Glutamine synthetase and glutamate dehydrogenase in triticale seeds: molecular cloning and genes expression

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Abstract In higher plants, glutamine synthetase (GS; EC 6.3.1.2) and glutamate dehydrogenase (GDH; EC 1.4.1.2) are the predominant enzymes in nitrogen metabolism. In this study, we cloned both the GS and GDH genes and analyzed their expression levels and variations in their activity in developing and germinating *x Triticosecale* (cv. Witon) kernels. The developing kernel samples were collected 3, 5, 7, 9, 13, 15, 20, 25, 30, 35, 40 and 45 days after flowering (DAF). The germinating kernel samples were collected after 8, 16, 24, 48 and 72 h of imbibition. There are two GS isoforms that are localized to different compartments: the cytosol (GS1) and the chloroplast (GS2). Five cDNAs encoding GS proteins in triticale plants were obtained using RT-PCR. We cloned the four genes encoding GS1, which we designated *TsGS1-1*, *TsGS1-2*, *TsGS1-3* and *TsGS1-4* and the only gene encoding GS2, which was designated *TsGS2-1*. We studied the changes in the enzymatic activity and the expression profiles of the GDH, GS1 and GS2 genes in both the developing and germinating seeds of triticale. Based on our results, there is likely cooperation between GDH and GS1 in the synthesis of glutamine and glutamate during the early stages of seed formation and in the scutella of kernels for up to 24 h of imbibition.

Keywords Gene expression · Glutamate dehydrogenase · Glutamine synthetase · Seed · Triticale

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Introduction

Nitrogen, which is the most important plant nutrient, is a factor which significantly limits the productivity of cereal crops. The NH_4^+ ion (ammonia) is the only form of nitrogen that can be incorporated into organic compounds by plants (Masclaux-Daubresse et al. 2005). In higher plants, ammonium is mainly assimilated through combined action of glutamine synthetase (GS, EC. 6.3.1.2) and glutamate synthase (GOGAT, EC 1.4.1.14). Alternatively, glutamate dehydrogenase (GDH, EC. 1.4.1.2-4) can also catalyze ammonium incorporation into glutamate by reductive amination of 2-oxoglutarate (Kwinta and Bielawski 1998; Mifflin and Habash 2002).

GS catalyzes the ATP-dependent condensation of NH_4^+ with glutamate to produce glutamine. In plants, GS can exist as distinct isoforms that are classified into groups according to their cellular localization. These isoforms include a cytosolic form, GS1, and a chloroplastic form, GS2 (Bielawski 1993; Mifflin and Habash 2002). In general, there is only one gene encoding GS2, with the exception of *Medicago truncatula* for which two genes were cloned (Seabra et al. 2010); however, a small, multi-gene family encodes for the GS1 proteins (Masclaux-Daubresse et al. 2010). At this time, at least three GS1 genes in rice (Tabuchi et al. 2005), five genes in maize (Martin et al. 2006) and seven genes in wheat (Bernard et al. 2008) have been identified. Studies have shown that both GS isoenzymes are regulated in a developmental manner in the leaves (Habash et al. 2001), and both isoenzymes have different metabolic roles. During the vegetative stage, GS2 assimilates ammonia originating from nitrate reduction and photorespiration (Wallsgrrove et al. 1987; Lam et al. 1996). GS1 isoforms are responsible for assimilating the ammonium produced by the reduction of nitrate in the root, and

are responsible for synthesizing glutamine for transporting nitrogen between different organs. During leaf senescence, cytosolic GS provides a key function in the assimilation and recycling of ammonia generated from various catabolic processes (Bernard et al. 2008). This role is important after anthesis, during seed development and filling in cereals when nitrogen is remobilized to the reproductive sinks. However, thus far, only two studies have firmly assigned cytosolic GS genes to leaf-related remobilization of assimilate to the seed (i.e., *OsGln1;1* in rice (Tabuchi et al. 2005) and *Gln1-3*, *Gln1-4* in maize (Martin et al. 2006).

GDH is found in all plant tissues but its metabolic role is still debated. In vitro, the enzyme catalyzes the reversible amination/deamination reaction between 2-oxoglutarate and glutamate (Kwinta and Bielawski 1998). However, in vivo the reversibility of the reaction catalyzed by GDH is disputed. There is evidence that GDH may operate in the catabolic direction, to fuel the TCA cycle under conditions of carbon limitation (Limami et al. 2008; Miyashita and Good 2008). GDH may also function in the direction of glutamate biosynthesis, under conditions of carbon and ammonium excess (Abiko et al. 2010; Lehmann et al. 2010). GDH is responsible for maintaining the balance between the metabolic pool of carbon and nitrogen compounds during each developmental phase, from seed germination to seed formation (Mifflin and Habash 2002). Immunohistochemical studies have determined that the NAD(H)-GDH protein is located mainly in the mitochondria, but occasionally this enzyme is found in the cytoplasm of phloem companion cells (Paczek et al. 2002; Tercé-Laforgue et al. 2004). The native GDH proteins consist of α and β subunits that combine in different ratios to form a hexamer and these combinations create the different GDH isoenzymes (Loulakakis and Roubelakis-Angelakis 1996). In most plant species, GDH is encoded by two distinct nuclear genes and each gene encodes a different subunit α or β (Melo-Oliveira et al. 1996; Restivo 2004). Only one gene coding for the GDH has been cloned from tomato, while the existence of two subunit types has been explained by its post-transcriptional modifications (Purnell et al. 1997). In our previous experiments, we have cloned and characterized one gene encoding GDH in triticale (Grabowska et al. 2011); however, its function in the developing kernels has not been studied in detail.

In the present work, our studies on GS and GDH were performed on developing and germinating seeds from triticale, which is an important feed grain. Triticale grain has a high content of protein and a higher content of lysine compared to wheat. Despite its nutritional advantages, triticale is still poorly characterized at the biochemical and molecular levels, particularly in terms of its nitrogen metabolism. Nitrogen content in the seeds further

determines germination efficiency and survival of young seedlings.

This is the first report to present a detailed account on the molecular cloning and characterization of glutamine synthetase genes in triticale. We analyze the expression patterns of these enzymes and assay the activity of two isoforms, TsGS and TsGDH1, in triticale seeds. We investigated the possible roles for GS and GDH during seed development and germination. The results are discussed in the context of our current knowledge of GS and GDH in other plant species.

Materials and methods

Plant material and growth conditions

Triticale *x Triticosecale* Wittm. cv. Witon were grown in soil in 10-l pots at ten plants per pot in a greenhouse. The soil was supplemented with 2.5 g N/pot in the forms of KNO_3 and NH_4NO_3 . The complete nutrient solution contained: 9 % Mg^{2+} , 0.9 % Mn^{2+} , 0.7 % Cu^{2+} , 0.6 % Zn^{2+} , 0.08 % B^{3+} , 0.01 % Mo^{2+} and 0.005 % Co^{2+} . Plants were vernalized for 6 weeks at 4 °C prior to transplanting in the greenhouse. For the seed development studies, individual spikes were tagged on the day of flowering, and seeds were collected from spikes harvested at different time points: 3, 5, 7, 9, 13, 15, 20, 25, 30, 35, 40 and 45 DAF (days after flowering). The seeds after 45 DAF were the dry grains. For the seed germination studies, dry seeds were surface-sterilized with a solution of 5 % hypochlorite and then germinated in the dark at 22 °C and 100 % relative humidity. Samples were collected after 8, 16, 24, 48, and 72 h of germination. The samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

Total RNA extraction

Total RNA was extracted from the seeds at 3, 5, 7, 9, 11, 13, 15, 20, 25, 30, 35, 40 and 45 DAF. Total RNA was also collected from the scutella and endosperms of dry seeds that were imbibed for 8, 16, and 24 h, and from the scutella, endosperms, shoots and roots of seeds imbibed for 48 and 72 h. The RNA was isolated using a guanidinium thiocyanate/acidic phenol extraction method (Chomczynski and Sacchi 1987), with an additional initial extraction as described Zdunek-Zastocka (2010). The RNA concentration and purity were determined spectrophotometrically by measuring absorption at 260, 230 and 280 nm. The integrity of the isolated RNA was analyzed on a 1.5 % (w/v) agarose/formaldehyde gel. To eliminate any genomic DNA contamination, the total RNA was treated with RNase-free DNAase I (Fermentas).

Cloning of the triticale full-length *glutamine synthetase* cDNA

Four micrograms of total RNA was isolated from a pool of differently developed seeds, scutella, endosperms, shoots and roots imbibed for 48 and 72 h. First-strand cDNA was synthesized using 200 U of Superscript II reverse transcriptase (Invitrogen) and 1 μ l biotinylated oligo-dT₂₅ (700 ng ml⁻¹). Conserved domains were identified by aligning the amino acid sequences of several GS1 and GS2 genes identified on the GenBank website. Based on the sequences of the conserved domains, primer pairs (GS1-F/GS1-R and GS2-F/GS2-R) were designed to amplify the core gene fragments (Table 1). PCRs were performed in a PTC-200 Peltier Thermal Cycler (MJ Research) using the following conditions: 2 min at 94 °C, 37 cycles of 30 s at 94 °C, 30 s at 62 °C, 45 s at 72 °C, and a final extension step for 5 min. The PCR was performed in a volume of 50 μ l containing 1 μ l of cDNA, 0.2 mM of each dNTP, 0.5 μ M of each primer, 1 $\times$ Green GoTaq[®] Flexi Buffer, 5 mM MgCl₂ and 1.25 U of GoTaq[®] Flexi DNA Polymerase (Promega). The resulting amplicon was cloned into the pGEM-T easy plasmid vector (Promega) and sequenced. Sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Kit on an ABI Prism 3730 DNA analyzer (Applied Biosystems). Trace files were checked and edited using Chromas 1.55 (Technelysium, USA).

The full-length cDNAs were obtained using the GeneRacer Kit (Invitrogen). The RACE-ready, first-strand cDNAs used as the templates for 5' RACE and 3' RACE

were prepared from total RNA that was isolated from a pool of differently developed seeds, scutella, endosperms, shoots and roots of seeds imbibed for 48 and 72 h. For the 5' RACE and 3' RACE reactions, we used either random primers or a GeneRacer OligodT Primer, respectively, according to the manufacturer's instructions. First-strand synthesis was performed using SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. The gene-specific primers used for RACE were designed from the aforementioned, partial *TsGS1* and *TsGS2* cDNA sequences. In Table 1, only primers used for the amplification of the longest 5' and 3' ends are displayed.

The PCR amplification of the 5' ends of *TsGS2-1*, *TsGS1-1*, *TsGS1-2*, *TsGS1-3* and *TsGS1-4* was performed using the GeneRacer 5' primer and gene-specific primers (GSP): rGS2/1-R, rGS1/1-R, rGS1/2-R, rGS1/3-R, rGS1/4-R, respectively, using the following conditions: 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at X °C (see appropriate temperatures for each primer in Table 1), 1 min at 68 °C, and a final extension for 10 min at 68 °C. The PCR was performed in a volume of 50 μ l containing 1 μ l of cDNA template, 0.2 mM of each dNTP, 0.2 μ M of GSP, 0.6 μ M of GeneRacer 5' Primer, 1 $\times$ high fidelity PCR buffer, 5 mM MgCl₂ and 2.5 U of Platinum[®] Taq DNA Polymerase High Fidelity (Invitrogen). The PCR amplifications of the 3' ends of *TsGS2-1*, *TsGS1-1*, *TsGS1-2*, *TsGS1-3* and *TsGS1-4* were performed using the GeneRacer 3' primer and GSP: rGS2/1-F, rGS1/1-F, rGS1/2-F, rGS1/3-F and rGS1/4-F, respectively. The PCR conditions

Table 1 Primer sequences used for cloning the triticale full-length glutamine synthetase cDNAs

Starter name	Forward and reverse primers (5' → 3')	Application	Product size (bp)	X-Annealing temperature (°C)
GS1-F	CGACGGCTCCAGCACCGGCCAGGC	1st PCR for <i>TsGS1-1</i> , <i>TsGS1-2</i> , <i>TsGS1-3</i> , <i>TsGS1-4</i>	702	62
GS1-R	TCGTTGCCCTCGCCGTAGGCGGCG			
GS2-F	GCAGGTCGGCCGTCCCGGCGAGGCAGCC	1st PCR for <i>TsGS2-1</i>	747	62
GS2-R	CTGCATCAATACCAACGCTGGGTCCAAC			
rGS2/1-F	AGGATCAGACAAGTCATTTGGCCGTGACAT	3' RACE for <i>TsGS2-1</i>	893	59
rGS2/1-R	CACCTTGAAGCCGGAGGTGGCACGGGCGGTC	5' RACE for <i>TsGS2-1</i>	282	62
rGS1/1-F	CGTGGATGCCGTGGAGAAGCTCAAGCTCAA	3' RACE for <i>TsGS1-1</i>	274	60
rGS1/1-R	CTGCTCAATACCATAACCATGGCTCCTCCTTGGA	5' RACE for <i>TsGS1-1</i>	390	61
rGS1/2-F	ACCGAGATCGCCGGAGTTGTCGTACATTT	3' RACE for <i>TsGS1-2</i>	574	63
rGS1/2-R	AGCCACCAACAGGCCAGCCGAGAGGCCAGTTGAT	5' RACE for <i>TsGS1-2</i>	497	59
rGS1/3-F	ATCAAGAAGGCGATCAAGAAGCTCGAGGCG	3' RACE for <i>TsGS1-3</i>	511	60
rGS1/3-R	CCGCTTGTTGCTCGGAATCGGCTCTCCGTTA	5' RACE for <i>TsGS1-3</i>	455	63
rGS1/4-F	ATCAAGACGGCCATCGAGAAGCTGGGCAAG	3' RACE for <i>TsGS1-4</i>	270	60
rGS1/4-R	GCCTCTGTTCGTGGGGATTGGCACACCTTGG	5' RACE for <i>TsGS1-4</i>	399	64

and the reaction mix were the same as described above. Amplification products were cloned into the pCR[®]4Blunt-TOPO[®] vector (Invitrogen) and sequenced.

Expression analysis by semi-quantitative RT-PCR

Semi-quantitative RT-PCR analysis was performed using the One-Step RT-PCR Kit (Novagen). The oligonucleotide primer sequences for *TsGS1-3*, *TsGS2-1*, and *TsGDH1*, and the annealing temperatures used for each individual RT-PCR, are displayed in Table 2. The RT-PCRs were performed using the following conditions: 30 min at 60 °C, 2 min at 94 °C, 31 cycles (for *TsGS1-3*, *TsGS2-1* and *TsGDH1*) and 10 cycles (for *rRNA*) of 30 s at 94 °C, 30 s at 56 or 60 °C, 30 s at 72 °C and a final extension step for 5 min at 72 °C. The PCR was performed in a total volume of 50 µl containing 100 ng of RNA, 0.2 mM of each dNTP, 0.5 µM of each primer, 1× Reaction Buffer, 5 mM Mn(OAc)₂, 15 units of RNase Inhibitor and 5 U *Tth* Polymerase. The subsequent RT-PCR-generated fragments were cloned into the pGEM-T Easy vector (Promega) and sequenced. To ensure that equal amounts of RNA template were added to each RT-PCR, amplification of the *18S rRNA* gene was performed as indicated in Table 2. The amplified products were analyzed by electrophoresis on 1.5 % agarose gels containing ethidium bromide. Densitometry analyses were conducted using the program Image J, version 1.44 (<http://www.rsb.info.nih.gov/ij>).

Enzyme assays and protein analysis

Glutamate dehydrogenase (GDH) was extracted by homogenizing 0.2 g of developing seeds (3, 5, 7, 9, 13, 15, 20, 25, 30, 35, 40 and 45 DAF) in 1 ml of extraction buffer containing 100 mM Tris–HCl (pH 7.8), 5 mM 2-mercaptoethanol and 20 µM PMSF. An Ultra-Turrax T25 (IKA) homogenizer was used for two, 60 s cycles at 20,000 rpm. The homogenates were centrifuged for 30 min at 10,000×g at 4 °C and the supernatant was used as the

enzyme extract. Supernatants were collected and kept on ice for GDH assays and protein concentration analysis. All assays were performed at 30 °C. GDH activity was determined in both the aminating (NADH–GDH) and deaminating (NAD⁺–GDH) directions by measuring the absorption change at 340 nm (Barash et al. 1973). The standard amination reaction mixture contained 100 mM Tris–HCl (pH 8.3), 200 mM NH₄Cl, 0.28 mM NADH, 32 mM 2-oxoglutarate, 0.15 ml of enzyme extract and deionized water, which were added to a final volume of 1.5 ml. The standard deamination reaction mixture contained 100 mM Tris–HCl (pH 9.2), 200 mM L-glutamate, 0.25 mM NAD⁺, 0.2 ml of enzyme extract and deionized water, which were added to a final volume of 1.5 ml. One unit of GDH activity was defined as the oxidation or reduction of 1 µmol of coenzyme (NADH or NAD⁺) per min at 30 °C g^{−1} of dry weight (DW).

Glutamine synthetase (GS) was extracted by homogenizing 0.2 g of developing seeds (3, 5, 7, 9, 13, 15, 20, 25, 30, 35, 40 and 45 DAF), scutella and endosperms of seeds imbibed for 8, 16, 24 h or from scutella, endosperms, shoots and roots of seeds imbibed for 48 and 72 h. Homogenization occurred in a 1 ml extraction buffer containing 100 mM Tris–HCl (pH 7.9), 1 mM EDTA, 20 mM MgSO₄, and 1 mM 2-mercaptoethanol. An Ultra-Turrax T25 (IKA) was used for 45 s at 20,000 rpm. The homogenates were centrifuged for 20 min at 10,000×g at 4 °C. The supernatant was used as the enzyme extract. Supernatants were collected and kept on ice for the GS and protein concentration assays. GS activity was measured using the synthetase assay based on the method described by O'Neal and Joy (1973) and optimized for triticale. Two hundred and fifty microliters of enzyme extract was added to 200 µl of assay mix that contained 265 mM glutamate and 60 mM hydroxylamine. The reaction began by the addition of 50 µl of 80 mM ATP and incubated at 30 °C for 30 min. The reaction was stopped by adding 125 µl of 0.5 M trichloroacetic acid. The samples were incubated at 20 °C, followed by centrifugation at 10,000×g for 10 min.

Table 2 Primer sequences and PCR conditions used for the semi-quantitative RT-PCR analysis

Gene	Forward and Reverse primers (5' → 3')	Product length (bp)	PCR annealing temperature (°C)	PCR cycle number
<i>TsGS1-3</i>	TCCCCTGTCCTCGCATTTCCAGAGA GGAGCTTGCTGGGGTCATCAACGGG	274	56	31
<i>TsGS2-1</i>	AGGTCGCCCCGCCCCCTTCCCTCCTC GGCCAGCACCTTGAAGCCGGAGGT	262	60	31
<i>TsGDH1</i>	AATCCCGAAAGATGATGGGACATTA ATCCATGCCATTGTCTGTGCGTTG	339	56	31
<i>18S rRNA</i>	CCAGGTCCAGACATAGTAAG GTACAAAGGGCAGGGACGTA	427	60	10

Five hundred microliters of supernatant was added to 250 μ l of 0.5 M ferric chloride and incubated for 15 min at 20 °C. The controls contained all of the reaction components except for the ATP. The formation of γ -glutamyl hydroxamate (GHM) was determined by measuring the absorbance at 540 nm using a spectrophotometer. The GS activity was expressed as the μ mol of GHM per min at 30 °C per DW.

The protein concentrations of the tissue extracts were determined with the Bradford method (Bradford 1976) using bovine serum albumin to create the standard curve. Each measurement was repeated three times, and the results are reported as the mean of two independent biological experiments.

Polyacrylamide gel electrophoresis

Discontinuous native-PAGE was performed as described by Ornstein (1964) and Davis (1964) and according to the manufacturer's protocol (Mini-Protein Tetra Cell, Bio-Rad). A 4 % stacking gel and a 7.5 % separating gel were used. Equal amounts (50 μ g) of soluble protein extracted from the developing seeds were added to each lane. Electrophoresis was performed at 120 V for 1 h at 4 °C in a Tris–glycine buffer system.

After electrophoresis, the protein bands containing GDH activity (deamination) were visualized by incubation in 100 mM Tris–HCl (pH 9.2) with 100 mM L-glutamate, 0.75 mM NAD, 0.4 mM nitroblue tetrazolium and 0.26 mM phenazine methosulfate for 15–60 min at 37 °C (Hartmann et al. 1973). As a control, an identical gel was incubated in the GDH stain solution minus the glutamate.

Bioinformatics analysis

Sequences were verified by a database search of the National Center for Biotechnology Information server using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov>). Sequencing dates from this article have been deposited on GenBank. The deduction of the amino acid sequences and calculation of the theoretical molecular masses and pIs were performed using ExPASy (<http://www.expasy.ch/tools/>). Multiple alignment of the amino acids sequences were generated using the program CLUSTALW (Thompson et al. 1997). Phylogenetic analysis was performed using the neighbor-joining (NJ) method, as implemented in the PhyML program (<http://www.phylogeny.fr/version2.cgi/index.cgi>) (Dereeper et al. 2010). The bootstrap value was generated using 100 non-parametric replicates. The sub-cellular localization of the deduced protein was predicted using TargetP (Emanuelsson et al. 2007).

Statistical analysis

The measurements of enzyme activities are presented as the mean $\pm$ SD (standard deviation) for three independent experiments, with two replications each. Statistical significance was determined using the Student's *t* test. The result was considered to be significant if the *P* value was less than 0.05 (MS Excel).

Results

Cloning of full-length *TsGS* cDNAs and sequence analysis of the *TsGS* and *TsGDH1* genes

Using RT-PCR, five *TsGS* genes were isolated and designated as *x Tritico-secale* cytosolic glutamine synthetase (*TsGS1-1*, *TsGS1-2*, *TsGS1-3*, *TsGS1-4*; GenBank accession numbers: JN188397, JN188396, JN188395, JN188394, respectively) and plastid glutamine synthetase (*TsGS2-1*, GeneBank accession number: JN188398). The PCR amplification of the core fragments of the *GS1* and *GS2* cDNAs was performed using two primer pairs: *GS1-F/GS1-R* and *GS2-F/GS2-R* (Table 1). The primers were subsequently used to amplify the 702 and 747 bp fragments of *GS1* and *GS2*, respectively. Ten additional primers (Table 1) were designed based on the confirmed sequences and used to isolate the 5' and 3' ends of the sequences.

The full-length *TsGS1-1* cDNA is 1,453 bp and contains a 1,071 bp open reading frame (ORF), a 32 bp 5' untranslated region (5' UTR) and a 350 bp 3' UTR. *TsGS1-1* encodes a protein containing 356 amino acids with a calculated molecular mass of 39.11 kDa and an isoelectric point of 5.53. The full-length *TsGS1-2* cDNA is 1,295 bp and contains a 1,071 bp ORF, a 49 bp 5' UTR and a 175 bp 3' UTR. *TsGS1-2* encodes a protein containing 356 amino acids with calculated molecular mass of 39.20 kDa and isoelectric point of 5.41. The full-length *TsGS1-3* cDNA is 1,440 bp and contains a 1,089 bp ORF, a 134 bp 5' UTR and a 217 bp 3' UTR. *TsGS1-3* encodes a protein containing 362 amino acids with a calculated molecular mass of 39.50 kDa and an isoelectric point of 5.66. The full-length *TsGS1-4* cDNA is 1,142 bp and contains a 1,065 bp ORF and a 77 bp 5' UTR. *TsGS1-4* encodes protein of 354 amino acids with a calculated molecular mass of 38.72 kDa and an isoelectric point of 5.35. Sequence analyses indicated that all *TsGS1* proteins contain two conserved domains. The first domain includes an N-terminal beta-Grasp domain from Tyr-23, Glu-17, Gly-17 or Asp-17 to Pro-97 for *TsGS1-1*, *TsGS1-2*, *TsGS1-3* and *TsGS1-4*, respectively. The second domain is the C-terminal catalytic domain from residue Pro-103 to Thr-350 (Fig. 1).

The full-length *TsGS2-1* cDNA is 1,687 bp long and contains a 1,284 bp open reading frame, a 147 bp 5' UTR and a 256 bp 3' UTR. *TsGS2-1* encodes a protein of 427 amino acids with a calculated molecular mass of 46.70 kDa and an isoelectric point of 5.75. Hits analyses indicated that *TsGS2-1* contains two conserved regions. These domains include an N-terminal beta-Grasp domain from Asp-72 to Pro-152 and a C-terminal catalytic domain from residue Pro-158 to Thr-405. *TsGS2-1* had the structure of the plant plastid GS isoform (Fig. 1). The TargetP program predicted that *TsGS2-1* contains a chloroplast target peptide (cTP) of 47 residues on its N-terminus (score 0.752, TargetP) (Fig. 1). With the cTP sequence removed, the calculated molecular mass of the mature *TsGS2-1* protein is 42.70 kDa and has an isoelectric point of 5.02.

The previously cloned full-length *TsGDH1* gene (Grabowska et al. 2011) encoded 411 amino acids. The deduced amino acid sequence *TsGDH1* contained two features present in all GDH proteins. First, a Glu/2-OG binding site from Ile-96 to Pro-109, and second, an NAD(H)-binding site from Phe-209 to Asp-237 (Britton et al. 1992). Additionally, *TsGDH1* contains a mitochondrial targeting peptide (mTP) on its N-terminus (score 0.323, TargetP).

Homology and phylogenetic analysis of plant glutamine synthetase

The sequence alignment analyses revealed that *TsGS1*s are highly homologous at both the nucleotide and the deduced amino acid levels. *TsGS1-1* shares a 97 % identity with *TsGS1-2*, and 79 and 81 % identities with *TsGS1-3* and *TsGS1-4*, respectively. The *TsGS1-2* sequence is 79 % identical to *TsGS1-3* and 82 % identical to *TsGS1-4*. *TsGS1-3* shares a 79 % identity with *TsGS1-4*. A similar identity percentage between *TsGS1* sequences was observed at the amino acid level. For *TsGS2-1*, sequence identity at the nucleotide and the deduced amino acid levels with all *TsGS1*s was approximately 60 %. All *TsGS*s genes are highly homologous to wheat genes. *TsGS1-1* shares a 99.6 % identity with *GS1a* (DQ124209), *TsGS1-2* shares a 99.9 % identity with *GS1b* (DQ124210), *TsGS1-3* shares a 99.1 % identity with *GSe2* (AY491971), *TsGS1-4* and *TsGS2-1* shares a 100 % identity with *GSr2* (AY491969) and *GS2c* (DQ124214), respectively.

To investigate the evolutionary relationships between the *TsGS*s and *GS*s from other plant species, a phylogenetic tree was constructed based on the deduced amino acid sequences of the *GS* genes. The phylogenetic analysis revealed that all plant *GS*s are closely related and can be grouped into two clusters (Fig. 2). All cloned *TsGS*s genes included orthologous genes from the major crops species: wheat, maize and rice. Triticale is a hybrid of wheat and

rye. The presence of ten *GS* genes in wheat may suggest the occurrence of additional genes in triticale. The phylogenetic analysis indicated that all members of *GS1* and *GS2* were classified into one group, respectively. *TsGS2-1* clustered with the *GS2*s from *M. truncatula* (AAO37651, ADM45299, ADM45300), *Triticum aestivum* (AAZ30060, AAZ30061, AAZ30062), *Oryza sativa* (P14655), *Zea mays* (P25462) and *Secale cereale* (AFB698778).

The *GS1* cluster is subdivided into three groups. The first group contained one of three rice *GS1* genes (P14656), two of the five maize *GS1* genes (P38561 and P38562), three of the seven wheat *GS1* genes (AAZ30057, AAZ30058 and AAZ30059), one of the two rye *GS1* genes (AFB69879) and two of the four triticale *GS1* genes (*TsGS1-1* and *TsGS1-2*). Other *GS1* members clustered in the second group with one of the five maize *GS1* genes (P38560), one of the three rice *GS1* genes (Q4W8D0), two of the seven wheat *GS1* genes (AAR84350 and AAR84349), one of the four triticale *GS1* genes (*TsGS1-3*) and one cotton *GS1* gene (ABW89466). The third class contained two of the five *GS1* genes found in both maize and wheat (maize: P38563, P38559; wheat: AAR84347, AAR84348), one of the two rye *GS1* genes (AFB69880), one of the three rice *GS1* genes (P14654) and one of the four triticale genes (*TsGS1-4*) (Fig. 2).

Expression analysis by semi-quantitative RT-PCR

Expression of the *TsGDH1*, *TsGS1-3* and *TsGS2-1* genes were investigated using semi-quantitative RT-PCR of the total RNA isolated from seeds during different developmental stages (Fig. 3). In addition, expression analysis was performed on the *TsGS1-3* and *TsGS2-1* genes in the scutella and endosperms of dry seeds, or seeds imbibed for 8, 16 or 24 h. Expression analysis was also performed on these two genes in the scutella, endosperms, shoots and roots of seeds imbibed for 48 and 72 h (Fig. 4). The expression of *TsGS1-1*, *TsGS1-2* and *TsGS1-4* genes displayed low transcript levels in the triticale seeds (data not shown). Based on these results and the phylogenetic analysis (Fig. 2), we chose to analyze the expression of the cytoplasmic *TsGS1-3* gene.

The *TsGDH1* mRNA was detected at all stages of seed development. The highest level (100 % relative intensity) in the seeds was observed at 15 DAF. The *TsGDH1* transcripts levels were high at 3 DAF and then gradually declined until 9 DAF. High levels were again observed at 13, 15 and 20 DAF. This was followed by a gradual decrease in transcript levels until 30 DAF, and subsequently, a further increase in transcript levels during the final stages of seed formation. The level of *TsGS1-3* transcript gradually increased during the early stages of kernel development, reaching its maximum at 13 DAF (100 % relative intensity), and then

Fig. 1 ClustalX multiple alignment of triticales glutamine synthetase amino acid sequences. Conserved amino acid residues are highlighted with *shadows*. The cTP sequences are enclosed in a *box*. The N-terminal beta-Grasp domain is shown by *dots* and the C-terminal catalytic domain is *underlined*

TsGS1-1	-----	-
TsGS1-2	-----	-
TsGS1-3	-----	-
TsGS1-4	-----	-
TsGS2-1	MAQAVVPAMQCCQVGVGRSAVPAQAPAGRVWGVRRRTARATSGFKVLA LGPETT	53
cTP (chloroplast Transit Peptide)		
TsGS1-1	--MALLTDLNLDLTDSEKIIAAYIWIGGSGMDLRKARTLGGPVTDPSKLP	51
TsGS1-2	--MALLTDLNLDLTDSEKIIAAYIWIGGSGMDLRKARTLGGPVTDPSKLP	51
TsGS1-3	--MSPLADLLSLDLSGCCGKIIAAYIWVGGTGMDVRKARTLGGPVDDPSKLP	51
TsGS1-4	--MASLADLVNLDLSDCGKIIIVAYLWVGSGGIDIRKARTVNGPIIDASQLP	51
TsGS2-1	GVIQRMQQLLDMDTTPFDKIIAAYIWVGSGGIDLRKSRITISKPEDESELP	106
N-terminal beta-Grasp domain		
TsGS1-1	KWNYDGSSTGQAPGEDSEVILYPQATFRDPFRKGNILVMCDQYTPAGVPIPT	104
TsGS1-2	KWNYDGSSTGQAPGEDSEVILYPQATFKDPFRKGNILVMCDQYTPAGVPIPT	104
TsGS1-3	KWNFDGSSTGQATGDDSEVILCPQATFRDPFRKGNILVICDQYAPNGEPIPS	104
TsGS1-4	KWNYDGSSTGQAPGEDSEVILYPQATFKDPFRGDNLLVMCDQYTPAGVPIPT	104
TsGS2-1	KWNYDGSSTGQAPGEDSEVILYPQATFKDPFRKGNILVICDQYTPAGVPIPT	159
TsGS1-1	NKRYNAAKIFSNEEDVAKEEFWYGIEQEYTLQKDDINWPLGWVPGGFPQGPGPY	157
TsGS1-2	NKRYNAAKIFSNEEDVAKEEFWYGIEQEYTLQKDDINWPLGWVPGGFPQGPGPY	157
TsGS1-3	NKRYNAARIFGHEEVKAEFEWYGIEQEYTLQKDDINWPLGWVPGGFPQGPGPY	157
TsGS1-4	NKRHNAAKIFNTEPKVAEEETWYGIEQEYTLQKDDINWPLGWVPGGFPQGPGPY	157
TsGS2-1	NKRHMAAQIFSDEKVTAEVWFEGIEQEYTLQKDDINWPLGWVPGGFPQGPGPY	212
TsGS1-1	YCSIGADKSFGRDIVDAHYKACLFAGVNIISGINGEVMPPQWQEFQVGPVVGISA	210
TsGS1-2	YCSIGADKSFGRDIVDSHYKACLFAGVNIISGINGEVMPPQWQEFQVGPVVGISA	210
TsGS1-3	YCAAGAEKSYGRDIVDAHYKACLYAGINISGINAEVMPQWQEFQVGPVVGISA	210
TsGS1-4	YCAAGADKAFGRDIVDAHYKACLYAGINISGINAEVMPQWQEFQVGPVVGISA	210
TsGS2-1	YCAVGSDEKSFGRDISDAHYKACLYAGIEISGINAEVMPQWQEFQVGPVVGISA	265
C-terminal catalytic domain		
TsGS1-1	GDOAVVARYLLERITEIAGVVVTEDPKPIPGDWNAGAGAHTNYSTESMRKDDGGF	263
TsGS1-2	GDOVWVARYLLERITEIAGVVVTEDPKPIPGDWNAGAGAHTNYSTESMRKDDGGF	263
TsGS1-3	GDEFWAARYILERITEIAGVVVSEDPKPIPGDWNAGAGAHTNYSTESMRSEGGY	263
TsGS1-4	SDQLWVARYILERITEVAGVVLSLDPKPIPGDWNAGAGAHTNYSTESMRREAGGY	263
TsGS2-1	GDHIWASRYILERITEQAGVVLTLDPKPIPGDWNAGAGCHTNYSTESMRREDGGF	318
TsGS1-1	KVIIVDAVEKLLKHKHEHIAAYGEGNERRLTGKHETADINTFSWGVANRGASVR	316
TsGS1-2	KVIIVDAVEKLLKHKHEHIAAYGEGNERRLTGKHETADINTFSWGVANRGASVR	316
TsGS1-3	BVIKKATKKLEARHMEHIAAYGEGNERRLTGRHETADINTFSWGVANRGASVR	316
TsGS1-4	BVIKTAIEKLGKRHAQHIAAYGEGNERRLTGHETADINTFKWGVADRGASVR	316
TsGS2-1	DVIKKAILNLSLRHDLHIAAYGEGNERRLTGLHETASISDFSWGVANRGCSIR	371
TsGS1-1	VGRETEQNGKGYFEDRRPASNMDPYVVTSMIAETTLWKP-----	356
TsGS1-2	VGRETEQNGKGYFEDRRPASNMDPYVVTSMIAETTLWKP-----	356
TsGS1-3	VGRDTEKEGKGYFEDRRPASNMDPYVVTSLIAETTLCKAGLSNGK-----	362
TsGS1-4	VGRDTEKDGKGYFEDRRPASNMDPYVVTSMIAETTL-----	354
TsGS2-1	VGRETEAKGKGYLEDRRPASNMDPYVTALLAETTLWEPTLEAEALAAKKLA	424
TsGS1-1	---	-
TsGS1-2	---	-
TsGS1-3	---	-
TsGS1-4	---	-
TsGS2-1	LKV 427	

gradually declined to very low levels at 45 DAF. The transcript level of *TsGS2-1* was at the highest at 7 DAF (100 % relative intensity), and then gradually declined to very low levels from 25 to 45 DAF (Fig. 3).

The expression levels of the *TsGS1-3* and *TsGS2-1* genes were also studied in dry and germinating triticales seeds imbibed for 8, 16, 24, 48 and 72 h. The transcripts of both genes were detected in all examined tissues. The highest

Fig. 2 Phylogenetic analysis of glutamine synthetase from different plant species. The number above each branch represents the bootstrap support for 100 replicates. The analysis was based on the deduced amino acid sequences of the GS genes. The names of the genes are given in *brackets*. The accession numbers ADM45299, ADM45300 and AAO37651 correspond to the *Medicago truncatula* (*Mt*) proteins; AAZ30060, AAZ30061, AAZ30062, AAZ30057, AAZ30058, AAZ30059, AAR84347, AAR84348, AAR84349 and AAR84350 correspond to the *Triticum aestivum* (*Ta*) proteins; ABF698778, ABF698779, ABF69880 correspond to the *Secale cereale* (*Sc*) proteins; P14656, P14654, Q4W8D0 and P14655 correspond to the *Oryza sativa* proteins (*Os*); P38559, P38560, P38561, P38562, P38563 and P25462 correspond to the *Zea mays* (*Zm*) proteins; ABW89466 corresponds to the *Gossypium hirsutum* (*Gh*) protein; AEM42903, AEM42902, AEM42901, AEM42900 and AEM42904 correspond to the *x* *Triticosecale* (*Ts*) proteins

expression levels were observed in the scutella of seeds imbibed for 24 h (100 % relative intensity), and the shoots of seeds imbibed for 72 h, for *TsGS1-3* and *TsGS2-1*, respectively. The transcript level of *TsGS1-3* was also high in the scutella of seeds imbibed for 8 and 16 h, and in the endosperms and the roots of seeds imbibed for 72 h. The lowest level of the *TsGS1-3* transcript was observed in the endosperms of dry seeds. *TsGS2-1* transcript levels were high in the shoots of seeds imbibed for 48 h, and in the scutella of seeds imbibed for 72 h (Fig. 4).

Glutamate dehydrogenase and glutamine synthetase activity

The process of kernel development is accompanied by the synthesis of many nitrogen storage substances, including amino acids and proteins. The development of tritcale seeds can be separated into three phases: seed formation, filled grains and maturation and desiccation.

During each stage of kernel development studied, both aminating (NADH-GDH) and deaminating activity (NAD⁺-GDH) were determined. During the initial phase of kernel development, the assayed activity levels of the NADH-dependent GDH were much higher than the deaminating activity (NAD⁺-GDH). Particular attention should be paid to the very high aminating activity of the enzyme, which was equal to 8.53 μmol of oxidized coenzyme (ox. coenz.) min⁻¹ g⁻¹ DW at 3 DAF. This activity rapidly dropped during the next few days (i.e., at 5 and 7 DAF) to reach the maximum value of 8.56 μmol ox. coenz. min⁻¹ g⁻¹ DW at 11 DAF (Fig. 5).

Similar trends were observed in the changes in deaminating activity of the NAD⁺-dependent GDH enzyme. The samples collected on consecutive days (i.e., 13–45 DAF) displayed a steady decline in their NADH-GDH activity. The lowest aminating activity of the enzyme (0.87 μmol ox.coenz. min⁻¹ g⁻¹ DW) was assayed in the seeds on 45 DAF. From 25 to 30 DAF, an increased activity of

Fig. 3 Expression of the *TsGDH1*, *TsGS1-3* and *TsGS2-1* genes in the developing triticale seeds. Quantitative, one-step, RT-PCR analyses were performed using 100 ng of total RNA. Amplification of the 18S *rRNA* gene was used to ensure that equal amounts of the template were added to each RT-PCR. Each figure represents one of at least three independent experiments that all yielded similar results. The relative gene expression intensities were quantified using NIH Image Software (Version 1.44). DAF days after flowering

Fig. 4 Expression of the *TsGS1-3* and *TsGS2-1* genes in different seed compartments, and their expression patterns during germination. E-0 endosperms of dry seeds; S-0 scutella from dry seeds; E-8 endosperms from seeds imbibed for 8 h; S-8 scutella from seeds imbibed for 8 h; E-16 endosperms from seeds imbibed for 16 h; S-16 scutella from seeds imbibed for 16 h; E-24 endosperms from seeds imbibed for 24 h; S-24 scutella from seeds imbibed for 24 h; E-48 endosperms from seeds imbibed for 48 h; S-48 scutella from seeds imbibed for 24 h; R-48 roots from seeds imbibed for 48 h; Sh-48 shoots from seeds imbibed for 48 h; E-72 endosperms from seeds

imbibed for 72 h; S-72 scutella from seeds imbibed for 72 h; R-72 roots from seeds imbibed for 72 h; Sh-72 shoots from seeds imbibed for 72 h. Quantitative, one-step, RT-PCR analyses were performed using 100 ng of total RNA. Amplification of the 18S *rRNA* gene was used to ensure that equal amounts of the template were added to each RT-PCR. Each figure represents one of at least three independent experiments that all yielded similar results. The relative gene expression intensities were quantified using NIH Image Software (Version 1.44)

NAD⁺-GDH, then gradual decrease was observed. In the dry grain, the GDH deaminating activity exceeded the aminating activity. During kernel formation (3–20 DAF), a

significant advantage of the GDH aminating activity over its deaminating activity was determined, and the ratio (aminating activity/deaminating activity) ranged from 8 to

Fig. 5 Changes in the in vitro activities of aminating and deaminating glutamate dehydrogenase (NAD(H)-GDH and NAD-GDH) in the developing triticale seeds. Results are the mean $\pm$ SD for three independent experiments with two replicates each

5.3. During the grain maturation phase, from 25 to 45 DAF, the ratio of NADH-GDH to NAD⁺-GDH gradually declined. Indeed, at 45 DAF the ratio was 0.77 (Fig. 5).

Preparations obtained from the developing kernels (3–45 DAF) were also analyzed electrophoretically under non-denaturing conditions. After staining the gels for NAD⁺-GDH activity, a single band of enzyme activity was visualized (Fig. 6).

For glutamine synthetase, a high level of activity in the developing seeds was observed as early as 3 DAF. On subsequent days of kernel formation, the activity increased and reached its maximum at 15 DAF (7.91 $\mu\text{mol GHM min}^{-1} \text{g}^{-1} \text{DW}$). At 20 and 25 DAF, the activity of the enzyme declined sharply relative to the maximum value (1.6- and 4.3-fold, respectively). In later stages of seed development, the GS activity gradually decreased and was only 0.19 $\mu\text{mol GHM min}^{-1} \text{g}^{-1} \text{DW}$ at 45 DAF (Fig. 7).

Glutamine synthetase activity was measured in the scutella and endosperms of dry seeds, as well as in seeds imbibed for 8, 16 and 24 h. GS activity was also measured in the scutella, endosperms, shoots and roots of seeds imbibed for 48 and 72 h. The lowest level of GS activity (0.15 $\mu\text{mol GHM min}^{-1} \text{g}^{-1} \text{DW}$) was observed in the endosperm of the dry seeds. During the next imbibition time points, the GS activity level in the endosperm gradually increased and reached its maximum level at 72 h (1.11 $\mu\text{mol GHM min}^{-1} \text{g}^{-1} \text{DW}$). In the scutella of dry seeds, the GS activity level was six times higher relative to

Fig. 6 Electrophoretic image of the distribution of GDH activity in developing triticale seeds. Each lane contains 30 μg of soluble proteins extracted from the seeds. The zymogram is representative of similar results obtained from three independent experiments. DAF days after flowering

the activity in the endosperm of the dry seeds (0.90 $\mu\text{mol GHM min}^{-1} \text{g}^{-1} \text{DW}$). During imbibition, the GS activity level in the scutellum gradually increased to 3.19 $\mu\text{mol GHM min}^{-1} \text{g}^{-1} \text{DW}$ at 72 h. The levels of the GS activity in the shoot after 48 and 72 h of imbibition were 1.89 and 4.44, respectively. After 48 and 72 h of imbibition in the root, the GS activity levels were 1.77 and 2.54 $\mu\text{mol GHM min}^{-1} \text{g}^{-1} \text{DW}$, respectively (Fig. 8).

Discussion

Nitrogen is an essential nutrient that plays a major role in determining the amount of protein stored in cereal grains. The content and quality of protein are major determinants of the nutritional value of cereal grains and grain-derived products. The accumulation of storage proteins in the seed involves N-remobilization from vegetative organs, which is a process involving the GS and GDH enzymes. Although both enzymes have been thoroughly investigated in vegetative plant organs (Masclaux-Daubresse et al. 2005), they have been poorly studied in seeds.

Similar to barley, the development of triticale seeds can be split into three main stages. The initial phase occurs approximately two weeks post-anthesis when the endosperm cellularizes and the organelles proliferate. The second phase is characterized by the rapid synthesis of storage products. The third phase occurs approximately 30 days after anthesis. During this phase, the dry matter accumulation rate decreases and the grains begin to desiccate (Ma and Smith 1992; Goldberg et al. 1994). Maturation, which is the final stage of seed development, is characterized by a dehydration phase that leads to a loss of more than 90 % of water content and a dramatic decrease in metabolic activity (Baskin and Baskin 1998). The induction of germination after seed rehydration induces an increase in both

Fig. 7 Changes in the in vitro activity of GS in the developing triticale seeds. Results are presented as the mean $\pm$ SD for three independent experiments with two replicates each

Fig. 8 Changes in the activity of GS in different compartments of germinated seeds after 8, 16, 24, 48 and 72 h of imbibition. Results are presented as the mean $\pm$ SD for three independent experiments with two replicates each

respiration and metabolic activity, thereby allowing the mobilization of *N* reserves.

This detailed study of the GS gene family in triticale has increased our understanding of the function of specific GS isoenzymes. As expected, the phylogenetic analysis indicated that the *TsGS2* gene was located on the same branch as other plant species' GS2 genes. Three isoforms of wheat GS2 (AAZ300060, AAZ300061 and AAZ300062) were located on the same branch as *TsGS2-1*. The *TsGS2-1* gene also clustered near three *M. truncatula* genes that are exclusively expressed in developing seeds (i.e., *MtGS2b-α* (ADM45299), *MtGS2b-β* (ADM45300) and *MtGS2a* (AAO37651)) (Seabra et al. 2010). The *TsGS1-3* gene, which was used in the expression analysis of the developing and germinating triticale seeds, clustered near three genes (i.e., *OsGln1;1* (P14656) in rice and *Gln1-3*, (P38562), *Gln1-4* (P38561) in maize). These three genes have similar physiological functions that are involved in controlling seed development (Tabuchi et al. 2005; Martin et al. 2006).

Two triticale genes, *TsGS1-1* and *TsGS1-2*, were located on the same branch of the phylogenetic tree in close proximity to *OsGln1;1*, *Gln1-3* and *Gln1-4*. The expression of these two genes displayed low transcript levels in the triticale seeds (data not shown). The phylogenetic analysis demonstrates that the *TsGS1-3* gene is closely related to *GhGS* (ABW89466) and *GS1-2* (P38560) from cotton and maize, respectively. A study by Yajun et al. (2008) demonstrated that *GhGS* may be associated with ovule development. Another gene located on the same branch of the phylogenetic tree as *TsGS1-3* is the *GS1-2* maize gene (Li et al. 1993). This gene is one of five genes encoding for the cytoplasmic forms of GS and encodes a unique isozyme, GS_{p1} (Muhitch 1989). GS_{p1} is strongly expressed in the basal maternal tissues of the developing seed (Rastogi et al. 1998). To date, examination of the tissue distribution of GS_{p1} has been limited to the endosperm, embryo, pedicle, roots and leaves (Muhitch 1989). Expression of a *GS1-2*

promoter/GUS heterologous gene resulted in the predicted tissue-specific expression patterns in the seeds. Indeed, the expression pattern of *GS1-2* within the kernel is consistent with the GS_{p1} isozyme's recognized role in nitrogen metabolism during grain filling (Muhitch 2003). The *TsGS1-3* gene is also closely related to *GSe1* (AAR84349) and *GSe2* (AAR84350) from wheat. The level of *GSe* mRNA was very low in root, leaf, peduncle and glume (Bernard et al. 2008). In our study a high level of expression of *TsGS1-3* gene was observed in the triticale seeds.

To determine the roles of GS and GDH in the developing and germinating seeds of triticale, we conducted studies to elucidate the relationship between the dynamic activity changes of these enzymes and the expression patterns of the GS and GDH genes.

The high activity of GS during the first phase of seed development confirms the key role of this enzyme in the assimilation of ammonia derived from nitrogen remobilization (Lea and Mifflin 2003). The aminating activity of GDH was highest during the first phase of kernel development, and this activity level decreased during the later stages of seed development. This result may indicate that GDH also participates in the assimilation of ammonia derived from nitrogen remobilization (Tercé-Laforgue et al. 2003). Similar results were obtained for both maize seeds (Hadzi-Taskovic Sukalovic 1984) and the Malno variety of triticale (Kwinta et al. 1999). Because the presented GS activity profile is the sum of both GS1 and GS2 activities, we conducted expression analyses on the genes encoding the cytoplasmic and the chloroplastic forms to determine the contribution of these isoenzymes to the assimilation of nitrogen during seed development. For *TsGS2-1*, the highest level of expression was observed at 7 DAF. At this same time point, the expression levels of both *TsGS1-3* and *TsGDH1* were lower.

In *M. truncatula* seeds from 3 to 33 DAF, Seabra et al. (2010) observed high transcript levels of one of the genes encoding the chloroplast GS isoenzyme. During the first phase of seed development in triticale, a possible function for the GS2 enzyme is in N-remobilization from vegetative organs to glutamine required for storage protein synthesis. For the *TsGDH1* and *TsGS1-3* genes, the maximum transcript levels were observed from 11 to 20 DAF, and both genes were simultaneously expressed. In addition, the high levels of *TsGDH1* transcript were also observed at 3 DAF. Similarly, Hansen et al. (2009) demonstrated that during the early stages of barley seed development, the expression of genes encoding GS1 was accompanied by the expression of glutamate dehydrogenase genes. While the high transcript level of the genes encoding GS explains the role of this enzyme in nitrogen assimilation (Bernard and Habash 2009), the high level of *TsGDH1* may be surprising. However, alternative pathways, such as the reaction

catalyzed by glutamate dehydrogenase, may be involved in the assimilation of nitrogen in the seeds. When the expression levels of *TsGS1-3* and *TsGS2-1* are low, *TsGDH1* gene expression was high, thus compensating for the low expression levels of the *GS* genes in seeds. Similar conclusions were drawn by Tabuchi et al. (2005). This suggests the potential participation of GDH in nitrogen assimilation in rice when *OsGS1-1* is missing.

During the third phase of seed development, when the dry matter accumulation rate decreases and grains begin to desiccate, a sharp decline in GS activity was observed in parallel with a decrease in *TsGS1-3* and the low expression levels of *TsGS2-1*. Simultaneously, the high transcript level of *TsGDH1* gene and the high deaminating activity (30 and 35 DAF) were observed. However, in dry seeds the deaminating activity was decrease. The high deaminating activity observed during the third phase of seed development (25–45 DAF) probably is part of the metabolic mechanism associated with the startup of protein storage. This is due to the fact that during kernel germination, amino acids, including glutamic acid, are the primary sources of nitrogen available to the growing plants. This correlates with the results of previous studies on the germination of triticale seeds (Grabowska et al. 2011).

Our previous experiments performed on germinating triticale seeds determined that the deaminating reaction catalyzed by GDH is predominant during the first phase of imbibition (Grabowska et al. 2011). The high transcript level of *TsGDH1* in the germinating triticale seeds coincides with the high expression levels of *TsGS1-3*. This may indicate a functional cooperation of these genes' protein products. The high activity of NAD^+ -GDH observed during the early stage of triticale germination (Grabowska et al. 2011) is responsible for the degradation of glutamic acid. It may be possible that this is the mechanism used to deliver ammonia to the reaction catalyzed by GS1, and to provide the carbon skeletons for respiration (Aubert et al. 2001).

The germinating triticale seeds displayed substantially higher levels of GS activity in the scutella compared to the endosperms. Within the seed, the scutellum is especially interesting because of its important role in nitrogen mobilization. Also, Marttila et al. (1993) observed an increase in the GS activity in the scutellum during barley seed germination. The transient induction of a seed-specific GS isoenzyme was also observed during the imbibition of sunflower seeds (de la Haba et al. 1992). Up to 48 h of imbibition, a higher level of *TsGS1-3* gene expression was observed in both the endosperm and the scutellum compared to the gene encoding the chloroplastic form of GS. This finding suggests that *TsGS1-3* is likely involved in the reassimilation of ammonia released from the amino acids that were the result of the proteolysis of storage proteins in

the seed. In cotyledons and the embryo axis of *M. truncatula*, Glevarec et al. (2004) also observed high levels of the transcript encoding the cytoplasmic form during the first phase of germination. In shoots from triticale seedlings, high levels of *TsGS2-1* expression, which correlated with the high activity of GS, were observed after 48 and 72 h of imbibition. This finding may suggest that GS2, rather than the cytoplasmic form, has a greater contribution to the total enzyme activity in the shoots of triticale seedlings. In the roots of triticale seedlings, however, the contribution of both GS isoforms to the overall activity of the enzyme appears to be similar, which is apparent from the expression analyses of *TsGS1-3* and *TsGS2-1*.

To the best of our knowledge, the present work demonstrates, for the first time, the enzymatic activity and the expression of the GS1, GS2 and GDH genes in seeds. We discuss the possible roles for these genes/enzymes during the development and germination of triticale seeds. The confirmation of a potential relationship between the *TsGSs* genes identified in this study and *TsGDH1* requires further research, including mutant analyses.

Authors contribution Agnieszka Grabowska and Joanna Kwinta designed research, performed experiments analyzed data, and wrote the paper. Agnieszka Grabowska PhD cloned all genes, performed semi-quantified gene expression, and conducted all bioinformatics analyses. Joanna Kwinta PhD performed enzymes assays, protein analysis, and polyacrylamide gel electrophoresis. Wiesław Bielawski supervised the project and gave technical support. All authors discussed the results and implications and commented on the manuscript at all stages. All authors take full responsibility for the content of the paper.

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