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Research article

Arabidopsis thaliana mutants devoid of chloroplast glutamine synthetase (GS2) have non-lethal phenotype under photorespiratory conditions

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ABSTRACT

Chloroplast located Glutamine Synthetase (GS2) is believed to play a major role in the reassimilation of ammonium generated by photorespiration, being GS2 knockout mutants unable to grow under photorespiratory conditions (low-CO_2 atmosphere) in the species characterized so far (Barley, Lotus). To investigate the importance of GS2 in *A. thaliana* nitrogen metabolism mutant plants devoid of this GS isoenzyme were characterized. It was shown that GS2 mutants although smaller, slightly chlorotic and with the nitrogen metabolism impaired, were able to grow and complete their life cycle under ordinary air conditions. Surprisingly, GS2 mutants were more tolerant to salt stress than wild-type plants. The lack of GS2 seems to be compensated by higher expression of some GS cytosolic isogenes, namely *GLN1;2* and *GLN1;3* and by glutamate dehydrogenase, whose activity and expression is enhanced in the GS2 mutant plants and might account for the increased tolerance to salt stress. Under conditions that minimize photorespiration (CO2-enriched atmosphere) plant growth and ammonium assimilation impairment is less evident in the GS2 mutant plants and is accompanied by an adjustment of levels of expression of the cytosolic isogenes, with an increase in the expression of *GLN1;3* and a decrease in the expression of the *GLN1;1* and *GLN1;2*. Altogether the results confirm a major role of GS2 in the assimilation of ammonium released during photorespiration, but suggest a redundancy of activity with cytosolic GSs and GDH and further support the involvement of the chloroplastic isoenzyme in primary nitrogen assimilation and plant growth and development in *A. thaliana*.

1. Introduction

Nitrogen (N) is a fundamental nutrient limiting plant growth and food production worldwide. The understanding of how nitrogen metabolism is regulated is critical to improve plant performance, to optimize N use efficiency and increase crop productivity, while reducing the N fertilizers demands in sustainable agriculture management strategies.

Glutamine synthetase (GS, EC 6.3.1.2) is a pivotal enzyme for plant nitrogen metabolism as it catalyses the first step of inorganic nitrogen assimilation into glutamine, being crucial for primary ammonium assimilation, but also for its reassimilation and recycling (Bernard and Habash, 2009; Lea and Miflin, 2011). Due to its central position in plant metabolism this enzyme is considered a bottleneck in plant growth and has received special attention to improve nitrogen use efficiency and crop yield (Thomsen et al., 2014).

GS exists in plants as a collection of isoenzymes, located either in the cytosol (GS1) or in plastids (GS2), consistent with the multiplicity of roles in plant metabolism and involvement in a wide variety of physiological processes throughout plant life cycle, often performing non-redundant and nonoverlapping roles (Bernard and Habash, 2009; Lea and Miflin, 2011; Thomsen et al., 2014). A small family of nuclear genes, with distinct cellular localization and temporal patterns of expression in the plant, encode the diversity of GS isoenzymes,

Abbreviations: GOGAT, Glutamate synthase; GDH, Glutamate dehydrogenase; GS, Glutamine synthetase; GS1, Cytosolic GS; GS2, Cloroplastid GS; MDA, Malondialdehyde; N, Nitrogen; NR, Nitrate reductase; SHMT, Serine hydroximethyltransferase; Wt, Wild type

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assimilating ammonium originated from a broad spectrum of primary sources, like nitrogen fixation, nitrate assimilation and ammonium uptake, or of secondary origins, as photorespiration, amino acid catabolism and phenylpropanoid pathway (Bernard and Habash, 2009; Lea and Miflin, 2011).

The expression and activity of GS is regulated in plants by a refined multilevel complex network involving transcriptional, post-transcriptional and post-translational mechanisms tightly controlled by both developmental signals and environmental factors (Lea and Miflin, 2011; Thomsen et al., 2014). Transcriptional regulation by nitrogen sources, carbon metabolites and light (Masclaux-Daubresse et al., 2005; Oliveira and Coruzzi, 1999), through specific transcriptional factors (Gutierrez, 2012; Marchive et al., 2013), is the main mechanism of control of GS localization and activity. But GS was found to be also post-transcriptionally modulated, by transcript stability and protein accumulation (Ortega et al., 2006), oxidative turnover (Ishida et al., 2002; Palatnik et al., 1999), phosphorylation and 14-3-3 interaction (Finnemann and Schjoerring, 2000; Lima et al., 2006a,b; Riedel et al., 2001), and more recently by tyrosine nitration and cystein nitrosylation (Melo et al., 2011) and by interaction with a uridylyltransferase-like protein, ACR11 (Osanai et al., 2017).

In *Arabidopsis thaliana* GS gene family embraces five well-studied genes coding for the different GS1 isoenzymes, *GLN1;1* to *GLN1;5* (Arabidopsis Genome Initiative, 2000; Ishiyama et al., 2004), which were studied mainly using knockout mutants (Dragicevic et al., 2014; Guan et al., 2015, 2016; Konishi et al., 2017, 2018; Lothier et al., 2011; Ji et al., 2019). GS2 is encoded by a single gene, *GLN2;0* and mutants defective in this isoenzyme are still uncharacterized in the plant model *Arabidopsis thaliana*. Cytosolic *Arabidopsis* GS isoenzymes have distinct tissue localizations, expression patterns, kinetic properties and foreseeable functions. G*LN1;1*, *GLN1;2* and *GLN1;3* are the three major GS1 isogenes, have high levels of expression in vegetative tissues (Dragicevic et al., 2014) and main contribution to N remobilization and seed yield (Moison et al., 2018; Ji et al., 2019). *GLN1;*1 has high affinity to ammonium and is expressed primarily in root epidermis (Lothier et al., 2011), anticipating a function in the transport of nitrogen forms from the external medium to inside the plant. Despite not extremely abundant, G*LN1;1* is induced to compensate the lack of other cytosolic isoenzymes, being regulated by environmental and development clues, like nitrogen starvation (Ishiyama et al., 2004) and senescence (Guo et al., 2004; Li et al., 2006; Moison et al., 2018) and is implicated in stress responses (Debouba et al., 2013; Dragicevic et al., 2014; Ji et al., 2019). *GLN1;2*, has low affinity for ammonium, its transcripts are abundant in almost all tissues except pollen and mature seeds, and exhibits the highest levels of expression in the leaf (Lothier et al., 2011; Schmid et al., 2005). This isoenzyme is essential for ammonium detoxification when it is present at high levels, for vegetative biomass when there is a high supply of nitrate (Guan et al., 2016; Lothier et al., 2011; Schmid et al., 2005) and for ammonium assimilation in roots, particularly when exposed to high concentrations of ammonium (Konishi et al., 2017, 2018). *GLN1;2* seems also to play a fundamental role in the mobilization of the nitrogen stored in the seeds, being essential for seed germination and seedling growth (Guan et al., 2015; Guan and Schjoerring, 2016). Because of its preferential location in phloem company cells, it is highly likely to be attributed to this isoform a role in nutrient translocation (Lothier et al., 2011). *GLN1;2* is the only GS1 gene significantly up-regulated by ammonia, strengthening the importance of this isoenzyme in ammonia detoxification (Guan et al., 2016; Ishiyama et al., 2004). G*LN1;3* is mostly expressed in proliferative tissues, apex, floral buds and in embryos (Schmid et al., 2005) and significantly expressed in root (Ishiyama et al., 2004; Konishi et al., 2017) and rosette leaves (Lothier et al., 2011). It is an ammonium low affinity GS1, down regulated by ammonium in roots (Ishiyama et al., 2004), located in the vasculature and root pericycle which points to an involvement of this isoenzyme in xylem loading (Konishi et al., 2017). *GLN1;*4 has high affinity for ammonium (Ishiyama et al., 2004), seems to be involved in nitrogen remobilization during developmental senescence (Pourtau et al., 2006) being almost exclusively, and highly expressed, in senescent leaves (Guo et al., 2004; Schmid et al., 2005) and is essential for pollen viability, only in the absence of *GLN1;1* and *GLN1;3*, (Ji et al., 2019). No data is available on the possible role of *GLN1;5*, which expression is limited to seeds.

The plastid located GS (GS2) is believed to be involved in plant N use efficiency and biomass production as a central regulator between the nitrogen and the carbon cycles (Hu et al., 2018; Li et al., 2011; Migge et al., 2000; Németh et al., 2018) and a key player in plant responses to abiotic stress (Diaz et al., 2010). It is the major GS isoenzyme expressed in leaves and other photosynthetic tissues, and is encoded by a single nuclear gene in most of the species, being mainly modulated by light and nitrogen sources (Edwards and Coruzzi, 1989; Lightfoot et al., 1988; Matt et al., 2001; Migge et al., 1996). The relative abundance of GS2 to GS1 in leaves is species-dependent, being much higher in C3 then in C4 plants (Becker et al., 2000). There is no evidences of plastid GS in conifers (Cánovas et al., 2007).

The reassimilation of ammonium produced during photorespiration in plants is the main function attributed to the GS located in the plastids (Blackwell et al., 1987; Kozaki and Takeba, 1996; Migge and Becker, 2000; Orea et al., 2002, 2014; Pérez-Delgado et al., 2015; Wallsgrove et al., 1987). Mutants lacking plastid GS grow normally in an atmosphere enriched with CO_2, which inhibits photorespiration, however when transferred to normal air, thus allowing photorespiration, GS2 mutant plants present severe stress symptoms and eventually plant death, (Orea et al., 2002; Pérez-Delgado et al., 2015; Wallsgrove et al., 1987), knowns as the photorespiratory phenotype (Timm and Bauwe, 2013). To date, mutants of plastid GS have been obtained only in barley (Wallsgrove et al., 1987) and *Lotus japonicus* (Orea et al., 2002, 2014; Pérez-Delgado et al., 2013, 2015). In barley the GS2 mutants are non-viable under photorespiratory conditions and GS1 is not able to compensate for the lack of GS2 as the mutants present normal levels of GS1 activity and protein (Wallsgrove et al., 1987). In *Lotus japonicus* GS2 mutant displayed necrotic phenotype after a period of incubation under normal CO_2 conditions and when shifted from high to low CO_2 conditions important genes from nitrogen metabolism are modulated, including cytosolic GS genes (Pérez-Delgado et al., 2013, 2015). In a *A. thaliana* screening for photorespiratory mutants Sommerville and coworkers were unable to isolate mutants for GS2 (Somerville and Ogren, 1980), remaining the lack of GS2 mutants a puzzling question in the field of the photorespiration (Lam et al., 1996; Timm and Bauwe, 2013).

GS2 has been also involved in nitrate assimilatory pathway, assimilating ammonium deriving from nitrate reduction, consistent with its induction by nitrate in roots and with the plastid located location of nitrite reductase (Prinsi and Espen, 2015). However, there is no correlation between the presence of GS2 in roots and nitrate assimilation (Woodall and Forde, 1996), and the *L. japonicus* GS2 mutants described so far are not affected in nitrate assimilation (Orea et al., 2002). Furthermore, in maize, GS1 also contributes to nitrate assimilation (Prinsi and Espen, 2015).

This work aims to clarify the importance of GS2 in plant nitrogen metabolism through the characterization of an *A. thaliana* mutant devoid of GS2. Herein it is shown that chloroplast located GS is not essential for plant survival under photorespiration conditions and that its function in the assimilation of ammonium from photorespiration can be partly replaced in *A. thaliana* by cytosolic GS, namely *GLN1;2* and *GLN1;3*, and by GDH. Furthermore, the lack of GS2 endows the mutant plants of a higher salt stress tolerance. The potential significance of GDH in plant response to salt stress is discussed. The results further underpin the role for GS2 in plant N use efficiency and biomass production.

2. Material and methods

2.1. Plant material

Arabidopsis thaliana ecotype Columbia-0 (Col-0) lines with T-DNA insertion in the 10° exon (SALK_051953 and SALK_071292), at 2890 and 2736 nucleotides downstream of the ATG of *GLN2;0* (AT5G35630) (Alonso et al., 2003) were obtained from the European Arabidopsis Stock Centre, Nottingham, UK.

Homozygous mutant plants were selected from two weeks old T1 progeny by PCR on genomic DNA using a primer annealing to the left border of the T-DNA (LBb1.3, 5′- ATTTTGCCGATTTCGGAAC-3′) and the following gene-specific primers: Fwd, 5′- TCACAGAACAAGCTGGT GTTG -3′ and rev, 5′- GTGACTTTGCCCACTACGATC -3′ in a two reaction PCR, one containing the two gene-specific primers and the other the LB T-DNA-specific primer combined with the RB gene-specific primer. Plants were backcrossed with wild-types (Wt) and homozygous mutants were selected again.

2.2. Plant growth conditions

Seeds of wild-type (Wt) and mutant lines were stratified for 48 h at 4 °C and grown in pots with soil substrate (SIRO Plant) under controlled growth chamber conditions with an 8h:16h light:dark cycle, light intensity of 150 μmol $m^{-2}s^{-1}$, 22 °C air temperature and CO_2 ~0.3% (v/v). Salt stress was induced by watering the plants with a 200 mM NaCl solution four weeks after germination. Leaves from five week old plants were collected, pooled on 5–8 plants samples, grinding in liquid nitrogen, aliquoted and stored at −80 °C for biochemical and gene expression analysis. To induce flowering, plants were transferred to a growth chamber with the same conditions but with a long-day cycle of 16h:8h light:dark. A photorespiration-suppressed atmosphere was created by generating in the chamber a 0.7% (v/v) CO_2 rich atmosphere and the plants were grown for four weeks with the growing conditions similar to those described above.

2.3. Protein isolation and enzyme activities

Soluble proteins were extracted from 0.2 to 0.3 g of frozen samples, by homogenization using extraction buffers [GS extraction buffer: 10 mM Tris-HCl pH7.5, 5 mM Na-glutamate, 10 mM $MgSO_4$, 1 mM dithiothreitol, 10% (v/v) glycerol, 1.5% (w/v) polyvinylpolypyrrolidone, 1 mM phenylmethylsulfonyl fluorideand, 0.05% (v/v) Triton X-100; GDH extraction buffer: 50 mM HEPES, pH 7.5, 10% (v/v) glycerol, 0.05% (w/v) BSA, 1 mM phenylmethylsulfonyl fluoride, 0.05% (v/v) Triton X-100, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 10 mM dithiothreitol or NR extraction buffer: 50 mM HEPES-KOH, pH 7.8, 1 mM PMSF, 10 mM $MgCl_2$]. The homogenates were centrifuged at 15,000 g at 4 °C for 15 min. Soluble protein concentration was measured by the Coomassie dye-binding assay (Bio-Rad) using BSA as a standard. GS activity was determined by quantification of the γ-glutamyl hydroxamate produced by the transferase reaction as previously described by Cullimore and Sims (1980). GDH activity was determined spectrophotometrically by monitoring NADH evolution based on the methodology reported by Groat and Vance (1981) and accordingly to described by Sarasketa et al. (2015). NR activity was measured following the consumption of NADH as described by Kaiser and Brendle-Behnisch (1991).

2.4. Gel electrophoresis and protein gel western blot analysis

Proteins were separated on 12% (w/v) SDS-PAGE, electroblotted onto nitrocellulose membrane (Schleicher & Schuell) using a Criterion blotter (Bio-Rad) and blocked with PBST (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH of 7.4 with 0.1% (v/v) Tween 20) containing 5% skim milk. The blocked membranes were incubated with a primary anti-GS antiserum raised against a GS isoform from *Phaseolus vulgaris* (kindly provided by Dr. Julie Cullimore from INRA Toulouse; Cullimore and Miflin, 1984) and subsequently detected with a secondary goat anti-rabbit peroxidase-conjugated IgGs (Vector Laboratories), as previously described (Melo et al., 2011). The immunocomplexes were detected by chemiluminescence, using the Amersham ECL Western Blotting Detection Reagent (GE Healthcare, Lifesciences) detection system, and a ChemiDoc imaging system (BioRad).

2.5. Chlorophyll, proline, malondialdehyde and ammonium content determination

Chlorophyll concentration was quantified in 200 mg of frozen leaf samples according to Sims and Gamon (2002). Ammonium content was determined in samples of 200 mg using the phenol hypochlorite test (reaction of Berthelot) according to the protocol described by Vega-Mas et al. (2015). Proline quantification was performed using 200 mg samples following the Bates et al. (1973) protocol. Malondialdehyde (MDA) production was quantified according to Heath and Packer (1968) using 200 mg of leaf samples.

2.6. DNA isolation and genomic PCR

Genomic DNA was isolated from 100 mg of frozen leaf tissue using CTAB miniprep method (Murray and Thompson, 1980) and quantified spectrophotometrically using Multiskan Go Microplate (ThermoFisher Scientific). PCR reactions for selection of the mutant homozygous were set using NZYTaq2x *Colourless Master Mix* (NZYTech) in a 20 μL volume, using the following PCR profile: initial desnaturation (95 °C/1 min), 40 cycles of desnaturation (95 °C/30 s), annealing (55 °C/30 s) and extension (72 °C/30 s).

2.7. RNA extraction, reverse transcription (RT) and qPCR analysis

Total RNA was isolated from 50 to 100 mg of frozen samples using 1 mL of NZYol (NZYTech), according to manufacturer's instructions, evaluated spectrophotometrically for purity and concentration using Multiskan Go Microplate (ThermoFisher Scientific) and assessed for integrity by agarose gel electrophoresis. After treatment with DNAse (ThermoFisher Scientific), according to manufacturer's guidelines, reverse transcriptase was performed using Superscript-RT IV (Invitrogen, Life Science), an oligo$(dT)_{16}$ primer R9:5′- CCAGTGAGCAGAGTGACG AGGACTCGAGCTCAAGCTTTTTTTTTTTT-TTTT-3′, and 1.25 μg of RNA, according to manufacturer's protocol.

Real-time PCR reactions were performed using CFX96™ Real-Time PCR Detection System (BioRad) and PowerUp SYBR Green Master Mix (Applied Biosystems) to quantify the gene expression using specific primers (Table 1), according to manufacturer. The genes analysed were selected by their expression coincident with the expression of *Gln2;0* in a eFP browser survey (Winter et al., 2007). Three technical replicates were performed for each primer pair and samples combination, in 10 μL reaction volume using 400 nM of each primer. The amplification conditions were as follows: UDG activation (50 °C/2 min), initial denaturation (95 °C/2 min) followed by 45 cycles of amplification and denaturation (95°Cfor 15s, 60 °C for 60 s) and melting curve generation (55 °C–95 °C read every 0.5 °C). Bio-Rad CFX Manager Software (version 3.1) was used for calculation of the cycle threshold (C_t) and primers efficiency. To normalize the gene expression data between the different biological samples the ratio between C_t value obtained for each primer pair and the housekeeping genes RUB1 conjugating enzyme 1 (*RCE*1) (AT4G36800) and *ACT8* (AT1G49240) was calculated.

2.8. Statistics

The significance of differences between data was determined using the Students *t*-test on Prisma6 software (Graphpad software Inc). All

Table 1
Sequences of primers used for qRT-PCR amplification.

Gene	Gene ID	Forward	Reverse
GLN1;1	AT5G37600	5′- CATCCAAACCCTTGGTATTTGT-3′	5′-GTAGCTGCGAAGGGTCAGTC-3′
GLN 1;2	AT1G66200	5′-TAACCTTGACATCTCAGACAACAGT-3′	5′-CTTTGGAAGTTTTGATGGATC-3′
GLN 1;3	AT3G17820	5′- TCCAACCAACAAGAGGCACA -3′	5′- ATACCGGCGTAAAGACAGGC -3′
GLN 1;4	AT5G16570	5′-GATTCCTAAGATATGTCTTCACTTGC-3′	5′- GCTTGAACCGTCGTAGTTCC -3′
GLN 1;5	AT1G48470	5′- GGATATGAGAAGCAAAGCAAGG -3′	5′- CTTCACATTGGGATGATCG -3′
GLN 2;0	AT5G35630	5′- GCAGCTTCTCCAACATGTCA -3′	5′- CCTGTCAGAGTAAGGTTTGGTG -3′
NR2	AT1G37130	5′- GAACTGGATGGTTGGGCAGA -3′	5′- TCGCATAATCGCCTCGGAAA -3′
GLU1	AT5G04140	5′- AGTCGGGAACACTTGCTTGT-3′	5′- TGTCATACCAGCAGCAACGT-3′
SHMT1	AT4G37930	5′- ACTCCTGCTCTCACTTCCAGA-3′	5′- GACTTCATGGCGCAGTTTCG-3′
GDH1	AT5G18170	5′- CAGGGCAGCGTTTTGTCATC-3′	5′- TTGAGCAAGGCCGGGATATC -3′
RCE1	AT4G36800	5′- CTGTTCACGGAACCCAATTC-3′	5′- GGAAAAAGGTCTGACCGACA -3′
ACT8	AT1G49240	5′- CTCAGGTATTGCAGACCGTATGAG-3′	5′-CTGGACCTGCTTCATCATACTCTG-3′

quantifications were performed in biological triplicates (tissue pooled from 5 to 8 plants grown in the same conditions) and, for each sample, technical triplicates were made.

3. Results and discussion

3.1. Arabidopsis thaliana gln2;0 knockout mutant is viable under photorespiratory-permitting conditions

To examine the effect of a loss of function of *GLN2;0* gene, the single gene coding for chloroplast located glutamine synthetase (GS2) in *A. thaliana*, homozygous T-DNA insertion mutants were isolated and analysed. The SALK_051953 (*gln2;0–1*) and SALK_071292 (*gln2;0–2*) mutants with T-DNA insertions located at the 10° exon of the *GLN2;0* gene (AT5G35630) were selected. Homozygous mutant plants were isolated from T1 progeny, after backcrossing with wild-type (Wt) plants, using a two genomic PCR reactions approach. One reaction was performed using two gene-specific primers that encompass the T-DNA insertion site so that amplification is only possible for Wt DNA. The other reaction used a primer annealing to the left border of the T-DNA and the right border gene-specific primer and detect the presence of the insertion. The homozygous plants were selected as only the amplicon from the second reaction was detected (Fig. 1A). Quantitative RT- PCR was performed to characterize the mutant at transcriptional level revealing the lack of *GLN2;0* transcript in both insertion lines (Fig. 1B). The *gln2;0* knockout mutants were grown in ordinary air conditions and were able to complete their life cycle in normal CO_2 concentration being only smaller and slightly chlorotic when compared to the Wt plants (Fig. 1C). Chlorophyll content measurement confirmed the lower levels in the mutant plants (Fig. 1D). Western blot analysis using an anti-GS antibody that recognizes both GS1 and GS2 forms (Cullimore and Miflin, 1984) revealed a total absence of GS2 protein in the homozygous plants (Fig. 1E). Mutant plants were completely devoid of detectable GS2 protein, while a higher amount of GS1 protein was detected in the *gln2;0* knockout mutants. As expected the T-DNA insertion in the GS2 gene prevented both accumulation of transcripts and protein. Furthermore a considerable reduction in total GS activity, around 50%, was reported in both GS2 mutant lines (Fig. 1 F). As both insertion lines showed to be knockout for *GLN2;0*, completely devoid of GS2 protein and display similar phenotype we proceed with this work using only one of them, *gln2;0–1*, hereinafter referred to as *gln2;0*.

In order to reveal the nitrogen and photorespiration metabolism status of the *gln2;0* mutants some genes with expression patterns similar to *GLN2;0* were selected and analysed in Wt and *gln2;0* knockout plants growing in normal CO_2 concentration. Three genes were selected: ferredoxin glutamate synthase 1 (Fd-GOGAT) (*GLU1*-AT5G04140), serine hydroximethyltransferase 1 (SHMT) (*SHMT1*-AT4G37930) and nitrate reductase 2 (NR) (*NR2*-AT1G37130), mostly expressed in young and mature rosette leaves (Winter et al., 2007). The GS2 mutant plants showed lower expression for two nitrogen metabolism enzymes, Fd-GOGAT and NR. Along with this, lower activity of NR (Fig. 2A) and higher ammonium content (Fig. 2C) showed impairment in the nitrogen metabolism in plants devoid of GS2. Also a gene from the photorespiratory metabolism, *SHMT1*, was affected decreasing its expression in the mutant plants.

The GS2 mutants reported so far, in barley and *L. japonicus*, have shown to be non-viable, or promptly presented severe symptoms of stress, when transferred to photorespiration-permitting conditions (Orea et al., 2002; Pérez-Delgado et al., 2015; Wallsgrove et al., 1987) assuming an essential role for GS2 in the reassimilation of ammonium produced by photorespiration. In the present work the *A.thaliana gln2:0* knockout mutants were able to complete their life cycle under photorespiratory conditions although with impairment in growth and nitrogen metabolism that indicates a non-essential role for GS2 and denoting specie-specific differences with respect to the importance of GS2 in nitrogen metabolism. The assigned function of GS2 in the reassimilation of ammonium released during photorespiration is presumably held in concert with the Fd-GOGAT *GLU1* gene, the main GOGAT isoform expressed in leaves and with the major role in photorespiration as well as with a potential role in primary nitrogen assimilation (Coschigano et al., 1998). The reduction of the Fd-GOGAT expression in *gln2:0* mutant plants emphasize this close connection between these two isoforms of the GS/GOGAT cycle. The down-regulation of NR in *gln2;0* mutant plants can further endorse for GS2 isoenzyme a putative role in nitrate assimilatory pathway, foreseen by some authors (Vézina and Langlois, 1989), but contrary to what was seen in *L. japonicus* GS2, mutants which were not affected in nitrate assimilation (Orea et al., 2002). Serine hydroximethyltransferase is a crucial enzyme in the photorespiration metabolism and is a part of the mitochondrial enzyme complex involved in ammonium production (Voll et al., 2006). In plants grown in photorespiration-permitting conditions the reduction in SHMT expression in the *A. thaliana* GS2 mutant reveals a markedly photorespiration impairment. As ammonium produced by SHMT is believed to be assimilated by GS2, the ammonium accumulation in *gln2;0* mutant plants would predictably lead to a feedback inhibition of SHMT expression. The repression of photorespiratory genes, as well as Fd-GOGAT, by photorespiratory conditions was also described in GS2 *L. japonicus* mutant plants (Pérez-Delgado et al., 2013).

3.2. Arabidopsis thaliana gln2;0 knockout mutant shows tolerance to salt stress

Four weeks old plants, *gln2;0* knockout mutants and Wt, were exposed to salt stress by watering with 200 mM NaCl solution. One week after the treatment Wt plants showed a pronounced stressed appearance, with rolled up and wilted leaves, while the *gln2;0* knockout plants showed a normal aspect, with no signs of wilting (Fig. 3 A). Proline (marker for hydric/salt stress) and MDA (marker for lipid membrane peroxidation) contents (Fig. 3B and C) were higher in salt stressed Wt

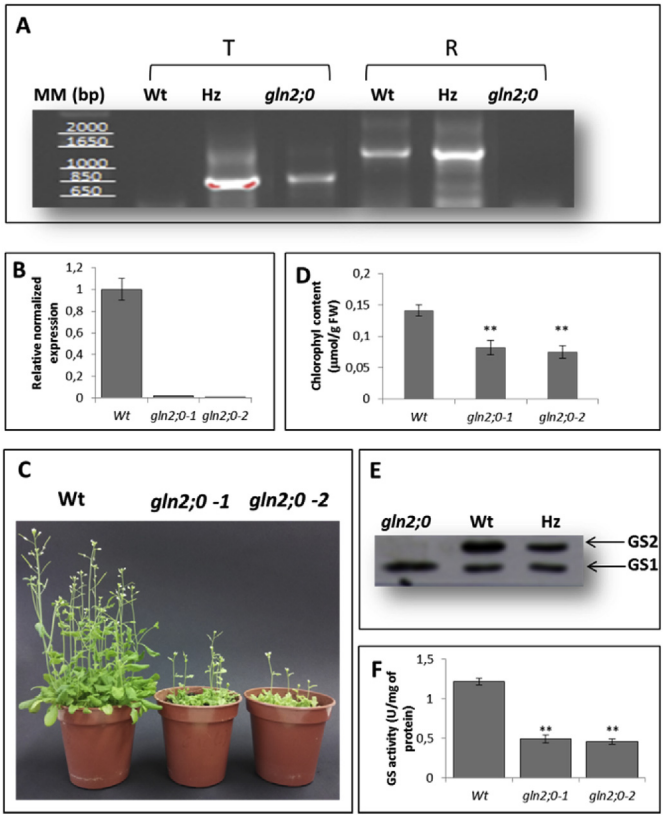

Fig. 1. Characterization of the *gln2;0* knockout mutants. (A) PCR based selection of homozygous mutant plants for the T-DNA insertion in the *gln2;0* gene. Genomic DNA from T1 SALK_051953 (*gln2;0–1*) and SALK_071292 (*gln2;0–2*) progeny and wild-type (Wt) plants were amplified with gene-specific primers, forward and reverse, (T) and T-DNA-specific primer combined with the reverse gene-specific primer (R). Hz, heterozygous plant; Wt, wild-type plant. Molecular mass markers (MW) are indicated. (B) RT–qPCR analysis of *Gln2;0* expression in the leaves of the *gln2;0–1* and *gln2;0–2* mutants and Wt plants. Values are expressed as fold changes in gene expression relative to *CRE1* and *ACT8* expression, determined from the C_t values and normalized to the Wt expression and are the mean ± SD from three biological replicates analysed in triplicate. (C) Plant growth and flowering phenotypes of *gln2;0–1* and gln2; 0–2 knockout mutants and Wt when grown under photorespiratory conditions. (D) Chlorophyll content in *gln2:0*-1 and *gln2;0–2* mutants and Wt plants. The values are expressed as means ± SD of 4 independent experiments assayed in triplicate (**, $P \leq 0.01$). FW, fresh weight. (E) Comparison of GS protein levels in 4 weak old leaves of wild-type (Wt), homozygous (*gln2;0*) and heterozygous (Hz) mutant plants. The proteins (10 µg) were separated by SDS-PAGE, transferred to nitrocellulose and the membranes proved with a GS antibody. GS2 and GS1 positions are indicated. (F) Comparison of GS activities in leaves of homozygous *gln2;0–1 and gln2;0–2* mutant and Wt plants. The values are expressed as means ± SD of 4 independent experiments assayed in triplicate (**, $P \leq 0.01$).

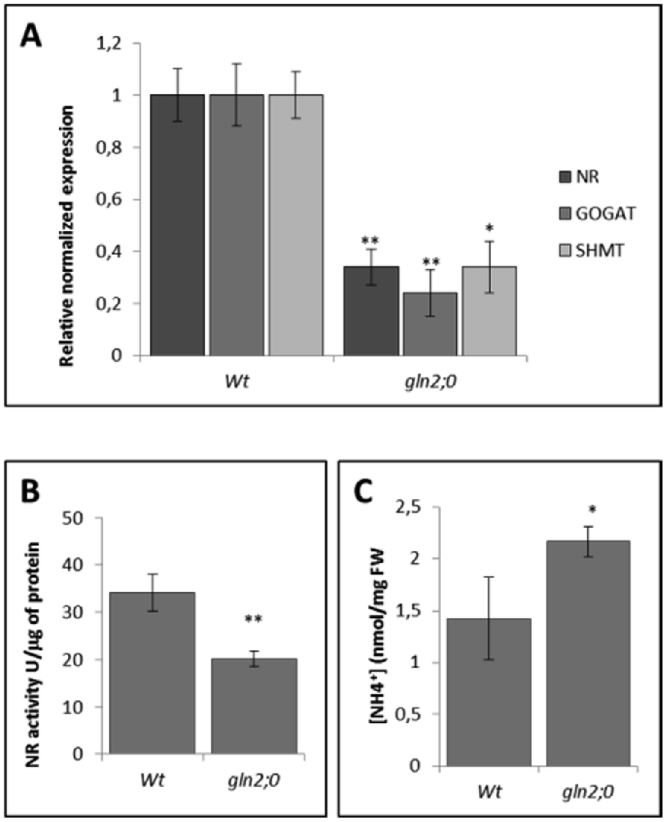

Fig. 2. Evaluation of *gln2;0* knockout mutant photorespiration and nitrogen metabolism. (A) Nitrate reductase (NR), ferredoxin-glutamate synthase (Fd-GOGAT) and serine hydroximethyltransferase (SHMT) expression in the leaves of *gln2;0* mutant and wild-type (Wt) plants. Values are expressed as fold changes in gene expression relative to *CRE1* and *ACT8* expression, determined from the C_t values and normalized to the Wt expression for each gene and are the mean ± SD from three biological replicates analysed in triplicate. For each gene the asterisk * indicates statically significant differences between Wt and mutant (*, $P \leq 0.05$; **, $P \leq 0.01$). (B) Nitrate reductase activity in leaves of *gln2;0* mutant and Wt plants. The values are expressed as means ± SD of 4 independent experiments assayed in triplicate (**, $P \leq 0.01$). (C) Quantification of ammonium contents in leaves of *gln2;0* mutant and Wt plants. The values are expressed as means ± SD of 4 independent experiments assayed in triplicate (*, $P \leq 0.05$). FW, fresh weight.

plants denoting a higher stress condition in accordance with the phenotype observed.

In rice, it was showed that an increase in GS2 level enhances salt tolerance in transgenic plants (Hoshida et al., 2000). In L. *japonicus* a key role for GS2 in drought resistance mechanisms and proline synthesis was suggested as GS2 mutant plants showed a lower proline accumulation under drought stress conditions, what could however be due to the reduced supply of precursors for plastidic proline biosynthesis (Diaz et al., 2010). In fact, in a later study the *L. japonicus* GS2 mutants showed increased levels of proline under drought stress conditions and under impaired photorespiration conditions (Perez-Delgado et al., 2013). Furthermore, in the same mutant plants, several genes for the biosynthesis of phenolic compounds were highly induced in the two stress conditions (Garcia-Calderon et al., 2015). Although the phenotype of the GS2 mutant plants was not described in these studies we can assume a strengthening of the stress resistance mechanisms in the absence of GS2. The results presented here showed a salt tolerance enhancement of the *gln2;0* mutants. As the *gln2;0* mutants are able to grow under photorespiration conditions there must be enzymes capable of assimilating the photorespiratory ammonium. One candidate could be glutamate dehydrogenase (GDH; EC 1.4.1.2), which can assimilate ammonium and contribute for an increase in stress tolerance (Lightfoot et al., 2007; Skopelitis et al., 2006). GDH is historically viewed as a major ammonium assimilatory enzyme but has a prime role in the glutamate deamination and amino acid catabolism in plants, producing ammonium in the mitochondria (Miyashita and Good, 2008). However, under stress conditions or senescence stage an *in vivo* function in re-assimilation of excess ammonium has been reported suggesting a non-redundant and complementary role to GS/GOGAT in ammonium assimilation (Lightfoot et al., 2007; Melo-Oliveira et al., 1996; Setién et al., 2013; Skopelitis et al., 2006; Terce-Laforgue et al., 2004, 2015).

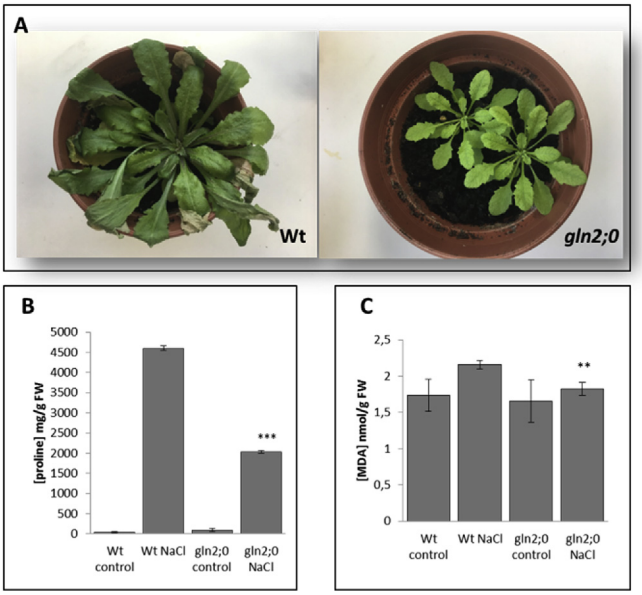

Fig. 3. Salt stress resistance of *gln2;0* knockout mutant. (A) Plant phenotype of *gln2;0* mutant and wild-type (Wt) plants, control or salt stressed (200 mM NaCl). (B) Proline content in *gln2;0* mutant and Wt plants, control or salt stressed. The results are expressed as means ± SD of 4 biological replicates assayed in triplicate* indicate statistically significant differences between proline contents in Wt and mutant growing in high salinity (***, $P \leq 0.001$). (C) Quantification of Malondialdehyde (MDA)) in *gln2;0* mutant and Wt plants, control or salt stressed, measured to assess lipid peroxidation. The results are expressed as means ± SD of 4 biological replicates assayed in triplicate.* indicates statistically significant differences between MDA contents in Wt and *gln2;0* mutant growing in high salinity (*, $P \leq 0.01$).

3.3. GDH expression and activity are enhanced in the Arabidopsis thaliana gln2;0 knockout mutant

To verify the involvement of GDH in the assimilation of ammonium in the absence of GS2, *gln2;0* mutant plants were analysed for the expression of *GDH1* (AT5G18170), the highest expressed GDH isoenzyme in *A. thaliana* leaves, and for the GDH NADH-dependent α-ketoglutarate aminating activity. Both transcripts and activity showed an increment in *gln2;0* mutants when compared with Wt plants (Fig. 4) suggesting a role of the enzyme in reassimilation and detoxification of photorespiratory ammonium in the absence of GS2. These results are in accordance with previously reported role of GDH operating in the assimilation of photorespiration-released ammonium when GS/GOGAT pathway was not able to fulfil its function. In fact, in *Nicotiana tabacum* plants down-regulated for Fd-GOGAT, it was showed that GDH expression and activity were induced in photorespiratory conditions (Terce Laforgue et al., 2004) and in *L. japonicus* GS2 mutant GDH expression and activity were also increased when the mutant plants were shifted from high to normal CO_2 conditions (Pérez-Delgado et al., 2015). Furthermore, the increase in GDH activity detected in this work might account for the higher tolerance of the *A. thaliana* GS2 mutant plants to salt stress, as this enzyme has been associated with increase drought stress tolerance and considered as an antistress enzymes under salinity stress conditions (Lightfoot et al., 2007; Setién et al., 2013; Skopelitis et al., 2006; Terce- Laforgue et al., 2015). The increase in GDH activity under salt stress conditions was related to glutamate production for proline synthesis (Skopelitis et al., 2006; Terce- Laforgue et al., 2015). However, in this work *gln2;0* mutant plants showed lower proline levels than the Wt plants under high salinity conditions, suggesting physiological differences in tolerance patterns. In addition to the clear increase of GDH expression and activity in the *gln2;0* mutant plants, both GDH transcripts and activity were stimulated by the salt stress in the Wt plants (Fig. 4), confirming the importance of GDH in ameliorate the impact of environmental stresses.

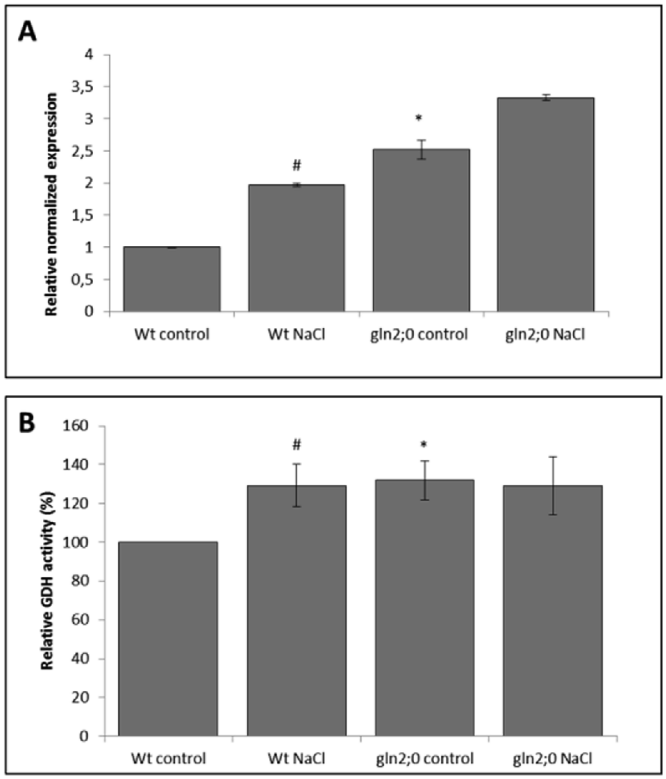

Fig. 4. Glutamate dehydrogenase (GDH) expression and activity in *gln2;0* knockout mutant. (A) GDH expression in the leaves of *gln2;0* mutant and wild-type (Wt) plants, control or salt stressed (200 mNaCl), determined by qRT-PCR. Values are expressed as fold changes in gene expression relative to *CRE1* and *ACT8* expression and normalized to the Wt control expression, determined from the C_t values, and are the mean ± SD from three biological replicates analysed in triplicate. (B) GDH activity in leaves of *gln2;0* mutant and Wt plants. The values are expressed as means ± SD of 4 independent experiments assayed in triplicate. * indicates statically significant differences between Wt and mutant plants in each condition (*, $P \leq 0.05$). # represents statically significant differences between treatments within the same genotype (#, $P \leq 0.05$).

3.4. GS cytosolic genes expression are enhanced in Arabidopsis thaliana gln2;0 knockout mutant

In addition to the GDH, also cytosolic GSs can potentially participate in the assimilation of photorespiratory ammonium in absence of the plastidial isoform, GS2. In fact the western blot analysis showed a visible increase in the protein level of GS1 in the *gln2;0* mutants (Fig. 1E). To further corroborate this result GS1 gene-specific oligonucleotides were synthesized based on sequences found in the available databases and the expression of the isogenes coding for GS1 isoforms was monitored by qRT-PCR. Both *GLN1;2* (AT1G66200) and *GLN1;3* (AT3G17820) showed increased expression in *gln2;0* mutant plants when compared to Wt plants, while no significant differences were detected in the expression of *GLN1;1* (AT5G37600) and *GLN1;4* (AT5G16570) (Fig. 5A). *GLN1;5* (AT1G48470) expression was not quantified as no expression was reported in leaves (Dragićević et al., 2014; Schmid et al., 2005). Both GS1 isoenzymes up-regulated in the *Arabidopsis gln2;0* mutants are considered to have low affinity/high capacity for ammonium and therefore detoxification roles (Guan et al., 2016; Ishiyama et al., 2004). Furthermore, these isogenes display tissue patterns of expression overlapping GS2, *Gln1;2* being the most highly GS1 isogene expressed in leaves and green tissues, while Gln1; 3 expresses mostly in proliferative tissues, where GS2 is also present

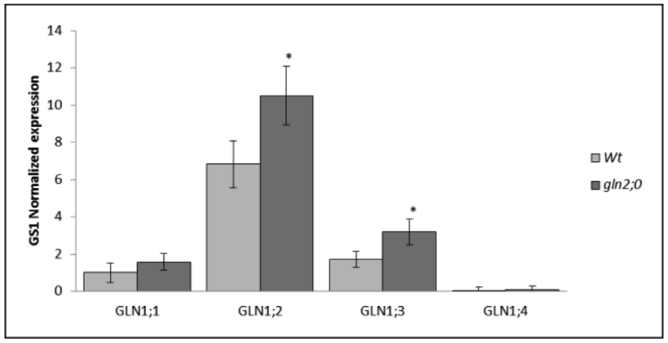

Fig. 5. GS1 genes expression in *gln2;0* knockout mutant. Changes in the transcript levels of the different GS1 genes in the leaves of *gln2;0* mutant and wild-type (Wt) plants determined by qRT-PCR. Values are expressed as fold changes in gene expression relative to *CRE1* and *ACT8* expression, determined from the C*t* values and normalized to the *GLN1;1* expression in the Wt, and are the mean ± SD from three biological replicates analysed in triplicate. For each gene * indicates statically significant differences between Wt and mutant (*, P ≤ 0.05).

(Schmid et al., 2005).

These results further confirm the species-specific differences with respect to the role of GS2, as in *A. thaliana* GS2 mutant plants GS1 isoenzymes are able to compensate the lack of plastid located GS and allow the survival of the plants under photorespiratory permitting conditions, contrary to what was described for other species like barley. *Lotus japonicus* mutant plants were described as having GS1 levels similar to Wt plants when grown in a CO_2-enriched atmosphere (Orea et al., 2002). Further analysis revealed an increase in the level of one cytosolic GS transcript, *LjGLN1.2*, when transferred to active photorespiratory conditions, both on mutant and Wt plants, but higher in the mutants (Pérez-Delgado et al., 2013, 2015). However, this higher GS1 level of expression in *L. japonicus* GS2 mutants does not seem to be sufficient to the long-term survival of the plants in photorespiration-permitting conditions.

Despite of the postulated specific and non-redundant roles of the GS isoenzymes in N metabolism (Lea and Miflin, 2011; Orsel et al., 2014), observed in species like *Oryza sativa* (Funayama et al., 2013; Tabuchi et al., 2005) or *Zea mays* (Martin et al., 2006), in *A. thaliana* functional complementation of GS1 isoenzymes was suggested in knockout mutants (Dragićević et al., 2014; Konishi et al., 2017). Furthermore, was shown that GS2 could also compensate the decrease in GS1 activity in the *gln1;1-gln1;2-gln1;3* triple mutant (Moison et al., 2018), reflecting a functional redundancy of the GS isoenzymes even located in different sub-cellular compartments. The present work further confirms the compensation mechanisms in GS isoenzymes functions in *A. thaliana.*

Recently Timm and Bauwe suggested the existence of a variety of photorespiratory phenotypes in *A. thaliana*, beyond the classic one, characterised by lethality under normal air but viability under elevated CO2 conditions (Timm and Bauwe, 2013). According to the classification of these authors GS2 mutants should be considered “Intermediate-to-slight photorespiratory phenotype”, likely resulting from a compensation mechanism similar to what happens to other photorespiratory enzymes like *HPR1* (Timm and Bauwe, 2013). The complete loss of peroxisomal hydroxypyruvate reductase (*HPR1*) only resulted in minor phenotypic alterations when plants were grown in normal air, due probably to a cytosolic bypass that almost completely compensates for the loss of *HPR1* (Timm and Bauwe, 2013).

3.5. Arabidopsis thaliana gln2;0 knockout mutant shows an impairment in ammonium assimilation under non-photorespiratory conditions

To further characterize the loss of function of the *GLN2;0* gene, Wt and GS2 knockout mutant plants were grown in a CO_2-enriched

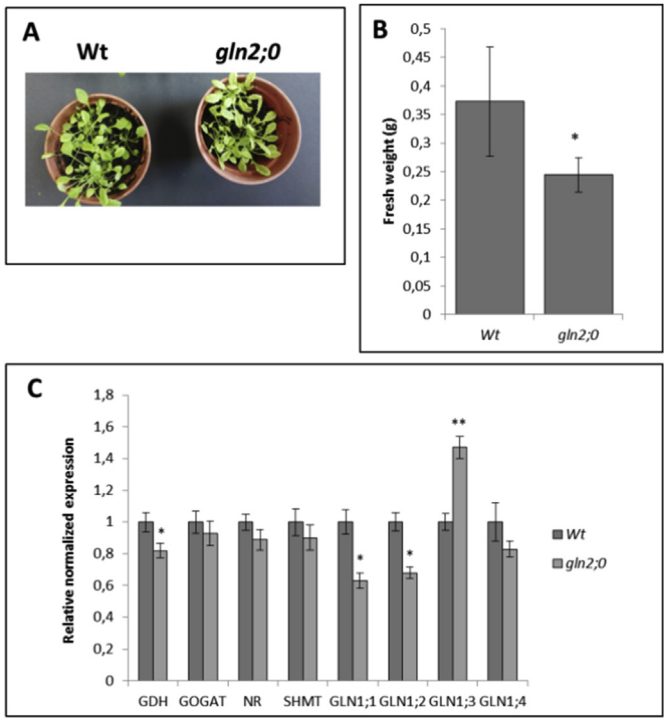

Fig. 6. Plant growth phenotype and gene expression in *gln2;0* knockout mutant growing under non-permitting photorespiratory conditions. (A) Plant phenotypes of *gln2;0* knockout mutants and wild-type (Wt) when grown under high CO_2 atmosphere (0.7%). (B) Wt and *gln2;0* fresh weight of plants grown under high CO_2 atmosphere (0.7%). The values are expressed as means ± SD (n = 20) (*, P ≤ 0.05). (C) Glutamate dehydrogenase (GDH), nitrate reductase (NR), ferredoxin-glutamate synthase (Fd-GOGAT), serine hydroximethyltransferase (SHMT) and GS1 (*GLN1;1*, *GLN1;2, GLN1;3, GLN1;4*) expression in the leaves of *gln2;0* mutant and Wt plants grown under high CO_2 atmosphere (0.7%), determined by qRT-PCR. Values are fold changes in gene expression relative to *CRE1* and *ACT8* expression determined from the C_t values, normalized to the Wt expression for each gene, and are the mean ± SD from three biological replicates analysed in triplicate. For each gene * indicates statically significant differences between Wt and mutant (*, P ≤ 0.05).

atmosphere (0.7%) and the phenotype and the expression of several genes of nitrogen and photorespiration metabolism analysed. Mutant plants growth in non-permitting photorespiration conditions were able to rescue the dwarf and chlorotic *gln2;0* phenotype, being the plants greener and higher than in normal air (Fig. 6A). However, a slight decrease in growth of the mutants compared to Wt plants, around 30% reduction in fresh weight (Fig. 6B), points to a role of GS2 in primary nitrogen assimilation and biomass production in addition to its function in the reassimilation of the photorespiratory ammonium. GS2 mutants described in barley (Blackwell et al., 1987; Wallsgrove et al., 1987) and *Lotus japonicus* (Orea et al., 2002; Pérez-Delgado et al., 2013, 2015) have a lethal phenotype when grown under photorespiration-permitting conditions but show a normal growth in photorespiration-suppressing conditions, suggesting an essential role for GS2 in the reassimilation of photorespiratory ammonium and discarding a role for GS2 in primary nitrogen assimilation. The phenotype of GS2 knockout mutants described here in emphasizes the major differences with respect to the role of GS2 in *A. thaliana* as *gln2;0* mutant plants are viable under photorespiration-permitting conditions and display defects in primary nitrogen assimilation when grown under conditions that suppress photorespiration, implying the involvement of GS2 in plant growth and yield. Earlier studies also pinpoint to the role to GS2 in nitrogen primary assimilation as it seems to be a rate-limiting enzyme for biomass production in tobacco seedlings (Migge et al., 2000), improves N-use efficiency and grain yield (Li et al., 2011; Hu et al., 2018)

and to play a central role as regulator of nitrogen and carbon metabolism (Németh et al., 2018) in wheat.

In plants grown under non-photorespiration conditions, expression analysis of genes from nitrogen and photorespiration metabolism shows a recovery in the *gln2;0* mutants plant metabolism, with the expression of Fd-GOGAT, NR and SHMT in mutant plants attaining the Wt levels (Fig. 6C). However, the ammonium assimilatory enzymes show significant differences, with an upregulation of *GLN1;3* isogene in the *gln2;0* mutant plants (as observed also in the photorespiration-permitting conditions, Fig. 5), while *GDH1*, *GLN1;1* and *GLN1;2* are down regulated in the mutant plants in non-permitting photorespiratory conditions. *GLN1;3* is a gene particularly expressed in proliferative active tissues (Schmid et al., 2005) pointing to the involvement of this gene in plant growth. The overexpression of this GS1 isogene to compensate GS2 lost function in both photorespiration- permitting and suppressing conditions discloses a role for GS2, further than its function in the assimilation of ammonium released during photorespiration, in primary nitrogen assimilation and plant development. In L. *japonicus*, although the comparison between the nitrogen metabolism of Wt and GS2 mutant plants in non-permitting photorespiration conditions was not explored, similar levels of expression of most genes analysed were detected in both genotypes, including GS1 isogenes and GDH, in plants grown in high CO_2 atmosphere, before the shift to normal CO_2 conditions (Pérez-Delgado et al., 2015).

4. Concluding remarks

The lack of plastid located GS (GS2) in *Arabidopsis thaliana* results in mutant plants viable under photorespiratory conditions, although impaired in nitrogen metabolism as seen by its dwarf and chlorotic phenotype, high ammonium content and reduction in the expression and activity of important enzymes of the N cycling, like nitrate reductase and GOGAT. This work emphasizes the major role of GS2 in the assimilation of ammonium released by photorespiration but shows the existence of a compensatory mechanism when the gene is mutated leading to plant survival of the *Arabidopsis thaliana* GS2 knockout mutant. Under conditions that suppress photorespiration the GS2 mutants continue to show some impairment in growth suggesting a role for GS2 also in primary nitrogen assimilation and highlighting the importance of GS2 in plant productivity. To overcome the lack of GS2 *A. thaliana* overexpresses some cytosolic GS isoforms, namely *GLN1;2* and *GLN1;3*, and GDH to fulfil its functions, assimilation of the ammonium resulting from photorespiration and primary nitrogen assimilation. *GLN1;3,* the GS1 isogene most expressed in proliferative active tissues, seems to replace GS2 in this last function, concur to plant growth, and upregulated in GS2 mutants either in permitting or supressing photorespiratory conditions. *GLN1:2* and GDH, genes highly expressed in leaves, similar to GS2 isogene (Schmid et al., 2005)displace the plastidic GS in the reassimilation of photorespiratory ammonium, being up regulated in photorespiratory conditions. The higher expression of GDH in absence of GS2 might account for the higher salt stress tolerance exhibited by knockout GS2 mutants, strengthening the role of GDH in ammonium detoxification, its complementary role to GS/GOGAT cycle and its importance in osmotic stress defences. Taken together this work disclose the role of plastid located GS in the model plant *A. thaliana,* give novel insights into the nitrogen metabolism and ammonium detoxification in plants and highlights the species-specificity function of this isoenzyme.

Contributions

Sónia Ferreira, Emanuel Moreira, made the mutant plant genotyping and biochemical characterization. Sónia Ferreira, Emanuel Barros and Isabel Amorim performed the qRT-PCR. Conceição Santos co-supervised all assays and data analyses. Paula Melo conceived the work, contributed to the qRT-PCR analysis and wrote the manuscript. All authors contributed to the manuscript revision.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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