RESEARCH PAPER

New isoforms and assembly of glutamine synthetase in the leaf of wheat (Triticum aestivum L.)

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Abstract

Glutamine synthetase (GS; EC 6.3.1.2) plays a crucial role in the assimilation and re-assimilation of ammonia derived from a wide variety of metabolic processes during plant growth and development. Here, three developmentally regulated isoforms of GS holoenzyme in the leaf of wheat (Triticum aestivum L.) seedlings are described using native-PAGE with a transferase activity assay. The isoforms showed different mobilities in gels, with GSII > GSIII > GS I. The cytosolic GS I was composed of three subunits, GS1, GSr1, and GSr2, with the same molecular weight (39.2 kDa), but different pI values. GS I appeared at leaf emergence and was active throughout the leaf lifespan. GS II and GS III, both located in the chloroplast, were each composed of a single 42.1 kDa subunit with different pI values. GS II was active mainly in green leaves, while GS III showed brief but higher activity in green leaves grown under field conditions. LC-MS/MS experiments revealed that GS II and GS III have the same amino acid sequence, but GS II has more modification sites. With a modified blue native electrophoresis (BNE) technique and in-gel catalytic activity analysis, only two GS isoforms were observed: one cytosolic and one chloroplastic. Mass calibrations on BNE gels showed that the cytosolic GS I holoenzyme was ~490 kDa and likely a dodecamer, and the chloroplastic GS II holoenzyme was ~240 kDa and likely a hexamer. Our experimental data suggest that the activity of GS isoforms in wheat is regulated by subcellular localization, assembly, and modification to achieve their roles during plant development.

Key words: Assembly, enzyme isoform, glutamine synthetase, nitrogen, protein modification, wheat.

Introduction

Glutamine synthetase (GS; EC 6.3.1.2) assimilates ammonium into glutamine, which is then used for the biosynthesis of all essential nitrogenous compounds (Miflin and Lea, 1977). All of the nitrogen within a plant, whether derived initially from nitrate, ammonium, N2 fixation, or catabolism of proteins, is channelled through reactions catalyzed by GS. Accordingly, GS plays a central role in nitrogen metabolism of vascular plants, and is a major checkpoint controlling plant growth and productivity (Brestic et al., 2014; Habash et al., 2007; Kichey et al., 2006; Lothier et al., 2011; Miflin and Habash, 2002; Simons et al., 2014; Tabuchi et al., 2005; Thomsen et al., 2014).

In vascular plants, two isoforms of GS were initially resolved by chromatography (Mann et al., 1979; McNally...
et al., 1983; McParland et al., 1976; O’Neal and Joy, 1973). Based on subcellular location, GS is classified as the cytosolic isoform (GS1) or the chloroplastic isoform (GS2). Electron microscopy analyses revealed that soybean (Glycine max) and common bean (Phaseolus vulgaris) GS enzymes are octamers (Llorca et al., 2006; McParland et al., 1976), whereas the crystallographic structures of GS in maize and Medicago truncatula are decamers (Torreira et al., 2014; Unno et al., 2006). GS2 is a single polypeptide (42–45 kDa) encoded by one nuclear gene, whereas GS1 is composed of polypeptides with the same molecular weight (38–40 kDa), but different pI values, and is encoded by three to five nuclear genes depending on the species. The GS isozymes have different metabolic roles, and their activities vary with plant development in different organs and cell types (Bernard et al., 2008; Coque et al., 2006; Finemann and Schjoerring, 2000; Gallais et al., 2006; Habash et al., 2001; Kamachi et al., 1991; Li et al., 1993; Ohashi et al., 2015; Orsel et al., 2014; Tabuchi et al., 2007). GS2 is the predominant isoenzyme in leaf mesophyll cells, where it assimilates ammonia originating from nitrate reduction and photorespiration (Kumagai et al., 2011; Tobin and Yamaya, 2001). GS1 has multiple metabolic functions, involving primary ammonium assimilation in the roots, and catabolism ammonia re-assimilation for transport and distribution throughout the plant, and localizes to the vascular cells of various tissue of Arabidopsis (Guan et al., 2015), wheat (Triticum aestivum L.) (Bernard et al., 2008; Kichey et al., 2005), rice (Oryza sativa) (Tabuchi et al., 2005), tobacco (Nicotiana tabacum) (Brugiere et al., 1999), and potato (Solanum tuberosum) (Pereira et al., 1995). During leaf senescence, GS1 functions in the assimilation and recycling of the ammonia generated from catabolic processes (Avila-Ospina et al., 2014; Bernard and Habash, 2009; Kamachi et al., 1992). This role, confirmed by quantitative trait locus analysis, or gene mutation or knockout, is particularly important during grain development in cereals when nitrogen is remobilized to the reproductive sinks (Brestic et al., 2014; Guan et al., 2015; Martin et al., 2006; Tabuchi et al., 2005). To achieve these multiple non-overlapping roles, GS isozymes are regulated at the levels of transcription, translation, subcellular localization, assembly of subunits into the holoenzyme, post-translational modification of the enzyme, and protein turnover (Hirel et al., 2001; Ishiyama et al., 2004; Kamachi et al., 1991; Li et al., 1993; Lima et al., 2006; Orsel et al., 2014; Ortega et al., 1999; Riedel et al., 2001; Tabuchi et al., 2007; Tobin and Yamaya, 2001). In wheat, seven genetic loci coding for three different forms of GS1 have been identified. TaGS1a, TaGS1b, and TaGS1c code for GS1;1, TaGSr1 and TaGSr2 code for GS1;2 (also called GSr), and TaGSel and TaGSel2 code for GS1:3 (also called GSe). Three alleles coding for GS2 (TaGS2a, TaGS2b, and TaGS2c) are known (Bernard et al., 2008; Thomsen et al., 2014). Here, three developmentally regulated GS holoenzymes in wheat are reported that can be separated by native-PAGE in plants.

There are several methods to analyze the oligomeric active state of a native protein, including gel filtration, analytical ultracentrifugation, electron microscopy, and X-ray crystallography. However, all of these methods require a substantial amount of protein and/or investment in expensive equipment. Blue native-PAGE (blue native electrophoresis (BNE)) and clear native-PAGE (clear native electrophoresis (CNE)) are performed with smaller amounts of protein and have been widely used to study membrane protein complexes (Filoni et al., 2013; Strecker et al., 2010; Wittig et al., 2007; Wittig and Schagger, 2009). The application of these techniques to determine the native molecular weights and oligomeric states of the GS isoforms in wheat is reported here.

**Materials and methods**

**Plant material and growth conditions**

Wheat (T. aestivum L.) cvs Yamui 34, 49, and 50 were used for isolation of GS isoforms during the growth of first leaves in April in Zhengzhou, China. The other cultivars shown in Supplementary Fig. S1 (at JXB online) were grown similarly, but at different times of the year. The seeds were put in a disk covered with wet gauze at 25 °C until they germinated; they were then sown in individual pots (25 cm upper diameter, 17 cm lower diameter, and 25 cm high) filled with vermiculite and grown outside under natural light/temperature. Each plant was sprayed with 300 ml sterile water every day. The leaves were sampled three times from the onset of seedling emergence to the first leaf turning yellow, harvesting 0.5 g per sample. Wheat cv. Yamui 49 was also grown under 10/14 h light/dark periods at 23 °C in a growth chamber, with 800 μmol m⁻² s⁻¹ photon flux density at the top of the canopy during the light period, and watered with 200 ml Hoagland solution (containing 1 mM KΗPO₄, 5 mM KNΟ₃, 1 mM MgSO₄, 0.5 mM CaSO₄, 4 mM Ca(NO)₂), 1 mM Mg(NO)₂, 0.5 mM CaCl₂, 1 μM H₂PO₄, 1 μM CaSO₄·5H₂O, 1 μM MnCl₂·4H₂O, 1 μM Na₂MoO₄, and 1 μM ZnSO₄·7H₂O) twice a week to keep the soil moist and supply sufficient nutrients. Fully expanded green leaves were collected for the preparation of intact chloroplasts and enzyme analysis when the wheat seedlings had four or five leaves.

**Preparation of leaf extract**

Sample (0.5 g each) were ground into powder in a chilled mortar with liquid N₂ and mixed with 1.5 ml Extraction Buffer (100 mM Tris, 1 mM EDTA, 1 mM MgCl₂, and 10 mM β-mercaptoethanol, pH 7.6). The extract was centrifuged at 13 000 g at 4 °C for 30 min. The supernatant was prepared for native gel analysis.

**Isolation of chloroplasts**

Intact chloroplasts were isolated essentially as described by Theg et al. (1989). The washed chloroplast pellet after the Percoll step was resuspended in 350 μl Extraction Buffer and incubated on ice for 10 min to break the organelles. The resuspension was used for immunoblotting or centrifuged at 13 000 g for 10 min and the supernatant used for enzyme assays immediately.

**In-gel detection of GS activity and molecular weight**

Four gel systems (running at 4 °C) were used to separate the proteins and detect GS activities in the gel. First, a discontinuous native-PAGE system was used according to Robert and Wong (1986). The native gel system employed a 1.5 mm×170 mm×100 mm gel, the analyzing gel was composed of 5% polyacrylamide (pH 8.7), and the stacking gel was 3% polyacrylamide (pH 6.7). Samples were normalized to 30 μl (~60 μg protein) from 0.5 g FW leaves in each lane, and electrophoresis was carried out at 80 V for the stacking gel and 120 V for the resolving gel at 4 °C. Second, the BNE system was used according to Wittig et al. (2006, 2007) with the following modifications. The sample gel contained 3.5% polyacrylamide and the
gradient resolving gel contained 4–13% polyacrylamide; the gel was 1.5 mm × 170 mm × 100 mm, the sample buffer was Extraction Buffer, and before loading sample in the gel, 100 μl sample was mixed with 10 μl 50% glycerol. The current was limited to 15 mA during electrophoresis. The gel was run with cathode buffer A (0.02% Coomassie Blue G250, 50 mM Tricine, and 5 mM imidazole, pH 7.0) until the blue dye front was up to half of the gel length; cathode buffer A was then removed and the gel was run with cathode buffer B (0.002% Coomassie Blue G250, 50 mM Tricine, and 5 mM imidazole, pH 7.0) until the blue dye front moved out of the gel. Third, the BNE protocol was modified as follows. The gradient gel was prepared as the second gel system. After the gel ran for 1 h with cathode buffer A, cathode buffer A was removed and the gel was run with cathode buffer C (50 mM Tricine and 5 mM imidazole, pH 7.0) until the blue dye front moved out of the gel. After electrophoresis, GS activity was detected in-gel by the conversion of 1-glutamine to γ-glutamyl hydroxamate (Barrett, 1980). The gel was immersed in 100 ml reaction buffer (100 mM Tricine, 1.3 mM EDTA, 20 mM sodium arsenate, 20 mM MgSO₄, 0.5 mM ADP, 25 mM hydroxylamine, and 50 mM 1-glutamate, pH 7.4) and incubated at 37 °C for 45 min with slow shaking, after which the reaction buffer was removed. The reaction was terminated by adding 50 μl stop solution (370 mM FeCl₃, 200 mM trichloroacetic acid, and 700 mM HCl) for ~3 min until GS activity appeared as a brownish band in the yellow background. The gel was washed twice with cool distilled H₂O and scanned immediately. The GS bands were marked with a blade and then the gel was stained with Coomassie Blue R250. The molecular mass of the GS isoforms was calculated by comparison with molecular weight standards (Life Technologies) using Quantity One software.

GS recovery and GS subunit identification

After the GS activity was detected in the gel, the band of interest was excised with a scalpel, rinsed with 0.5 M EDTA, pH 7.6, and ground in a chilled mortar with this same solution. The homogenate was centrifuged at 12 000 g at 4 °C for 20 min and then the extraction was mixed with an equal volume of 0.1 M Tris-buffered phenol (pH 8.0). After being centrifuged (12 000 g) at 4 °C for 20 min, the protein in the phenol phase was precipitated with 4 vols 0.1 M ammonium acetate in methanol overnight at −20 °C. The proteins recovered by centrifugation were washed once with 1 ml cold methanol and twice with 1 ml cold aceton, and then resolved in SDS sample buffer for analysis. A discontinuous SDS-PAGE system was implemented according to Laemmli (1970), with a 12.5% polyacrylamide analyzing gel and a 6% polyacrylamide stacking gel, and electrophoresis was performed at room temperature. Proteins were transferred to polyvinylidene difluoride membranes for blot analysis. GS polypeptides were detected using polyclonal antisera (generously provided by Bertrand Hirel) raised against GS2 of tobacco (Bernard et al., 2008).

Protein extraction for two-dimensional immunoblots

Protein was extracted using a modification of the phenol-based method (Finnemann and Schjoerring, 2000). Wheat leaves were homogenized in an ice-cold mortar and pestle in SDS sample buffer (0.1 M Tris-Cl, 2% SDS, 5% 2-mercaptoethanol, and 30% sucrose, pH 8.0) and then mixed with the same volume of Tris-buffered phenol (pH 8.0). The homogenate was centrifuged at 10 000 g for 5 min at 4 °C. Protein in the upper phenol phase was precipitated with 5 vols 0.1 M ammonium acetate in methanol for 30 min at −20 °C. The protein recovered by centrifugation was washed twice with cold 80% acetone and then dissolved in SDS sample buffer or rehydration buffer (8 M urea, 4% CHAPS, 2% IPG buffer, pH 4–7, and 20 mM DTT). Protein was quantified by the Bio-Rad protein assay with BSA as standard. For two-dimensional gel electrophoresis, wheat leaf proteins (600 μg) were loaded on to pH 4–7 Immobiline Drystrips (7 cm; Amersham) by passive rehydration overnight at room temperature. The rehydrated strips were resolved in a Multiphor II apparatus (Pharmacia Biotech) by isoelectric focusing for 8000 Vh at 10 °C. The resolved strips were consecutively equilibrated in DTT solution (50 mM Tris, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, and 1% DTT, pH 8.8) and iodoacetamide solution (50 mM Tris, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, and 2.5% iodoacetamide, pH 8.8) for 15 min, and the secondary SDS–PAGE was run with 12.5% gels. After electrophoresis, immunodetection was performed as described above.

Protein identification by LC-MS/MS

After GS activity was detected in a native-PAGE gel, each band of interest was excised with a scalpel and washed with 75% ethanol. The samples were sent to the Genome Center at the University of California-Davis for identification of GS proteins and modifications by LC-MS/MS, and analyzed with Scaffold 4.0 software.

Results

Three isoforms of GS are active during wheat leaf development

To elucidate the role of GS isoforms during wheat development, leaf extracts from three cultivars of wheat seedlings at different developmental stages were separated by native-PAGE and GS isoforms were detected using transferase activity staining. Three isoforms of GS holoenzyme were identified in the wheat leaf. GS₁, GS₁I, and GS₁II emerged sequentially with the development of the first leaf (Fig. 1). Conversely, GS₁II, GS₁I, and GS₁ disappeared in turn with leaf senescence (Fig. 1). GS₁ had the highest mobility in native-PAGE, followed by GS₁II and then GS₁. GS₁ was present during the seed germination stage and increased progressively in activity until leaf senescence. GS₁II appeared with leaf expansion and maintained the highest activity in green leaves, but disappeared when the leaf turned yellow. GS₁II had a shorter period of activity, albeit with higher activity, in the growing green leaf, i.e. from the stage of fast leaf expansion (Fig. 1, panels 2dpe and 5 dpe) to the full-length size (Fig. 1, panel 7dpe). It was deduced that GS₂ was likely cytosolic (GS₁) because it was present from the onset of germination until leaf senescence. GS₂ was considered likely to be chloroplastic (GS₂) because it was the dominant GS in green leaves. GS₁I has not been described before.

Subunit composition and subcellular localization of GS isoforms

To characterize the GS isoforms further, chloroplasts were isolated and GS activity therein detected using native-PAGE. Both GS₁I and GS₁II were found in chloroplasts (Fig. 2A) and immunoblots revealed them to be derived from only one GS polypeptide of 43.6 kDa (Fig. 2B). Based on these findings, they are considered to be chloroplastic GS2-type isoforms. By contrast, GS₁ was not found in chloroplasts and so might be cytoplasmic. To confirm the identities of the GS isoforms,
leaf proteins were separated by native-PAGE, and the bands displaying GS activity were recovered from the gel by chemical extraction, separated by SDS-PAGE, and probed with anti-tobacco GS2.

**Identification of GS protein sequences and modifications**

To determine unambiguously the proteins corresponding to GS\(_I\), GS\(_{II}\), and GS\(_{III}\), bands containing these activities were excised from native gels and analyzed by LC-MS/MS (Supplementary Table S1 at JXB online). Protein identification revealed that the GS\(_I\) band contained fragments of three previously described cytosolic GS isoforms, GS\(_1\), GS\(_{r1}\), and GS\(_{r2}\) (equivalent to GS\(_{1\;1}\) and two forms of GS\(_{1\;2}\)), although no GSe (equivalent to GS\(_{1\;3}\)) was detected. The complete sequences of GS\(_{II}\) and GS\(_{III}\) were obtained in the LC-MS/MS experiment; they were identical with a theoretical molecular weight of 42.1 kDa, identical to GS\(_{2\;a}\), GS\(_{2\;b}\), and GS\(_{2\;c}\). The LC-MS/MS data (parent error <5 ppm) indicated that GS\(_{II}\) had many more modifications than did GS\(_{III}\), including acetylation, oxidation, dioxidation, and deamidation. In comparison, GS\(_{III}\) had fewer sites of oxidation, one site of acetylation, and more sites of deamidation (Table 1).

**Oligomers of GS isoenzymes**

The authors next turned their attention to elucidation of the oligomeric state of the wheat GS isoforms. In BNE, protein complexes are separated according to size in acrylamide gradient gels and their sizes can be calibrated with standards. During the initial BNE experiments, the presence of the Coomassie Brilliant Blue G250 (referred to hereafter as G250) interfered with the activity stain for GS\(_I\) (Supplementary Fig. S2 at JXB online). To overcome this the BNE protocol was modified to include a 1 h separation of proteins in the presence of G250 and then an additional 3 h separation in which the cathode buffer was replaced with one lacking the dye. This allowed a separation of the protein complexes by molecular weight and subsequent detection by the transferase activity stain. Fig. 4 shows the results of such an analysis and reveals that the GS\(_I\) holoenzyme has a molecular weight of ~490 kDa. Given the molecular weight of the monomer (39 kDa), this indicates that the GS\(_I\) holoenzyme is likely...
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Interestingly, GS2, containing both GS\textsubscript{II} and GS\textsubscript{III} activities, ran as a single band with a molecular weight of 240 kDa, suggesting that the holoenzyme is most likely a hexamer. These results additionally suggest that while GS1 and GS2 have distinct migrations in native-PAGE gels, in part due to different oligomeric states of their respective holoenzymes, the different mobilities of GS\textsubscript{II} and GS\textsubscript{III}, both GS2 isoforms, in the same native gel system must be due in part to their different modifications as described in Table \ref{table:1}.

**Discussion**

In vascular plants, only two isoforms of the GS holoenzyme have been resolved by standard chromatography (Mann \textit{et al.}, 1979; McNally \textit{et al.}, 1983; McParland \textit{et al.}, 1976; O’Neal and Joy, 1973) and by native gel electrophoresis (Nagy \textit{et al.}, 2013; Péscsvéradi \textit{et al.}, 2009). Here, three isoforms of GS were separated using native gels in wheat seedlings (Figs 1 and 2); the third isoform, GS\textsubscript{III}, has not been reported before. The fact that all three isoforms were observed in >20 cultivars (Supplementary Fig. S1 at JXB online) confirms they are generally present in wheat seedlings. In general, GS\textsubscript{III} is readily observed in plants grown in the field, but is more difficult to detect in those grown in growth chambers. This might be ascribed to the lower light intensity of the growth chamber environment (<1000 μmol m\textsuperscript{-2} s\textsuperscript{-1} photon flux density), as opposed to sunlight which can provide ~2000 μmol m\textsuperscript{-2} s\textsuperscript{-1} photon flux density of photosynthetically active radiation in the field. The appearance of GS\textsubscript{III} is also regulated with the leaf development independent of whether the wheat seedlings were grown without (Fig. 1) or with (Supplementary Fig. S3 at JXB online) nitrogen. These two factors may be responsible for it not having been identified in previous studies (Nagy \textit{et al.}, 2013; Péscsvéradi \textit{et al.}, 2009). For instance, GS\textsubscript{III} was not found in green leaves of 14-d-old seedlings growing in chambers, whereas it was abundant in green leaves of 21-d-old seedlings from the same chamber (Fig. 2A).

The early estimates for the molecular weight of GS oligomers came from direct measurements (gel filtration, sedimentation equilibrium) with purified protein (Mann \textit{et al.}, 1979; McParland \textit{et al.}, 1976). It is difficult to obtain sufficient quantities of purified protein from plants for assembly and structure studies, and, consequently, these previous GS structural studies used proteins heterologously expressed in \textit{Escherichia coli} (Llorca \textit{et al.}, 2006; O’Neal and Joy, 1973; Seabra \textit{et al.}, 2009; Torreira \textit{et al.}, 2014; Unno \textit{et al.}, 2006). BNE techniques provide an independent method for separating protein complexes with high resolution and from which

![Table 1. Protein modifications detected in GS\textsubscript{II} and GS\textsubscript{III}](https://example.com/tables/table1.png)

| Isoform | Oxidation | Acetyl | Deamidation | Dioxidation |
|---------|-----------|--------|-------------|------------|
| GS\textsubscript{II} | 7 | 3 | 7 | 6 |
| Amino acid | M W Q N | A L G | Q N | W M |
| GS\textsubscript{III} | 4 | 1 | 9 | 5 |
| Amino acid | M W Q N | L | W Q N | W M |

![Fig. 3. Two-dimensional analysis of GS isozymes in wheat leaves.](https://example.com/figures/fig2.png)

Fig. 3. Two-dimensional analysis of GS isozymes in wheat leaves. (A) Two-dimensional gel of proteins extracted from the wheat leaf; stained with Coomassie Blue R250. The rectangle shows the region putatively containing GS spots. (B) Two-dimensional immunoblots of GS subunits in wheat leaf. IEF, isoelectric focussing. Leaf proteins (50 μg) were separated by two-dimensional electrophoresis, and the region thought to contain GS (larger than the rectangle) was electroblotted and probed with antibodies against tobacco GS2.

![Fig. 4. Identification of the native molecular weight of GS isoforms using BNE.](https://example.com/figures/fig3.png)

Fig. 4. Identification of the native molecular weight of GS isoforms using BNE. (A) Soluble proteins (~100 μg) from wheat seedling chloroplasts (Cp), leaves (L), and roots (R) were separated by BNE on a 4–13% polyacrylamide gradient gel, and GS isoforms were detected with a transferase activity assay. (B) The GS bands were marked and the gel stained with Coomassie Blue R250. M, high-molecular-weight markers (mass in kDa given to the right of the gel). (This figure is available in colour at JXB online.)
their molecular weights can be determined. When soluble proteins from leaves or isolated chloroplasts were separated by BNE and GS isoform activities were detected in the gel, only one GS isoform (corresponding to chloroplastic GS2) was observed (Supplementary Fig. S2 at JXB online). This is likely because cytosolic GS1 activity appears to be sensitive to G250 and because Rubisco runs in the same part of the gel, overwhelming the transferase signal. When leaf or chloroplast soluble proteins were separated using the BNE procedure, but omitting G250 from the cathode buffer, two GS isoforms were detected in leaf extracts (GS1 and GS2) and one in the chloroplast (GS2), although chloroplastic GS in leaves and chloroplasts displayed slightly different mobility (Supplementary Fig. S4 at JXB online). Finally, the BNE protocol was modified, first running the gel with cathode buffer containing 0.02% G250 for 1 h to ensure that all proteins carried same net charge and then changing to cathode buffer without G250 to reduce the influence of G250 on GS activity. Although cytosolic GS activity was weak, it was detectable. Furthermore, chloroplastic GS in leaf extracts and chloroplast extracts had the same mobility in the gel.

Based on this modified BNE procedure, the cytosolic GS, with the activity in GS1, was likely to be a dodecamer, the same as GSr1 in soybean nodules analyzed by analytical ultracentrifugation and native-PAGE (Masalkar and Roberts, 2015) and GS in prokaryotes (Eisenberg et al., 2000). Our data suggest that wheat chloroplastic GS, with activities in GS II and GS III, was a hexamer, which differs markedly from other plants. For example, GSs from soybean (McParland et al., 1976) and common bean (Llorca et al., 2006) are octamers as determined by electron microscopy; GSs from maize and M. truncatula and GS1β from soybean are decamers (Masalkar and Roberts, 2015; Torreira et al., 2014; Unno et al., 2006) as determined by X-ray crystallography. Separated by CNE and detected by in-gel GS activity assay, GS from spinach stroma is a decamer (Kimata-Ariga and Hase, 2014). Confidence in the methodology for oligomeric state determination is strengthened by Supplementary Fig. S5 (at JXB online) in which maize GS can be seen running as a decamer. Nonetheless, the authors recognize that the oligomeric state reported here should be further evaluated by additional techniques, and future plans call for expression of recombinant TaGS1 and TaGS2 and analysis by X-ray crystallography. The authors are also working to compare GS proteins in M. truncatula, soybean, Arabidopsis, spinach, and common bean with those in wheat using the modified BNE system.

The different pI values for the wheat GS proteins have different origins. GSII, cytosolic GS, is encoded by a multi-gene family, GS1 and GSR, and the pI values detected here are close to those predicted by analysis of the respective gene sequences (Bernard et al., 2008). However, no GS2 was identified by MS analysis, perhaps because its expression was too low to be detectable in leaves and roots during the wheat seedling stage (Bernard et al., 2008). In contrast, chloroplastic GS is encoded by three alleles (TaGS2a, TaGS2b, and TaGS2c), and the different pI values must arise from different post-translational modifications. Lima (2006) reported that phosphorylated GS2 of M. truncatula interacts with 14-3-3 proteins, which leads to selective proteolysis and thus inactivation of the plastid isoform. In E. coli, GS is reported to be inactivated by adenylation (Liaw et al., 1993), and oxidation of soybean root GS has been reported to lead to its inactivation and increased susceptibility to degradation (Ortega et al., 1999). No evidence for phosphorylation of GS2 was found here, but numerous other modifications were detected, and they were different for GS II and GS III (Supplementary Fig. S6 at JXB online). For instance, GS II had three acetylation sites in its N-terminal region, whereas GS III had one such site. GS II had seven sites of oxidation, while GS III had four, even though GS II activity was higher than that of GS III in all but the most active stages of leaf development (Fig. 1). Whether the various modifications regulate GS2 enzyme activity or stability remains to be established.

Recently, an analysis of GS in Arabidopsis was presented in which 11 different GS1 isoforms were detected in a 7% resolving gel using a phosphate release assay and no GS2 was observed (only this group detected GS activity using this method) (Dragicevic et al., 2014). This is clearly different from the situation described herein for wheat and emphasizes the potential diversity of GS assembly configurations in different plant species.

When GS isoenzymes were originally discovered, their putative functions were deduced from their pattern of expression in different tissues during plant development and further confirmed by genetic methods (Bao et al., 2014; Gadaleta et al., 2011; Gadaleta et al., 2014; Guo et al., 2013; Habash et al., 2007; Martin et al., 2006). GS1, vascular-localized cytosolic GS, is proposed to be involved in the re-assimilation of ammonium released during leaf senescence and in transporting ammonium from source organs to sink organs, e.g. from fully expanded leaves to new leaves (Bernard et al., 2008; Kichey et al., 2006; Kichey et al., 2007). GS2, however, was found in both mitochondria and chloroplasts in Arabidopsis (Taira et al., 2004), suggesting that this isoform is active in re-assimilation of the large pool of ammonium released by photosynthesis. It is noteworthy that neither GS activity nor GS subunits in mitochondria purified from wheat leaves were detected in the present report (data not shown). This, along with the detection of GS II primarily in leaves grown under relatively high light intensity, would be consistent with a function of chloroplastic GS2 in original nitrogen assimilation, especially under conditions of abundant energy availability that would promote the conversion of nitrate to ammonium in the plastid. Although the physiological role of the newly described GS III remains to be elucidated, findings presented here suggest that there is a complex and flexible regulation for GS isoforms in wheat that is coupled to nitrogen utilization and plant growth.

**Supplementary data**

Supplementary data are available at JXB online.

**Figure S1.** GS isoforms in the leaf of different wheat cultivars.
Figure S2. GS isoforms in wheat chloroplasts, leaves, and roots.

Figure S3. GS isoforms as a function of leaf development in wheat.

Figure S4. GS isoforms in wheat chloroplast, leaf, and roots.

Figure S5. GS isoforms in wheat leaf and roots, and maize leaf and roots.

Figure S6. Amino acid modifications sites in GSII and GSIII.

Table S1. Identification of the composition of GS\textsubscript{I}, GS\textsubscript{II}, and GS\textsubscript{III} by MS analysis.

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