commun 12:4944

Harding LW Jr, Mallonee ME, Perry ES, Miller WD, Adolf JE, Gallegos CL, Paerl HW (2019) Long-term trends, current status, and transitions of water quality in Chesapeake Bay. Sci Rep 9:6709

He YX, Gui L, Liu YZ, Du Y, Zhou Y, Li P, Zhou CZ (2009) Crystal structure of Saccharomyces cerevisiae glutamine synthetase Gln1 suggests a nanotube-like supramolecular assembly. Proteins 76:249–254

Heldt H, Piechulla B (2011) Plant biochemistry, 4th edn. Elsevier, Academic Press, Cambridge

Hirel B, Krapp A (2021) Nitrogen utilization in plants I biological and agronomic importance. In: Jez J (ed) Encyclopedia of biological chemistry III, 3rd edn. Elsevier, Amsterdan, pp 127–140

Hirel B, Tétu T, Lea PJ, Dubois F (2011) Improving nitrogen use efficiency in crops for sustainable agriculture. Sustainability 3:1452–1485

James D, Borphukan B, Fartyal D, Achary VMM, Reddy MK (2018) Transgenic manipulation of glutamine synthetase: a target with untapped potential in various aspects of crop improvement. In: Gosal SS, Wani SH (eds) Biotechnology of crop improvement. Springer International Publishing, Cham, pp 367–416

Ji Y, Li Q, Liu G, Selvaraj G, Zheng Z, Zou J, Wei Y (2019) Roles of cytosolic glutamine synthetases in *Arabidopsis* development and stress responses. Plant Cell Physiol 60:657–671

Jing ZP, Gallardo F, Pascual MB, Sampalo R, Romero J, Torres de Navarra A, Cánovas FM (2004) Improved growth in a field trial of transgenic hybrid poplar overexpressing glutamine synthetase. New Phytol 164:137–145

Kaminski KP, Kørup K, Andersen MN, Sønderkær M, Andersen MS, Kirk HG, Nielsen KL (2015) Cytosolic glutamine synthetase is important for photosynthetic efficiency and water use efficiency in potato as revealed by high-throughput sequencing QTL analysis. Theor Appl Genet 128:2143–2153. https://doi.org/10.1007/s00122-015-2573-2

Kew RBG (2016) The state of the World's Plant Report. Royal Botanic Gardens, Kew

Kishorekumar R, Bulle M, Wany A, Gupta KJ (2020) An overview of important enzymes involved in nitrogen assimilation of plants. In: Gupta KJ (ed) Nitrogen metabolism in plants: methods and protocols. Springer, New York, pp 1–13

Krajewski WW, Collins R, Holmberg-Schiavone L, Jones TA, Karlberg T, Mowbray SL (2008) Crystal structures of mammalian glutamine synthetases illustrate substrate-induced conformational changes and provide opportunities for drug and herbicide design. J Mol Biol 375:217–228

Kuvykin IV, Vershubskii AV, Priklonskii VI, Tikhonov AN (2009) Computer simulation study of pH-dependent regulation of electron transport in chloroplasts. Biophysics 54:455–464

Lea PJ, Ireland RJ (1999) Nitrogen metabolism in higher plants. In: Singh BK (ed) Plant amino acids. Marcel Dekker Inc, New York, pp 1–47

Lea PJ, Miflin BJ (2018) Nitrogen assimilation and its relevance to crop improvement. In: Foyer CH, Zhang H (eds) Nitrogen metabolism in plants in the post-genomic era. Annual plant reviews, vol 42. Wiley-Blackwell, Chichester, pp 1–40

Lim H, Jämtga S, Oren R, Gruffman L, Kunz S, Näsholm T (2022) Organic nitrogen enhances nitrogen nutrition and early growth of *Pinus sylvestris* seedlings. Tree Physiol 42:513–522

Lima L, Seabra A, Melo P, Cullimore J, Carvalho, (2006a) Phosphorylation and subsequent interaction with 14-3-3 proteins regulate plastid glutamine synthetase in *Medicago truncatula*. Planta 223:558–67

Lima L, Seabra A, Melo P, Cullimore J, Carvalho H (2006b) Post-translational regulation of cytosolic glutamine synthetase of *Medicago truncatula*. J Exp Bot 57:2751–2761

Liu H-L, Wang X-BG-B, Wang et al (2021) The nearly complete genome of *Ginkgo biloba* illuminates gymnosperm evolution. Nat Plants 7:748–756. https://doi.org/10.1038/s41477-021-00933-x

Liu Y, Wang S-B, Li L-Z et al (2022) The *Cycas* genome and the early evolution of seed plants. Nat Plants 8:389–401. https://doi.org/10.1038/s41477-022-01129-7

Llorca O, Betti M, González JM, Valencia A, Márquez AJ, Valpuesta JM (2006) The three-dimensional structure of an eukaryotic glutamine synthetase: functional implications of its oligomeric structure. J Struct Biol 156:469–479

Martin A, Lee J, Kichey T et al (2006) Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production. Plant Cell 18:3252–3274. https://doi.org/10.1105/tpc.106.042689

Martin F, Kohler A, Murat C, Veneault-Fourrey C, Hibbett DS (2016) Unearthing the roots of ectomycorrhizal symbioses. Nat Rev Microbiol 14:760–773

Masclaux-Daubresse C, Carrayol E, Valadier MH (2005) The two nitrogen mobilisation- and senescence-associated GS1 and GDH genes are controlled by C and N metabolites. Planta 221:580–588

Mathis R, Gamas P, Meyer Y, Cullimore JV (2000) The presence of GSI-like genes in higher plants: support for the paralogous evolution of GSI and GSII genes. J Mol Evol 50:116–22

Melo PM, Silva LS, Ribeiro I, Seabra AR, Carvalho HG (2011) Glutamine synthetase is a molecular target of nitric oxide in root nodules of *Medicago truncatula* and is regulated by tyrosine nitration. Plant Physiol 157:1505–1517

Mondal R, Kumar A, Chattopadhyay SK (2021) Structural property, molecular regulation, and functional diversity of glutamine synthetase in higher plants: a data-mining bioinformatics approach. Plant J 108:1565–1584

Moran-Zuloaga D, Dippold M, Glaser B, Kuzyakov Y (2015) Organic nitrogen uptake by plants: reevaluation by position-specific labeling of amino acids. Biogeochemistry 125:359–374

Nacry P, Bouguyon E, Gojon A (2013) Nitrogen acquisition by roots: physiological and developmental mechanisms ensuring plant adaptation to a fluctuating resource. Plant Soil 370:1–29

Näsholm T, Kielland K, Ganeteg U (2009) Uptake of organic nitrogen by plants. New Phytol 182:31–48

Neale DB, McGuire PE, Wheeler NC et al (2017) The Douglas-fir genome sequence reveals specialization of the photosynthetic apparatus in pinaceae. G3 (Bethesda) 7:3157–3167. https://doi.org/10.1534/g3.117.300078

Neale DB, Zimin AV, Zaman S et al (2022) Assembled and annotated 26.5 gbp coast redwood genome: a resource for estimating evolutionary adaptive potential and investigating hexaploid origin. G3 (Bethesda) 12:jkab380. https://doi.org/10.1093/g3journal/jkab380

Niu S-H, Li J, Bo W-H et al (2022) The chinese pine genome and methylome unveil key features of conifer evolution. Cell 185:204–217e14. https://doi.org/10.1016/j.cell.2021.12.006

Nystedt B, Street NR, Wetterborm A et al (2013) The Norway spruce genome sequence and conifer genome evolution. Nature 497:579–584. https://doi.org/10.1038/nature12211

Obara M, Sato T, Sasaki S, Kashiba K, Nagano A, Nakamura I, Ebitani T, Yano M, Yamaya T (2004) Identification and characterization of a QTL on chromosome 2 for cytosolic glutamine synthetase content and panicle number in rice. Theor Appl Genet 110:1–11

Oliveira IC, Coruzzi GM (1999) Carbon and amino acids reciprocally modulate the expression of glutamine synthetase in Arabidopsis. Plant Physiol 21:301–310

One Thousand Plant Transcriptomes Initiative (2019) One thousand plant transcriptomes and the phylogenomics of green plants. Nature 574:679–685

Ortega JL, Roche D, Sengupta-Gopalan C (1999) Oxidative turnover of soybean root glutamine synthetase. In vitro and in vivo studies. Plant Physiol 119:1483–1496

Ortega JL, Temple SJ, Sengupta-Gopalan C (2001) Constitutive overexpression of cytosolic glutamine synthetase (GS1) gene in transgenic alfalfa demonstrates that GS1 may be regulated at the level of RNA stability and protein turnover. Plant Physiol 126:109–121

Ortega JL, Moguel-Esponda S, Potenza C, Conklin CF, Quintana A, Sengupta-Gopalan C (2006) The 3' untranslated region of a soybean cytosolic glutamine synthetase (GS1) affects transcript stability and protein accumulation in transgenic alfalfa. Plant J 45:832–846

Ortigosa F, Valderrama-Martín JM, Urbano-Gámez JA, García-Martín ML, Ávila C, Cánovas FM, Cañas RA (2020) Inorganic nitrogen form determines nutrient allocation and metabolic responses in maritime pine seedlings. Plants (Basel) 9:481

Ortigosa F, Lobato-Fernández C, Shikano H, Ávila C, Taira S, Cánovas FM, Cañas RA (2022) Ammonium regulates the development of pine roots through hormonal crosstalk and differential expression of transcription factors in the apex. Plant Cell Environ 45:915–935

Pascual MB, El-Azaz J, de la Torre F, Cañas RA, Ávila C, Cánovas FM (2016) Biosynthesis and metabolic fate of phenylalanine in conifers. Front Plant Sci 7:1030

Pérez-Rodríguez MJ, Suárez MF, Heredia R et al (2006) Expression patterns of two glutamine synthetase genes in zygotic and somatic pine embryos support specific roles in nitrogen metabolism during embryogenesis. New Phytol 169:35–44. https://doi.org/10.1111/j.1469-8137.2005.01551.x

Pesole G, Bozzetti MP, Lanave C, Preparata G, Saccone C (1991) Glutamine synthetase gene evolution: a good molecular clock. Proc Natl Acad Sci USA 88:522–526. https://doi.org/10.1073/pnas.88.2.522

Plett D, Garnett T, Okamoto M (2017) Molecular genetics to discover and improve nitrogen use efficiency in crop plants. In: Hossain MA, Kamiya T, Burritt DJ, Tran LSP, Fujiwara T (eds) Plant macronutrient use efficiency. Academic Press, London, pp 93–122

Robertson DL, Tartar A (2006) Evolution of glutamine synthetase in heterokonts: evidence for endosymbiotic gene transfer and the early evolution of photosynthesis. Mol Biol Evol 23:1048–1055

Rueda-López M, Crespillo R, Cánovas FM, Avila C (2008) Differential regulation of two glutamine synthetase genes by a single dof transcription factor. Plant J 56:73–85

Sakakibara H, Kobayashi K, Deji A, Sugiyama T (1997) Partial characterization of the signaling pathway for the nitrate-dependent expression of genes for nitrogen-assimilatory enzymes using detached maize leaves. Plant Cell Physiol 38:837–847

Scott AD, Zimin AV, Puiu D et al (2020) A reference genome sequence for giant sequoia. G3 (Bethesda) 10:3907–3919

Seabra AR, Vieira CP, Cullimore JV, Carvalho HG (2010) Medicago truncatula contains a second gene encoding a plastid located glutamine synthetase exclusively expressed in developing seeds. BMC Plant Biol 10:183

Shafreen M, Vishwakarma K, Shrivastava N, Kumar N (2021) Physiology and distribution of nitrogen in soils. In: Cruz C, Vishwakarma K, Choudhary DK, Varma A (eds) Soil nitrogen ecology. Soil biology, vol 62. Springer, Cham. https://doi.org/10.1007/978-3-030-71206-8_1

Sharma N, Sinha VB, Prem Kumar NA, Subrahmanyam D, Neeraja CN, Kuchi S, Jha A, Parsad R, Sitaramam V, Raghuram N (2021) Nitrogen use efficiency phenotype and associated genes: roles of germination, glowering, root/shoot length and biomass. Front Plant Sci 11:587464

Simon B, Sengupta-Gopalan C (2010) The 3' untranslated region of the two cytosolic glutamine synthetase (GS(1) genes in alfalfa (*Medicago sativa*) regulates transcript stability in response to glutamine. Planta 232:1151–1162

Stevens KA, Wegrzyn JL, Zimin A et al (2016) Sequence of the sugar pine megagenome. Genetics 204:1613–1626. https://doi.org/10.1534/genetics.116.193227

Suárez MF, Avila C, Gallardo F, Cantón FR, García-Gutiérrez A, Claros MG, Cánovas FM (2002) Molecular and enzymatic analysis of ammonium assimilation in woody plants. J Exp Bot 53:891–904

Taylor AF, Alexander IAN (2005) The ectomycorrhizal symbiosis: life in the real world. Mycologist 19:102–112

Tegeder M, Masclaux-Daubresse C (2018) Source and sink mechanisms of nitrogen transport and use. New Phytol 217:35–53

Thomsen HC, Eriksson D, Møller IS, Schjoerring JK (2014) Cytosolic glutamine synthetase: a target for improvement of crop nitrogen use efficiency? Trends Plant Sci 19:656–663

Tikhonov AN (2013) pH-dependent regulation of electron transport and ATP synthesis in chloroplasts. Photosynth Res 116:511–534

Unno H, Uchida T, Sugawara H, Kurisu G, Sugiyama T, Yamaya T, Sakakibara H, Hase T, Kusunoki M (2006) Atomic structure of plant glutamine synthetase: a key enzyme for plant productivity. J Biol Chem 281:29287–29296

Urriola J, Rathore KS (2015) Overexpression of a glutamine synthetase gene affects growth and development in sorghum. Transgenic Res 24:397–407

Valderrama-Martín JM, Ortigosa F, Ávila C, Cánovas FM, Hirel B, Cantón FR, Cañas RA (2022) A revised view on the evolution of glutamine synthetase isoenzymes in plants. Plant J 110:946–960

Valderrama-Martín JM, Ortigosa F, Aledo J, Ávila C, Cánovas FM, Cañas RA (2023) Pine has two glutamine synthetase paralogs, GS1b.1 and GS1b.2, exhibiting distinct biochemical properties. Plant J 113:1330–1347. https://doi.org/10.1111/tpj.16113

Verzeaux J, Hirel B, Dubois F, Lea PJ, Tétu T (2017) Agricultural practices to improve nitrogen use efficiency through the use of arbuscular mycorrhizae: Basic and agronomic aspects. Plant Sci 264:48–56

Wan T, Liu Z-MIJ, Leitch et al (2021) The *Welwitschia* genome reveals a unique biology underpinning extreme longevity in deserts. Nat Commun 12:4247. https://doi.org/10.1038/s41467-021-24528-4

Wang XQ, Ran JH (2014) Evolution and biogeography of gymnosperms. Mol Phylogenet Evol 75:24–40

Wang YY, Cheng YH, Chen KE, Tsay YF (2018) Nitrate transport, signaling, and use efficiency. Annu Rev Plant Biol 69:85–122. https://doi.org/10.1146/annurev-arplant-042817-040056

Warren CR, Adams MA (2002) Possible causes of slow growth of nitrate-supplied *Pinus pinaster*. Can J For Res 32:569–580. https://doi.org/10.1139/x01-225

Warren RL, Keeling CI, Saint Yuen MM et al (2015) Improved white spruce (*Picea glauca*) genome assemblies and annotation of large gene families of conifer terpenoid and phenolic defense metabolism. Plant J 83:189–212. https://doi.org/10.1111/tpj.12886

Watanabe A, Takagi N, Hayashi H, Chino M, Watanabe A (1997) Internal Gln/Glu ratio as a potential regulatory parameter for the expression of a cytosolic glutamine synthetase gene of radish in cultured cells. Plant Cell Physiol 38:1000–1006

Wei Y, Xiong S, Zhang Z, Meng X, Wang L, Zhang X, Yu M, Yu H, Wang X, Ma X (2021) Localization, gene expression, and functions of glutamine synthetase isozymes in wheat grain (*Triticum aestivum* L.). Front Plant Sci 12:580405

Wu CS, Chaw SM, Huang YY (2013) Chloroplast phylogenomics indicates that Ginkgo biloba is sister to cycads. Genome Biol Evol 5:243–254. https://doi.org/10.1093/gbe/evt001

Xiong X-Y, Gou J-B, Q-G, Liao et al (2021) The *Taxus* genome provides insights into paclitaxel biosynthesis. Nat Plants 7:1026–1036. https://doi.org/10.1101/2021.04.29.441981

Zhou X, Wang A, Hobbie EA et al (2021) Mature conifers assimilate nitrate as efficiently as ammonium from soils in four forest plantations. New Phytol 229:3184–3194. https://doi.org/10.1111/nph.17110

Zimin A, Stevens KAMW, Crepeau et al (2014) Sequencing and assembly of the 22-Gb loblolly pine genome. Genetics 196:875–890

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