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Elucidation of the role of glutamine synthetase seed isoform *GLN1;5* in *Arabidopsis thaliana* (L.) with a reverse genetics approach

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Abstract: Glutamine synthetase (E.C. 6.3.1.2) is a key enzyme of plant nitrogen metabolism that assimilates ammonia into glutamine. The *Arabidopsis thaliana* genome encodes one chloroplastic (*GLN2*) and five cytosolic (*GLN1;1 – GLN1;5*) isoforms with different expression patterns, kinetic properties, regulation and functions. Physiological roles of different isoforms have been elucidated mainly by studying knockout mutants. However, the role of *GLN1;5,* which is expressed in dry seeds, remains unknown. To clarify the function of *GLN1;5,* we studied a *GLN1;5* knockout line (GLN1;5KO) homozygous for T-DNA insertion within the *GLN1;5. GLN1;5* deficiency results in a phenotype with slightly delayed bolting and fewer siliques. The dry weight of GLN1;5KO seeds was 73.3% of wild-type (WT) seed weight, with seed length 90.9% of WT seeds. Finally, only 18.33% of the mutant seeds germinated in water within 10 days in comparison to 34.67% of WT seeds. KNO_3 strongly stimulated germination of both GLN1;5KO and WT seeds, while germination in the presence of increasing NH_4Cl concentrations potentiated the differences between the two genotypes. It can be concluded that GLN1;5 activity supports silique development and grain filling and that it has a role in ammonium reassimilation in the seed, as well as assimilation and/or detoxification of ammonium from the environment.

Keywords: germination; glutamine synthetase; grain filling; knockout mutant; phenotype

Abbreviations: glutamine synthetase *1;1* knockout mutant (GLN1;1KO); glutamine synthetase *1;2* knockout mutant (GLN1;2KO); glutamine synthetase *1;5* knockout mutant (GLN1;5KO); cytosolic glutamine synthetase isoforms (GS1); plastidic glutamine synthetase isoforms (GS2)

INTRODUCTION

Glutamine synthetase (GS or GLN, E.C. 6.3.1.2) is a central enzyme of plant nitrogen metabolism that catalyzes ATP-dependent assimilation of ammonia into glutamine, which serves as amido-donor and N-transporting amino acid. The ammonia may originate from the soil, nitrate reduction, photorespiration, as well as from different catabolic processes including protein degradation during senescence [1]. Higher plants have one plastidic (GS2) and one or more cytosolic (GS1) isoforms. The *Arabidopsis* nuclear genome encodes one chloroplastic (*GLN2*) and five cytosolic isoforms named *GLN1;1* through *GLN1;*5 [1,2]. The *Arabidopsis* GS1 genes have different spatiotemporal expression [3-5] and different kinetic properties [2]. They are differentially regulated by both internal cues such as amino acids and carbon skeletons [6] and plant hormones [7], and by external signals, including ammonia [2], nitrates [8] and light [6,9]. For these reasons, specific and non-redundant functions have been suggested for *Arabidopsis* GS isoforms [1,4,10].

The plastidic *GLN2* isoform is expressed primarily in photosynthetic tissues and is involved in the reassimilation of photorespiratory ammonia, but also in nitrate assimilation in both leaves and roots [1,11].

In leaves, this isoform is light-inducible [6,9], while in roots its expression is stimulated by nitrates [8].

According to sequence clustering, isoforms *GLN1;1*, *GLN1;2*, and *GLN1;4* are grouped together, while *GLN1;3* and *GLN1;5* belong to another cluster [1]. *GLN1;1* and *GLN1;4* are characterized as high affinity isoforms, whereas *GLN1;2* and *GLN1;3* have low affinities for substrates [2]. Finally, based on overall expression, *GLN1;1*, *GLN1;2* and *GLN1;3* can be classified as major and *GLN1;4* and *GLN1;5* as minor isoforms [3,12]. Major isoforms can combine in all stoichiometric relations to form heterodecamers, thus expanding the possibilities for their regulation – a feature that has yet to be determined for minor isoforms [12].

The physiological roles of major isoforms *GLN1;1*, *GLN1;2* and *GLN1;3* and to some extent that of *GLN1;4*, have been investigated in great detail. *GLN1;1* is expressed in most organs, but primarily in dry seeds, senescent leaves and flowers [3,13], as well as in the root surface layer [2,4]. This isoform accumulates during N limitation and is downregulated by ammonium excess in roots, so it is thought to be responsible for ammonium assimilation under limited N supply [2,11] and for primary root development during germination [4]. In addition, *GLN1;1* is a stress- and senescence-responsive isoform, since it is upregulated during salinity stress and senescence [1,13,14] and is inducible by abscisic acid (ABA) [7]. *GLN1;2* is the main isoform in vegetative tissues and some reproductive organs, being expressed more than any other *GLN1* isoform in both shoots and roots [4,5,7,12]. Specifically, *GLN1;2* is expressed in the vasculature of roots, leaves, petals and stamens, as well as in companion cells, old parenchymal cells, mesophyll, cortex, leaf and sepal epidermis, trichomes, nodes between the pedicel and the developing silique and other tissues [1,2,4,5]. *GLN1;2* is significantly upregulated by ammonium and nitrate, establishing its role as essential for ammonium assimilation and amino acid synthesis, ammonium homeostasis and detoxification in both roots and shoots under ample ammonium or nitrate supply [1,2,5,11]. In addition, *GLN1;2* plays an important role in N remobilization during seed production and seedling establishment [4]. *GLN1;3* is significantly expressed in the roots [2], rosette leaves [1], young seedlings [12] and generally in proliferating tissues [3]. It contributes to ammonium assimilation as a low affinity isoform that is inhibited by a high concentration of glutamate [2,5]. *GLN1;4* is a minor isoform with very low expression in young seedlings [12], roots [2,7,11] and rosette leaves [1], but with higher expression in senescent cauline leaves [3], so it is thought to be involved in N remobilization during senescence [1].

In the abovementioned studies, the expression of *GLN1;5* was not detected at all, or was at the limit of detection in *Arabidopsis* roots or shoots under different conditions [1,2,7,8,11,12]. This isoform is only expressed during seed maturation and in dry seeds [3,13], but it disappears during germination unless the seeds are exposed to salt stress [13]. The physiological role of this isoform is unknown. Since the most valuable data on physiological functions of major *Arabidopsis* GS isoforms have been collected using knockout mutants and double mutants of these genes [1,4,5], we decided to exploit *GLN1;5* knockout mutants and to study their phenotype with the aim of elucidating the physiological function of *GLN1;5*.

MATERIALS AND METHODS

Plant material

Arabidopsis seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC). *Arabidopsis thaliana* (L.) Heynh., ecotype Columbia (NASC:N60000), was used as the WT plant; NASC:N660998 line (SALK_086579C, [15]) had a T-DNA insertion in the *GLN1;5* gene. Homozygous *GLN1;5* knockout mutants (GLN1;5KO) were selected from T1 progeny as described previously [12]. Pools of obtained WT and mutant (T2) seeds were used for RNA extraction, for germination tests and for production of plantlets for morphometric measurements.

RNA isolation and RT-PCR

Total RNA was isolated from 10 mg of *Arabidopsis* seeds following the protocol developed by Meng and Feldman [16], with 1% sodium dodecyl sulfate (SDS) instead of sarkosyl. Following the DN*ase* I treatment (#EN0521, Fermentas), RNA was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit

(#K1622, Fermentas). Since standard PCR was unsuccessful, presumably due to the presence of some inhibiting compounds from the seeds, the cDNA was amplified using the Maxima SYBR Green mix (#K0221, Fermentas), which is commonly used for qPCR. The optimized buffer of undisclosed composition of this mix allowed for successful amplification with the cDNA template prepared from 50 ng RNA, 0.3 mM specific *GLN1;1* or *GLN1;5* primers and the PCR program as described by Dragićević et al. [12].

Southern hybridization

Southern hybridization was performed to estimate the copy number of T-DNA within the genome of mutant *A. thaliana* line. Genomic DNA was isolated from ~500 mg of leaf tissue by the cetyl trimethylammonium bromide (CTAB) miniprep method [17]. DNA was treated with RNase A (#EN0531, Fermentas) and quantified spectrophotometrically. Twenty µg of genomic DNA of the mutant *A. thaliana* line were digested with HindIII, EcoRI or BamHI endonucleases (Fermentas), while DNA isolated from WT plants was digested with HindIII. Digested DNA samples were loaded on a 1% agarose gel (Sigma-Aldrich Co., USA), separated electrophoretically and blotted onto a positively-charged nylon membrane (Roche, Indianapolis, IN, USA) by capillary transfer. A 362-bp fragment was used as a probe for detection of the *NPTII* gene, which is located in close proximity to the right T-DNA border. The probe was labeled by PCR with digoxigenin (DIG)-dUTP (Roche), using the forward primer: 5'-GATGTTTCGCTTGGTGGTCG-3' and the reverse primer: 5'-ATTCGGCTATGACTGGGCAC-3'. Hybridization was performed in DIG Easy Hyb buffer (Roche) for 16 h at 50°C. The membrane was then washed 2×5 min in each of the following buffers: 2×saline-sodium citrate buffer (SSC)+0.1% SDS and 1×SSC+0.1% SDS at 50°C and 0.5×SSC+0.1% SDS and 0.1×SSC+0.1% SDS at 68°C. Hybrids were detected with anti-digoxigenin antibody (Roche), visualized with chemiluminescent substrate CDP-Star (Roche) and recorded on X-ray film (Kodak, Rochester, NY, USA). For fragment size estimation, a DNA molecular weight marker II (Roche) labeled with digoxigenin was used.

Morphometric measurements

WT and GLN1;5KO seeds were stratified for 72 h at 4°C and set to germinate in 10x10 cm pots with Floradur B Pot Medium (Coarse, Floragard, Odenburg, Germany). The plantlets were grown in a greenhouse (location: Belgrade, lat: 44.817048, long: 20.487303, time of sowing: middle of May 2015) under 80±5% humidity at 25±2°C. The obtained plants were used for morphometric measurements and DNA extraction. Growth and development of 30 WT and 30 mutant plantlets was followed over 9 weeks. Emergence of the main inflorescence stem was recorded for all plantlets as days after sowing (DAS); the length of the main stem was periodically recorded.

The number of developed siliques was recorded 7 weeks after sowing, separately for the main stem and side branches. Siliques from both groups were further sorted into those shorter and those longer than 5 mm. The dimensions of 40 immature (10 days after emergence) and 40 mature siliques (collected 9 weeks after sowing) and seeds from them (n=40) were measured using *ImageJ* ver. 1.5. The dry weights of 1000 mature seeds were also recorded for WT and mutant lines, each in 3 replicates.

Seed germination

In order to determine whether nitrate (in the form of KNO3) and ammonium (applied as NH4Cl) differentially affect the germination of WT and GLN1;5KO seeds, batches of 100 seeds, each in 3 replicates, were placed in 6-cm Petri dishes with either 2 mL of distilled water or the test substance. KNO3 and NH4Cl were applied in a range of 0.1-10 mM concentrations, with pH adjusted to 7. The seeds were germinated in a growth chamber at 25±2°C under white light (32.5 µmol m−2 s−1) applied as a 16 h photoperiod. The number of germinated seeds (with radicle >2mm) was counted after 1, 2, 3, 4, 5, 7 and 10 days following the onset of imbibition.

Statistical analyses

All statistical analyses were performed in R software. Seed germination data was analyzed using semiparametric time-to-event (Cox proportional hazards)

statistics as proposed by McNair et al. [18] utilizing R package survival (Therneau TM, 2015) [19]. The proportional hazards assumption was checked for all explanatory variables by testing if the scaled Schoenfeld residuals are significantly correlated with transformed survival time [20]. Ties were handled using the Efron approximation as suggested by McNair et al. [18]. The concentration variable did not meet the proportional hazards assumption in both NO_3^- and NH_4^+ data, so this variable was stratified (separate baseline hazard functions were fit for each strata). The non-independence of observations within a Petri dish was accounted for by using robust estimates (observations were clustered within Petri dishes and generalized estimating equations were utilized as incorporated by the R survival package). The effect of genotype on time-to-inflorescence-stem emergence was estimated by creating Kaplan-Meier curves for each genotype and testing whether they differed significantly using the G-rho family of tests [21]. Welch's t-test was used to estimate the effects of genotype on silique count, seed dimensions and 1000-seed weight. The effect of genotype on inflorescence stem height was analyzed by multiple regression. All other measured parameters were examined graphically and tested with appropriate statistical methods but did not show differences between the genotypes.

RESULTS

Molecular characterization of the GLN1;5KO mutant line

In order to investigate whether T-DNA insertion completely prevents the formation of *GLN1;5* mRNA, RT-PCR was performed. Total RNA was successfully isolated from dry WT and mutant seeds using a slightly modified Meng and Feldman [16] protocol (Fig. 1A). RT-PCR analysis showed that *GLN1;5* was indeed expressed in dry seeds, although at a significantly lower level in comparison to *GLN1;1*, which was used as a positive control. As expected, the WT seeds expressed both isoforms, while GLN1;5KO seeds expressed only *GLN1;1* (Fig. 1B).

To estimate the copy number of T-DNA insertions within the GLN1;5KO line, the DNA was digested with three restriction enzymes and Southern

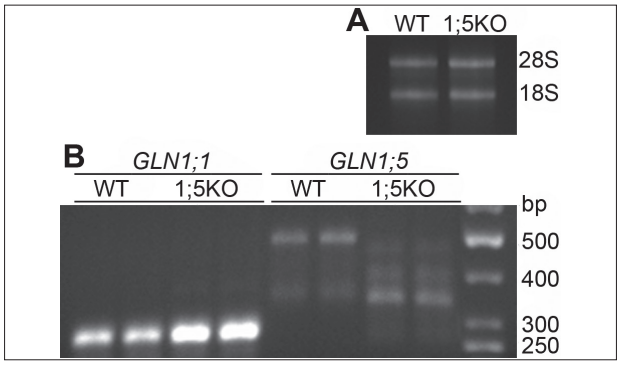

Fig. 1 Expression of glutamine synthetase isoforms *GLN1;1* and *GLN1;5* in WT and GLN1;5KO seeds. **A** – Total RNA isolated from seeds of WT and GLN1:5KO plants; **B** – Expression of glutamine synthetase isoforms *GLN1;1* and *GLN1;5* in WT and GLN1;5KO seeds by RT-PCR. The expected size of the amplicons is 286 bp and 495 bp for *GLN1;1* and *GLN1;5*, respectively

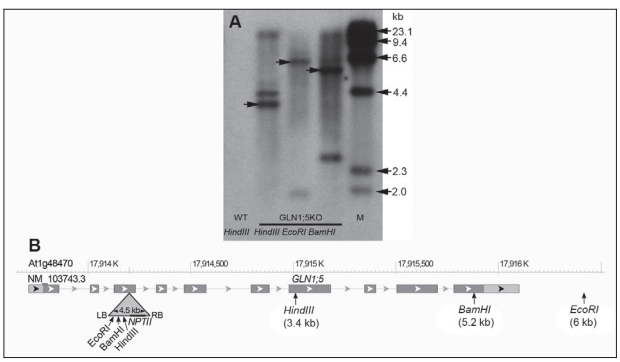

Fig. 2 Southern analysis of genomic DNA of the mutant *A. thaliana* line GLN1:5KO for T-DNA copy number estimation. **A** – Genomic DNA was isolated from untransformed (WT) and mutant plants (GLN1;5KO) and 20 μg DNA was digested with either HindIII, EcoRI or BamHI restriction endonuclease. M – DNA molecular weight marker labeled with digoxigenin. **B** – Schematic representation of the *GLN1;5* gene (At1g48470) with indicated exons (gray rectangles with white arrowheads), 5'-UTR and 3'-UTR (light gray rectangles with black arrowheads), T-DNA insertion (triangle) and restriction sites. The scale indicates the gene position on chromosome 1. LB and RB – left and right border, respectively; *NPTII* – neomycin phosphotransferase II

hybridization was performed using neomycin phosphotransferase II (*NPTII*) 362-bp fragment located near the right T-DNA border as a probe (Fig. 2). Based on the SALK_086579C LB flanking sequence, the expected size of the restriction fragments should be 3.4 kb after HindIII, 5.2 kb after BamHI and 6 kb after EcoRI digestion (Fig. 2B). The probe hybridized with 2-3 T-DNA insertions in the mutant genome depending on the used restriction enzyme. The restriction fragments obtained by Southern hybridization were

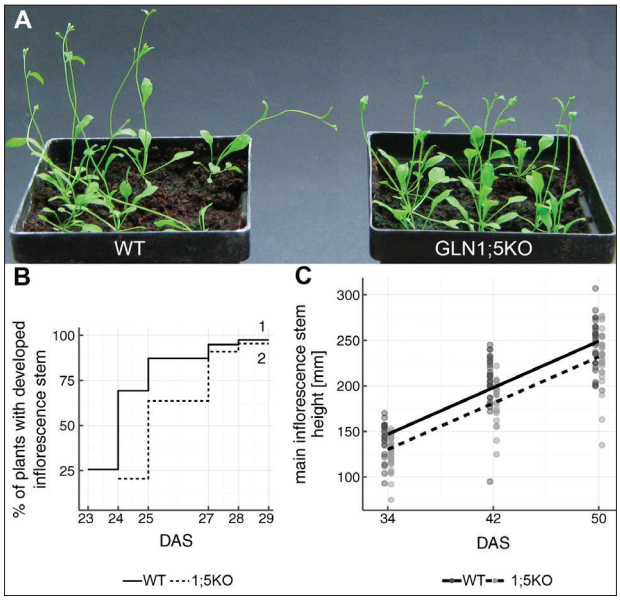

Fig. 3 The effect of GLN1;5 knockout on inflorescence stem emergence and growth. **A** – WT and GLN1;5KO plantlets growing in pots, 34 DAS. Bar is 1 cm. **B** – Kaplan-Meier cumulative incidence (calculated as 1 – Kaplan-Meier estimator) of plants with elongated inflorescence stem for WT and GLN1;5KO genotypes. The numbers near the curves indicate censored events (number of plants that failed to develop inflorescence stems during the measured period). **C** – Multiple regression plot for main inflorescence stem height.

0.3-0.4 kb longer than predicted; specifically, HindIII produced 3.8 and 4.3 kb fragments; EcoRI a 6.3 kb and a <2 kb fragment, while BamHI digestion gave 5.6 and a 2.5 kb fragments (the underlined values are indicated by arrows on Fig. 2A).

The GLN1;5 knockout mutation slightly delayed bolting

The GLN1;5KO plantlets visibly lagged behind the WT plants as regards the inflorescence emergence (Fig. 3A). The effect of genotype on bolting was evaluated by constructing Kaplan-Meier estimators of time-to-inflorescence-stem formation for each genotype (Fig. 3B). This showed that almost all WT plants formed the inflorescence stem over the period of 23-29 DAS, just as expected for WT Columbia, for which the average time for bolting (principal growth stage 5) is 26 ± 3.5 DAS [22]. The significance of the bolting time-lag was estimated by the log-rank test, which indicated that the curves for the inflorescence emergence for WT and GLN1;5KO plantlets (Fig. 3B) were significantly different (for $\rho=0$, $p=1.66\cdot10^{-4}$, Table 1). This difference specifically relates to the early days of bolting, because when a higher weight is given to the initial part of the curves ($\rho=1$), the difference is highly significant ($p=5.86\cdot10^{-6}$), but later on the curves for WT and GLN1;5KO plantlets do not differ significantly (for $\rho=-1$, $p=0.27$, Table 1).

Table 1. The G-ρ family statistic for Kaplan-Meier estimates of the effect of genotype on inflorescence stem emergence[1].

ρ	p-value
0	$1.66\cdot10^{-4}$
1	$5.86\cdot10^{-6}$
-1	0.27

[1]For ρ=0 this is the log-rank test.

The GLN1;5KO genotype also affected the height of the inflorescence stem as estimated by multiple regression (Fig. 3C and Supplementary Table S1), which showed that GLN1;5KO plants were on average 17 mm shorter than the WT plants at 34 DAS (Supplementary Table S1). However, this is just a consequence of the initial delay in inflorescence emergence, and not an effect on the inflorescence stem growth rate. Namely, the growth rate of both genotypes seemed to be the same (the slopes of the regression lines on Fig. 3C) and was, on average, 6.367 mm/day (Supplementary Table S1). The number of nodi on the main inflorescence stem, as well as the number of formed side branches did not differ between the two genotypes (data not shown).

GLN1;5 knockouts develop fewer siliques with smaller seeds

To estimate the effect of *GLN1;5* deficiency on silique development, the number and the size of siliques were compared for GLN1;5KO and WT plants. The number of siliques shorter than 5 mm and those longer than 5 mm was determined for the main inflorescence stem and the side branches separately. While the two genotypes have a comparable number of shorter siliques on both main inflorescence stem and side branches, the number of siliques >5 mm was significantly smaller in the GLN1;5KO plants (Fig. 4). The WT plants developed, on average±SD, 7.55±2.31 siliques on the main inflorescence stem and 26.51±8.13 siliques on side branches, while the mutant plants developed

4.81±2.51 and 15.19±8.39 siliques on main and side branches, respectively, which was significantly less according to Welch's t-test at the confidence level of p≤0.001 (Fig. 4). To evaluate the effects of *GLN1;5* deficiency on seed yield structure elements, different parameters were measured and expressed relative to WT values (% of WT) for each seed yield structure element (Table 2). The number of siliques developed on the main and side branches measured 7 weeks after sowing were significantly decreased in the GLN1;5KO mutant in comparison to the WT (63.7% and 57.3% of WT, respectively, Table 2). The length and width of immature and mature siliques (9 weeks after sowing) and the number of seeds in them did not differ between the two genotypes (Table 2). Although the seeds developed on GLN1;5KO plants did not differ visually from the WT seeds (Fig. 5A), they were 26.67% lighter in comparison to WT seeds (Table 2). Specifically, while a sample of 1000 WT seeds weighed on average±SD 22.27±0.70 mg, many GLN1;5KO

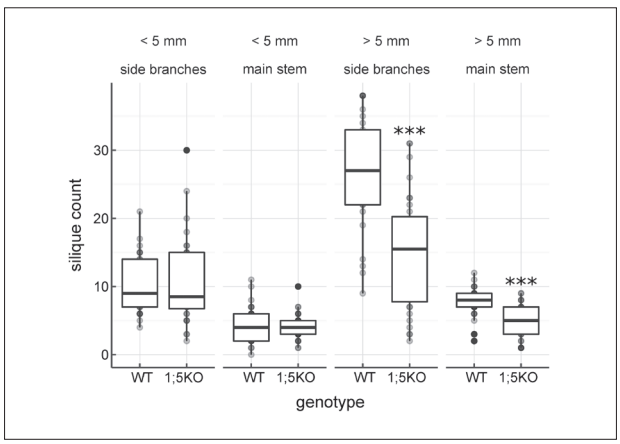

Fig. 4 Box-plots of silique counts on main and side branches. Siliques were separated according to length in two groups: shorter and longer than 5 mm. The differences in the means of GLN1;5KO mutants and WT plants were assessed by Welch's t-test: *** denotes a p-value smaller than 0.001; 29-32 plants were assessed per genotype

Table 2. Comparison of the effects of different GS1 knockout mutations on seed yield structure elements. Data obtained for GLN1;5KO mutants are compared to previously published data for GLN1;1KO and GLN1;2KO mutants and GLN1;1:GLN1;2KO double mutants [4][1].

	Knockout mutant			
Seed yield structure element [% of WT]	**GLN1;1**	**GLN1;2**	**GLN1;1: GLN1;2**	**GLN1;5**
Number of siliques on the main stem	94 [9 was]	87 [9 was]	85 [9 was]	63.7 [7 was]
Number of siliques on the side stems	92 [9 was]	85 [9 was]	75 [9 was]	57.3 [7 was]
Silique width	N/A	N/A	N/A	100 [9 was (2)]
				104 [9 was (3)]
Silique length	N/A	N/A	N/A	99.9 [9 was (2)]
				96.9 [9 was (3)]
Seeds per silique	94 [10 wag]	83 [10 wag]	84 [10 wag]	103.6 [9 was (2)]
				102.8 [9 was (3)]
Seed dry weight	94 [12 wag]	90 [12 wag]	82 [12 wag]	73.3 [9 was]
Seed length	109.7 [10 wag]	97.4 [10 wag]	91.4 [10 wag]	96.5 [9 was (2)]
				90.9 [9 was (3)]
Seed width	100.9 [10 wag]	97.6 [10 wag]	96 [10 wag]	98.4 [9 was (2)]
				101.1 [9 was (3)]

[1]The effect of the mutations is expressed relative to wild type values (% of WT) for each seed yield structure element. Superscripts indicate the time of recording (was – weeks after sowing; wag – weeks after germination).
[2]Immature siliques, measured 10 days after emergence.
[3]Mature siliques.

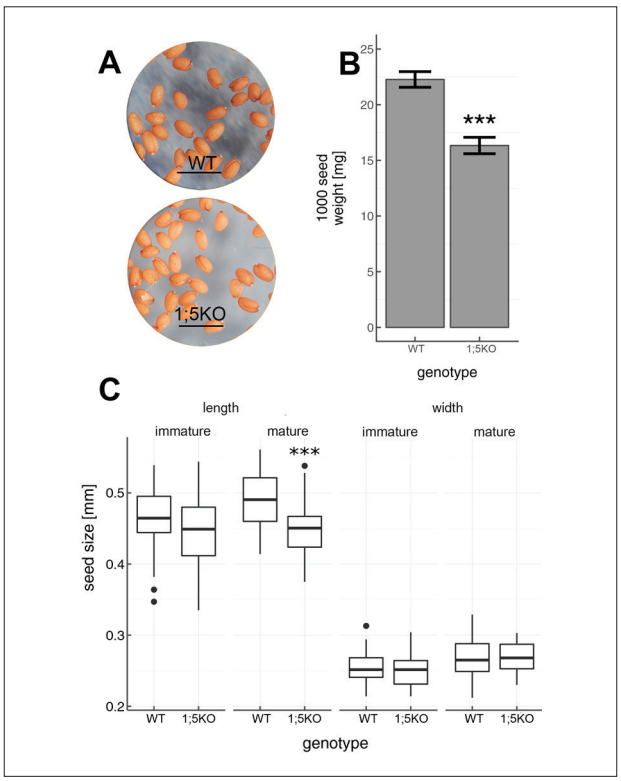

Fig. 5 Seed size in WT and GLN1;5KO mutants. **A** – Appearance of WT and mutant seeds. Bar represents 1 mm. **B** – The weight of 1000 seeds for WT and GLN1;5KO plants. The differences in the means of GLN1;5KO mutants and WT plants were assessed by Welch's t-test: *** denotes a p-value smaller than 0.001. Three measurements of 1000 seeds were averaged; the error bar represents ±standard deviation. **C** – Box-plots of seed length and width formed in ten days old and in mature yellow siliques. The differences in the means of GLN1;5KO mutants and WT plants were assessed by Welch's t-test: *** denotes a p-value smaller than 0.001; 40 seeds were assessed per silique type, per genotype.

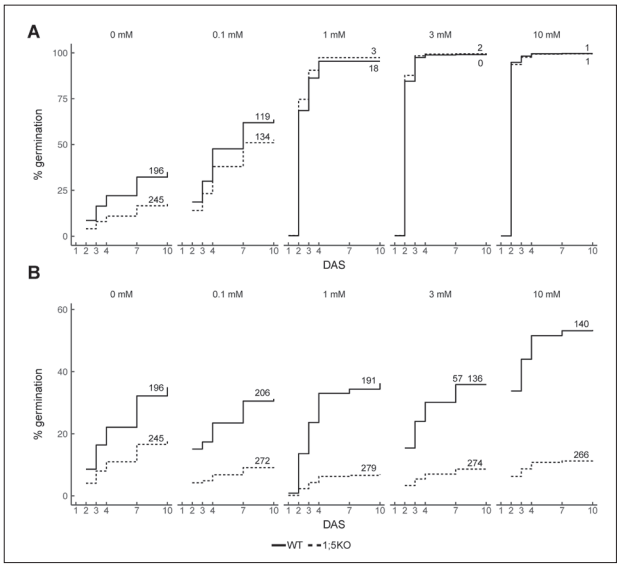

Fig. 6. Cumulative event survival curves of Cox PH models for **A** – KNO_3 and **B** – NH_4Cl. The numbers near the curves indicate censored events (number of seeds that failed to germinate within the measured time).

seeds weighed only 16.33±0.74 mg (Fig. 5b). In addition, both immature and mature mutant seeds (from 10-day-old and mature siliques, respectively) were shorter in comparison to immature and mature WT seeds, but this difference was statistically significant only for mature seeds (Table 2, Fig. 5C). Finally, the width of immature and mature WT and mutant seeds did not differ (Table 2, Fig. 5C).

Effect of GLN1;5 knockout on seed germination

In order to elucidate whether *GLN1;5* has a role in seed germination, we compared the germination of WT and GLN1;5KO seeds in water and in increasing concentrations of nitrates (KNO_3) or ammonia (NH_4Cl). After a period of 10 days, 34.67% of WT seeds germinated in water in comparison to only 18.33% of mutant seeds (Fig. 6).

Germination in KNO_3 solutions of increasing concentration decreased the difference in germination between WT and GLN1;5KO seeds that was observed in water (Fig. 6A, Table 3 and Supplementary Table S2). In fact, while there was still a small difference between the two genotypes for germination in 0.1 mM nitrate (73.88 %, Table 3) at any concentration >1 mM KNO_3, the two types of seeds germinated at similar rates.

Table 3. The ratio [%] of germination potentials of GLN1;5KO and WT seeds during the first 10 days of germination[1].

	treatment	GLN1;5KO / WT [%]*
H_2O	0	46.63
KNO_3 [mM]	0.1	**73.88**
	1	**118.64**
	3	**112.48**
	10	**93.54**
NH_4Cl [mM]	0.1	26.21
	1	**16.14**
	3	**20.31**
	10	**15.7**

[1]Bold values represent significant differences ($p<0.01$) between the corresponding treatment and H_2O (control). The values were calculated from the hazard ratios obtained by semi parametric time-to-event analyses (Cox proportional hazards) presented in Supplementary Table S2.

Unlike germination in nitrate solutions, germination in increasing NH_4Cl concentrations potentiated the differences between the two genotypes (Fig. 6B, Table 3). Ammonium up to 10 mM did not inhibit germination of WT seeds and even slightly stimulated it (Fig. 6B). However, germination of GLN1;5KO seeds in any NH_4Cl solution appeared to be slightly lower in comparison to germination in water (Fig. 6B).

DISCUSSION

The GLN1;5KO mutant is a SALK_086579C line, with a T-DNA insertion in the 3rd exon of the *GLN1;5* gene (AT1G48470). The production of homozygous mutant plants and their testing by genomic PCR and RT-PCR were described in detail previously [12]. Total RNA is isolated from WT and GLN1;5KO seeds, since literature data indicate that *GLN1;5* is expressed in dry seeds [3,13]. *GLN1;1*, is known to be highly expressed in dry seeds [13], and hence it was used as a positive control. RT-PCR confirmed that the insertion completely prevents *GLN1;5* mRNA formation. It was already shown that the expression of these two isoforms rapidly declines during the course of imbibition and germination [13], so this was not studied here.

Southern hybridization confirmed the presence of T-DNA inserts containing an NPT II fragment in GLN1;5KO genomic DNA, but not in the DNA of WT plants. The fact that the restriction fragments obtained by Southern hybridization were 0.3-0.4 kb longer than

predicted implied that the T-DNA RB could be longer, perhaps containing a part of the vector sequence, which was the main reason behind the mapping of only LB positions during the Signal project [15]. The results of the Southern analysis suggested the possibility of more than one insertion in the GLN1;5KO line.

Seed germination and early seedling establishment in *Arabidopsis* require N remobilization, when N stored in the cotyledons is used to sustain the growth of new sink leaves [4]. In knockout mutants lacking GLN1;2, in double GLN1;1:GLN1;2KO mutants and, to a lesser extent, in GLN1;1KO mutants, the N remobilization from cotyledons and during early seedling establishment is impaired, resulting in smaller seedling size of mutants as compared to WT plants [4]. The authors concluded that *GLN1;2* and, to a lesser extent *GLN1;1,* have a function in N remobilization from cotyledons during seed germination and seedling establishment. However, it is unlikely that *GLN1;5*, as a minor seed isoform, has a function similar to that of *GLN1;2* and *GLN1;1*, particularly because GLN1;5KO did not affect the number of rosette leaves (data not shown) but merely the time of bolting.

The studied effect of genotype on bolting showed a significant difference in the inflorescence emergence for WT and GLN1;5KO plantlets, which specifically relates to the early days of bolting. This result can be interpreted as a short delay (≈1 day) in the inflorescence emergence for GLN1;5KO as compared to WT. However, since all plants, regardless of the genotype, eventually form the inflorescence stems, this difference is likely of marginal physiological importance. It cannot be excluded that *GLN1;5* expressed at a very low level in young seedlings [12] may have supported the reallocation of N related to inflorescence emergence.

Recent work on cereals and on *Arabidopsis* has revealed that specific GS1 isoforms have important roles with respect to seed yield structure. In maize, at least three out of five cytosolic GS1 isoforms affect kernel development. Using knockout mutants, Martin et al. [23] showed that the maize gln1-4 knockout phenotype displayed reduced kernel size, while gln1-3 mutants had reduced kernel number. Interestingly, both isoforms are expressed in leaves, where GS1-3 is present in mesophyll cells, whereas GS1-4 is specifically localized in the bundle sheath cells [23]. In addition, GS1-2, which is specifically expressed in the basal maternal tissues of the developing kernel, including the surrounding pericarp and specifically in the pedicel parenchyma but not in the endosperm and embryo, is suggested to have a role in nitrogen metabolism during grain fill [24]. A rice knockout mutant lacking *OsGS1;1* showed severe retardation in growth rate and grain filling when grown at normal nitrogen concentrations [25].

In *Arabidopsis*, the main isoform *GLN1;2,* expressed in the vasculature of different organs and other tissues, plays an important role in N remobilization during both seed production and seedling establishment [4]. The authors showed that *GLN1;2* knockout mutants, *GLN1;1:GLN1;2* double mutants, and to lesser extent *GLN1;1* mutants, exhibit a decrease for several seed yield structure elements. To evaluate the physiological importance of GLN1;5 for silique development, the effect of GLN1;5 deficiency was compared to the previously described effects of GLN1;1 and GLN1;2 deficiency [4] on the number of siliques developed on the main and side branches of different knockout mutants relative to WT plants (Table 2). It is clear that, relative to WT plants, the GLN1;5KO plants develop fewer siliques than GLN1;1KO or GLN1;2KO mutants and even double GLN1;1:GLN1;2 mutants.

The fact that the length and width of immature and mature siliques and the number of seeds in them did not differ between the two genotypes suggests that GLN1;5 activity did not contribute to the growth of siliques nor to their final size. Guan et al. [4] found that GLN1;1 and GLN1;2 deficiency reduces the number of seeds per silique, but the authors did not assess the dimensions of the siliques. It should be noted that silique dimensions and the number of seeds per silique in our experimental setup were somewhat smaller than listed as a standard for WT Columbia [24], probably due to different growth conditions.

According to Guan et al. [4], both GLN1;1 and GLN1;2 isoforms contribute to seed development, since their deficiency negatively affects the seed dry weight, but GLN1;5KO mutants had lighter seeds, relative to WT, than either GLN1;1KO, GLN1;2KO or even GLN1;1:GLN1;2KO double mutants. The seed length reduction in GLN1;5KO plants relative to WT is comparable to that found for GLN1;1:GLN1;2KO

double mutants [4]. Finally, the width of immature and mature WT and mutant seeds did not differ; mutants investigated by Guan et al. [4] also had unaltered seed width. Our data suggest that the isoform *GLN1;5* was involved in seed formation and grain fill, since several seed yield structure elements were significantly decreased in the GLN1;5KO mutants, including the number of developed siliques and seed dry weight. In *Arabidopsis* seeds, most of the N is incorporated in the form of storage proteins, which accumulate during seed maturation and late maturation [26]. The seed filling requires both N uptake and assimilation and N remobilization from maternal tissues to seeds as new sinks [4]. Since *GLN1;5* transcripts accumulate during seed maturation to reach maximum in mature dry seeds [3], we can speculate that this isoform was responsible for N assimilation within the seeds. Other isoforms expressed primarily in maternal tissues, particularly *GLN1;2*, are responsible for N remobilization to support the seed development [4].

The degradation of seed storage proteins during germination produces ammonium that has to be reassimilated into Gln to be remobilized to support early seedling establishment. Since Gln acts as a sink for ammonium released from storage protein degradation and amino acid deamination and as a source for *de novo* amino acid synthesis by transamination, GS enzymes have a key role in N metabolism during germination [27]. In the absence of an external N source, germination and early seedling establishment depend exclusively on the N stored in seeds, mostly in the form of storage proteins [26]. As mentioned, *GLN1;5* transcripts accumulate during seed maturation and are stored in dry seeds, to be rapidly degraded over the course of imbibition [3,13]. However, transcript levels do not always correlate with the presence and activity of specific GS isoforms in a tissue, since posttranslational modifications such as phosphorylation and interaction with 14-3-3 proteins can significantly modulate GS activity, stability and turnover [28]. In other words, GLN1;5 proteins may be present and active in N remobilization in germinating seeds after their transcripts have been degraded. Along with *GLN1;5,* the isoform *GLN1;2* has an important role in seed reserve N remobilization, since *GLN1;2* knockout mutants are impaired in seed germination, N remobilization from cotyledons and early seedling establishment [4]. Similarly, in maize, two isoforms – *GLN3* and *GLN4,* were shown to have important roles in germination [27].

WT and GLN1;5KO mutant seeds germinate at similar rates in KNO_3 solutions, since nitrates strongly stimulate germination. One interpretation of this finding is that nitrates compensate for *GLN1;5* deficiency simply as an external N source. While KNO_3 in the medium may indeed be a nutrient, it should be noted that nitrates generally promote germination independently of nitrate reduction [29,30], therefore having a signaling rather than merely a supplying role. Namely, nitrates, various organic nitrogenous compounds, NO-donors as well as NO by itself, potentiate germination of different photoblastic seeds, including *Arabidopsis* seeds, based on the release of NO as a signaling molecule [30,31]. In *Arabidopsis*, where seed dormancy is largely due to mechanical constraints imposed by the seed coat, this stimulatory effect of nitrates and the abovementioned compounds on germination is based on interaction with phytochrome A signaling [31] and with ABA and gibberellin signaling pathways [29]. Since the stimulatory effect of nitrates on *Arabidopsis* seed germination is so pronounced, it is hard to distinguish whether any part of this stimulation was actually due to compensation of *GLN1;5* deficiency in mutant seeds or to stimulatory signaling that overrides other factors.

Germination of WT seeds in increasing NH_4Cl concentrations was not impaired, although ammonium in excess is toxic. It seems that WT seeds are able to efficiently cope with it and probably use it as a nitrogen source. However, lower germination of mutant seeds in any NH_4Cl solutions in comparison to germination in water provides good evidence that the role of *GLN1;5* is not only to reassimilate ammonia within the seed (as discussed for germination in water), but also to assimilate and detoxify ammonia from the environment. The *GLN1;2* isoform that was shown to be involved in germination [4] is also the main isoform that can be upregulated to relieve ammonium toxicity [2,11]. Even though the expression of *GLN1;5* declines during imbibition in water, if *Arabidopsis* seeds are germinated under salt stress (50-200 mM NaCl), the expression of *GLN1;5* is upregulated [13], which also suggests a possible role for *GLN1;5* in alleviating stress.

Comparison of the WT and GLN1;5KO phenotypes suggests several physiological roles for the *Arabidopsis* GS isoform *GLN1;5*. The GLN1;5 activity supports silique development and grain fill, probably by N assimilation within the seed, since it is expressed during seed maturation and in dry seeds. Even though the transcript levels of *GLN1;5* declined during imbibition, we speculate that the GLN1;5 enzyme was active in seeds during imbibition and germination, and that it had a role in ammonium reassimilation within the seed, as well as assimilation and/or detoxification of ammonium from the environment. Finally, small levels of *GLN1;5* that are expressed in young seedlings may have a minor role in promoting inflorescence stem emergence.

To the best of our knowledge, the work presented herein is a first attempt toward the elucidation of the physiological role of the *GLN1;5* isoform based on a reverse genetics approach.

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Author contributions: MD performed statistical analyses and interpreted the data. KĆ and MB were involved in morphometric data collection and RNA and DNA isolation. SZK performed the Southern analysis. AS wrote the manuscript and was involved in data interpretation. ST designed the experiments, carried out most of the work regarding morphometry and seed germination and was involved in data interpretation and finalizing the manuscript. All coauthors approved the final version of the manuscript.

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Supplementary Data

Supplementary Tables S1 and S2.
Available at: http://serbiosoc.org.rs/NewUploads/Uploads/Dragicevic%20et%20al_Supplementary%20data_4099.pdf